SIGNALING FUNCTIONS OF THE DEUBIQUITINATING ENZYME CYLD IN LYMPHOCYTE ACTIVATION AND OSTEOCLASTOGENESIS

A Dissertation in Genetics

by

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ABSTRACT

Mammalian immune system is composed of innate and adaptive immune responses, which function cooperatively to combat microbial infections. The innate immune responses are largely mediated by phagocytic cells, including macrophages and neutrophils, whereas the adaptive immune responses rely on antigen-specific lymphocytes, the T and B cells. Defective immune cell function can cause diverse immunodeficiencies, while deregulated immune cell activation leads to chronic inflammation, autoimmunity and cancer. Therefore, activation of immune cells is subject to tight regulation by both positive and negative mechanisms. One increasingly recognized mechanism of immunoregulation is protein ubiquitination, a reversible process that is counter regulated by ubiquitin conjugating enzymes and deubiquitinating enzymes (DUB).

This thesis project concerns a recently identified deubiquitinating enzyme, CYLD. CYLD was originally identified as a tumor suppressor mutated in patients with cylindroma, benign tumors of skin appendages. *In vitro* studies suggest that CYLD deubiquitinates the signaling molecules TRAF2 and IKKγ, thereby negatively regulates activation of the transcription factor NF-κB by innate immune stimuli, Toll-like receptors (TLRs) and tumor necrosis factor receptors (TNFRs). However, our recent studies using CYLD knockout mice fail to reveal an important role for CYLD in the regulation of TLR and TNFR signaling in innate immune cells. Instead, these genetic analyses reveal a critical role for CYLD in regulating adaptive immune function, particularly the development of T cells in the thymus. Studies to be presented in this thesis have further elucidated pleotropic functions of CYLD in the regulation of peripheral activation of T and B lymphocytes and
the prevention of chronic inflammation. Additionally, CYLD also regulate a specific aspect of macrophage function, osteoclastogenesis. The major findings are summarized in the following:

1. **CYLD negatively regulates ubiquitin-dependent kinase Tak1 and prevents abnormal T cell responses.** Although CYLD deficiency causes impaired thymocyte development therefore reduced peripheral T cell number, T cells isolated from spleen and mesenteric lymph nodes of CYLD knockout mice are hyper-responsive upon TCR and CD28 stimulation. *In vivo*, CYLD-deficient T cells infiltrate into colon and mediate intestinal inflammation, which resembles human inflammatory bowel disease (IBD). At the molecular level, CYLD targets a ubiquitin-dependent kinase, Tak1, by inhibiting its ubiquitination and auto-activation. In the absence of CYLD, Tak1 is constantly ubiquitinated and activated, leading to activation of downstream kinases, IKK and JNK. These findings emphasize a critical role for CYLD in preventing the spontaneous activation of Tak1 axis of T cell signaling, thereby maintaining normal T cell function.

2. **CYLD regulates the peripheral development and naïve phenotype maintenance of B cells.** CYLD knockout mice develop B cell hyperplasia in secondary lymphoid organs especially in mesenteric lymph nodes, which is dramatically enlarged at 12 week of age. B cells deficient with CYLD spontaneously display surface activation markers and are hyper-responsive to IgM and LPS stimulation both *in vitro* and *in vivo*, indicating their abnormal activation status. EMSA results obtained from freshly isolated spleen and mesenteric lymph node B cells reveal hyper activation of NF-κB due to loss of CYLD. The
constitutive NF-κB activation in CYLD knockout B cells is due to constant phosphorylation and degradation of NF-κB inhibitory protein IκBα by its kinase IKKβ. This finding suggests that CYLD is a crucial negative regulator of IKK activation in B cells. However, unlike in T cells, loss of CYLD results in hyper activation of Erk but not JNK and p38 MAPKs, which indicates different upstream molecular target(s) of CYLD in B cells.

3. **CYLD regulates osteoclast function and osteoporosis.** Macrophage, one of the major players in innate immunity, is also the precursor of bone-resorbing osteoclasts. Macrophage can differentiate into osteoclast in the presence of both M-CSF and RANKL. In contrast to our previous finding that macrophage activation by innate immune stimuli (LPS and TNF-α) is normal in the absence of CYLD, the function of macrophage as osteoclast precursor is regulated by CYLD. CYLD-deficient bone marrow derived macrophages differentiate into osteoclasts *in vitro* considerably more efficiently than wildtype cells. Moreover, the CYLD knockout mice display an osteoporotic phenotype, characterized by loss of trabecular bone mass. Mechanistically, CYLD physically interacts with and deubiquitinates TRAF6, the key signaling component of RANK signaling, therefore controlling downstream NF-κB and AP-1 activation. These findings establish a negative-regulatory role of CYLD in RANK pathway and osteoclast differentiation. In agreement with these functional data, the expression of CYLD protein is potently induced by RANKL but not by LPS or TNF-α, thus emphasizing the importance of CYLD protein level in its function in macrophages.
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LIST OF ABBREVIATIONS

APC  antigen-presenting cell
BAFF  B-cell activation factor of the TNF family
BCR  B cell receptor
BV  bone volume
CARD  membrane associated guanylate kinase
CBM  CARMA1-BCL10-MALT1
CD  connectivity density
CD  Crohn’s disease
CFSE  carboxyl fluorescent succinimidyl ester
ChIP  chromatin immunoprecipitation
DA  degree of anisotropy
DAG  diacyl glycerol
DC  dendritic cell
DUB  deubiquitinating enzyme
EMSA  electrophoresis mobility shift assay
ERK  extracellular signal-regulated kinase
H&E  hematoxylin and eosin
HTLV  human T-cell leukemia virus
IB  immunoblotting
IBD  inflammatory bowel disease
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IkB</td>
<td>inhibitor kB</td>
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<td>IKK</td>
<td>IkB kinase</td>
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<tr>
<td>IL-6</td>
<td>interleukin 6</td>
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<tr>
<td>IP</td>
<td>immunoprecipitation</td>
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<td>IP3</td>
<td>inositol 1,4,5-triphosphate</td>
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<td>ITAM</td>
<td>immunoreceptor tyrosine activation motif</td>
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<td>JAMM</td>
<td>JAB1/MPN/Mov34 metalloenzyme</td>
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<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
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<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MAGUK</td>
<td>membrane associated guanylate kinase</td>
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<td>MAPK</td>
<td>MAP kinase</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>microCT</td>
<td>microcomputed tomography</td>
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<tr>
<td>OC</td>
<td>osteoclast MLN mesenteric lymph node</td>
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<tr>
<td>MJD</td>
<td>Machado-Joseph Domain</td>
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<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
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<tr>
<td>NF-ATc1</td>
<td>nuclear factor of activated T cells c1</td>
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<td>NIK</td>
<td>NF-κB inducing kinase</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<td>NP-LPS</td>
<td>nitro-phenol-conjugated lipopolysaccharide</td>
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<tr>
<td>OTU</td>
<td>ovarian tumor related</td>
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<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<td>PDB</td>
<td>Paget’s disease of bone</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor-κB ligand</td>
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<td>RHD</td>
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<td>RPA</td>
<td>RNase protection assay</td>
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<td>Src family tyrosine kinase</td>
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<td>supramolecular activation clusters</td>
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<td>TGF-β activating kinase 1</td>
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<tr>
<td>Tb.N</td>
<td>trabecular number</td>
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<td>Tb.Sp</td>
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<td>Tb.Th</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor</td>
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<td>Th1(2)</td>
<td>T helper 1(2)</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
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<td>tumor necrosis factor-alpha</td>
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<td>TRAF</td>
<td>TNF receptor associated factor</td>
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<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
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<td>Abbr</td>
<td>Description</td>
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<tr>
<td>TV</td>
<td>total volume</td>
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<tr>
<td>UCH</td>
<td>C-terminal hydrolases</td>
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<tr>
<td>UBA</td>
<td>ubiquitin association domain</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin-specific proteases</td>
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CHAPTER I

LITERATURE REVIEW
1.1 Immune System Overview

Immune system is the self-protective device eukaryotes have developed during evolution in response to the threat from harmful microorganisms in the environment. In the constant battle with pathogenic microbes, the immune system has evolved multiple defense mechanisms in adaptation to the diversity of pathogens, including bacteria, virus, fungi, protozoa, parasites, irritants and allergens. In vertebrate animals, these defense mechanisms can be classified into two distinct yet corporative branches: innate immunity and adaptive immunity.

1.1.1 Innate immunity

Innate immunity is the main mechanism for immediate responses to infection by foreign pathogens. The epithelial cells lining the skin and internal mucosal milieus, such as the respiratory and the gastrointestinal tracts, form a physical barrier to prevent entry by most microbes. Pathogens that break this barrier and enter the host body can be quickly detected and attacked by professional phagocytic cells, most importantly macrophages and neutrophils. This early phase of immune reaction is facilitated by the complement system, which is composed of a large number of plasma proteins helping to opsonize pathogens, recruit inflammatory cells, and directly engage in killing of microbes (88).

The professional phagocytic cells recognize pathogenic microorganisms through a limited number of germline-encoded receptors known as pattern recognition receptors (PRR) (4). PRRs are evolutionarily conserved and detect pathogen-associated molecular patterns (PAMPs), which are usually characteristic structures of pathogens but not part of
the host cells. PAMPs include cell wall components, proteins, nucleic acids and synthetic chemical compounds (4). In addition to mediating phagocytosis, the PRRs signal to the phagocytes to produce soluble innate immune factors, including proinflammatory cytokines, chemokines, and anti-microbial peptides, which participate in the recruitment and activation of additional immune cells and direct anti-microbial reactions. One major family of signaling PRRs that has been extensively studied is the Toll-like receptors (TLRs). The Toll proteins were first found in *Drosophila* for their importance in dorsal-ventral patterning and effective immune response against fungal infection (111, 138). The homologues of the *Drosophila* toll protein, TLRs, were subsequently identified in mammals, with 11 members in human and 13 members in mouse discovered to date (96). TLRs differ in their ligand specificities, subcellular localization, expression patterns, as well as target genes. For example, TLR4 is expressed on the surface of the innate immune cells and many other cell types and responds to Gram-negative bacteria through recognition of the lipid A moiety of bacterial lipopolysaccharide (LPS), whereas TLR3 is an intracellular receptor that recognizes double-stranded viral RNA in the endosome (5, 158). TLR3 signaling produces type-1 interferon (IFN), key cytokines in anti-viral response; however, TLR4 ligation induces a large variety of pro-inflammatory cytokines, such as TNF-a and IL-6, in addition to type-1 IFNs.

### 1.1.2 Adaptive Immunity

Although the innate immune system provides the immediate and rapidly induced mechanisms of host defense, it is usually insufficient to clear pathogenic infections but
rather functions to hold the pathogens in check and facilitate the development of adaptive immune responses. The central players of adaptive immunity are lymphocytes: T cells and B cells. Unlike innate immune cells, which detect the PAMPs, lymphocytes recognize highly specific antigens derived from microorganisms using their antigen receptors. Lymphocyte antigen receptors are composed of the invariant domain and the highly variable antigen recognition domain. The B-cell receptor (BCR) is a cell-surface immunoglobulin, which is the membrane-bound form of the antibody the same B cell secretes and uses to detect intact antigen or antigen-complement complex. The T-cell receptor (TCR), on the other hand, recognizes antigens bound by the major histocompatibility complex (MHC) molecules on the surface of professional antigen-presenting cells (APCs). Therefore, T cell activation needs the help of APCs.

APCs are specialized to take up foreign substances and present the antigens to T cells, which provide a bridge between innate and adaptive immunity. It is generally believed that dendritic cells (DC) are the primary APCs for initiating an adaptive immune response, although macrophages and B cells may also serve the function in some circumstances. Immature DCs function as phagocytic cells in innate immunity. After engulfing the pathogen, they are activated and undergo a maturation process, which allows them to efficiently degrade, process and present antigens on their surface MHC molecules. The microbial activation of DCs also triggers their migration to the local lymphoid organs, where they can ‘crosstalk’ with the naïve T cells and pass the information for an infection. Consequently, antigen-specific lymphocytes undergo clonal expansion and acquire effector functions. The antigen presentation and clonal expansion processes delay the initiation of adaptive immune response for three to five days after infection.
Functionally, conventional T cells can be divided into two groups: CD4+ T cells and CD8+ T cells, which express CD4 or CD8 as TCR co-receptors, respectively. CD8+ T cells eliminate pathogens through direct cytotoxic function, whereas CD4+ T cells function as helper cells. Depending on the type of pathogens and cytokine milieu, activated CD4+ T cells differentiate into distinct subtypes of effector T cells that carry out specialized immune functions (189). T helper 1 (Th1) cells mediate macrophage activation, thereby promoting immune destruction of intracellular pathogens, whereas Th2 cells provide help for B cell activation, leading to production of antibodies for attacking extracellular pathogens (1, 65). A more recently identified subset of helper T cells, Th17 cells, play a role in recruiting innate immune cells, particularly neutrophils. However, a better-known function of Th17 cells is to mediate autoimmunity and inflammation (106). Effector T cells are subject to tight regulation by apoptosis, which ensures that an immune response is terminated when the pathogens are eliminated. A small portion of effector T cells can become memory cells that mediate rapid responses to re-exposure to their specific antigens.

The major function of B cells is to produce antigen-specific antibodies that mediate humoral immune responses to destroy extracellular pathogens. There are three major subtypes of B cells, defined based on their developmental origin, surface phenotype, and anatomic localization in the periphery; these include follicular B cells, marginal zone B cells and B1 cells (145). Follicular B cells reside in the lymphoid follicles and are the major sources of antigen induced antibody production under the T cell help. Activated follicular B cells undergo clonal expansion and form germinal centers. Germinal center B cells are selected through somatic hypermutation for high affinity antibody production. Only those cells with strong antigen binding BCR survive and differentiate into antibody producing
plasma cells or memory cells (160). Marginal zone B cells and B1 cells are mainly localized in marginal zone of spleen and peritoneal cavity, respectively. These cells are considered ‘innate B cells’ since they can rapidly respond to certain types of antigens and function largely independent of T cell help (15, 128).

The function of lymphocytes relies largely on their antigen receptors. Contrary to innate immune receptors, which are designed for fixed molecular patterns, lymphocyte antigen receptors provide tremendous diversity for antigen recognition by virtue of rearranged gene segments of the receptor chains. The lymphoid-specific V(D)J recombinases RAG1 and RAG2 mediate the somatic recombination events during lymphocyte development. The random selection and combination from multiple V (variable) segments result in unique antigen binding site structure and antigen specificity for every single cell, therefore, generating a receptor repertoire of sufficient diversity to recognize antigens from any potential pathogens.

1.1.3 Immune tolerance and autoimmunity

The endless diversity of lymphocyte antigen receptor repertoire favors the defense against infections; however, it also increases the risk of self-antigen recognition and improper immune responses against host body. To avoid potential autoimmune responses, immature lymphocytes are selected during development according to the binding affinity and avidity between their antigen receptors and self-antigens presented by the environmental stromal cells. Strong interaction leads to accelerated apoptosis. This negative selection process is termed as central tolerance, which happens in bone marrow for B cells and thymus for T cells. In periphery, auto-reactive lymphocytes escaped from negative selection
or specific for certain tissues are kept in an unresponsive or anergic stage by incomplete antigen receptor activation, which is defined as peripheral tolerance.

A major mechanism of peripheral tolerance is mediated by regulatory T cells (Tregs). Tregs are a small population of CD4+CD25+ T cells specifically expressing Foxp3, a member of the forkhead winged-helix family transcription factors encoded by the X chromosome. Peripheral Tregs bear two origins: naturally developed from autoreactive thymocytes or induced in the periphery in the presence of TCR signal and cytokine TGF-β. It is not clear whether there is functional difference between Tregs with distinct origin. Nevertheless, Tregs appear to function as the essential mediator of immune tolerance, since mutation of Foxp3 in human is associated with IPEX, a multiorgan autoimmune syndrome (14). Similarly, mice with Foxp3 deletion lack Treg population and suffer from an overwhelming autoimmune symptom (60), (61). Foxp3 is not only required for Treg development, constitutive expression of Foxp3 is also necessary for the effector function of mature Tregs in suppressing autoimmunity (243). Tregs mediate their immunoregulatory role through a number of independent mechanisms, including production of immunosuppressive cytokines, IL-10 and TGF-b, and direct inhibition of T cell activation through inhibitory surface co-stimulatory molecule CTLA-4 (87).

1.1.4 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a term referring to a number of intestinal autoimmune inflammatory diseases (52). There are two most prevalent forms of IBD, Crohn’s disease (CD) and ulcerative colitis (UC). Although both disease forms share some
clinical features, such as diarrhea, rectal bleeding, abdominal pain, weight loss, they can be distinguished by unique histopathologies and mechanism of inflammation. Crohn disease is characterized by a transmural, granulomatous inflammation occurring anywhere in the alimentary canal but usually centered in the terminal ileum and ascending colon, whereas ulcerative colitis has the key feature of superficial inflammation causing epithelial ulceration centered in the rectum and colon (23, 156). Susceptibility to IBD involves both genetic and environmental factors. Although the exact etiologies of IBD remain unclear, it is generally believed that the disease is due to loss of tolerance of mucosal immune system to the normal luminal flora (156).

Both innate and adaptive immune factors contribute to IBD symptoms. The importance of innate immune system in IBD etiology is suggested by the findings that mutations in TLRs and Nod proteins, major innate microbial detectors, are linked to IBD (249). The innate immune system appears to play an important role in preventing the productive invasion by luminal flora, thereby preventing the triggering of inflammatory responses. However, deregulated innate immune cell responses may cause overproduction of proinflammatory cytokines, thus promoting the development of IBD. Indeed, a major characteristic of human and animal models of IBD is excessive production of proinflammatory cytokines (186). Among the adaptive immune factors, CD4+ helper T cells function as important mediators of IBD (43, 82, 134, 249). Th1-derived cytokines participate in the induction of Crohn’s disease (CD), whereas the Th2-derived cytokines are associated with ulcerative colitis (23, 156, 204). Strong evidence suggests that Th17 cells are also tightly associated with the development of human and animal models of IBD (265, 266).
In addition to intestinal inflammation, IBD patients often experience secondary pathological disorders. For example, based on a recently report from The Crohn’s and Colitis Foundation of America, between 30 and 60 percent of IBD patients suffer from bone loss. The bone loss in IBD patients is at least partly due to the elevated production of pro-inflammatory cytokines and bone-resorptive cells (osteoclasts) (203, 211).

1.2 Osteo-immune system

Bone metabolism is regulated by hormone, calcium and other local factors in the bone microenvironment. Accumulated evidence suggests that the immune system also influences bone homeostasis. Many bone diseases are caused or influenced by immune factors. Additionally, the bone metabolism system and immune system share certain molecular components. Thus, the term ‘osteoinmunology’ has been proposed to describe the crosstalk between bone and the immune system (89).

Human bone is characterized by calcified hard tissue of type I collagen and highly organized deposits of calcium phosphate (190). Although it seems to be metabolically inert, adult bones constantly undergo reconstruction with newly synthesized bones replacing the old ones. This homeostasis process is defined as bone remodeling, which depends on the dynamic balance of bone formation and resorption, mediated by osteoblast and osteoclast, respectively. Imbalance of bone remodeling is often linked to metabolic bone diseases. Excess bone formation causes osteopetrosis, whereas hyper bone resorption leads to bone destruction diseases, such as osteoporosis and rheumatoid arthritis (RA). Osteoporosis is an asymptomatic systemic disease of the skeleton characterized by decreased bone mass and
loss of microarchitectural integrity (38). RA is an inflammatory joint disease characterized by synovial tissue inflammation, often leading to impairment of joint cartilage and bone (232). The bone destruction in these diseases, regardless of the involvement of inflammation, is mediated by the bone resorbing osteoclasts.

Osteoclast formation and function have a close relationship with the immune system. Osteoclasts are large, multi-nucleated cells formed by the fusion of precursor cells from macrophage-monocyte lineage. The differentiation of osteoclasts requires two cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). M-CSF is a critical proliferation and survival factor for osteoclast precursor cells as well as macrophages. Mutation of the M-CSF gene results in defects in both macrophage and osteoclast formation (260). M-CSF also stimulates RANK expression on the surface of osteoclast precursor cells, thereby enabling them to efficiently respond to RANKL, the crucial osteoclast differentiation factor. RANKL is a member of the tumor necrosis factor (TNF) superfamily. In bone microenvironment, RANKL is produced by stromal cells and osteoblasts, as a mechanism of controlling osteoclast activity. Mice with genetic ablation of RANKL or its receptor RANK, share the same phenotype with a complete block of osteoclast development and severe osteopetrosis (54, 101).

Excessive immune responses break the balance between osteoblast and osteoclast. It has been documented that infection, inflammation and autoimmune disorders are often associated with systemic or local bone loss. Recent studies place activated T cells and their products as the key positive regulators of osteoclast formation and activity (238). RANKL expressed by activated T cells may have a direct contribution (222). Inflammatory cytokines secreted by macrophages with T cell help, such as TNF-α, IL-1 and IL-6, also facilitate
osteoclastogenesis through synergy with RANKL signals (25, 206, 264). These osteolytic cytokines create a microenvironment that favors osteoclast activity and provide a link between prolonged immune response and bone destruction.

1.3 NF-κB Signaling Pathway

Immune cells communicate with their environment and fulfill their function through a variety of signal transduction events. Cells sense the environmental changes by virtue of stimulus recognition by the surface receptors. Upon binding to their ligands, the receptors deliver a specific signal that initiates downstream signaling events, ultimately leading to activation of transcription factors and induction of gene expression. Different combinations of transcription factors target unique sets of effector genes and lead to distinct cell fates. In T cells, ligation of T cell receptors in the presence of co-stimulatory signals induces the activation of several transcription factors, including NFAT, AP-1 and NF-κB, which lead to the induction of interleukin-2 (IL-2) and subsequent T cell proliferation and differentiation. However, TCR ligation without co-stimulation only activates some of these transcription factors, in particular NFAT, which programs T cell anergy (126). Thus, elucidating the mechanism that regulates the signaling pathways mediated by different immune receptors is important for understanding how immune cells respond to microbial stimuli and mediate the different phases of host responses.
1.3.1 NF-κB family transcription factors

NF-κB family transcription factors were originally identified as proteins that bound to the enhancer element of the immunoglobulin κ light chain gene in B cells (192). It is now clear that they are ubiquitously expressed and regulate a wide variety of biological processes. In the immune system, NF-κB family members are critically involved in both innate and adaptive immune cell activation. They are also required for the development and survival of lymphocytes as well as the formation of certain secondary lymphoid organs (31, 37, 91, 185). In addition, NF-κB is indispensable for RANKL mediated osteoclast development (62, 84).

Mammalian cells have five NF-κB family members, including RelA (also called p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100) (Fig. 1-1). According to structural and functional differences, the five members can be subdivided into two groups, with c-Rel, RelA, RelB in the first group and NF-κB1, NF-κB2 in the second group. Although all five members share a Rel homology domain (RHD) at the N-terminus for dimerization and DNA binding, the first group contains a trans-activation domain at the C-terminus that is absent in the second group. Additionally, c-Rel, RelA and RelB are synthesized as mature proteins, whereas NF-κB1 and NF-κB2 are transcribed in the form of precursor proteins p105 and p100, which undergo processing to generate the functional subunit p50 and p52, respectively. The processing of p105 is constitutive and co-translational, whereas the processing of p100 is inducible and mediated by a mechanism that is dependent on the ubiquitin/proteasome machineries (36, 193, 251). Functionally, NF-κB proteins activate gene transcription as hetero-dimers. Different receptors and signals tend to activate different hetero-dimers. The underlying mechanism and functional difference between individual
hetero-dimers are not clear. Interestingly, p50 and p52, but not the others, can fulfill additional function as repressors of κB transcription in the form of homo-dimers (130).

1.3.2 IκBs and NF-κB activation

In the absence of stimulus, NF-κB proteins are sequestered in the cytoplasm by the IκB family of inhibitory proteins. IκB proteins possess an N-terminal regulatory domain, followed by six or more ankyrin repeats and a PEST domain at the C-terminus. The ankyrin repeats allow IκB proteins to interact with NF-κB dimers, thereby masking the nuclear localization signal (NLS) of NF-κB (240). In mammals, there are seven IκB family members including IκBα, IκBβ, IκBγ, IκBε, Bcl3, as well as p105 and p100, the precursor protein of NF-κB1 and NF-κB2, respectively (Figure 1) (95). Among all the IκB members, IκBα is the prototypical one that has been extensively studied.

Upon stimulation, IκBα is rapidly phosphorylated at serines 32 and 36 in the N-terminus (28). This phosphorylation event triggers the conjugation of poly-ubiquitin chain primarily on lysines 21 and 22 by the E3 ubiquitin ligase β-TrCP (183, 201, 245, 258). Degradation of ubiquitinated IκBα by the 26S proteasome causes NLS exposure, as well as subsequent nuclear translocation and activation of NF-κB. It has been clearly demonstrated that phosphorylation of IκBα is a prerequisite for its degradation. Mutation of serines 32 and 36 to alanines abolishes the inducible ubiquitination of IκBα. On the contrary, mutants with glutamic acid substitution for these two serines are constitutively ubiquitinated. It is also agreed that ubiquitination is required for IκBα degradation, since mutation of the ubiquitin conjugation site, lysine 21 and 22, results in the loss of both ubiquitination and protein degradation, but not inducible phosphorylation of IκBα (183). Therefore, inducible
NF-κB activation is mediated by the sequential processes of IκBα phosphorylation and degradation.

IκBα not only mediates NF-κB sequestration in the resting state, but also plays a critical role in the feedback inhibition of NF-κB. After moving into the nucleus, NF-κB transcription factors not only activate pro-inflammatory gene transcription, but also initiate rapid re-synthesis of IκBα proteins (29, 208). Newly synthesized IκBα terminates the transcription activity of NF-κB by means of binding and exporting them from the nucleus back to the cytoplasm (6). This negative regulation loop ensures rapid but transient NF-κB activation.

Biochemical and genetic evidence suggests that different IκB proteins may exert distinct functions in NF-κB regulation. In particular, IκBα mainly engages in the regulation of c-Rel, RelA and p50, whereas, p100 specifically regulates RelB. As the precursor of p52, p100 forms a dimer with RelB. In response to cellular stimuli, p100 undergoes phosphorylation, which triggers proteosome-mediated degradation of the C-terminal IκB consensus region of p100 (251). This process, defined as p100 processing, generates functional p52/RelB dimers. Unlike the transient nuclear translocation of c-Rel, RelA and p50 mediated by IκBα degradation, the p52/RelB activation is characterized by slow but persistent kinetics. To better distinguish the two separate NF-κB pathways, they are designated as canonical and non-canonical NF-κB pathways, respectively (Fig. 1-2).
Figure 1-1. NF-κB and IκB family members
Figure 1-2. Canonical and non-canonical NF-κB pathway
1.3.3 IκB kinase holoenzyme and its activation

Since phosphorylation of IκB is the general mechanism of both canonical and non-canonical NF-κB activation, the kinase that fulfills this function, termed as IκB kinase (IKK), stands out as the essential player in the NF-κB signaling pathway. IKK was identified in 1997 as a ~300kDa holoenzyme complex composed of two catalytic subunits – IKKα and IKKβ (also referred to as IKK1 and IKK2) (50, 163, 262), and a regulatory subunit IKKγ (also called NEMO) (171, 255). Under native conditions, IKK exists in a huge protein complex with a molecular mass between 700 to 900kDa. The precise components of this super protein complex are not clear, potentially including IKK binding partners and/or IKK holoenzyme dimers or trimers.

IKKγ is a 48kDa protein with no intrinsic kinase activity. Yet its regulatory role in signal induced activation of IKK, especially IKKβ, is indispensable. IKKγ knockout mice are embryonic lethal due to massive hepatic apoptosis (172). Cells lacking IKKγ have blocked NF-κB activation (115, 172, 255). In human, mutations of this gene are associated with two diseases – Incontinentia Pigmenti (IP) and Anhidrotic Ectodermal Dysplasia with Immunodeficiency, both owing to defective NF-κB activation (51, 200). How exactly IKKγ regulates the activation of IKK holoenzyme is poorly understood. One explanation is that the existence of multiple protein-protein interaction domains enables IKKγ to be an adaptor linking upstream signaling molecules with the IKK complex (49). Alternatively, upstream signaling molecules may trigger IKKγ oligomerization, which activates IKK (83, 159). The function of IKKγ appears to be regulated by its phosphorylation. Mutation of Ser85 and Ser141 results in attenuated IKKβ activation and IκBα phosphorylation in response to
several innate immune stimuli (34, 218). However, it is not clear whether phosphorylation is required for IKKγ function or for maintaining proper structure.

The two catalytic subunits, IKKα and IKKβ, are serine/threonine kinases of 85 and 87kDa, respectively. These two proteins are highly homologous, with 50% sequence identity and over 70% sequence similarity. Considerable evidence suggests that IKK activation is phosphorylation dependent. Phosphorylation of two-conserved serine residues located in the activation loop (serines 176 and 180 for IKKα; serines 177 and 181 for IKKβ) causes conformational change that activates the kinase (95). Previous data have shown that a large amount of kinases can phosphorylate IKK in vitro, yet IKK can also be activated under over-expression conditions without stimulation. Therefore, it cannot be concluded whether this phosphorylation event is self mediated or conducted by some other kinases.

Given the fact that IKK is the central player in the NF-κB pathway, its activation must be tightly controlled for normal immune function. The mechanism of IKK negative regulation is not well characterized, but evidence indicates that auto-phosphorylation at the C-terminus is involved (47). Replacement of 10 auto-phosphorylated serines to alanines (mimic dephosphorylated state) at the C-terminus of IKKβ significantly prolonged its activation when stimulated by TNF-α. Conversely, serine to glutamic acid mutation of these 10 sites (mimic phosphorylated state) greatly reduced IKKβ activity. There are several theories to address the potential mechanism of this regulation process. C-terminal phosphorylation may change the conformation of IKKβ, leading to decreased kinase activity. Probably occurring right after IκBα phosphorylation, the progressive phosphorylation of this serine cluster may serve as a timing device that limits the duration of IKK activation. Alternatively, the confirmation change may create a docking site for some phosphatase(s)
that remove the phosphorylation in the activation loop. Phosphatase PP1 and PP2A may serve this function, since inhibition of those two phosphatases with okadaic acid results in the autophosphorylation and activation of IKK, even in unstimulated cells (50). These findings also raise the question of whether IKK is inactive or constitutively activated but equally suppressed in the unstimulated condition.

1.3.4 Distinct physiological function of IKKα and IKKβ

As the catalytic subunits of the IKK complex, both IKKα and IKKβ can phosphorylate IκB in vitro (95). Surprisingly, genetic analysis using knockout mouse models have shown that their physiological function in vivo is quite distinct. Similar to IKKγ knockout mice, IKKβ deficient animals die from TNF-α induced hepatic apoptosis due to lack of NF-κB survival signal (113, 116, 217). The embryonic lethality phenotype can be corrected by crossing IKKβ knockout mice with TNF receptor 1 deficient mice, although the double knockout mice still die within one month after birth (113). In contrast to the major function of IKKβ in TNF signaling, IKKα has indispensable role in epidermal differentiation. IKKα knockout mice display multiple developmental defects and die within 4 hours after birth, mostly due to abnormal epidermal differentiation (79, 215). The epidermis of IKKα deficient mice is five to ten times thicker than normal, which is the result of excessive proliferation and defective differentiation associated with a block in NF-κB nuclear translocation.

Consistent with functional variation, IKKα and IKKβ respond to different cellular stimuli. IKKβ can be activated by most NF-κB stimulators, including proinflammatory cytokines, lymphocyte antigen receptors and microbial components. IKKβ kinase activity is
required for inducible IkBα degradation, which mediates rapid but transient activation of canonical NF-κB pathway (113, 116, 217). Unlike IKKβ, IKKα does not respond to proinflammatory stimuli, but can be activated by a limited number of inducers, such as lymphotoxin, B cell activating factor (BAFF), CD40 ligand and RANKL (33, 37, 39, 46). Interestingly, IKKα is dispensable for IkBα degradation but is required for phosphorylation and processing of p100, the initiation step of the slow but persistent non-canonical NF-κB pathway (79, 193). In summary, canonical and non-canonical NF-κB activation rely on their specific IkB kinase IKKβ and IKKα, respectively.

1.4 Physiological and Pathological Function of NF-κB

1.4.1 NF-κB pathway in lymphocyte development and function

The physiological functions of NF-κB and its upstream signaling components have been extensively studied in mice by gene targeting approaches. The embryonic lethality resulting from deletion of the major NF-κB subunit RelA, have hampered the study of intrinsic NF-κB function in lymphocytes. Early studies using transgenic mice expressing an IkB super-suppressor (a mutant that cannot be degraded) showed that early T cell development is partially blocked. And mutant thymocytes are more apoptotic in ex vivo assays (229). A similar phenotype is seen in B lymphopoiesis with exogenous IkB super-suppressor expression (58). The proliferative defect of the IkB transgenic lymphocytes is at least partially due to the role of NF-κB in regulating the activation of signal transducer and activator of transcription 5a (STAT5a) (137), a transcription factor that is required for T cell proliferation induced by IL-2 and IL-4. The role of NF-κB in T cell function has been
further studied using mice expressing dominant negative (DN) IKKα and/or IKKβ (with kinase activity disrupted). In contrast to the IκB transgenic studies, IKKDN in T lineage cells does not affect T cell development even when the two mutants are co-expressed (167, 185). Similarly, NF-κB is also dispensable for B cell development in the bone marrow (152). The potential problem of these studies is that the lineage specific transcription of IKKDN occurs at later developmental stages, which may not reveal IKK function in early developmental events.

In contrast to the confusion in lymphocyte development, the function of NF-κB in peripheral lymphocytes is more clearly demonstrated. Canonical NF-κB is indispensable for peripheral lymphocytes maintenance. Expression of mutant IKKβ causes severe reduction of both T and B cell number (152, 185). There are two critical survival signals for B cells: the BCR signal and the BAFF signal. BCR provides a survival signal at least partly through activation of canonical NF-κB, because deletion of CARMA1, BCL-10 and MALT1, signaling molecules linking BCR to NF-κB, results in reduction in peripheral B cells (224, 252). The BAFF signal activates the non-canonical NF-κB pathway (37) and synergizes with the BCR signal in the activation of canonical NF-κB (74). Both canonical and non-canonical pathways are required for B cell maturation and survival. Functional inactivation of either branch causes partial B cell deficiency (91, 152, 193, 253), while loss of BAFF signal largely blocks the generation of mature B cells (184, 196). One of the mechanisms of BAFF function is to induce NF-κB dependent expression of anti-apoptotic proteins, such as BCL2, BCL-XL and A1 (37, 78, 179, 219). Interestingly, Sasaki et al. have shown recently that constitutive activation of canonical NF-κB through the expression of a constantly active IKKβ mutant can substitute for non-canonical NF-κB and completely replace the BAFF
signal (180), emphasizing the essential role of NF-κB in B cell maintenance. These findings further suggest the potential functional redundancy between the two branches of the NF-κB signaling pathway.

NF-κB is also important for lymphocyte activation, differentiation and subsequent effector functions. Canonical NF-κB is required for T and B cell proliferation, cytokine production and antibody responses (in B cells) (94, 114, 168). NF-κB regulates a large number of genes associated with T cell activation, including those encoding the T cell growth factor IL-2, and the alpha subunit of the IL-2 receptor (IL-2Rα) (11, 77). Induction of IL-2 and IL-2Rα initiates an autocrine loop that drives clonal expansion of activated T cells. Canonical NF-κB also regulates IFN-γ production and the Th1 response (40, 220). The function of non-canonical NF-κB in T cell activation is less well understood and appears to involve both positive and negative mechanisms (86).

Consistent with the critical role of NF-κB in lymphocyte survival and function, its aberrant activation is often the causative factor or at least connected to many inflammatory diseases and lymphoid malignancies. Constitutive IKK activation has been noted in human T-cell leukemia virus (HTLV) type-I-induced T cell leukemia and diffuse large B cell lymphoma (44, 227). As the key transcriptional regulator of pro-inflammatory cytokines, NF-κB is one of the major drug targets to treat inflammatory bowel disease (45, 59).

1.4.2 NF-κB pathway in innate immune cell function and osteoclastogenesis

The function of NF-κB in the immune system extends far beyond the regulation of lymphocyte activation. Another immunomodulatory function of NF-κB is to regulate innate immune cell activation. For example, NF-κB is a key signaling factor mediating induction
of pro-inflammatory cytokines by innate immune receptors, such as the IL-1 receptor, TNF receptor and Toll-like receptors. Additionally, NF-κB mediates induction of co-stimulatory molecules in dendritic cells, thereby regulating DC maturation and initiation of adaptive immune responses. The expression of various chemokines is also under the control of NF-κB. This function of NF-κB is attributed to its role in lymphoid organogenesis and cell trafficking involved in inflammatory responses.

In addition to its function in the immune system, NF-κB participates in various other biological processes. One such function is the regulation of osteoclast development from macrophage-monocyte precursors (9). The requirement of NF-κB for osteoclastogenesis is first implicated by the observation that mice lacking both NF-κB1 and NF-κB2 are osteopetrotic due to an osteoclast developmental defect (62, 84). Consistently, NF-κB activation is one of the early signaling events downstream of RANK. It induces the expression of a set of osteoclast specific genes, such as tartrate-resistant acid phosphatase (TRAP) and Cathepsin K. More importantly, NF-κB controls the induction of NFATc1, a master transcription factor in osteoclastogenesis (8, 85, 214). Chromatin immunoprecipitation (CHIP) analysis has shown that NF-κB is recruited to the NFATc1 promoter immediately after RANKL stimulation (8). Consistently, an NF-κB inhibitor can suppress RANKL-induced NFATc1 expression. Since the effect of the NF-κB inhibitor on osteoclastogenesis is more significant when it is administered in the early stage of development than in the late stage, NF-κB may be particularly important for early gene induction by RANKL (212).

Within the two arms of the NF-κB signaling pathway, the canonical pathway is proved to be important for osteoclast differentiation both in vitro and in vivo, as demonstrated by
using IKKβ conditional knockout mice (176). The significance of non-canonical NF-κB, however, is not as clear. There is no doubt that blocking of this signaling pathway in vitro causes osteoclast developmental arrest, but mice with either IKKα or NIK (the IKKα kinase) deficiencies have no bone phenotype unless they are challenged with osteoclastogenic stimuli (10, 148). It is possible that non-canonical NF-κB is important under pathological conditions, such as osteoporosis and rheumatoid arthritis, when excessive stimuli are present.

Bone destructive diseases are often associated with hyper NF-κB activation. The Paget’s disease of bone (PDB) is a disease of highly exaggerated bone remodeling initiated with increased osteoclast activity. The hyper osteoclast activation is strongly linked to the genetic mutation of p62, an adaptor protein positively regulating the NF-κB pathway. A truncation or point mutation in the ubiquitin-binding domain (UBA) of p62 results in enhanced NF-κB signaling with unknown mechanism (57). NF-κB also bridges inflammation with bone destruction. Elevated NF-κB activity has also been detected in synovium and lymphocytes from patients suffering rheumatoid arthritis (RA), an inflammatory joint disease largely attributed to activation of lymphocytes and osteoclasts (72, 89). These findings establish NF-κB as a potential drug target for inflammatory bone diseases. Indeed, a cell permeable peptide, which inhibits canonical NF-κB by interrupting IKKγ interaction with IKKα and IKKβ, can efficiently block osteoclast formation in vitro and inflammatory bone destruction in vivo (90).
1.5 Receptor Signaling Leading to Canonical NF-κB Activation

A variety of immune receptors utilize the NF-κB signaling pathway to fulfill their intracellular functions. Different immune receptors signal through distinct proximal components, but they all converge on IKK, the protein kinase that mediates IκBα phosphorylation and NF-κB nuclear translocation. In contrast, the molecular pathway that activates the IKK complex appears to be specific to each receptor.

1.5.1 Canonical NF-κB activation by lymphocyte antigen receptors

Extensive effort has been made to understand NF-κB activation by lymphocyte antigen receptors. T and B cell receptors are the major adaptive immune receptors to recognize foreign antigen. The T cell receptor (TCR) and B cell receptor (BCR) are multi-protein complexes composed of highly divergent antigen-binding subunits and invariant signaling chains. The antigen detection subunits of TCR on mainstream T cells includes α and β chains, which form a dimer that recognizes antigenic peptides presented by the MHC molecules on the surface of APCs (88). The antigen recognition triggers signaling events through the TCR signaling subunits, including CD3 and TCRζ chains (88). Similarly, BCR is composed of antigen recognition cell-surface immunoglobulin and invariant Igα and Igβ chains responsible for transducing signals (88). Unlike the MHC-restricted manner of TCR, BCR recognizes epitopes on intact antigens. Additionally, BCR is able to internalize the antigen, a process required for the antigen presentation function of B cells. Engagement of TCR and BCR is insufficient to activate T and B cells. As part of the lymphocyte activation receptor complex, co-stimulatory molecules are required.
The early signaling events in T and B cells share various common features, both involving the formation of immunological synapses, activation of receptor-proximal tyrosine kinases and subsequent recruitment of adaptor proteins. Immunological synapses are ordered and membrane-associated complexes, with clustered TCRs surrounded by a central signaling zone. These ‘supramolecular activation clusters’ (SMAC) can be further divided into central SMAC (cSMAC) and peripheral SMAC (pSMAC). The TCR-CD28 complex and most signaling intermediates relevant to NF-κB activation are located to cSMAC (188). Similar synaptic structures are formed after BCR engagement (12). The initial signaling event after receptor engagement is the activation of receptor complex-bound Src family protein tyrosine kinases (SFKs): Lck and Fyn in T cells and Blk, Fyn and Lyn in B cells. SFKs then phosphorylate the conserved immunoreceptor tyrosine activation motifs (ITAMs) on CD3 or Igα and Igβ chains, leading to subsequent recruitment of Syk family tyrosine kinases, ZAP70 in T cells and Syk in B cells. ZAP70 and Syk, in turn, phosphorylate several adaptor proteins, including the transmembrane adaptor LAT and LAB and the cytosolic adaptor SLP76 and SLP65 in T and B cells, respectively. The phosphorylated adaptor proteins serve as docking sites for other signaling molecules, such as the p85 regulatory subunit of phosphatidylinositol-3 kinase (PI3K) and phospholipase-Cγ (PLC-γ), and facilitate the formation of a multiprotein signaling complex (188). PLC-γ cleaves membrane-bound phosphatidylinositol-(4,5)-bisphosphate to two critical secondary messengers, inositol 1,4,5-triphosphate (IP3) and diacyl glycerol (DAG). IP3 mediates calcium mobilization and activation of the downstream transcription factor NFAT, whereas DAG activates protein kinase C (PKC). Among the 9 members of the PKC family, PKCθ and PKCβ are required for NF-κB activation in T and B cells, respectively (188).
How PKC activates IKK has remained a mystery until the discovery of an intermediate signaling complex composed of CARMA1, BCL10 and MALT1. B-cell lymphoma 10 (BCL10), as its name indicates, was first identified as a target of chromosomal translocation in certain aggressive B cell lymphomas (244). Overexpression of BCL10 results in constitutive NF-κB activation. MALT1, a distant relative of caspase, is also targeted by chromosomal translocation in mucosa-associated lymphoid tissue (MALT) lymphomas, resulting in a chimeric fusion protein that strongly activates NF-κB (125, 228). CARMA1, identified as BCL10 binding partner, is the only lymphocyte specific member of the membrane associated guanylate kinase (MAGUK) protein family (16, 63, 235). Deleting either one of the three proteins in mice impairs NF-κB activation and antigen-induced proliferation in T and B cells (56, 174) (73, 173, 175). To examine the relative signaling position of these proteins, BCL10–/– T cells were used to test the integrity of TCR signaling. TCR proximal signaling is intact in BCL10–/– T cells, with normal tyrosine phosphorylation and calcium influx when stimulated with anti-CD3 antibody. However, when the cells are treated with phorbol myristate acetate (PMA) and ionomycin, mitogens that bypass TCR and directly activate PKC and calcium signal, NF-κB activation is still defective, suggesting BCL10 as a signaling component that functions downstream of PKC (174). Interestingly, the role of Bcl10 in NF-κB activation is specific for antigen receptors, since the Bcl10 deficiency in T cells does not affect NF-κB activation by the pro-inflammatory cytokines TNF-α and IL-1 (174).

Biochemical studies suggest that within the CARMA1-BCL10-MALT1 (CBM) complex, CARMA1 and BCL10 directly interact with each other via caspase-recruitment domain (CARD) present in both CARMA1 and Bcl10 (16, 63, 235). On the other hand,
MALT1 is recruited to the complex through BCL10 (175). CARMA1 is constantly associated with the lipid raft. Upon TCR stimulation, CARMA1 is phosphorylated by PKC0 and undergoes oligomerization, which in turn triggers subsequent biochemical events including recruitment of the BCL10-MALT1 and IKK complexes to the lipid raft (188). Within the lipid raft, BCL10-MALT1 induces Lys-63-linked polyubiquitination of IKKγ in an ubiquitin-conjugating enzyme 13 (UBC13)-dependent manner (267). The E3 ubiquitin ligase TRAF6 also may be involved in the IKKγ ubiquitination induced by Bcl10-MALT1 (207). However, T cells deficient in TRAF6 can still activate NF-κB, albeit with delayed kinetics (98). It is likely that TRAF2 has a redundant role with TRAF6 in TCR signaling, as RNAi knockdown of both TRAFs in Jurkat T cells led to more profound NF-κB defect than the loss of either one alone (207). A discrepancy exists in the previous data for the mechanism of MALT1-dependent NF-κB activation (207, 267). A recent publication by Oeckinghaus et al. provides a new explanation (149). They found MALT1 is K63 poly-ubiquitinated by associated TRAF6 in response to TCR signal. The ubiquitin chains of MALT1 provide a docking surface for the recruitment of IKKγ, which is also K63 poly-ubiquitinated and thereby activated by TRAF6.

Despite the uncertainties regarding the mechanism of IKKγ ubiquitination, this molecular event appears to be essential for IKK activation. Emerging evidence suggests that ubiquitination of IKKγ and its upstream signaling components serve as a dynamic mechanism to recruit IKK to its activating kinase TAK1. Transforming growth factor (TGF)-β-activated kinase 1 (TAK1) was first characterized in TGF-β signaling (254), and was subsequently found broadly involved in both innate and adaptive immune signaling pathways. The TAK1 complex contains the kinase TAK1 and its binding proteins TAB1,
TAB2 and TAB3 (2). As a unique member of MAP3K family, TAK1 needs the TABs for its kinase activity. Among the TABs, only TAB1 can directly activate TAK1 \textit{in vitro}, while TAB2 and TAB3 appear to serve as an ubiquitin-binding linker bringing TAK1 to other signaling molecules. TAK1 activation requires poly-ubiquitination by TRAF6 and Ubc13/Uev1A, which act as E3 and E2, respectively. The ubiquitination activated TAK1 complex then phosphorylates IKK\(\beta\) at serine 177 and 181 in the activation loop, leading to IKK activation (234). In addition to activation of IKK, TAK1 also activates MKK3 and MKK6, thereby triggering the JNK and p38 signaling pathways involved in activation of the transcription factor AP-1. The importance of TAK1 in lymphocyte activation has been demonstrated using T or B lineage conditional knockout mice. T cell specific TAK1 deficient mice have a severe defect in thymocyte development and activation, coupled with a block of both NF-\(\kappa\)B and JNK signaling pathways (120, 182, 233). In effector T cells, however, I\(\kappa\)B\(\alpha\) degradation in response to TCR ligation is minimally affected in the absence of TAK1 (233). The signaling function of TAK1 in B cells is receptor dependent. TAK1 is required for activation of both NF-\(\kappa\)B and MAPKs by innate immune receptors, including IL-1\(\beta\) receptor, TNF-\(\alpha\) receptor and Toll-like receptor, but is only responsible for JNK activation in the BCR signaling pathway (181). The lymphocyte antigen receptor signaling pathway leading to canonical NF-\(\kappa\)B activation is summarized in Fig. 1-3, using TCR signaling as a representative pathway.

1.5.2 Canonical NF-\(\kappa\)B activation by RANK

RANKL, a type II homotrimeric transmembrane protein belonging to the TNF family, provides the major signal for osteoclast differentiation. Activation of its receptor RANK, a
type I homotrimeric transmembrane protein, leads to osteoclast specific gene expression (24). The RANK signal activates five major downstream pathways: NF-κB, MAPKs (Jnk, Erk, p38) and Akt. The RANK signal stimulates transcription factor AP-1 through MAPK activation. Similar to other TNF receptor family members, RANK lacks catalytic activity in the intracellular signaling domain, and interacts with TNF receptor associated factors (TRAFs) that act as adaptors to downstream signaling cascades (9). TRAFs are a family of adaptor proteins evolutionally conserved from C. elegans to mammals (231). Among all the 7 TRAF members, only TRAF6 is genetically proven to be a critical molecule in RANK signaling and osteoclastogenesis. Loss of TRAF6 causes osteopetrosis in mice owing to defective osteoclast development (123, 142). TRAF6 contains a C-terminal TRAF domain, essential for homo- and heterotrimerization of TRAF molecules, as well as interaction with receptors and other signaling molecules (110). The N-terminus of TRAF6 is a RING finger domain followed by a zinc finger repeat domain. The N-terminus of TRAF6 is essential for the activation of downstream signaling cascades (248). RANKL and RANK association triggers the trimerization of RANK followed by recruitment of TRAF6. The interaction with RANK induces TRAF6 trimerization and poly-ubiquitination at lysine 63, which is self-mediated through the RING finger domain as an ubiquitin ligase in the presence of ubiquitin conjugating enzyme Ubc13/Uev1A (104, 105). Mutation analysis revealed that intact TRAF6 ubiquitination is needed for RANKL-mediated osteoclastogenesis (105).

There are three major signaling cascades downstream of TRAF6, the central molecule in RANK signaling. First, TRAF6 facilitates c-Src function in the Akt pathway (246). C-Src, a member of Src family tyrosine kinases (SFKs), interacts with TRAF6 and RANK upon receptor engagement. TRAF6 subsequently enhances the kinase activity of c-Src,
leading to phosphorylation of adaptor protein Cbl. Phosphorylated Cbl then recruits PI3Kinase to the receptor complex and mediates activation of Akt (7). Second, TRAF6 mediates canonical NF-κB activation through the adaptor protein p62 (55). P62 is identified as an atypical PKC-interacting protein and has been suggested to be involved in NF-κB activation downstream of IL-1 and TNFR pathways (140). As a scaffolding protein, p62 contains multiple protein interaction domains starting from the N terminus, including a PB1 domain that binds to atypical PKCs, a ZZ zinc finger domain that binds to RIP, the critical adaptor protein in TNFR pathway, and a short stretch of amino acids that binds to TRAF6. There is also an ubiquitin association (UBA) domain located at the C-terminus of p62 (141).

The involvement of p62 in osteoclastogenesis was first implicated from the discovery that a truncation or point mutation of p62 is genetically linked to the Paget’s disease of bone, an osteolytic disease resulting from hyper activated osteoclasts (57). Genetic deletion of p62 in mice revealed that this protein is dispensable for basal bone remodeling but is required for hormone-induced osteoclastogenesis, indicating the potential role of p62 in pathological bone loss (55). Full-length p62 participates in TRAF6 mediated canonical NF-κB activation but the mechanism is not well characterized. One possibility is that p62 brings the atypical PKCs and TRAF6-IKK complex together, enabling atypical PKC to phosphorylate the IKK activation loop, as suggested in some other signaling pathways (140). Alternatively, p62 may provide a docking site for the ubiquitinated signaling molecules through its protein interaction domains and UBA domain in an atypical PKC independent mechanism (140). Interestingly, a mutation in the ubiquitin-binding domain produces gain-of-function mutants. The UBA mutants of p62 strongly activate NF-κB, which is directly associated with PDB etiology. The mechanism of this gain of function is not fully understood. One study
suggested that the UBA domain targets TRAF6 to the proteasomal compartment (259). I will discuss this potential explanation further in chapter 4. Third, TRAF6 controls AP-1 activity and NF-κB transactivation through the TAK1 complex (80, 135). As in the antigen receptor signaling, TAK1 functions downstream of TRAF6 in the RANK pathway. However, TAK1 does not regulate IκBα degradation and NF-κB DNA binding in this case. Instead, TAK1 stimulates the MKK6-p38 pathway, which activates transcription factor AP-1 and contributes to NF-κB transactivation by phosphorylating p65 on Serine 536 (80). The canonical NF-kB pathway downstream of RANK is summarized in Fig. 1-4.
Figure 1-3. Canonical NF-κB pathway downstream of TCR
Figure 1-4. NF-κB pathway downstream of RANK
1.6 Ubiquitin Regulation of Canonical NF-κB

1.6.1 Ubiquitination

Covalent tagging of ubiquitin molecules onto target proteins, a process known as ubiquitination, is a fundamental mechanism that regulates diverse biological processes, including cell cycle progression, apoptosis, DNA repair, and immune responses (75, 122, 154). Ubiquitin is a highly conserved and ubiquitously expressed 76 amino-acid protein. Its conjugation to the target protein is mediated by the cooperative action of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). Eukaryotic cells contain a single E1, multiple E2s (about 25 in mammals), and considerably more E3s. E3s mediate specific binding to substrate proteins, thus determining the substrate specificity in a ubiquitin reaction. E3s can be subdivided into three groups: HECT (homologous to E6-AP COOH-terminus), RING and Ufd2-related (U-box) E3s, distinguished by their functional domains (13). The ubiquitin conjugation by E3 could be either mono- or poly-meric, each facilitating specific biological functions. Monoubiquitination mainly serves as a sorting signal for intracellular protein trafficking and endocytosis (71, 76) but also mediates other cellular processes, such as DNA repair, DNA replication and gene transcription (198). Polyubiquitination is traditionally thought to mediate proteasomal degradation, but it is also known to mediate various non-degradative functions, including signal transduction.
1.6.2 Regulation of NF-κB activation by ubiquitination

Polyubiquitin chains are formed through isopeptide bonds between the C-terminal glycine residue of one ubiquitin and a lysine residue of another. According to the position the lysine residue is conjugated, usually on position 48 or 63, polyubiquitination can be classified as K48-linked polyubiquitination and K63-linked polyubiquitination. K48 polyubiquitination regulates signal transduction by controlling the abundance of signal regulatory proteins via a proteasomal dependent mode. K48 ubiquitination negatively regulates antigen receptor signaling by targeting the receptor and multiple downstream effector molecules, such as Lck, Lyn, ZAP70, Syk, for proteasomal degradation (122). At the same time, K48 ubiquitination-mediated degradation of IκB proteins also activates NF-κB transcription factors (35). K63 ubiquitination is proteolysis-independent and mostly stimulates signal transduction, including NF-κB activation. Activation of NF-κB by the TCR involves K63-ubiquitination events, which appear to mediate the recruitment of Tak1 and IKK complexes to the CARMA1-BCL10-MALT1 signaling complex and the subsequent activation of Tak1 and IKK. The discovery that the E2 complex Ubc13/Uev1A is a binding partner of TRAF6 indicates that the latter may function as an E3 (48). Indeed, TRAF6 contains a N-terminal RING domain found in a family of E3s. Biochemical studies confirmed TRAF6 is an ubiquitin ligase functioning together with Ubc13/Uev1A to conjugate K63 polyubiquitin chains on its targets, including TRAF6 itself (48). The E3 activity and auto-ubiquitination are important for TRAF6 to activate NF-κB, since a single mutation on its auto-ubiquitination site abolishes RANKL induced NF-κB activation and osteoclastogenesis (104, 105). Auto-ubiquitination of TRAF6 is triggered by its oligomerization, which is mediated by the upstream CARMA1-BCL10-MALT1 (CBM)
complex in the presence of Ubc13/Uev1A (207). Additionally, MALT1 is ubiquitinated by TRAF6 upon TCR stimulation. Polyubiquitin chains on MALT1 provide a docking surface for the recruitment of IKKγ into the CBM-TRAF6 cascade, leading to subsequent polyubiquitination and activation of IKK by TRAF6 (149). IKKγ, in this case, acts as an ubiquitin receptor binding preferentially to K63 polyubiquitin chains as previously reported (272, 273). The IKKγ ubiquitin-binding domain has previous been shown to mediate trimerization (3), the prerequisite for IKKγ ubiquitination. Mutations in this domain impair IKK activation, but affect IKKγ as both ubiquitin receptor and ubiquitin target (2). Further studies need to be done to determine the individual role of IKKγ ubiquitin binding and ubiquitination in IKK activation. Similarly, the function of TAK1, the IKKβ kinase, is also ubiquitination dependent. The TAK1 complex is composed of the kinase TAK1 and two adaptor proteins TAB1 and TAB2. Although TAB1 can activate TAK1 in vitro (177), it is TAB2 that is required for TAK1 activation by TRAF6 (234). TAB2 contains a highly conserved C-terminal zinc-finger domain that binds preferentially to K63 polyubiquitin chains. Mutations in this domain of TAB2 that impair poly-ubiquitin binding abolish the ability of TAB2 to activate TAK1 and IKK. Substitution of the zinc-finger domain with a heterologous ubiquitin-binding domain restores TAB2 function in TAK1 and IKK activation. Therefore, it is likely that TAB2 activates TAK1 and IKK by binding to the K63 ubiquitin chains of TRAF6 (93). Little is known about how binding to polyubiquitinated TRAF6 activates TAK1, but it is clear that TRAF6 ubiquitination is required for TAK1 activation (234).
1.7 Deubiquitinating Enzymes

As predicted from its critical roles in regulating diverse cellular events, ubiquitination is a reversible and tightly regulated event. A subclass of proteases has been identified that reverses the protein ubiquitination process by cleaving the isopeptide linkages of poly-ubiquitins. These proteases are termed deubiquitinating enzymes (DUBs) according to their functional relevance. Although nearly 100 DUBs have been identified in the human genome, they are far less understood than the ubiquitin ligases in terms of functional mechanism and target specificity (146).

DUBs can be divided on the basis of sequence homology into five distinct subgroups: ubiquitin C-terminal hydrolases (UCH), ubiquitin-specific proteases (USP/UBP), Machado-Joseph Domain (MJD), ovarian tumor related (OTU), and the JAB1/MPN/Mov34 metalloenzyme (JAMM) motif DUBs. Among the subgroups, most of DUBs belong to the cysteine proteases except JAMM motif DUBs, which are Zn$^{2+}$-containing metallo proteases. The structure of different subgroups of DUBs reveal significant diversity in the catalytic domain, suggesting the functional specificity of individual subgroup. Genome-wide analysis indicated that the human genome encodes approximately 95 DUBs, including 58 USP, 4 UCH, 5 MJD, 14 OTU and 14 JAMM domain-containing genes, 79 of which are functional (146). It is noticeable that the majority of DUBs belong to the USP type. Computational analysis revealed that the numbers of this DUB population, as well as the E3 ubiquitin ligases, which determine substrate specificity, are both increased during evolution, implicating potential functional relevance between these two protein populations (191). The following section will focus one of the USP family of DUBs, CYLD.
CYLD is a DUB of the cysteine protease family that was first identified as a tumor suppressor mutated in familiar cylindromatosis, benign tumors of skin appendages (18). The cylindromatosis patients carry germ-line mutations in one CYLD allele, and the other allele is lost in the tumor cells. The tight association of cylindroma formation with loss of heterozygosity in the CYLD gene establishes CYLD as a tumor suppressor.

The CYLD gene encodes a protein of 956 amino acids. Sequence analysis revealed four recognizable sequence motifs in the CYLD protein (Fig. 1-5) (18). The N-terminus of CYLD contains three CAP-GLY domains and two proline rich domains, which has been reported to mediate microtubule association and protein-protein interactions, respectively. There are two short sequence motifs homologous to the cysteine and histidine boxes of the USP type of DUBs at the C-terminus of the CYLD protein. In addition, four Cys-X-X-Cys motifs reside at the C-terminus, possibly forming the metal-binding finger-like domains. CYLD protein is evolutionarily conserved from *C. elegans* to human, especially at the C-terminus cysteine and histidine boxes (18, 102). Recombinant CYLD exhibits DUB activity capable of digesting poly-ubiquitin chains *in vitro* (30), and the DUB activity of CYLD requires intact cysteine and histidine boxes (DUB domain) (30). Interestingly, most of CYLD mutations identified in cylindromatosis patients fall in the C-terminus of the protein involving the DUB domain (18), suggesting the functional importance of CYLD DUB activity.

Although CYLD is clearly demonstrated as a tumor suppressor, its biological function is largely unknown. Yeast two-hybrid and transfection studies suggest that CYLD
physically interacts with the TRAF2 and IKKγ (30, 102, 226), the regulatory subunit of the IKK complex. Transfected CYLD suppresses the constitutive ubiquitination of overexpressed TRAF2, TRAF6 and IKKγ, and inhibits NF-κB activation downstream of TNF receptor and Toll-like receptor 4, two important receptors for innate immunity (30, 102, 226).

It is difficult to unravel the physiological function of CYLD using an in vitro system, therefore, our lab generated CYLD knockout mice as a model system to study CYLD function especially in the immune system (166). The functions of CYLD in lymphocyte activation and osteoclastogenesis form the basis of in this thesis.

![Figure 1-5. CYLD domain structure](image)

Figure 1-5. CYLD domain structure
CHAPTER II

DEUBIQUITINATING ENZYME CYLD NEGATIVELY REGULATES THE UBIQUITIN-DEPENDENT KINASE TAK1 AND PREVENTS ABNORMAL T-CELL RESPONSES

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ABSTRACT

Deubiquitinating enzyme CYLD has recently been implicated in the regulation of signal transduction, but its physiological function and mechanism of action are still elusive. Here we show that CYLD plays a pivotal role in regulating T-cell activation and homeostasis. T cells derived from Cyld knockout mice display hyper-responsive phenotype and mediate spontaneous development of intestinal inflammation. Interestingly, CYLD targets a ubiquitin-dependent kinase, Tak1, and inhibits its ubiquitination and autoactivation. Cyld-deficient T cells exhibit constitutive activity of Tak1 and its downstream kinases, JNK and IKKβ. These results emphasize a critical role for CYLD in preventing spontaneous activation of the Tak1 axis of T-cell signaling and thereby maintaining normal T-cell function.
INTRODUCTION

T cells serve as a central component of the adaptive immune system. Defect in T-cell activation results in severe immune deficiencies, whereas deregulated T-cell activation is associated with chronic inflammations and autoimmunity (197). Thus, the process of T-cell activation is subject to tight regulation by positive and negative mechanisms. One recently discovered mechanism of T-cell regulation is ubiquitination, which plays an important role in both the development and activation of T cells (121). Protein ubiquitination is a reversible process that is counter-regulated by ubiquitin conjugating enzymes and deubiquitinating enzymes (DUB) (241). While the ubiquitin conjugating enzymes have been extensively studied, little is known about how the DUBs participate in immune regulation, particularly in the regulation of T-cell function. We have recently shown that a DUB, CYLD, regulates TCR proximal signaling in thymocytes and is required for thymocyte development (166), although its role in peripheral T-cell function is enigmatic.

CYLD was originally identified as a tumor suppressor that is mutated in familial cylindromatosis (18). Since patients carry heterozygous mutation of the Cyld gene with loss of heterozygosity occurring only in tumor cells, the physiological function of CYLD was not resolved by the patient studies. The DUB function of CYLD was first revealed by in vitro work showing that CYLD inhibits the ubiquitination of certain tumor necrosis factor receptor-associated factors (TRAFs) and the regulatory subunit of IκB kinase (IKKγ) (30, 102, 226). When transfected in cell lines, CYLD inhibits the activation of NF-κB and MAP kinases (MAPK) stimulated by innate immune receptors, such as the toll-like receptors (TLRs) and tumor necrosis factor receptors (TNFRs) (30, 102, 162, 164, 226, 261).
However, recent studies using Cyld knockout (Cyld<sup>−/−</sup>) mice suggest that the signaling function of CYLD is complex and may vary among different cell types and stimulation conditions (129, 166, 263). As such, it is still not clear how CYLD regulates NF-κB and other signaling pathways under physiological conditions.

NF-κB represents a family of transcription factors that plays a central role in regulating the activation and homeostasis of T cells (118). The NF-κB factors are normally sequestered in the cytoplasm through physical interaction with inhibitory proteins, predominantly IκBα (68). Activation of NF-κB by diverse immunologic stimuli typically involves degradation of IκBα, which in turn is triggered through IκBα phosphorylation by the IκB kinase (IKK) (68). IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit termed IKKγ or NEMO. IKKβ plays the primary role in mediating canonical pathway of NF-κB activation, whereas IKKα regulates an alternative pathway involving processing of the NF-κB2 precursor protein p100 (68, 193, 251).

Crosslinking of the TCR and CD28 costimulatory molecule in T cells leads to activation of the canonical NF-κB pathway; this signaling cascade involves several intermediate molecules, including CARMA1, Bcl10, and MALT1 (118). Recent genetic evidence suggests that IKK activation by the TCR signal requires TGF-beta-activated kinase 1 (Tak1) (120, 182, 233), a ubiquitin-dependent kinase (234) known to phosphorylate and activate IKKβ (147). In addition to activating IKK, Tak1 also mediates TCR-stimulated activation of c-Jun N-terminal kinase (JNK) (120, 182, 233). These findings suggest that Tak1 is a master kinase that mediates activation of both IKK and JNK, although how Tak1 is regulated in T cells remains elusive. We show in the present study that CYLD physically interacts with Tak1 and inhibits its ubiquitination and catalytic activity. The loss of CYLD
in T cells results in constitutive activation of Tak1 and its downstream kinases, JNK and IKKβ. Consistently, the Cyld<sup>−/−</sup> T cells display a hyper-responsive phenotype and mediate spontaneous development of intestinal inflammation in Cyld<sup>−/−</sup> mice. These findings establish CYLD as a specific DUB that prevents spontaneous activation of the Tak1 axis of TCR signaling and thereby maintains normal T-cell responses.
RESULTS

CYLD-deficient T cells are hyper-responsive to TCR stimulation in vitro

To examine how CYLD regulates peripheral T-cell activation, we began by analyzing the activation of Cyld−/− T cells in vitro. Interestingly, the loss of CYLD resulted in hyper-proliferation of lymph node T cells when stimulated with agonistic anti-CD3 and anti-CD28 antibodies (Fig. 2-1A). Compared to the wildtype T cells, the Cyld−/− T cells also produced markedly larger amounts of cytokines, including interferon-γ (IFN-γ) (Fig. 2-1B), and IL-2 (Fig. 2-1C). Similar results were obtained with splenic T cells (Supplementary Fig. 2-1).

To determine whether the hyper-responsive phenotype of Cyld−/− T cells occurred in naïve or memory population, we purified these cells by flow cytometric cell sorting. Remarkably, the loss of CYLD resulted in hyper-proliferation and aberrant cytokine production in both naïve and memory T cells (Fig. 2-1D and E). These results suggest a pivotal role for CYLD in negatively regulating peripheral T-cell activation.

Cyld−/− mice spontaneously develop autoimmune symptoms and colonic inflammation

Since abnormal T-cell responses are often associated with chronic inflammations and autoimmunity (197), we examined whether Cyld−/− mice develop immunological abnormalities. Even at early ages (8 week), the Cyld−/− mice exhibited prominent lymphocyte infiltration into the peri-portal region of the liver (Fig. 2-2A). More prominently, the colons of the Cyld−/− mice contained markedly more and larger lymphoid follicles or colonic patches (Fig. 2-2B, lower, arrows). Colonic patch hypertrophy is a hallmark of hapten-induced colitis and implicated in the development of inflammation in
human inflammatory bowel disease (IBD) (52). Of note, the lymphocytes from the colonic patches often breached into the mucosal layer and were associated with crypt damage and focal architectural distortion (Fig. 2-2B, lower, arrowhead). Moreover, as seen in animal models of IBD and human IBD, inflammatory cell infiltration was also detected in the lamina propria of the colon in areas that lack the colonic patches (Fig. 2-2C, arrow 1). Crypt damage (Fig. 2-2C, arrow 2) and thickening of the muscularis layer (Fig. 2-2C, arrow 3) were also readily detected in these inflamed areas. Consistent with the involvement of CD4 T cells in IBD, we detected abundant infiltrating CD4 T cells in both the colonic patches and the inflamed mucosa (Fig. 2-2D). These IBD-like pathological phenotypes were not detected in the control mice (Fig. 2A-D). Indeed, blind analyses of the colonic sections revealed that the Cyld–/– mice had significantly higher IBD histological scores than the background scores of their wildtype littermates (Fig. 2-2E).

A molecular hallmark of IBD is the expression of proinflammatory mediators in the colon, which contributes to the inflammation and destruction of the colonic tissue (178). To further examine the similarity between the intestinal inflammation of Cyld–/– mice and IBD, we analyzed the cytokine gene expression in the colons of Cyld–/– and wildtype mice. The constitutive expression of a number of proinflammatory genes was detected in the colons of Cyld–/– mice but not in the colons of wildtype mice (Fig. 2-2F). The Cyld–/– mice also expressed larger amounts of T-cell derived cytokines, including IL-4, IL-12 (Fig. 2-2F). The level of an anti-inflammatory cytokine, IL-10, was moderately enhanced in the Cyld–/– colon (Supplementary Fig. 2-2A). Elevated expression of IL-10 has also been detected in other models of murine colitis as well as human IBD patients (27, 132, 144), although the underlying functional significance in IBD pathogenesis is unclear. Nevertheless, our results
suggest that the intestinal inflammation in Cyld–/– mice is associated with aberrant expression of proinflammatory cytokines. In addition to the histological and biochemical features of IBD, the Cyld–/– mice had significant weight loss (Fig. 2-2G), a characteristic clinical symptom of IBD patients (156). Of note, these results were obtained with mice housed under pathogen-free conditions with wildtype littermates being used as controls. Thus, the Cyld–/– mice develop spontaneous colonic inflammation and extra-intestinal abnormalities that resemble IBD.

By analyzing human genome sequence, we found that human Cyld gene is located in a major IBD-susceptibility locus (IBD-1) on chromosome 16 adjacent to a known IBD-regulatory gene, Nod2 (Fig. 2-2h). This finding suggests an intriguing possibility that CYLD may also be involved in regulation of human IBD.

**Adoptive transfer of Cyld−/− T cells induces autoimmune symptoms and colonic inflammation in recipient mice**

Given the hyper-responsive phenotype of Cyld−/− T cells, it was important to examine whether these mutant T cells contribute to the development of IBD-like symptoms. We adoptively transferred T cells from either Cyld−/− or wildtype mice into RAG1−/− mice that lack endogenous lymphocytes. The RAG1−/− recipients of both Cyld−/− and wildtype T cells had efficient T-cell repopulation in the spleen six weeks following the adoptive transfer (Fig. 2-3A). However, the RAG1−/− recipients of Cyld−/− T cells, but not those of wildtype T cells, displayed lymphocyte infiltration into the peri-portal regions of the liver (Fig. 2-3B). Moreover, the RAG1−/− recipients of Cyld−/− T cells spontaneously developed colitis, characterized by inflammatory cell infiltration, thickening of the mucosa, and goblet cell
depletion (Fig. 2-3C). The IBD histology scores of these mice were markedly higher than the background detected in recipients of wildtype T cells (Fig. 2-3D). In addition to these histological features, the recipients of \textit{Cyld}^{−/−} T cells had significant weight loss (Fig. 2-3E) and expressed high levels of proinflammatory cytokines in the colon (Supplementary Fig. 2-2B). Severe bowel thickening was also observed in some of the recipients of \textit{Cyld}^{−/−} T cells but not in the controls (Fig. 2-3F). These findings demonstrate that the T cells from CYLD knockout mice are sufficient to induce IBD-like features.

**Constitutive activation of JNK and NF-κB in \textit{Cyld}^{−/−} T cells**

To understand the molecular mechanism mediating the abnormal T-cell responses in \textit{Cyld}^{−/−} mice, we examined the TCR signaling. We have previously shown that CYLD positively regulates LCK function and TCR-proximal signaling in thymocytes (166). However, since LCK is largely dispensable for peripheral T-cell activation (136), it was intriguing to examine whether the loss of CYLD affected TCR-proximal signaling of peripheral T cells. In naïve T cells, the CYLD deficiency had no significant effect on TCR/CD28-mediated phosphorylation of ZAP-70 and the MAPK ERK (Fig. 2-4A). Interestingly, in the \textit{Cyld}^{−/−} T cells, JNK became constitutively activated (Fig. 2-4A, lane 4). The constitutive activation of JNK was also detected using \textit{Cyld}^{−/−} total T cells. Thus, in peripheral T cells, CYLD is not required for TCR-proximal signaling but serves as a crucial negative regulator of JNK.

To further investigate the signaling basis for the hyper-responsive property of \textit{Cyld}^{−/−} T cells, we examined the activation of transcription factor NF-κB. As expected, crosslinking of TCR/CD28 led to the activation of nuclear DNA-binding activity of NF-κB (Fig. 2-4B).
Remarkably, as seen with JNK, high levels of active NF-κB were detected in Cyld−/− T cells even under unstimulated conditions (Fig. 2-4B, lane 5). Of note, the constitutive activation of NF-κB occurred in both the naïve and memory Cyld−/− T cells (Fig. 2-4C), further arguing for an intrinsic role for this DUB in negatively regulating the JNK and NF-κB signaling axes in T cells. Antibody supershift assays revealed that the constitutively activated NF-κB complex in Cyld−/− T cells was predominantly composed of the canonical NF-κB members, p50 and RelA (Fig. 2-4D). Parallel IB assays revealed markedly enhanced expression of non-canonical NF-κB members, RelB and p100, at the levels of both protein (Fig. 2-4E) and RNA (Fig. 2-4F). Since the genes encoding these two proteins are regulated by NF-κB (26, 124), this result further supported the conclusion that CYLD deficiency causes constitutive activation of NF-κB. We noticed that p100 remained largely unprocessed (not converted to p52, Fig. 2-4E) despite its upregulation. Since p100 is a specific inhibitor of RelB, this finding explained why RelB was not detected in the active nuclear NF-κB complex (Fig. 2-4D). Thus, the loss of CYLD is associated with constitutive activation of canonical NF-κB and JNK, a result that provides a mechanistic insight into the hyper-responsive phenotype of Cyld−/− T cells.

**Constitutive activation of IKKβ in Cyld−/− T cells and Cyld-knockdown Jurkat T cells**

To understand the mechanism of constitutive NF-κB activation associated with the CYLD deficiency, we examined the level and fate of the primary NF-κB inhibitor IκBα. In Cyld−/− T cells, the steady state level of IκBα was significantly lower than in wildtype T cells (Fig. 2-5A, top panel). This abnormality was not due to reduced expression of the IκBα gene. In fact, the Cyld−/− T cells expressed a markedly higher level of IκBα mRNA
than the wildtype T cells (Fig. 2-5B). Interestingly, despite their lower level of total IκBα protein, the mutant T cells had more phosphorylated IκBα (Fig. 2-5A, middle panel). These findings suggested that IκBα might be undergoing chronic degradation and resynthesis in the CYLD-deficient T cells. Indeed, inhibition of protein synthesis by cycloheximide (CHX) led to rapid depletion of IκBα in Cyld−/− T cells but only had a weak effect on the IκBα level in wildtype T cells (Fig. 2-5C, compare lanes 2 and 4). The chronic degradation of IκBα appeared to be dependent on IKKβ, since this event was blocked by a selective IKKβ inhibitor, PS1145 (Fig. 2-5C, lane 7). Consistent with these results, the Cyld−/− T cells displayed a high level of constitutive IKKβ catalytic activity (Fig. 2-5D, top panel, lanes 1 and 2). Thus, the loss of CYLD results in constitutive activation of the NF-κB signaling pathway in T cells.

To determine whether the constitutive activation of IKKβ occurred in naïve Cyld−/− T cells, we began by analyzing the IKKβ kinase activity in thymocytes. As seen with peripheral T cells, Cyld−/− thymocytes exhibited constitutive IKKβ activity (Fig. 2-5D). We next purified naïve peripheral T cells by flow cytometric cell sorting. Since the low number of highly purified naïve T cells precluded us from performing in vitro kinase assays, we attempted to detect in vivo phosphorylation of IKKβ based on its band shift on SDS gels. Indeed, when fractionated on a low-percentage gel, IKKβ exhibited a clear band shift in Cyld−/−, but not control, naïve T cells (Fig. 2-5E). Consistently, the naïve T cells had a reduced level of IκBα and an upregulated level of RelB and p100 (Fig. 2-5E). Thus, IKKβ is chronically activated in naïve T cells of Cyld−/− mice.

We next utilized an alternative approach to confirm that loss of CYLD in T cells causes constitutive activation of IKKβ. By infecting Jurkat T cells with a retroviral vector
encoding a *Cyld*-specific small hairpin RNA (shCYLD), we were able to generate a bulk of cells with greatly reduced level of CYLD (Fig. 2-5F, bottom panel). As seen with the primary T cells, the Cyld-knockdown Jurkat cells displayed hyper-activation of IKKβ (Fig. 2-5F, top panel), and this abnormality was associated with the reduction in IκBα protein level (Fig. 2-5F, panel 3). Taken together, these results suggest that CYLD plays a crucial role in preventing spontaneous activation of IKKβ or its upstream signaling steps.

**CYLD controls the activity of Tak1**

The involvement of CYLD in the regulation of both JNK and IKKβ suggests a role for this DUB in controlling the activity of a more upstream molecule. However, since the CYLD deficiency did not cause activation of ERK, the target of CYLD might be an intermediate signaling factor specifically mediating the activation of JNK and IKKβ. In this regard, recent genetic studies highlighted a critical role for Tak1 in mediating TCR-stimulated activation of IKK and JNK (120, 182, 233). We therefore examined whether Tak1 is under the control of CYLD. Indeed, high constitutive activity of Tak1 was detected in both peripheral T cells and thymocytes of *Cylḍ*+ mice (Fig. 2-6A). Moreover, RNAi-mediated *Cyld* knockdown in Jurkat T cells also resulted in constitutive activation of Tak1 (Fig. 2-6A). These results establish a pivotal role for CYLD in controlling Tak1 function and explain how CYLD negatively regulates IKKβ and JNK.

Prior *in vitro* studies suggest that Tak1 is a ubiquitin-dependent kinase (234). Although how the catalytic activity of Tak1 is regulated *in vivo* is not well understood, a recent study suggests that polyubiquitination of Tak1 mediates its autoactivation (223). It was thus logical to examine whether CYLD targets Tak1 and regulates its ubiquitination.
Interestingly, endogenous CYLD and Tak1 indeed formed a complex in T cells, which was readily detected by coIP assays using the anti-CYLD antibody (Fig. 2-6B). This molecular interaction was specific, since Tak1 was not precipitated by anti-CYLD from the Cyld−/− T cells (Fig. 2-6B, lane 2). Specific CYLD/Tak1 interaction was also readily detected in transfected cells (Fig. 2-6C). To determine whether Tak1 is a functional target of CYLD, we analyzed the effect of CYLD on Tak1 ubiquitination. As expected (223), transfected Tak1 underwent constitutive polyubiquitination (Fig. 2-6D). Importantly, the ubiquitination of Tak1 was efficiently inhibited by CYLD. Furthermore, consistent with the constitutive Tak1 activation in Cyld−/− T cells, transfected CYLD potently suppressed the catalytic activity of Tak1 (Fig. 2-6E).

To further assess the role of CYLD in regulating Tak1 ubiquitination, we analyzed the ubiquitination of endogenous Tak1. Remarkably, the loss of CYLD resulted in significant elevation of Tak1 ubiquitination in T cells (Fig. 2-6F), thus providing evidence for the involvement of CYLD in modulating Tak1 ubiquitination in vivo. We also analyzed the effect of Cyld deficiency on the ubiquitination of IKKγ, since this molecular event can be inhibited by transfected CYLD (30, 102, 226). A significant basal level of IKKγ ubiquitination was detected in wildtype T cells, which was moderately enhanced in Cyld−/− T cells (Fig. 2-6F). Thus, Tak1 appears to be a primary target of CYLD in T cells, although the involvement of CYLD in regulating other signaling components of the NF-κB pathway cannot be excluded.
DISCUSSION

In this study, we demonstrate a critical role for CYLD in controlling the catalytic activity of Tak1 and its downstream kinases in T cells. This function of CYLD was demonstrated using different cell systems: total T cells, naïve T cells, and Cyld knockdown Jurkat T cells. Consistently, the Cyld<sup>−/−</sup> T cells are hyper-responsive to TCR/CD28 stimulation and the Cyld<sup>−/−</sup> mice spontaneously develop intestinal inflammation. Furthermore, adoptive transfer of Cyld<sup>−/−</sup> T cells into RAG1 knockout mice is sufficient to induce the inflammatory phenotype, thus suggesting that the abnormal response of T cells in Cyld<sup>−/−</sup> mice plays a primary role in the spontaneous development of intestinal inflammation.

Genetic and biochemical evidence has established Tak1 as a pivotal kinase that mediates activation of IKK and JNK by diverse cellular stimuli (120, 147, 151, 181, 182, 195, 233). Using T-cell specific Tak1 knockout mice, several groups have recently shown that Tak1 is required for TCR-stimulated activation of IKK and JNK and T-cell development (120, 182, 233). Due to the lack of Tak1-deficient peripheral T cells in these mutant animals, the signaling function of Tak1 in naïve T cells could not be assessed. In vitro deletion of Tak1 in pre-activated T cells (similar to effector T cells) indicates that it is essential for JNK activation but functionally redundant for IKK activation under these conditions (233). Despite the potential variations in the necessity of Tak1, its potent activity in IKK/JNK activation suggests the requirement for a tight mechanism of regulation. Our data emphasize a critical role for CYLD in Tak1 regulation. The loss of CYLD in both primary T cells and Jurkat T cell line causes constitutive activation of Tak1 as well as its downstream targets, JNK and IKK. Since ERK is not affected by the CYLD deficiency, it is
unlikely that CYLD negatively regulates a further upstream component in the TCR signaling pathway. Indeed, our data suggest that CYLD directly targets Tak1 and inhibits Tak1 ubiquitination. In keeping with a previous finding that Tak1 ubiquitination mediates its autoactivation (223), we have shown that CYLD potently inhibits the catalytic activity of Tak1 in transfected cells. Thus, CYLD has a pivotal role in preventing the spontaneous activation of Tak1 and its downstream kinases, JNK and IKK.

Prior studies suggest that A20 is a DUB that functions to prevent prolonged activation of IKK in innate immune cells (21, 109, 239). To understand the functional difference between CYLD with A20, we examined whether CYLD regulates the kinetics or magnitude of signal-induced activation of IKKβ in innate immune cells. Surprisingly, the loss of CYLD did not result in basal activation of IKKβ or Tak1 in macrophages (Supplementary Fig. 2-3A and B). This result suggests cell-type specific function of CYLD or functional redundancy of CYLD with other DUBs in certain cell types, such as macrophages. The CYLD deficiency also did not alter the level of LPS-stimulated activation of IKKβ or prolong the activation of IKKβ following an extended time-course of LPS stimulation (Supplementary Fig. 3C). Thus, unlike A20 (21), CYLD does not play an essential role in controlling the basal or inducible activation of IKKβ in macrophages. Another major difference between CYLD and A20 is that CYLD does not seem to play a role in turning off the inducible IKK activation. Despite its essential role in suppressing the constitutive activity of IKKβ in T cells, the loss of CYLD in Jurkat T cells did not significantly prolong the IKK activation induced by TNF-α or mitogens (Supplementary Fig. 2-4A). Consistently, the level of CYLD protein was not enhanced by the mitogens (Supplementary Fig. 2-4B) or TNF-α. Thus, whereas A20 regulates the kinetics of inducible IKK activation
in innate immune cells, CYLD is critical for regulating spontaneous, and probably initial inducible, activation of Tak1 and IKK in T cells.

We have previously shown that CYLD positively regulates the late stage of thymocyte development (166). CYLD physically interacts with LCK and facilitates the binding of active LCK to its target ZAP-70, thereby enhancing the TCR-induced tyrosine phosphorylation of ZAP-70. We found in the current study that the naive Cyld−/− peripheral T cells did not show defect in TCR-proximal signaling. This finding is consistent with a previous report that LCK is required for thymocyte development but is largely dispensable for peripheral T-cell activation (136). Thus, the constitutive activation of Tak1, coupled with the diminished effect of CYLD on TCR-proximal signaling, contributes to the hyper-responsive phenotype of Cyld−/− T cells. Since Tak1 is also activated in Cyld−/− thymocytes, a question is raised with regard to its effect on thymocyte development. Based on the results of Tak1 knockout studies (120, 182, 233), activation of Tak1 should promote thymocyte development. However, the Cyld−/− mice even produce reduced numbers of mature thymocytes (166). While this phenotype is clearly due to the attenuation of LCK function and TCR-proximal signaling, it is also likely that the constitutive activation of Tak1 may contribute to the thymocyte defect by causing excessive negative selection. Future studies will examine this possibility by crossing Cyld−/− mice with TCR transgenic mice.

One surprising finding with the Tak1 conditional knockout mice (Tak1 flox/flox) was their spontaneous development of intestinal inflammation at old ages, despite the critical role for Tak1 in NF-κB activation (182, 233). However, this phenotype has been attributed to the requirement of Tak1 in regulatory T-cell (Treg) development. The Tak1 flox/flox mice produce scarce number of peripheral T cells, which turns out to be “leaked” cells that have escaped
from Cre-mediated Tak1 locus deletion (182). Thus, the effector T cells that mediate inflammation are actually Tak1-expressing cells activated due to the lack of Tregs (182). In contrast, the T-cell abnormality of Cyl d^{−/−} mice is intrinsic to the mainstream (responder) T cells. Both naïve and memory Cyl d^{−/−} T cells are hyper-responsive to TCR/CD28 stimulation, and the adoptively transferred T cells mediate colitis in RAG1^{−/−} mice. Furthermore, our preliminary studies did not detect any defect in Treg development in Cyl d^{−/−} mice (data not shown). Thus, loss of Treg function or abnormality in responder T cells may contribute to the development of intestinal inflammation.

The spontaneous development of colonic inflammation of Cyl d^{−/−} mice suggests the intriguing possibility that CYLD may serve as an important regulator of IBD. A recent study reveals that Cyl d^{−/−} mice are also more sensitive to dextran sodium sulfate-induced colitis (263). In addition to colonic inflammation, we have shown that the CYLD^{−/−} mice display other features of IBD, such as weight loss, lymphocyte infiltration into the liver, and early onset of inflammatory symptoms. In concert with the animal studies, the CYLD gene is located within a major IBD-susceptibility locus (Fig. 2G). This locus, IBD-1, also contains the NOD2 gene that encodes an intracellular pattern-recognition molecule known to mediate NF-κB activation in macrophages and intestinal epithelial cells (205). Genetic mutations of human NOD2 gene increase the risk of, although are insufficient for, developing IBD (81, 150). Consistently, ablation or mutation of the NOD2 gene in mice does not cause spontaneous intestinal inflammation (100, 127, 153), although the NOD2 mutation sensitizes mice to dextran sodium sulfate-induced intestinal inflammation (127). These findings suggest the involvement of specific environmental factors or secondary genetic factors for the development of IBD in patients carrying the NOD2 gene mutations.
Notably, the CYLD gene is located adjacent to the NOD2 gene (Fig. 2G). At least in mice, the loss of CYLD has no effect on the expression of NOD2 (Supplementary Fig. 2-5). It remains to be examined whether mutations in the CYLD gene occur in some of the IBD patients who are epidemiologically linked with the IBD1 locus. Since the NOD2 gene is located immediately upstream of the CYLD gene, it is also possible that certain genetic mutations located at the 3’ region of the NOD2 gene may affect the expression of the CYLD gene. Although these possibilities need to be examined by further studies, a recent work does suggest the association of human IBD with reduced expression of CYLD (41). Given that NOD2 and CYLD regulate the innate and adaptive immune responses, respectively, it is intriguing to examine whether the combined genetic mutations of these two genes have synergistic effect on the development of colitis in mice.
MATERIALS AND METHODS

**Mice.** *Cyld* knockout mice (in C57BL6/DBA genetic background) were generated as described(166). *Cyld*+/– mice were intercrossed to generate *Cyld*+/+ and *Cyld*−/− littermates. Genotyping was performed by PCR using tail DNA and the following primers. *Cyld* forward primer, CCA GGC ACT TTG AAT TGC TGT C; *Cyld* reverse primer 1, CGT TCT TCC CAG TAG GGT GAA G; *Cyld* reverse primer, GCA TGC TCC AGA CTG CCT TGG When the three primers were used together, the PCR yielded a 209 bp product for *Cyld*+/+ mice, a 209 bp and a 255 bp product for *Cyld*+/– mice, and a 255bp product for *Cyld*−/− mice.

*RAG1*+/– mice (in C57BL6 genetic background) were purchased from Taconic. All mice were housed in specific pathogen-free cages and monitored periodically (every 3 month) for the lack of common pathogens. Animal experiments were in accordance with protocols approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

**Plasmids, antibodies and reagents.** GST-IKKβ was constructed by cloning a cDNA fragment encoding amino acid 166-197 of human IKKβ into the pGex-4T-3 vector (Pharmacia). pCMV-HA-Tak1 and pCMV-flag-Tab1 were kindly provided by Dr. Kunihiro Matsumoto (194). Anti-mouse CD3ε (145-2C11), anti-mouse CD28, anti-CD4-PE-CY5.5 (L3T4), anti-CD44-FITC (IM7), and anti-CD25-PE (PC61.5) were purchased from eBioscience. Anti-CD19-PE-Cy7 (1D3) and other conjugated antibodies used for flow cytometric analyses and the unconjugated anti-CD4 (L3T4) were purchased from BD Biosciences was purchased from BD Pharmigen. Goat anti-hamster Ig was purchased from
Southern Biotechnology. The anti-IKKβ (H470), anti-RelB (N-17), anti-Lamin B (H-90), and anti-Actin (C2) were purchased from Santa Cruz. Anti- Tak1 was provided by Drs. Kunihiro Matsumoto and Jun Ninomiya-Tsuji. Recombinant MKK6 was from Upstate, and cycloheximide was obtained from Sigma. All other antibodies and reagents have been described previously(165, 166, 236).

RNAi-mediated CYLD knockdown. Jurkat T cells were infected with either the empty pSUPER-retro-puromycin vector (Oligoengine) or the same vector encoding CYLD shRNA, followed by puromycin selection as reported(165). The bulk of infected cells, named Jurkat-pSUPER and Jurkat-shCYLD, were used in experiments.

Histology and immunohistochemistry. Colons were removed from sacrificed mice and flushed with Iscove’s media. Distal and proximal halves of the colons were opened longitudinally, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for hematoxylin-eosin staining. Slides were analyzed blindly and scored for the degree of inflammation (0-40 scale) as described(242). Liver, lung, and salivary gland sections were prepared similarly, and pictures were taken from typical liver and colon sections. For immunohistochemistry, the distal and proximal portions of colons were freshly frozen in Tissue-Tec OCT compound (VWR) using liquid nitrogen prechilled 2-methylbutane. The frozen tissues were stored at -70°C until sectioning. 4-6 micron cryostat sections were prepared and stained with rat anti-mouse CD4 (eBioScience), and the bound anti-CD4 was detected by biotinylated rabbit anti-rat immunoglobulin and peroxidase-
conjugated streptavidin with dianimobenzidine as chromagen (VECTASTAIN Elite ABC Kit, Vector).

**Flow cytometry.** Splenic and lymph node cell suspensions were prepared by gentle homogenization using a tissue homogenizer. Mononuclear cells were isolated by centrifugation over lymphocyte separation media (Cellgro) and were subjected to flow cytometry analyses as previously described(166). To isolate naïve and memory T cells, unfixed mesenteric lymph node cells were stained with anti-CD44-FITC, anti-CD25-PE, and anti-CD19-PE-Cy7. Naïve (CD44lo-medCD25-CD19-) and memory (CD44hiCD25-CD19-) T cells were purified using MofloTM cell sorter (Dako). Purity of the isolated populations was greater than 98%.

**Cell proliferation and ELISA.** Purified T cells were stimulated in 5 replicate wells in 96-well plates (1 x 10⁵ cells/well) with the indicated amounts of plate-bound anti-CD3 and anti-CD28 (1 μg/ml). After the indicated times of stimulation, cell-culture supernatants were collected and subjected to ELISA assays (eBioScience) to measure the concentration of cytokines, whereas the cells were labeled for 6 hr with ³H-thymidine for proliferation assays based on thymidine incorporation.

**T-cell adoptive transfer.** Wildtype and Cyld⁻/⁻ T cells were purified using CD90-conjugated magnetic beads (Miltenyl Biotec) from mesenteric lymph nodes, and 7 x 10⁶ cells were injected intravenously into RAG1⁻/⁻ mice. Recipient mice were monitored for weight loss and sacrificed after 6 weeks. Colon, liver, and salivary glands were removed for
histology analyses. The efficiency of adoptive transfer was assessed by flow cytometry analyses of T cells in the spleen.

**Ubiquitination assays.** 293 cells were transfected in 12-well plates with the indicated plasmid expression vectors. Ubiquitination of transfected and endogenous proteins was analyzed as previously described (165).

**IB, electrophoresis mobility shift assay (EMSA), and in vitro kinase assays.** Mesenteric lymph node T cells were purified by using CD90-labeled magnetic beads and incubated on ice for 15 min with the indicated concentrations of anti-mouse CD3 and anti-mouse CD28. The cells were washed once with 500 μl of cold Iscove’s media and stimulated by crosslinking the receptor-bound anti-CD3 and anti-CD28 for the indicated times in a 37°C water bath with goat anti-hamster Ig (45 μg/ml). Jurkat cells were collected by centrifugation and resuspend in fresh media followed by stimulation with the indicated inducers. Untreated and stimulated thymocytes, lymph node T cells, and Jurkat cells were lysed in a kinase cell lysis buffer supplemented with phosphatase inhibitors and subjected to in vitro kinase assays and IB as previously described(227). For EMSA, thymocytes and T cells were either not treated or stimulated with plate-bound anti-CD3 plus soluble anti-CD28 for the indicated times. Nuclear extracts were prepared and subjected to EMSA using a 32P-radiolabeled κB oligonucleotide as previous described(209).

**RNase protection assay (RPA) and RT-PCR.** Total cellular RNA was isolated from the colon using the TRI reagent (Molecular Research Center, Inc.). RPA was performed using
the BD RiboQuant Reagents and a custom template set according to the manufacturer’s instructions (BD Biosciences). Semi-quantitative RT-PCR was performed using specific primers for the indicated murine genes (primer sequences are available upon request).

**Statistics.** Statistical significance was determined by t test.
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Figure 2-1. Hyperresponsiveness of Cyld<sup>−/−</sup> T cells. (A-C) Wild-type (+/+ and Cyld<sup>+</sup>/) mesenteric lymph node T cells were either not treated (NT) or stimulated with the indicated amounts of plate-bound anti-CD3 plus soluble anti-CD28. Cell proliferation (A) and cytokine production (B, C) were measured by thymidine incorporation and ELISA, respectively. Data are presented as mean ± s.d. of three independent experiments. (D, E) Purified naïve and memory T cells were stimulated with plate-bound anti-CD3 (2.5 μg/ml for naïve and 1 μg/ml for memory T cells) and anti-CD28 (2.5 μg/ml for naïve and 1 μg/ml for memory T cells) for 48 h followed by measuring cell proliferation and IFN-γ production as in A and B.
Figure 2-1
Figure 2-2. Spontaneous development of autoimmune symptoms and colonic inflammation in Cyld<sup>−/−</sup> mice. (A-C) Hematoxylin-eosin staining of tissue sections of the liver (A) and the distal portion of the colon (B, C) from 8 week old control (+/+) and Cyld<sup>−/−</sup> mice. Inflammatory cell infiltration in the periportal vein of liver (A, arrow), colonic patches in the colon (B, arrows), and inflammatory cell infiltration in the colonic mucosa (C, arrow 1) are indicated. Original magnification, x20 (A, C) and x2 (B). (D) Immunohistochemistry staining of colon sections with anti-CD4, showing massive CD4 T-cell infiltration in the Cyld<sup>−/−</sup> colon. Data in A-D are representative of four different experiments, each with four wildtype and four Cyld<sup>−/−</sup> mice. (E) Histological scores of mucosal inflammation in wildtype and Cyld<sup>−/−</sup> mice. Data were from three wildtype and three Cyld<sup>−/−</sup> mice (8 week of age), each with two colon sections. Similar results were obtained from 3 additional experiments. (F) RPA showing the constitutive expression of several proinflammatory genes in the colon of Cyld<sup>−/−</sup> mice (#1 and #3) but not the wildtype mice (#2 and #4). The house-keeping genes, L32 and Gapdh, were included as loading control. (G) Body weight of wildtype and Cyld<sup>−/−</sup> mice, showing the reduced body weights of male and female Cyld<sup>−/−</sup> mice (8 week old). * indicates P-value <0.05. (H) Adjacent localization of Cyld and Nod2 genes on human chromosome 16. The nucleotide number was based on the sequence of homo sapiens chromosome 16 clone RP11-327F22 (accession number: AC007728). The map was drawn on scale, and the transcriptional direction of the genes is indicated by arrows. Note that the two genes have the same transcriptional direction and are separated by only 8988 nucleotides.
Figure 2-2
Figure 2-3. Induction of autoimmunity and colitis by adoptively transferred $Cyld^{+/−}$ T cells. $RAG1^{−/−}$ mice (8 weeks old) were intravenously injected with T cells isolated from the mesenteric lymph nodes of wildtype (+/+) or $Cyld^{+/−}$ mice. (A-C) After 6 weeks, the recipient mice were sacrificed for flow cytometry analyses of transferred T cells in the spleen (A), histology analyses of lymphocyte infiltration in the liver (B), and inflammation of the colon (C). An arrow in C (lower) indicates a colonic patch detected in a recipient of $Cyld^{+/−}$ T cells. Data are representative of three mice per group. (D) Histology scores measuring the degree of colonic inflammation in the recipient wildtype (+/+) and $Cyld^{+/−}$ T cells. Data were from three mice per group. (E) Body weights of recipient mice. Body weights were measured 6 weeks after T-cell transfer, showing the significant weight loss in the recipients of $Cyld^{+/−}$ T cells. (F) Picture to compare the colons of recipients of wildtype (+/+) and $Cyld^{+/−}$ T cells.
Figure 2-3
Figure 2-4. Constitutive activation of JNK and NF-κB in Cyld−/− T cells. (A) purified naïve lymph node T cells from control (+/+) and Cyld−/− mice were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) for the indicated times. Immunoblotting (IB) assays were performed using the indicated phospho-specific (α-P) and pan-antibodies to determine the phosphorylation of ZAP-70, ERK, and JNK. (B) Hyperactivation of NF-κB in Cyld−/− T cells. Wildtype (+/+) and mutant (-/-) total lymph node T cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) for the indicated times. Nuclear extracts were subjected to EMSA to determine the activity of NF-κB. Actin IB was included as a loading control. (C) Nuclear extracts were isolated from purified naïve and memory T cells and subjected to EMSA to determine the constitutive activity of NF-κB. An EMSA of NF-Y was included as a control. (D) EMSA was performed using the nuclear extract isolated from untreated Cyld−/− T cells in the presence of the indicated antibodies. (E) Whole-cell extracts were isolated from wildtype (+/+) and Cyld−/− mesenteric T cells and subjected to IB assays to detect the indicated NF-κB members. (F) RT-PCR was performed to detect mRNA of relB and Gapdh using total RNA isolated from wildtype (+/+) or Cyld−/− mesenteric lymph node cells.
Figure 2-4
Figure 2-5. *Cyld* deficiency results in constitutive activation of IKKβ and chronic degradation of IκBα. (A, B) Chronic degradation and resynthesis of IκBα in *Cyld*+/− T cells. Wildtype (+/+ ) and mutant (−/−) total T cells were subjected to IB assays (A) or RT-PCR (B) to detect the total and phosphorylated IκBα (P-IκBα) and the IκBα messenger RNA, respectively. (C) IκBα degradation in *Cyld*+/− T cells is mediated by IKKβ. Wildtype and mutant T cells were incubated for 50 min in the absence (−) or presence (+) or cycloheximide (CHX, 10 μg/ml). In lane 7, the cells were preincubated with the IKKβ inhibitor PS1145 (10 μM) for 60 min before the start of cycloheximide treatment. Expression of IκBα, as well as CYLD and tubulin, was analyzed by IB. (D) Chronic activation of IKKβ in *Cyld*+/− T cells. IKKβ was isolated by IP (using anti-IKKβ) from untreated wildtype (+/+ ) and *Cyld*+/− lymph node T cells and thymocytes, and the activity of IKKβ was measured by *in vitro* kinase assays using both GST-IκBα and GST-IKKβ substrates (top two panels). IB was performed to monitor the IKKβ protein level (bottom panel). (E) Whole-cell extracts isolated from purified naïve T cells (untreated) were subjected to IB. (F) Whole-cell extracts isolated from untreated Jurkat-pSUPER and Jurkat-shCYLD cells were subjected to IKKβ kinase assay (panel 1) followed by IB to detect the IKKβ protein on the kinase assay membrane (panel 2). Cell lysates were subjected to IB to detect the indicated proteins (panels 3-5).
**Figure 2-6. CYLD physically interacts with Tak1 and inhibits the ubiquitination and autoactivation of Tak1.** (A) Tak1 was isolated by IP from lymph node T cells, thymocytes, or Jurkat T cells followed by kinase assays using recombinant MKK6 as substrate (upper). The kinase assay membrane was further subjected to IB to detect the Tak1 protein (lower). (B) CYLD complex was isolated by IP from wildtype (+/+ or $Cyld^{-/-}$) thymocytes followed by IB to detect CYLD and its associated Tak1. Tak1 level in cell lysates was monitored by IB (bottom). (C) 293 cells were transfected with CYLD (150 ng) either in the absence (–) or presence (+) of Tak1 (100 ng). Tak1 complex was isolated by IP, followed by IB to detect Tak1 and associated CYLD. CYLD expression level was analyzed by IB (bottom). (D) 293 cells were transfected with Tak1 (100 ng) either in the absence (–) or presence (+) of CYLD (150 ng in lane 3 and 300 ng in lane 4), along with HA-tagged ubiquitin (200 ng). Tak1 was isolated by IP followed by IB (with anti-HA) to examine its ubiquitin conjugation. (E) 293 cells were transfected with Tak1 (100 ng) in the absence (–) or presence (+) of two doses of CYLD (150 ng and 300 ng). The cells were also transfected with the Tak1 partner protein Tab1 (100 ng). Tak1 was isolated by IP, followed by kinase assays using recombinant MKK6 as substrate (panel 1). The kinase assay membrane was further subjected to IB to detect the precipitated Tak1 (panel 2). CYLD expression in lysates was monitored by IB (panel 3). (F) Tak1 and IKKγ were isolated by IP from wildtype (+/+ and CYLD-deficient (−/−) T cells and subjected to IB (using anti-ubiquitin) to detect the ubiquitinated Tak1 and IKKγ. The membrane was reprobed with anti-Tak1 to monitor the precipitated Tak1 protein (lower panel). Since IKKγ comigrates with the Ig heavy chain, a direct IB was performed to monitor its expression level (lower panel).
Supplementary Figure 2-1. Hyperresponsiveness of Gyld⁻/⁻ splenic T cells. Wild-type (+/+) and Gyld⁻/⁻ splenic T cells were either not treated (NT) or stimulated with plate-bound anti-CD3 plus soluble anti-CD28. Cell proliferation (A) and cytokine production (B, C) were measured by thymidine incorporation and ELISA, respectively. Data are presented as mean ± s.d. of three independent experiments.
Supplementary Figure 2-2. Expression of cytokine RNA in the colon. (A) Total RNA was prepared from wildtype (+/+) or Cylt⁻⁻ mice (males, 8 weeks of age) and subjected to semi-quantitative RT-PCR analyses to detect the mRNA of IL-10 and Gapdh. (B) Total RNA was prepared from OCT-embedded frozen colons derived from RAG1 knockout mice adoptively transferred with T cells isolated from wildtype (+/+) or Cylt⁻⁻ mice (same tissues used in Fig. 3). Semi-quantitative RT-PCR assays were performed to analyze the RNA expression of the indicated genes.
Supplementary Figure 2-3. Loss of CYLD does not result in constitutive activation of IKKβ and Tak1 nor affects the kinetics of inducible IKK activation in macrophages. (A) IKKβ was precipitated from untreated wildtype (+/+) or Cyld<sup>−/−</sup> bone marrow derived macrophages and subjected to kinase assays using GST-IkBα or GST-IKKβ as substrate. The cell lysates were subjected to IB to detect expression levels of IKKβ, IkBα, and tubulin. (B) Tak1 was isolated by IP from untreated bone marrow derived macrophages and subjected to kinase assay using MKK6 as substrate. The level of Tak1 was detected by IB. (C) Wildtype (+/+) and Cyld<sup>−/−</sup> bone marrow derived macrophages were stimulated with LPS (500 ng/ml) for the indicated times followed by kinase assays to detect IKKβ catalytic activity and IB to monitor the expression of IKKβ and tubulin.
Supplementary Figure 2-4. CYLD is not important for turning off the IKK activation signal in Jurkat T cells. (A) Jurkat-sSUPER and Jurkat-shCYLD cells were stimulated with either TNF-α (20 ng/ml) or PMA (50 ng/ml) plus ionomycin (1 μM) for the indicated times followed by IKKβ kinase assays and IB. (B) Lack of induction of CYLD along with mitogen stimulation. Jurkat-sSUPER and Jurkat-shCYLD cells were stimulated with PMA plus ionomycin for the indicated times followed by IB to detect CYLD and tubulin. A non-specific band is labeled ns.
Supplementary Figure 2-5. NOD2 expression is not affected by Cylt deficiency. Whole-cell lysates were prepared from bone marrow derived macrophages of wildtype (+/+) and Cylt knockout (−/−) mice and subjected to IB using antibodies for NOD2 or tubulin.
CHAPTER III

DEUBIQUITINATING ENZYME CYLD REGULATES THE
PERIPHERAL DEVELOPMENT AND NAIVE PHENOTYPE
MAINTENANCE OF B CELLS

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ABSTRACT

Deubiquitinating enzymes (DUB) form a family of cysteine proteases that digest ubiquitin chains and reverses the process of protein ubiquitination. Despite the identification of a large number of DUBs, their physiological functions remain poorly defined. Here we provide genetic evidence that CYLD, a recently identified DUB, plays a crucial role in regulating the peripheral development and activation of B cells. Disruption of the CYLD gene in mice results in B cell hyperplasia and lymphoid organ enlargement. The CYLD-deficient B cells display surface markers indicative of spontaneous activation and are hyperproliferative upon in vitro stimulation. When challenged with antigens, the CYLD−/− mice develop exacerbated lymphoid organ abnormalities and abnormal B cell responses. Although the loss of CYLD has only a minor effect on B-cell development in bone marrow, this genetic deficiency disrupts the balance of peripheral B cell populations with a significant increase in marginal zone B cells. In keeping with these functional abnormalities, the CYLD−/− B cells exhibit constitutive activation of the transcription factor NF-κB due to spontaneous activation of IκB kinase beta and degradation of the NF-κB inhibitor IκBα. These findings demonstrate a critical role for CYLD in regulating the basal activity of NF-κB and maintaining the naïve phenotype and proper activation of B cells.

Keywords: CYLD, IKK, NF-κB, B-cell activation, deubiquitinating enzyme
INTRODUCTION

Ubiquitination is a posttranslational mechanism that regulates the degradation and biological function of diverse proteins (70, 154). Protein ubiquitination is catalyzed by well-defined enzymatic machinery, composed of an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). Recent studies on the E3 ubiquitin ligases demonstrate an important role for protein ubiquitination in the regulation of immune responses (122). In particular, ubiquitination is involved in the development, activation, and differentiation of lymphocytes. Defects in E3 ubiquitin ligases are associated with severe immunological disorders, such as the loss of immunological tolerance and development of autoimmunity (122). Emerging evidence suggests that protein ubiquitination is a tightly controlled and reversible process that is counter regulated by deubiquitinating enzymes (DUBs), a family of cysteine proteases digesting ubiquitin chains (146). Like the E3s, the DUBs exist in large numbers, thus suggesting a high level of functional diversity and substrate specificity in their functions (146). However, despite the extensive studies on E3s, the physiological functions of DUBs are poorly defined. We have recently described the function of a DUB, CYLD, in regulating thymocyte development (166). CYLD positively regulates thymic TCR signaling and is required for the generation of CD4 and CD8 mature thymocytes (166). These findings provide the first example for how a DUB can function in the adaptive immune system. However, it is unclear whether CYLD also regulates other aspects of immune function, particularly the activation and homeostasis of lymphocytes.
CYLD was originally identified as a tumor suppressor mutated in familial cylindromatosis (18), an autosomal dominant predisposition to benign tumors of the skin appendages (117). More recent in vitro work suggests that CYLD functions as a DUB of TRAFs and the regulatory subunit of IκB kinase (IKKγ) (30, 102, 226, 261). Ubiquitination of these signaling molecules appears to serve as a mechanism that activates their signal transduction functions (35). Consistently, in vitro work demonstrates that CYLD inhibits the activation of NF-κB and MAP kinases (MAPKs) by toll-like receptors (TLRs) and TNFRs (30, 102, 162, 164, 226, 261). However, how CYLD regulates signal transduction under physiological conditions is still poorly understood. Recent studies using CYLD knockout (CYLD<sup>−/−</sup>) mice suggest that the signaling function of CYLD is complex, which may involve distinct target proteins in different cell types and signaling pathways (129, 166). For example, the loss of CYLD in primary macrophages has no significant effect on the activation of NF-κB induced by TNF-a and TLR ligands (166). On the other hand, CYLD modulates the signaling function of a protein tyrosine kinase, Lck, in thymocytes (166) and the nuclear translocation of an NF-κB coactivator protein, Bcl-3, in keratinocytes (129). Clearly, the precise signaling role of CYLD, especially that in the regulation of NF-κB, warrants further studies.

NF-κB represents a family of transcription factors that regulates diverse genes involved in the activation and survival of lymphocytes (114). In mammals, the NF-κB family includes RelA, RelB, c-Rel, NF-κB1 (or p50), and NF-κB2 (or p52), which form different homo- and heterodimers. The NF-κB members are normally sequestered in the cytoplasm as inactive complexes by physical interaction with specific inhibitors, including IκBα and related proteins (20). Activation of NF-κB involves phosphorylation-triggered
degradation of IκBα and nuclear translocation of NF-κB complexes, particularly the p50/RelA and p50/c-Rel dimers. A multi-subunit IKK complex responds to diverse cellular stimuli and mediates the phosphorylation of IκBα (95). In addition to this canonical pathway of NF-κB activation, a noncanonical pathway exists to mediate activation of two specific NF-κB members, RelB and NF-κB2 (20). Accumulating evidence suggests that the deregulated activation of NF-κBs can cause severe immunological disorders, such as lymphoid malignancies and autoimmunity (92, 210, 250). As such, both the basal and inducible activity of NF-κB are likely subject to negative mechanism of regulation, although the physiological negative regulators of NF-κB remain poorly defined.

In the present study, we show that CYLD plays a critical role in preventing uncontrolled NF-κB activation in B cells. Consistently, CYLD-deficient B cells are hyperproliferative when stimulated in vitro and display elevated levels of antigen responses in vivo. The CYLD+/− mice develop B-cell hyperplasia and lymphoid organ abnormalities, which can be further exacerbated when these animals are challenged with antigens. We further show that CYLD also regulates peripheral B-cell development, since the loss of CYLD results in abnormal production of marginal zone B cells. These findings establish CYLD as a key regulator of B-cell activation and development and reveal a physiological function of CYLD in NF-κB regulation.
RESULTS

CYLD−/− mice display lymphoid organ abnormalities and B-cell hyperplasia

To investigate the role of CYLD in regulating immune system function, we began by analyzing the peripheral lymphoid organs of the CYLD−/− and wildtype mice. As early as 8 weeks of age, the CYLD−/− mice displayed striking enlargement of the mesenteric lymph nodes (MLNs), and this abnormality became even more profound at older ages (Fig. 3-1A). On the other hand, the CYLD+/− and wildtype mice did not show obvious size differences in other lymph nodes or the Peyer’s patches, and only a small percentage of the CYLD−/− mice had slightly enlarged spleens. Thus, a prominent lymphoid abnormality of the CYLD−/− mice is the enlargement of MLNs.

To examine the effect of CYLD on lymphocyte homeostasis, we performed flow cytometry analyses to measure the frequency of B and T cells in MLNs. The CYLD−/− MLNs exhibited a profound increase in the percentage of B cells and a reduction in the percentage of T cells (Fig. 3-1B). The absolute number of MLN B cells was even more drastically increased in the CYLD−/− animals (Fig. 3-1C) due to the severe lymphadenopathy (see Fig. 3-1A). We previously reported that the spleen of CYLD−/− mice contained more B cells and reduced numbers of T cells (166). Consistently, flow cytometry analyses of multiple animals revealed significantly higher frequency and numbers of B cells and reduced frequency and numbers of T cells in the spleens of CYLD−/− mice (Fig. 3-1D).

In addition to the mainstream B cells (B2 cells), we also analyzed the frequency of B1 cells, which are predominantly located in the peritoneal cavity. The CYLD−/− mice only showed a slight increase in this population of B cells in the peritoneal cavity (Fig. 3-1E) and...
no difference in spleen and MLNs (data not shown). Taken together, these results suggest that the loss of CYLD causes hyperplasia of mainstream B cells and abnormalities of peripheral lymphoid organs, especially MLNs.

**CYLD plays a minor role in regulating B-cell development in the bone marrow**

Peripheral B cells are derived from immature B cells generated in the bone marrow. Because of the peripheral B-cell hyperplasia in CYLD<sup>−/−</sup> mice, we examined whether the loss of CYLD resulted in elevated generation of immature B cells in the bone marrow. FACS analyses of bone marrow CD19<sup>+</sup> cells (B cells) detected three major populations: the early stages of developing B cells (ProPre B cells, IgM<sup>−</sup>IgD<sup>−</sup>), the immature B cells (IgM<sup>+</sup>IgD<sup>−</sup>), and the recirculating mature B cells (IgM<sup>+</sup>IgD<sup>+</sup>). The CYLD<sup>−/−</sup> mice did not produce more immature B cells but rather had a moderate reduction in this population of B cells (Fig. 3-2A and B). This result suggests that CYLD plays a minor and positive role in B-cell development at the immature B stage. Thus, the peripheral B-cell hyperplasia of CYLD<sup>−/−</sup> mice was not due to the overproduction of immature B cells within the bone marrow.

**CYLD regulates marginal zone B-cell development**

In the spleen, immature B cells go through transitional stages and eventually become follicular mature B cells or marginal zone B cells (32). To examine how the loss of CYLD affects peripheral B-cell maturation, we analyzed the splenic B-cell populations based on their defined surface markers (32). Young CYLD<sup>−/−</sup> mice (8 week) did not display profound alterations in B-cell maturation (Fig. 3-2C and D), although they had a slight reduction in the CD21<sup>hi</sup>CD23<sup>lo</sup> T1 cells (Fig. 3-2D). Additionally, we detected a small increase in the CD21<sup>hi</sup>CD23<sup>lo</sup> marginal zone B-cell population in these mutant animals (Fig. 3-2D).
Interestingly, the increase in marginal zone B cells became much more prominent in older CYLD−/− mice (14 weeks), as assessed based on the staining of both CD21/CD23 (Fig. 3-2E) and another marginal zone B-cell marker, CD1d (Fig. 3-2F). These results suggest a role for CYLD in regulating the peripheral development of B cells to marginal zone population.

Spontaneous activation of B cells in CYLD−/− mice

We next examined whether the B-cell hyperplasia in CYLD−/− mice was associated with abnormal B-cell activation. This possibility was first indicated in our analyses of B-cell maturation markers. Although the CYLD deficiency in young mice did not profoundly alter the frequency of transitional and mature B-cell subpopulations in the spleen (see Fig. 3-2D), the CYLD−/− splenic B cells displayed considerably higher intensity of CD23 and CD21 (Fig. 3-3A, Total, dotted lines). This abnormality occurred primarily in follicular B cells (Fig. 3-3A, FO) but not marginal zone B cells (Fig. 3-3A, MZ). Since follicular B cells contain both mature and transitional populations, we further identified the CD21/CD23 overexpressing cells based on the expression of AA4.1. Loss of CYLD caused CD21/CD23 upregulation in both transitional (AA4.1 positive) and mature (AA4.1 negative) B cells (Fig. 3-3A, bottom panel). Further, this abnormality was also detected on B cells isolated from the MLNs (Fig. 3-3B). Parallel RT-PCR analyses showed that the CYLD−/− B cells expressed substantially higher levels of CD23 mRNA than the wildtype B cells, suggesting a role for CYLD in regulating CD23 gene expression (Fig. 3-3C).

Since CD21 and CD23 have been implicated in B-cell activation and humoral immune responses (133, 157, 169, 202), the findings described above, together with the B-cell hyperplasia, suggest the possibility that the loss of CYLD may lead to abnormal B-cell activation. To further confirm this possibility, we analyzed the expression of two other
known B-cell activation markers, CD80 and CD86, which function as costimulatory molecules modulating the activation of T and B cells (67, 155). As expected, wildtype B cells expressed low levels of CD80 and CD86 (Fig. 3-3D, solid lines). In contrast, the $\text{CYLD}^{-/-}$ B cells expressed markedly higher levels of CD80 and CD86 (Fig. 3-3D, dotted lines), thus further suggesting the activation phenotype of these mutant B cells. The spontaneous activation of $\text{CYLD}^{-/-}$ B cells was also indicated by their larger size, as demonstrated by the forward scatter analysis in flow cytometry (Fig. 3-3E). Taken together, these results suggest a key role for CYLD in maintaining the naïve phenotype of B cells and provide an explanation for the B-cell hyperplasia in $\text{CYLD}^{-/-}$ mice.

**Hyperresponsiveness of CYLD$^{-/-}$ B cells**

As a more direct approach to determine the effect of CYLD deficiency on B-cell activation, we analyzed the proliferative response of the $\text{CYLD}^{-/-}$ B cells to stimulation via different receptors. Thymidine incorporation assays revealed that the $\text{CYLD}^{-/-}$ splenic B cells had significantly higher proliferative ability than the wildtype B cells when stimulated with the BCR inducer anti-IgM (Fig. 3-4A). The $\text{CYLD}^{-/-}$ B cells were also hyperresponsive to LPS (Fig. 3-4A), a bacterial cell wall component stimulating B cells via TLR4 and the TLR-related molecule RP105 (230). On the other hand, the $\text{CYLD}^{-/-}$ and wildtype B cells only exhibited a moderate difference in their responses to an agonistic antibody to CD40 (Fig. 3-4A), a key costimulatory molecule that mediates B cell activation by helper T cells (19).

To further confirm the hyperproliferative phenotype of $\text{CYLD}^{-/-}$ B cells, we analyzed the division rate of $\text{CYLD}^{-/-}$ and control B cells using the carboxyl fluorescent succinimidyl
ester (CFSE) labeling technique (Fig. 3-4B). Consistent with the thymidine incorporation results, the CYLD<sup>−/−</sup> B cells displayed significantly higher proliferation ability than the control B cells when stimulated with anti-IgM (Fig. 3-4B, middle panel, dotted line). After 48 hr of anti-IgM stimulation, the majority of the CYLD<sup>−/−</sup> B cells had undergone five or more cell cycles, whereas most of the wildtype B cells had four or fewer divisions. The CYLD<sup>−/−</sup> B cells displayed even more drastic hyperproliferative ability when stimulated with LPS (Fig. 3-4B, bottom panel). Thus, the CYLD deficiency causes hyperresponses of B cells to BCR and TLR stimulations.

**Antigen exposure causes exacerbated lymphoid organ abnormalities and elevated B-cell responses in CYLD<sup>−/−</sup> mice**

To determine the effect of CYLD deficiency on *in vivo* immune responses, we immunized the CYLD<sup>−/−</sup> mice with sheep red blood cells (SRBC), which are frequently used as a model antigen for analyzing B-cell expansion and germinal center formation. Interestingly, upon SRBC immunization, the CYLD<sup>−/−</sup> mice, but not wildtype mice, developed splenomegaly (Fig. 3-5A). This abnormality was associated with prominent enlargement of B-cell follicles in the white pulp of the spleen (Fig. 3-5B, B220), although the size of T-cell zones was comparable between the CYLD<sup>−/−</sup> and wildtype mice (Fig. 3-5B, CD3). The mutant spleen also contained larger and increased numbers of germinal centers, indicating heightened expansion of B cells (Fig. 3-5B, PNA). Since the CYLD<sup>−/−</sup> B cells were hyperresponsive to LPS *in vitro* (see Fig. 3-4), we also immunized the mice with the T-independent antigen NP-LPS (nitro-phenol-conjugated LPS). As seen with SRBC, NP-LPS immunization caused prominent splenomegaly in CYLD<sup>−/−</sup> mice (Fig. 3-5A). We next
examined whether the CYLD<sup>+</sup> mice developed splenomegaly when they were exposed to natural pathogens. Age- and sex-matched mutant and wildtype mice were transferred to conventional housing conditions, which allowed exposure of the animals to common pathogens within the food and air. Indeed, after 6 weeks of conventional housing, over 50% of the CYLD<sup>+/–</sup> mice, but none of the wildtype mice, developed severe splenomegaly (Fig. 3-5A). The MLNs of these mutant animals were also further enlarged compared with the mice housed under specific pathogen-free condition. These results suggest that immune responses causes exacerbated lymphoid abnormalities in CYLD<sup>+/–</sup> mice and further emphasize a role for CYLD in controlling B-cell activation and homeostasis.

Deregulated B-cell activation can cause abnormal immune responses through different mechanisms, such as activation of T cells and aberrant production of antibodies (53). Analysis of serum Ig level in unimmunized mice did not reveal significant differences between the wildtype and CYLD<sup>+/–</sup> mice (Fig. 3-5C). We next examined how the loss of CYLD affected the antigen-specific antibody responses by immunizing the mice with NP-conjugated T-independent (LPS) and T-dependent (KLH, keyhole limpet hemocyanin) antigens. Consistent with the hyper activation of CYLD<sup>+/–</sup> B cells by LPS in vitro, the CYLD<sup>+/–</sup> mice mounted elevated levels of anti-NP-LPS IgM responses than the wildtype mice, although this T-independent antigen did not induce significant responses of other antibody isotypes (Fig. 3-5D). In response to NP-KLH, the CYLD<sup>+/–</sup> mice produced markedly more IgG1 and IgG2a than the wildtype mice (Fig. 3-5B). Since the production of IgG1 and IgG2a is critically dependent on CD80 and CD86 (22), this result is in agreement with the hyperexpression of CD80 and CD86 on CYLD<sup>+/–</sup> B cells. The basal immunoglobulin level of CYLD<sup>+/–</sup> mice was similar to that of the wildtype mice. Taken
together with the data presented in Fig. 3-3, these findings suggest that CYLD plays a critical role in negative regulation of B-cell responses in vivo.

**Loss of CYLD results in constitutive activation of NF-κB in B cells**

To understand the molecular mechanism by which CYLD negatively regulates B-cell responses, we examined the effect of CYLD deficiency on BCR signaling using purified splenic and lymph node B cells. We detected a moderate enhancement of ERK MAPK activation in CYLD<sup>−/−</sup> B cells. More prominently, the activation of NF-κB induced by anti-IgM and LPS was significantly enhanced in CYLD<sup>−/−</sup> B cells (Fig. 3-6A, compare lanes 2 and 3 with lanes 5 and 6). Notably, the CYLD<sup>−/−</sup> B cells also exhibited a markedly higher level of basal NF-κB activity than the wildtype B cells (Fig. 3-6A, compare lanes 1 and 4). This finding suggested that the loss of CYLD might promote constitutive activation of NF-κB. To assure that the hyper basal activation of NF-κB in CYLD<sup>−/−</sup> B cells was not due to the in vitro incubation, we prepared nuclear extracts from the CYLD<sup>−/−</sup> and wildtype B cells immediately after purification and repeated the EMSA. Consistent with prior reports (119), the wildtype B cells isolated from both mesenteric lymph nodes and spleen exhibited constitutive NF-κB activity (Fig. 3-6B, lanes 1 and 3). Importantly, the constitutive NF-κB activity was markedly elevated in the CYLD<sup>−/−</sup> B cells (lanes 2 and 4). Thus, in agreement with their hyperproliferative phenotype, the CYLD<sup>−/−</sup> B cells had aberrant activation of NF-κB.

To further examine the mechanism of NF-κB constitutive activation in CYLD<sup>−/−</sup> B cells, we performed antibody supershift assays to analyze the composition of the active NF-κB complexes. The C2 NF-κB complex appeared to contain mostly p50, since it was
completely shifted by the anti-p50 antibody but did not appreciably react with the other anti-NF-κB antibodies (Fig. 3-6C). On the other hand, C1 partially reacted with all NF-κB members, including p50, RelA, c-Rel, as well as the noncanonical NF-κB members, p52 and RelB. These immune reactions were specific, since neither C1 nor C2 reacted with a preimmune serum (Fig. 3-6C, lane 1).

Immunoblotting (IB) assays were then carried out to examine whether the loss of CYLD affected the expression level of NF-κB members. The CYLD−/− and wildtype B cells expressed comparable amounts of canonical NF-κB members (p50, RelA, c-Rel) (Fig. 3-6D). In contrast, the noncanonical NF-κB members, p100 and RelB, were significantly induced in the absence of CYLD (Fig. 3-6D). On the other hand, the CYLD deficiency did not enhance the processing of p100, since the level of p52 was only slightly increased in CYLD−/− B cells (Fig. 3-6D). The upregulation of p100 and RelB was consistent with the constitutive activation of NF-κB, since the genes encoding these noncanonical NF-κB members are under the regulation of NF-κB (26, 209). Thus, the loss of CYLD causes posttranslational activation of canonical NF-κB, which in turn appears to mediate hyperexpression of noncanonical NF-κB members.

CYLD deficiency promotes chronic phosphorylation and degradation of IκBα in B cells

Since the hyper activation of NF-κB under unstimulated conditions is a major feature of CYLD−/− B cells, we investigated the underlying mechanism by examining the fate of the NF-κB inhibitor IκBα. The steady level of IκBα was significantly lower in CYLD−/− cells compared with wildtype cells (Fig. 3-7A). However, this result was not due to the reduction
in \(I\kappa B\alpha\) gene expression, since the level of \(I\kappa B\alpha\) mRNA was even higher in the mutant B cells (Fig. 3-7B). The existence of a higher amount of \(I\kappa B\alpha\) mRNA in \(CYLD^{-/-}\) B cells was consistent with the hyper activation of NF-\(\kappa B\), as the expression of \(I\kappa B\alpha\) gene is under the control of NF-\(\kappa B\) (208). These findings suggested that \(I\kappa B\alpha\) might be undergoing chronic degradation and resynthesis in \(CYLD^{-/-}\) B cells. Indeed, inhibition of protein synthesis by cycloheximide led to rapid loss of \(I\kappa B\alpha\) in \(CYLD^{-/-}\) B cells but only a slight reduction in the wildtype B cells (Fig. 3-7C, top panel). We noticed that the \(I\kappa B\alpha\) from \(CYLD^{-/-}\) B cells migrated as doublet bands on SDS gels when the cell lysates were prepared in the presence of phosphatase inhibitors (Fig. 3-7C, top panel, lane 4). We thus examined whether the loss of CYLD resulted in constitutive phosphorylation of \(I\kappa B\alpha\). A weak basal level of \(I\kappa B\alpha\) phosphorylation was detected in wildtype B cells (Fig. 3-7C, middle panel, lane 1). Interestingly, however, a markedly higher level of phospho-\(I\kappa B\alpha\) was detected in \(CYLD^{-/-}\) B cells (Fig. 3-7C, middle panel, lane 4), despite the lower level of total \(I\kappa B\alpha\) protein in these mutant cells (Fig. 3-7C, top panel, lane 4). A selective IKK inhibitor, PS1145, blocked the chronic phosphorylation of \(I\kappa B\alpha\) and prevented the loss of \(I\kappa B\alpha\) in cycloheximide-treated \(CYLD^{-/-}\) B cells (Fig. 3-7C, top and middle panels, lane 6), suggesting the requirement of IKK\(\beta\)-mediated \(I\kappa B\alpha\) phosphorylation in triggering the chronic degradation of \(I\kappa B\alpha\). Indeed, parallel \textit{in vitro} kinase assays revealed hyperactivation of IKK\(\beta\) in \(CYLD^{-/-}\) B cells (Fig. 3-7D). Thus, the CYLD deficiency promotes constitutive activation IKK\(\beta\) and degradation of \(I\kappa B\alpha\).
Loss of CYLD bypasses the requirement of BAFF for CD23 induction

BAFF receptor (BAFF-R) provides a major signal in B cells that targets the activation of noncanonical NF-κBs (37, 97). Engagement of BAFF-R by its ligand BAFF stimulates both B-cell survival and upregulation of CD21 and CD23 (66). BAFF-induced B-cell survival is mediated through nuclear exclusion of PKCδ (131), although how BAFF induces CD21/CD23 expression is not completely understood. Since the CYLD−/− B cells displayed hyper-expression of CD21 and CD23 (see Fig. 3-3) and aberrant activation of the canonical and noncanonical NF-κBs (Fig. 3-6), we examined whether the loss of CYLD bypasses the requirement of BAFF for CD21/CD23 expression. We focused on CD23, since it was more drastically elevated in CYLD−/− B cells (see Fig. 3-3). In agreement with prior studies (66), the expression of CD23 was largely diminished when wildtype B cells were cultured in vitro for 48 hr in the absence of BAFF (Fig. 3-8, solid line, compare 0 hr with media 48 hr), whereas addition of recombinant BAFF to the cell culture efficiently maintained the expression of CD23 (Fig. 3-8, solid line, BAFF 48 hr). In contrast to wildtype B cells, CYLD−/− B cells did not require exogenous BAFF for CD23 expression (Fig. 3-8, dotted line, media 48 hr). Further, the CD23 expression in CYLD−/− B cells appeared to be already at maximal levels, since it was not further enhanced by recombinant BAFF (Fig. 3-8, dotted line, BAFF 48 hr). These results suggest that the loss of CYLD triggers BAFF-independent expression of CD23.
DISCUSSION

Protein ubiquitination plays an important role in modulating different aspects of immune responses (122). Although E3 ubiquitin ligases have been extensively studied, the role of their opposing enzymes, DUBs, is poorly defined. In the present study, we have shown that a recently identified DUB, CYLD, is crucial for regulation of B-cell activation and homeostasis. Loss of CYLD in mice results in lymphoid organ enlargement and accumulation of B cells with activated phenotypes. These symptoms are exacerbated when the mice are exposed to antigens, suggesting the involvement of abnormal immune reactions in triggering the lymphoid abnormalities. Indeed, when stimulated in vitro, the CYLD−/− B cells are hyperproliferative and exhibit aberrant signaling properties, particularly the activation of NF-κB. Since the CYLD−/− B cells exhibit spontaneous NF-κB activation and express surface activation markers, CYLD appears to be required for maintaining the naïve phenotype of B cells.

CYLD is known to positively regulate thymic TCR signaling and thymocyte development (166). Our present work suggests that like E3 ubiquitin ligases, the DUB CYLD may possess different cellular targets and mediate multiple functions in the immune system. In thymocytes, a major target of CYLD is the Src kinase Lck. By physically interacting with Lck and inhibiting Lck ubiquitination, CYLD facilitates the recruitment of active Lck to its downstream target ZAP-70 and thereby promotes TCR-proximal signaling. Although B cells have an Lck homologue, Lyn, we have not been able to demonstrate the binding of CYLD to Lyn under endogenous conditions. Further, CYLD is not required for B-cell development or BCR signaling. It is more likely that CYLD targets a different
molecule involved in NF-κB activation in B cells. Although the precise mechanism by which CYLD regulates NF-κB in B cells requires further investigations, we have obtained evidence that the major NF-κB inhibitor, IκBα, undergoes chronic phosphorylation and degradation in CYLD−/− B cells. Further, the IκBα degradation appears to be mediated through its phosphorylation by IKKβ, since a selective IKK inhibitor is able to block the degradation of IκBα and since IKKβ is constitutively activated in CYLD−/− B cells. Prior in vitro studies suggest that CYLD inhibits the ubiquitination of the IKK regulatory subunit, IKKγ, and negatively regulates NF-κB activation by innate immune stimuli (30, 102, 226), although this function of CYLD has not been confirmed using primary innate immune cells (166). The results of the current study raise the intriguing question of whether IKKγ serves as a target of CYLD in primary B cells. Unfortunately, we were unable to detect endogenous CYLD/IKKγ physical interaction in primary B cells, and others failed to detect this molecular interaction in cell lines (226). We also did not detect hyper-ubiquitination of IKKγ in CYLD−/− B cells. Whether these results are due to technical challenge with endogenous proteins or CYLD functions through novel targets remain to be further studied. Nevertheless, our data suggest that chronic phosphorylation of IκBα by IKKβ is a mechanism that mediates the aberrant activation of NF-κB caused by CYLD deficiency. Given the critical role of NF-κB in regulating lymphocyte activation (114), the constitutive NF-κB activity likely contributes to the activated phenotype of CYLD−/− B cells.
MATERIALS AND METHODS

Mice. Cyld knockout mice were generated as described (166). Cyld\(^{+/−}\) mice were intercrossed to generate Cyld\(^{−/−}\) and Cyld\(^{+/+}\) littermates. Genotyping was performed by PCR using tail DNA and the following primers. Cyld forward primer 1, CCA GGC ACT TTG AAT TGC TGT C; Cyld reverse primer 1, CGT TCT TCC CAG TAG GGT GAA G; Cyld reverse primer 2, GCA TGC TCC AGA CTG CCT TGG

When the three primers were used together, the PCR yielded a 209 bp product for Cyld\(^{+/+}\) mice, a 209 bp and a 255 bp product for Cyld\(^{+/−}\) mice, and a 255bp product for Cyld\(^{−/−}\) mice. Unless specified, mice were housed in specific pathogen-free cages and monitored periodically for the lack of common pathogens. For studies that involved housing of mice under conventional conditions, age- and sex-matched CYLD\(^{−/−}\) and wildtype mice were transferred from ventilated cages to conventional cages and housed for 6 weeks. Animal experiments were in accordance with protocols approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Antibodies and reagents. The anti-CYLD antibody was generated by injecting rabbits with a GST-fusion protein containing an N-terminal region of human CYLD (amino acid 136–301). Phospho-IκBα (Ser32) antibody was from Cell Signaling. Antibodies for actin (C-2), IKKβ (H470), tubulin (Tu-02), p50 (C-19), c-Rel (sc-70), and RelB (C-19) were purchased from Santa Cruz Biotechnology, Inc. Fluorescence-labeled anti-mouse antibodies used in flow cytometry included APC-anti-C19Rp (AA4.1), APC-anti-CD3 (145-2C11), PE.CY7-anti-CD19 (1D3), FITC-anti-CD21 (7G6), PE-anti-CD23 (B3B4), FITC-anti-CD80 (16-
10A1), PE-anti-CD86 (GL1), FITC-anti-IgD (11-26c.2a), and PerCP-Cy5.5-anti-IgM (R6-60.2). Anti-C19Rp, anti-CD80 and anti-CD86 were purchased from eBioscience, and the rest of the conjugated antibodies were from BD Biosciences. Unconjugated anti-IgM and anti-CD40, used for B-cell stimulation, were purchased from Jackson Immunoresearch and BD Biosciences, respectively. SRBC and human recombinant BAFF were purchased from Cocalico Biologicals, Inc. and Biosource, respectively. GST-IKKβ was cloned by inserting a cDNA fragment encoding amino acids 166-197 of human IKKβ into pGEX-4T vector (Pharmacia). Recombinant protein was produced in E. coli and purified using GST-sepharose. Cycloheximide was obtained from Sigma, and all other antibodies and reagents have been described previously (139, 166, 236).

**Flow cytometry.** Bone marrow cells were prepared as previously described (237). Spleen and MLN cell suspensions were prepared by gentle homogenization using a tissue homogenizer. Mononuclear cells were isolated by centrifugation over lymphocyte separation media (Cellgro). Peritoneal cells were isolated by flushing the peritoneal cavity using 10 ml of PBS. Flow cytometry was performed as previously described (166). The data shown in Fig. 3D were collected using FACSCalibur, and all the other data were generated using FACSCanto. For analyses of in vitro cultured B cells, the cells were incubated for 48 hr in Iscove’s media either in the presence or absence of BAFF (100 ng/ml) and then subjected to flow cytometry.

**Cell proliferation assays.** B cells were purified from splenocytes using anti-B220 conjugated magnetic beads (Miltenyl Biotec) and were stimulated in 4 replicate wells of 96-
well plates (1 x 10^5 cells/well) with anti-IgM (10 μg/ml), anti-CD40 (2 μg/ml), or LPS (3 μg/ml). After the indicated times of stimulation, the cells were labeled for 5 hr with ^3H-thymidine for proliferation assays based on thymidine incorporation.

For CFSE cell proliferation assay, purified splenic B cells were washed once with PBS (prewarmed to 37ºC) and incubated with CFSE (1.25 μg/ml in PBS) for 10 min at 37ºC. After two washes with Iscove’s media, the cells were stimulated as described above followed by flow cytometry to measure the CFSE intensity.

**Mouse immunization, immunohistochemistry, and antibody analyses.** Mice were injected i.p. with 0.2 ml of SRBC (1 x 10^9/ml in PBS) and sacrificed 6 days later. Spleens were frozen in Tissue-Tec OCT compound (VWR) using liquid nitrogen prechilled 2-methlbutane. The frozen tissues were stored at -70ºC until processed to produce 6-8 micron cryostat sections. The sections were stained with rat anti-mouse B220 (eBioScience) followed by biotinylated anti-rat immunoglobulin (Vector) or with biotin-conjugated hamster anti-mouse CD3 (eBioScience), biotin-conjugated PNA (Vector). The immunostaining were then detected with peroxidase-conjugated streptavidin using diaminobenzidine as chromagen (VECTASTAIN Elite ABC Kit, Vector).

For analyses of antibody responses, mice were injected i.p. with 0.2 ml of NP-KLH or NP-LPS (0.1 mg/ml in PBS). Sera were collected at the indicated times after immunization and subjected to ELISA to detect NP-specific antibodies using the SBA Clonotyping System (Southern Biotechnology, Inc.).
**IB and EMSA.** Purified B cells were stimulated with anti-IgM (2.5 μg/ml) or LPS (2.5 μg/ml) for the indicated times. Total and subcellular extracts were prepared from the cells and subjected to IB and EMSA as previously described (64, 187). In the case of protein phosphorylation analyses, cells were lysed in a kinase cell lysis buffer supplemented with phosphatase inhibitors (227). For antibody supershift assays, the nuclear extracts were premixed with 0.5 μl of the indicated antibodies for 8 min at room temperature and then mixed with the ³²P-radiolabeled κB oligonucleotide in EMSA buffer.

**In vitro kinase assays.** IKKβ was isolated by IP from untreated MLN B cells followed by analyzing its catalytic activity by in vitro kinase assays (227) using GST-IKKβ as substrate.

**RT-PCR.** Total cellular RNA was isolated from purified MLN B cells using the TRI reagent (Molecular Research Center, Inc.). Semi-quantitative RT-PCR was performed using the following primers to amplify murine CD23, IκBα and Gapdh.

*CD23* forward, 5’-GTG AGG ACT GTG TGA TGA TGC-3’; *CD23* reverse, 5’-GAG GAG AAA TCC AGA AGA GTG-3’; *IκBα* forward, 5’-CTT TTT GTT GTG AAA CTG AAG AGC TG-3’; *IκBα* reverse, 5’-CTT CAC AAA AGC AAC ATA GTG GC-3’; *Gapdh* forward, 5’-CTC ATG ACC ACA GTC CAT GCC ATC-3’; *Gapdh* reverse, 5’-CTG CTT CAC CAC CTC ATG ACC ACA GTC CAT GCC ATC-3’.
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Figure 3-1. Lymphoid organ abnormalities and B-cell hyperplasia in CYLD–/– mice. (A) Individual lymph nodes were dissected from the mesentery of wildtype (+/+) and CYLD–/– mice. This phenotype was observed in over 80% of the mice analyzed between 8 and 12 weeks of age (30 mice were analyzed). (B and C) MLN cells were stained for B (CD19) and T (CD3) cell markers. The percentages of T and B cells are shown in a representative flow cytometry profile (B), and both the percentages and absolute numbers of B cells are quantitated based on multiple animals with 8-12 weeks of age (C). (D) Splenocytes from +/+ and -/- mice (8-12 weeks of age) were analyzed by flow cytometry to determine the percentage of B cells (CD19+). The absolute numbers of B cells were calculated based on their percentage and the total numbers of splenocytes. Data are presented as mean ± s.d. of 5 animals in one experiment and are representative of 5 independent experiments. (E) Peritoneal cells were analyzed by flow cytometry based on expression of CD19 and B220 to detect the number of CD19hiB220lo B1 cells in wildtype and CYLD–/– animals (12 weeks of age).
Figure 3-1
Figure 3-2. Role of CYLD in B-cell development and maturation. (A and B) Bone marrow cells from CYLD knockout (-/-) and wildtype (+/+) mice were stained with fluorescence-conjugated anti-IgD and anti-IgM and subjected to flow cytometry to analyze the percentage of recirculating (RC), immature (IM), and early developing states of (ProPre) B cells (A). The data presented in B are mean ± s.d. of 4 animals in one experiment and are representative of 4 independent experiments. (C and D) Splenocytes from young wildtype and CYLD knockout mice (8 weeks old) were stained with the indicated antibodies and analyzed by flow cytometry to determine the percentage of different subpopulations of B cells as previously defined [Su, 2004 #2903]. FM, follicular mature (IgM\text{int}IgD\text{hi}); T1, transitional 1 (IgM\text{hi}/IgD\text{lo}, CD21\text{lo}/CD23\text{lo}); T2, transitional 2 (IgM\text{hi}/IgD\text{hi}); MZ, marginal zone (IgM\text{hi}/IgD\text{lo}, CD21\text{hi}/CD23\text{lo}); FO, follicular (CD21\text{int}CD23\text{hi}). Data are representative of 16 animals in four independent experiments. (E and F) Splenocytes from older wildtype and CYLD knockout mice (14 weeks) were stained with the indicated antibodies and analyzed by flow cytometry as in C and D.
Figure 3-2
**Figure 3-3. CYLD$^{-/-}$ B cells display activation markers.** (A) Splenocytes from wildtype (+/+) and CYLD knockout (-/-) mice were stained with fluorescence-conjugated antibodies for CD19, CD21, CD23, and AA4.1 and subjected to flow cytometry. The intensity of CD21 and CD23 was measured by gating on total B cells (Total, CD19$^+$), follicular B cells (FO, CD21$^{int}$CD23$^+$), and marginal zone B cells (MZ, CD21$^b$CD23$^l$). The CD23 expression in follicular B cells was further gated, based on AA4.1 expression, to immature (AA4.1+) and mature (AA4.1-) B cells (bottom panel). (B) Mesenteric lymph node cells were stained with fluorescence-labeled antibodies for CD19, CD21, and CD23 and subjected to flow cytometry to determine the level of CD21 and CD23 on CD19$^+$ cells. (C) RT-PCR analyses to measure the steady level of CD23 mRNA in purified MLN B cells. The house-keeping gene Gapdh was included as control. (D and E) Splenocytes were stained with antibodies for CD19, CD80, and CD86, and subjected to flow cytometry to measure the expression level of CD80 and CD86 on total splenic B cells (CD19$^+$) (D). The size of the wildtype and CYLD$^{-/-}$ B cells was measured by forward scatter (E).
Figure 3-3
Figure 3-4. CYLD+/– B cells are hyperproliferative upon in vitro stimulation.  (A) Wild-type (+/+ ) and CYLD knockout (-/-) splenic B cells were cultured for 48 hr either in media or media supplemented with anti-IgM (10 μg/ml), LPS (3 μg/ml), or anti-CD40 (2 μg/ml). Cell proliferation was measured by thymidine incorporation.  (B) Splenic B cells were labeled with CFSE and cultured for 48 hr in media or media supplemented with anti-IgM or LPS.  Cell proliferation was measured by flow cytometry based on the dilution of CFSE during cell division.  The intensity of CFSE is reduced to one half following each cell division.
Figure 3-4
Figure 3-5. Antigen exposure causes exacerbated splenomegaly and heightened B-cell responses in CYLD−/− mice. (A) Wildtype (+/+) and CYLD knockout (-/-) mice (6 weeks of age) were immunized (i.p.) with SRBC for 6 days or NP-LPS for 21 days or housed in conventional cages for 6 weeks. Pictures of representative spleens are shown for each group. (B) Immunohistochemistry of spleen sections from SRBC-immunized mice showing the staining of B-cell follicles cells (B220), T-cell zones (CD3), and germinal centers (PNA). (C) ELISA to measure the basal level of serum Ig isotypes in unimmunized wildtype (+/+) and CYLD knockout (-/-) mice (3 month of age). (D) ELISA to determine antibody responses. Wildtype (+/+) and CYLD knockout (-/-) mice (6 weeks of age) were immunized (i.p.) with NP-LPS or NP-KLH. Sera were collected after 2 weeks and subjected to ELISA to determine the titer of NP-specific antibody isotypes. Data are presented as mean ± s.d. of four animals.
Figure 3-5
**Figure 3-6. Deregulated activation of NF-κB in CYLD−/− B cells.** (A) B cells purified from the spleen of wildtype (+/+ ) and CYLD knockout (−/−) mice (8 weeks) were stimulated for 4.5 hr with media control, anti-IgM (2.5 μg/ml), or LPS (2.5 μg/ml), and nuclear extracts were subjected to EMSA using a 32P-radiolabeled κB probe. (B) Freshly purified B cells were lysed immediately for EMSA in order to detect the level of constitutive NF-κB activity in wildtype and mutant B cells. (C) Antibody supershift assay. EMSA was performed using a nuclear extract isolated from untreated CYLD−/− MLN B cells (used in B) either in the absence (none) or presence of pre-immune or different anti-NF-κB antibodies. The two NF-κB complexes (C1 and C2) and the supershifted bands are indicated. The immunoreactivity is determined by both the supershifts and the reduction in the intensity of C1 and C2 complexes. (D) Total cell lysates of untreated MLN or splenic (SP) B cells were subjected to IB using the indicated antibodies.
Figure 3-6
Figure 3-7. CYLD deficiency causes constitutive IKKβ activation and IκBα degradation. (A) IB was performed using total cell lysates prepared from untreated MLN (lanes 1 and 2) or splenic (lanes 3 and 4) B cells to show expression of IκBα and the loading control tubulin. (B) RT-PCR to show expression of IκBα and Gapdh mRNA in untreated MLN B cells. (C) IKKβ activation. IKKβ was precipitated from freshly isolated (untreated) CYLD+/− and wildtype control B cells and subjected to in vitro kinase assays (upper). The kinase assay membrane was analyzed by IB to show the IKKβ protein level (lower). (D) IκBα phosphorylation and degradation in CYLD−/− B cells. Purified splenic B cells were incubated for 60 min either in the absence (-) or presence (+) of a protein synthesis inhibitor, cycloheximide (CHX; 20 μg/ml). In lanes 3 and 6, the cells were preincubated for 60 min with an IKK inhibitor, PS1145 (10 μM), before the cycloheximide treatment. IB was performed using antibodies against total IκBα, phosphorylated IκBα (P-IκBα), or tubulin.
Figure 3-7
Figure 3-8. CYLD deficiency causes BAFF-independent expression of CD23. B cells purified from the spleens of wildtype (+/+) and CYLD knockout (-/-) mice were either immediately subjected to flow cytometry (0 hr) or incubated for 48 hr in vitro in media control (media 48 hr) or media supplemented with BAFF (100 ng/ml, BAFF 48 hr) and then subjected to flow cytometry. The surface expression of CD23 on B cells is presented as histograms.
Figure 3-8
CHAPTER IV

DEUBIQUITINATING ENZYME CYLD REGULATES RANK SIGNALING AND OSTEOCLASTOGENESIS

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I would like to thank my collaborators for their contributions to the following experiments

ABSTRACT

Osteoclastogenesis is a tightly regulated biological process, whose deregulation can lead to severe bone disorders such as osteoporosis. How the osteoclastic signaling events are regulated is incompletely understood. We show that genetic deficiency of CYLD, a recently identified deubiquitinating enzyme, causes aberrant osteoclast differentiation and severe osteoporosis. The CYLD-deficient osteoclast precursors are hyper-responsive to receptor activator of nuclear factor κB ligand (RANKL), producing extensively more and larger osteoclasts. CYLD expression is drastically upregulated in pre-osteoclasts, where it negatively regulates RANK signaling by inhibiting TRAF6 ubiquitination and activation of downstream signaling events. Interestingly, CYLD physically interacts with a signaling adaptor, p62, and thereby is recruited to TRAF6. These findings establish CYLD as a crucial negative regulator of osteoclastogenesis and suggest its involvement in the p62/TRAF6 signaling axis.
INTRODUCTION

Normal bone physiology is regulated by the harmonic actions of osteoblasts and osteoclasts (OCs), cells that mediate bone formation and bone resorption, respectively (69). Osteoclasts are multinucleated cells that are derived from macrophage precursors. Osteoclast differentiation is induced by receptor activator of NF-κB ligand (RANKL) in the presence of the macrophage growth factor, M-CSF (25, 221). Binding of RANKL to its receptor, RANK, stimulates receptor trimerization and recruitment of signaling adaptors, most importantly TNF receptor-associated factor 6 (TRAF6) (9). TRAF6 transduces the RANK-mediated signal by initiating a number of downstream signaling pathways, including those that activate the transcription factors NF-κB and AP-1 (9, 213). These transcription factors in turn induce the expression of a master osteoclastogenic regulator, nuclear factor of activated T cells c1 (NF-ATc1), and trigger a cascade of gene expression events required for osteoclast differentiation (213, 221, 256). Genetic deficiencies in TRAF6 or its downstream signaling factors attenuate osteoclast differentiation and bone resorption, a condition known as osteopetrosis (221, 225). On the other hand, excessive production or activation of osteoclasts can lead to uncontrolled bone resorption or osteoporosis. Thus, a fundamental understanding of RANK signaling is important for rational design of therapeutic approaches for the treatment of bone disorders.

Recent studies suggest that ubiquitination of TRAF6 is an important mechanism mediating its signaling functions (35, 104, 105). Lysine 63-linked polyubiquitin chains facilitate the association of TRAF6 with target signaling factors, such as IκB kinase (IKK), a multisubunit enzyme mediating activation of NF-κB (68). The regulatory subunit of IKK,
IKKγ (also known as NEMO), has intrinsic ubiquitin-binding activity and is thought to recruit the IKK catalytic subunits, IKKα and IKKβ, to ubiquitinated upstream regulators (272, 273). How the ubiquitination and signaling function of TRAF6 are regulated under physiological conditions, particularly during osteoclastogenesis, is incompletely understood. Nevertheless, an adaptor protein, p62 (also known as Sequestosome 1), has been shown to physically associate with TRAF6 and play both positive and negative roles in RANK signaling. Complete loss of p62 attenuates RANK signaling and osteoclastogenesis (55). On the other hand, mutations of p62 that disrupt its C-terminal ubiquitin association (UBA) domain cause aberrant RANK signaling and hyper-production of osteoclasts (103, 107, 161, 221, 259). Such genetic alterations of p62 are etiologically associated with development of Paget’s disease of bone (PDB), a severe bone disorder characterized by formation of giant osteoclasts, excessive bone resorption, and irregular bone formation (42, 107, 108). The positive signaling role of p62 appears to involve recruitment of atypical protein kinase Cs (PKCs) to TRAF6, which contributes to IKK activation by RANK (55). Although how p62 negatively regulates RANK signaling is unclear, one implication is that p62 may be involved in interaction with negative regulators.

An emerging family of signaling regulators involved in diverse biological processes are deubiquitinating enzymes (DUBs), which digest ubiquitin chains and reverse the process of protein ubiquitination ((146). One DUB, CYLD, has been implicated as an important regulator of immune response and oncogenesis (166, 269, 270, 271, 18, 129, 263). The signaling function of CYLD appears to be cell-type specific. Thus, CYLD negatively regulates the activation of IKK and c-Jun N-terminal kinase (JNK) in lymphocytes but has no obvious role in regulating these signaling events in macrophages (166, 269). In the
present study, we show that the expression level of CYLD is extremely low in macrophages but is markedly upregulated along with RANKL-induced osteoclastogenesis. We provide genetic evidence that CYLD is a crucial negative regulator of RANK signaling in pre-osteoclasts. The loss of CYLD in mice causes production of enlarged and hypernucleated osteoclasts, associated with severe osteoporosis. Interestingly, the adaptor protein p62 physically interacts with CYLD and promotes the binding of CYLD to TRAF6, and this novel molecular interplay requires the C-terminal region of p62. These findings establish CYLD as the first DUB that regulates osteoclastogenesis and suggest a potential mechanism mediating the negative signaling function of p62.
RESULTS

CYLD knockout mice develop osteoporosis.

To investigate the physiological functions of CYLD, we generated *CYLD* knockout mice (166). During the preparation of bone marrow cells, we noticed that the femurs of *CYLD* knockout (*CYLD*−/−) mice appeared to be more fragile. This finding prompted us to compare the bone mass and structure of *CYLD*+/- and wildtype (*CYLD*+/+) mice. Femurs of *CYLD*+/- and *CYLD*−/− mice were subjected to microcomputed tomography (microCT) analyses. The *CYLD*−/− mice exhibited severe loss of trabecular bone, as shown in representative images of 3-dimentional microCT reconstruction (Figure 4-1A). Consistently, the volume of trabecular bone per unit of metaphysis (BV/TV) was reduced almost by half in the *CYLD*−/− mice (Figure 4-1B). The mutant animals also displayed obvious abnormalities in several other parameters of trabecular bone architecture, including decreased trabecular number (Tb.N) and trabecular thickness (Tb.Th), but increased trabecular separation (Tb.Sp), which together suggested a reduced and more separated trabecular bone network. Structure model index (SMI) is scored from 0 to 3 for indication of increased fragility. Compared to wildtype femurs, the *CYLD*+/- femurs had dramatically higher SMI numbers and lower degree of anisotrophy (DA) and connectivity density (CD), indicating more fragile bone in the mutant animals (Figure 4-1B). In agreement with the trabecular bone data, analysis of the cortical bone at the midshaft level revealed a moderate but significant decrease in cortical bone thickness (Figure 4-1C, Tb. Th). The total volume (TV) of cortical bone and the volume of bone fraction (BV) were also reduced. However, the proportion of bone (BV/TV) remains the same between wildtype and *CYLD*+/- femurs,
indicating decreased diameter of $CYLD^{-/-}$ femurs but not defect in osteoblast function. These results suggest the loss of CYLD in mice leads to osteoporosis.

**CYLD$^{-/-}$ mice exhibit osteoclast abnormalities.**

To address the mechanism by which CYLD regulates bone density, we performed histological analyses of femurs from $CYLD^{+/+}$ and $CYLD^{-/-}$ mice. Consistent with the microCT results, hematoxylin and eosin (H&E) staining of femoral sections revealed a severe reduction in trabecular bone in $CYLD^{-/-}$ mice (Figure 4-2A). Moreover, the $CYLD^{-/-}$ trabecular bone contained increased osteoclast activity, as revealed by positive staining with tartrate-resistant acid phosphatase (TRAP) (Figure 4-2B). Additionally, the osteoclasts from $CYLD^{+/+}$ mice were markedly enlarged in size (Figure 4-2C). On the other hand, bone nodule assay to test the ability of osteoblasts to form mineralized bone in vitro did not show obvious difference between the $CYLD^{-/-}$ and wildtype mice (Supplemental Figure 4-1A). Furthermore, the $CYLD^{-/-}$ mice only had a weak increase in serum concentration of osteocalcin, an indicator of osteoblast activity (Supplemental Figure 4-1B). Thus, as seen with mice expressing a p62 mutant (103), the $CYLD^{-/-}$ mice have abnormalities predominantly in osteoclasts, leading to osteoporosis.

**CYLD negatively regulates osteoclastogenesis.**

The osteoclast abnormalities in $CYLD^{-/-}$ mice led us to examine the role of CYLD in regulating osteoclast differentiation. Bone marrow derived macrophages (BMDMs) were cultured in M-CSF growth medium either in the absence or presence of RANKL. As expected, RANKL induced the generation of TRAP-positive osteoclasts at a concentration
of 100 ng/ml (Figure 4-3A). Remarkably, the same concentration of RANKL induced a drastically higher number of osteoclasts in the CYLD−/− BMDMs (Figure 4-3A). The CYLD−/− osteoclasts were also notably larger (Figure 4-3A) and contained substantially more nuclei than the control osteoclasts (Figure 4-3B). A dose-responsive assay revealed that loss of CYLD also rendered the cells responsive to lower doses of RANKL (Figure 4-3C). These results were not due to alteration in the surface expression of RANK (Supplemental Figure 4-2). The level of M-CSF receptor (M-CSFR) is also similar between the wildtype and CYLD−/− cells (Supplemental Figure 4-2). Consistent with these results, overexpression of CYLD in wildtype osteoclast precursor cells strongly inhibited the osteoclastogenesis (Supplemental Figure 4-3).

We next performed real-time PCR to examine the effect of CYLD deficiency on the induction of osteoclastogenic markers, including the early markers TRAP and cathepsin K and the late marker calcitonin receptor. Following RANKL stimulation, the expression of TRAP and cathepsin K was increased within 1-2 days, whereas the induction of calcitonin receptor occurred at 2-3 days (Figure 4-3D). Importantly, induction of both the early and late osteoclastogenic markers was greatly potentiated in CYLD−/− cells (Figure 4-3D). Together, these results suggest a crucial role for CYLD in negatively regulating osteoclastogenesis.

**CYLD negatively regulates RANK signaling in pre-osteoclasts.**

To elucidate the molecular mechanism by which CYLD negatively regulates osteoclastogenesis, we examined the effect of CYLD deficiency on RANKL-stimulated signaling events. Stimulation of wildtype BMDMs with RANKL results in rapid activation
of MAP kinases (MAPKs), including ERK, JNK, and p38 (Figure 4-4A) as well as NF-κB (Figure 4-4B). The **CYLD** deficiency had no obvious effect on the early activation of MAPKs (Figure 4-4A). Similarly, during the initial phase of RANKL stimulation in BMDMs, the loss of CYLD did not significantly affect the activation of NF-κB (Figure 4-4B). Another transcription factor, AP-1, was not detected under the short-term stimulation conditions. Thus, CYLD is not involved in regulation of the initial phase of RANK signaling in BMDMs.

We next examined the role of CYLD in RANK signaling in developing osteoclasts, hereafter referred to pre-osteoclasts. Active NF-κB and AP-1 were detected in the nuclei of pre-osteoclasts exposed to RANKL for 2 days (Figure 4-4C). Interestingly, the activity of both NF-κB and AP-1 was markedly enhanced in **CYLD**–/– cells. Recent studies suggest that osteoclast development involves induction of NF-ATc1, a master regulator of osteoclastogenesis (213, 214). Since NF-κB and AP-1 have been implicated in the induction of NF-ATc1, we examined whether the loss of CYLD also promoted NF-ATc1 induction. Indeed, substantially higher levels of NF-ATc1 were induced by RANKL in **CYLD**–/– cells than in the control cells (Figure 4-4D). On the other hand, the loss of CYLD did not affect the induction of TRAF6 or constitutive expression of the protein kinase Tak1 (Figure 4-4D). These results suggest that CYLD regulates the sustained activation of NF-κB and AP-1 and induction of NF-ATc1 in pre-osteoclasts, which provides a molecular insight into the role of CYLD in regulating osteoclastogenesis.
**Induction of CYLD expression along with osteoclast differentiation.**

Because of the distinct roles of CYLD in BMDMs and pre-osteoclasts, we examined its expression pattern along with RANKL-induced osteoclast differentiation. Consistent with its dispensable role during the early phase of RANK signaling, the level of CYLD was considerably low in BMDMs (Figure 4-5A). Interestingly, CYLD expression was markedly induced after 1 day of RANKL stimulation and further upregulated after prolonged stimulation (Figure 4-5A). The RANKL-induced upregulation of CYLD protein was associated with induction of *CYLD* mRNA, as determined by parallel real-time PCR assays (Figure 4-5B). This pattern of CYLD expression provides a good explanation for its specific signaling function in pre-osteoclasts.

Since RANKL stimulates rapid activation of both canonical NF-κB and MAPKs in BMDMs, the delayed induction of CYLD expression suggested the requirement of additional regulators. As an initial approach to address this issue, we examined whether CYLD expression is also induced by other macrophage inducers, including the bacterial endotoxin LPS and the proinflammatory cytokine TNF-α. In contrast to RANKL, neither TNF-α nor LPS significantly induced the expression of CYLD (Figure 4-5C). On the other hand, LPS potently induced the expression of a known proinflammatory mediator, iNOS, and both TNF-α and LPS induced the expression of the *nfkb2* gene product p100 (Figure 4-5C). Thus, the induction of CYLD appeared to be specifically mediated by the osteoclast inducer RANKL.

An important feature of RANKL-stimulated signaling is the induction of non-canonical NF-κB (148), a process that is regulated by NF-κB inducing kinase (NIK) and involves processing of the NF-κB2 precursor protein p100 to generate p52 (251). To
examine the involvement of noncanonical NF-κB in CYLD induction, we examined RANKL-stimulated expression of CYLD in NIK−/− and control BMDMs. As expected, RANKL stimulated the processing of p100 (generation of p52) in wildtype but not NIK−/− cells (Figure 4-5D). Interestingly, the RANKL-induced expression of CYLD was severely attenuated, although not completely blocked, in NIK−/− cells. Thus, optimal induction of CYLD by RANKL requires the noncanonical NF-κB signaling pathway, which may function in cooperation with the canonical NF-κB and other signaling pathways.

**CYLD is assembled into the TRAF6 complex and negatively regulates TRAF6 ubiquitination in pre-osteoclasts.**

TRAF6 is a master signaling molecule controlling multiple downstream pathways induced by RANKL. Ubiquitination of TRAF6 plays an important role in its signaling function, including the regulation of osteoclastogenesis (35, 104, 105). The finding that CYLD negatively regulates RANK signaling and osteoclastogenesis prompted us to examine whether the loss of CYLD altered ubiquitination of TRAF6 in pre-osteoclasts. As expected, polyubiquitinated TRAF6 was detected in RANKL-induced pre-osteoclasts (Figure 4-6A). Importantly, substantially more ubiquitinated TRAF6 was accumulated in CYLD−/− cells (Figure 4-6A). Moreover, the TRAF6 ubiquitination in CYLD−/− cells was more persistent than in CYLD+/+ cells, as revealed by chasing the cells in RANKL-free media (Figure 4-6A, lanes 5 and 6). Consistent with these results, transfected CYLD potently inhibited RANK-induced TRAF6 ubiquitination (Supplemental Figure 4-4). Thus, CYLD plays a non-redundant role in suppressing the ubiquitination of TRAF6 during
RANKL-induced osteoclast differentiation, a finding that provides a molecular insight into the mechanism by which CYLD regulates osteoclastogenesis.

We next examined the physical association between CYLD and TRAF6. The expression level of both CYLD and TRAF6 was up-regulated in RANKL-induced pre-osteoclasts (Figure 4-6B, middle and bottom panels). Moreover, TRAF6 and CYLD were co-precipitated (Figure 4-6B, top panel), indicating their presence in the same complex. Together, these results emphasize a physiological role of CYLD in negatively regulating TRAF6 ubiquitination mediated by RANKL.

**P62 physically interacts with CYLD and promotes the CYLD/TRAF6 association.**

Prior studies have identified p62 as a component of the TRAF6 complex during osteoclastogenesis (55). Like CYLD, p62 is markedly induced during RANKL-stimulated osteoclast differentiation (Figure 4-6C and ref. (55)). Since p62 is known as an adaptor of TRAF6, we examined whether CYLD was physically associated with p62. Indeed, a stable complex of p62 and CYLD was readily identified from pre-osteoclasts by coimmunoprecipitation (coIP) assays (Figure 4-6C). The p62/CYLD interaction was also demonstrated using transfected 293 cells (Figure 4-6D). Interestingly, a p62 mutant lacking its C-terminal UBA domain (p62ΔUBA) was largely defective in CYLD binding (Figure 4-6D). This phenotype of p62ΔUBA was not due to the variation in its expression, since this mutant was expressed even at higher levels than the wildtype p62.

The strong interaction of p62 with CYLD led us to examine whether p62 facilitates the assembly of CYLD into the TRAF6 complex. We found that in 293 cells, endogenous TRAF6 did not appreciably associate with CYLD (Figure 4-6E, top panel, lane 2), a finding
that was consistent with a previous report (102). Remarkably, however, expression of p62 in the cells induced the complex formation between CYLD and TRAF6 (Figure 4-6E, top panel, lane 3 and Supplemental Figure 4-5). Furthermore, this function of p62 was correlated with its CYLD-binding activity, since it was not seen with the p62ΔUBA mutant (lane 4). Thus, p62 appears to function as an adaptor recruiting CYLD to TRAF6, thus providing a molecular insight into the negative function of p62 in osteoclastogenesis. On the other hand, the association of p62 with TRAF6 was not affected by CYLD (Supplemental Figure 4-6). CYLD was also not involved in RANK-mediated activation of PKCζ (Supplemental Figure 4-6), which is thought to involve the positive signaling function of p62. These findings suggest that CYLD may specifically mediate the negative signaling function of p62 by deubiquitinating TRAF6.
Osteoclastogenesis is a tightly controlled biological process that involves both positive and negative regulation. Compared to the positive players, the negative regulators of this process are relatively less well understood. Prior studies have identified the suppressive role of SHIP-1 and IRAK-M in osteoclast formation. Both proteins are abundantly expressed in macrophages, the osteoclast precursors, and have inhibitory roles in innate immune signaling (99, 199). SHIP-1 controls osteoclastogenesis by targeting M-CSF receptor signaling, while IRAK-M acts by inhibiting IL-1 receptor pathway (112, 216, 268). Our present study suggests that CYLD, unlike SHIP-1 or IRAK-M, functions as a specific negative regulator of osteoclast development by suppressing RANK signaling.

We have previously shown that loss of CYLD in macrophages has no obvious effect on the signaling events mediated by innate immune stimuli, such as TNF-α and LPS (166, 269). Similarly, we have found in the present study that CYLD does not regulate RANKL-stimulated initial signaling in BMDMs. In sharp contrast, CYLD has a crucial role in controlling RANKL-stimulated signaling in pre-osteoclasts. This functional diversity of CYLD is likely due to its differential expression. The level of CYLD is very low in BMDMs but is drastically up-regulated in pre-osteoclasts. Interestingly, the induction of CYLD expression is specific for RANKL signal, as it is not seen with the proinflammatory stimuli TNF-α and LPS, which potentially explains the functional specificity of CYLD in RANK signaling. Although how RANKL specifically induces the expression of CYLD is incompletely understood, our data suggest the requirement of non-canonical NF-κB
signaling pathway. Thus, as depicted in Figure 4-7, CYLD is a novel target gene of RANK signaling that is involved in the feedback inhibition.

The expression pattern of CYLD is reminiscent of p62 (55), a signaling adaptor with positive and negative roles in regulating osteoclastogenesis. P62 physically associates with TRAF6 and appears to promote RANK signaling by recruiting atypical PKCs to TRAF6 (55). Under overexpression conditions, p62 also induces the self-ubiquitination activity of TRAF6, although the physiological relevance remains unclear (247). How p62 negatively regulates RANK signaling has remained poorly understood, although this function of p62 is known to require its C-terminal region (103, 107, 161, 221, 259). Our present study shows that p62 physically interacts with CYLD and promotes the binding of CYLD to TRAF6. CYLD is assembled into the TRAF6 complex and plays an essential role in preventing excessive ubiquitination of TRAF6 in pre-osteoclasts. Moreover, the C-terminal region of p62 is essential for its adaptor function to promote CYLD/TRAF6 interaction. These findings suggest an intriguing molecular interplay between p62 and CYLD and shed light on how p62 negatively regulates RANK signaling (Figure 4-7).

A recent study demonstrates that transgenic mice expressing a mutated form of p62 develop osteoporosis, although the p62 mutation is insufficient to cause the full PDB phenotype (103). In particular, the p62 mutation causes abnormal osteoclastogenesis but does not enhance osteoblast numbers and activity, as seen in PDB. These findings suggest that individuals with p62 mutations may be predisposed to, but insufficient for, the development of PDB. Our data indicate that CYLD may mediate an important part of the negative signaling functions of p62. As seen with the p62-mutant transgenic mice, CYLD–/– mice spontaneously develop osteoporosis, although they do not show significant
abnormalities in osteoblast development and function. Our in vitro studies further reveal that the *CYLD* deficiency strongly promotes osteoclastogenesis, causing the formation of increased numbers of large and multinucleated osteoclasts. These phenotypes are associated with aberrant RANK signaling. It is likely that the enhanced RANK signaling in *CYLD*−/− cells promotes formation of mononuclear pre-osteoclasts, which in turn fuse to form the multinucleated osteoclasts. Since larger osteoclasts have been reported in diseases associated with excessive bone resorption, such as PDB, our present study suggests *CYLD* as a potential genetic factor involved in the development of bone disorders.
METHODS

**Mice.** *CYLD* knockout mice were generated as described (166). Heterozygous (*CYLD*+/−) mice were intercrossed to generate *CYLD*−/− and *CYLD*+/+ littermates, and genotyping was performed by PCR using tail DNA (269). *NIK* knockout mice were provided by Amgen Inc. All mice were housed in specific pathogen-free cages and monitored periodically (every 3 month) for the lack of common pathogens. Animal experiments were performed in accordance with protocols approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

**Plasmids, antibodies, and reagents.** Expression vectors encoding pEGFP-p62 and pEGFP-p62ΔUBA were provided by Dr. Jiake Xu and described previously (259), and the expression vectors for FLAG-tagged p62 and FLAG-tagged RANK were provided by Dr. Gregory D. Longmore and Dr. Yongwon Choi. pCLXSN(GFP), pCLXSN(GFP)-CYLD, pcDNA-HA-CYLD, and pcDNA-HA-CYLD 1-932 (a catalytically inactive mutant) were described previously (164, 165). PE-conjugated anti-mouse RANK (clone IK22/5) and anti-mouse M-CSFR (CD115, clone AFS98) were from eBioscience. Antibodies for iNOS (M-19), TRAF6 (H274), Lamin B (H-90), p62 (SQSTM1, D-3), anti-PKCζ (C-20), and Tubulin (TU-02) were purchased from Santa Cruz. The antibody for phospho-PKCζ (anti-P-PKCζ, ζ Thr410) was from Cell Signaling Technology. Anti-ubiquitin, anti-NF-ATc1 (NF-AT2), and anti-Tak1 were provided by Drs. Vincent Chau, Nancy Rice, and Jun Ninomiya-Tsuji, respectively. Other antibodies have been described previously (237, 251). LPS (E. coli 0127:B8) and murine TNF-α were from Sigma and PeproTech, respectively. Recombinant...
M-CSF and GST-RANKL were generous gifts from Dr. Steven Teitelbaum.

**Cell line and transfection.** Human embryonic kidney cell line 293T was cultured in DMEM media with 5% FBS. The cells were seeded in 12-well plates and transfected using Lipofectamine-2000 (Invitrogen).

**MicroCT.** Femurs of age-matched CYLD⁺/⁺ and CYLD⁻/⁻ male mice (14 weeks of age; 7 mice/group) were subjected to analysis by using a Scanco vivaCT 40 scanner (Scanco Medical, Bassersdorf, Switzerland). Distal femoral metaphysis, between 263 μm to 1040 μm from the growth plate, was assessed for trabecular bone architecture. Femoral mid-diaphysis was analyzed for cortical bone morphology. Data were analyzed for statistical significance using Mann-Whitney test (GraphPad Prism, San Diego, CA).

**Histology.** Femurs of age-matched CYLD⁺/⁺ and CYLD⁻/⁻ male mice (14 weeks of age) were fixed in 10% neutral buffered formalin for 2 days, decalcified in 10% EDTA for 10 days, and embedded in paraffin. 6 μm sections were cut and subjected to hematoxylin-eosin (H&E) and tartrate-resistant acid phosphatase (TRAP) staining using a TRAP assay kit (Sigma).

**Bone marrow culture, retroviral infection, and in vitro osteoclastogenesis.** BMDMs were prepared from femurs and tibia of age-matched CYLD⁺/⁺ and CYLD⁻/⁻ mice (4-10 weeks of age) as previously described (237). For *in vitro* osteoclast differentiation, the cells were cultured in 6-well plates (1 x 10⁶/well) in DMEM growth media supplemented with
15-30 ng/ml M-CSF and the indicated amounts of GST-RANKL. After 4-7 days, osteoclasts were visualized by TRAP staining using a commercial kit (Sigma).

Retroviral infection of bone marrow cells was essentially as described (170, 237) except for the use of pCL-Eco (143) as the packaging plasmid. Briefly, bone marrow cells were cultured in macrophage growth media using 6-well plates. The cells were infected twice, on day 3 and 4, with pCLXSN(GFP) or pCLXSN(GFP)-CYLD and cultured in DMEM growth media supplemented with M-CSF and GST-RANKL. Osteoclasts were visualized by fluorescence microscopy.

**Flow cytometry.** BMDM were stained with PE anti-mouse RANK or PE anti-mouse M-CSFR and subjected to flow cytometry analyses as previously described (166).

**IB, electrophoresis mobility shift assay (EMSA), and ubiquitination assay.** Whole-cell lysates were prepared from BMDM or pre-osteoclasts in a cell lysis buffer supplemented with phosphatase inhibitors, and subjected to IB as previously described (227). Nuclear extracts were prepared and subjected to EMSA using a $^{32}$P-radiolabeled oligonucleotide probes for NF-kB (CAA CGG CAG GGG AAT TCC CCT CTC CTT) and AP-1 (GAT CTA GTG ATG AGT CAG CCG).

For ubiquitination assays, cells were lysed in kinase lysis buffer supplemented with 1mM N-ethylmaleimide (NEM). TRAF6 was immunoprecipitated using TRAF6 specific antibody, and the ubiquitin-conjugated TRAF6 was detected by IB using anti-ubiquitin antibody.
**Real-time quantitative RT-PCR.** Total RNA was isolated from pre-osteoclasts using TRI reagent (Molecular Research Center, Inc.) and subjected to cDNA synthesis using RNase H-reverse transcriptase (Invitrogen) and oligo (dT) primers. The initial quantity of cDNA samples was calculated from primer-specific standard curves with iCycler Data Analysis software. Real-time quantitative PCR was performed using iCycler Sequence Detection System (Bio-Rad) and RT² Real-Time™ SYBR green PCR master mix (Superarray). The expression of individual genes was calculated by a standard curve method and normalized to the expression of actin for Fig 4-3B and GAPDH for Fig 4-5B. Data are presented as fold change between the RANKL-induced cells (both CYLD\(^{+/+}\) and CYLD\(^{-/-}\)) and the unstimulated CYLD\(^{+/+}\) cells. The gene-specific primer sets (all for murine genes) were:

**CYLD,** 5’-GGACAGTACATCCAAGACC-3’ and 5’-GAACCTGCATGCGGTTGCTC-3’;
**TRAP,** 5’-TGGTCCAGGAGCTTAACTGC-3’ and 5’-GTCAGGAGTGGGAGCCATATG-3’;
**cathepsin,** K 5’-GTGGGTGTGTTCAAGTTTCTGC-3’ and 5’-GGTGAGTCTTTCTCATTAGC-3’;
**calcitonin receptor,** 5’-CTCCAACAAGGTGCTTGGA-3’ and 5’-GAAGCAGTAGATAGTCGCA-3’;
**Actin,** 5’-CGTGAAAAGATGACCCAGATCA-3’ and 5’-CACAGCCTGGATGGCTACGT-3’;
**GAPDH,** 5’-CTCATGACCACAGTCCCATGCCATC-3’ and 5’-CTGCTTCACCACCTTCTTGATGTC-3’.
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Figure 4-1. Bone loss in CYLD<sup>−/−</sup> mice. Age-matched CYLD<sup>+/−</sup> and CYLD<sup>−/−</sup> male mice (14 weeks of age, 7 per group) were subjected to microCT analysis. (A) Representative images of three-dimensional microCT reconstruction of trabecular bone 263 μm above the distal femoral growth plate showing the severe bone loss in CYLD<sup>−/−</sup> mice. (B) Parameters of trabecular bone mass, including bone volume fraction (BV/TV), trabecular numbers (Tb. N), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp), connectivity density (CD), structure model index (SMI), and degree of anisotrophy (DA). (C) Parameters of cortical bone mass, including bone volume fraction (BV/TV), total volume (TV), bone volume (BV), and cortical bone thickness (Tb. Th).

*p<0.05, **p<0.02, ***p<0.002. Error bars represent standard error.
Figure 4-1
Figure 4-2. *CYLD*<sup>−/−</sup> mice contain enlarged osteoclasts. (A) H&E staining of femoral sections of age-matched *CYLD*<sup>+/+</sup> and *CYLD*<sup>−/−</sup> mice (14 weeks old), showing reduced trabecular bone (white arrowheads) in *CYLD*<sup>−/−</sup> femur. White arrows indicate bone marrow. (B) Increased TRAP activity in *CYLD*<sup>−/−</sup> bone compared to *CYLD*<sup>+/+</sup> (black arrows). 30x magnification. (C) Higher magnification (150x) images to show the enlarged TRAP positive osteoclasts in two different *CYLD*<sup>+/+</sup> and *CYLD*<sup>−/−</sup> mice.
Figure 4-2
Figure 4-3. Enhanced osteoclast differentiation from CYLD<sup>−/−</sup> bone marrow cells. (A) Bone marrow cells derived from age-matched CYLD<sup>+/+</sup> and CYLD<sup>−/−</sup> mice were cultured in M-CSF media either in the absence (NT) or presence of 100 ng/ml of GST-RANKL for 4 days and then subjected to TRAP staining. The images of RANKL-stimulated cells are presented as lower (20x) and higher (100x) magnifications. (B) Average number of nuclei per osteoclast calculated based on counting in 15 CYLD<sup>+/+</sup> and 15 CYLD<sup>−/−</sup> osteoclasts. (C) Bone marrow cells derived from CYLD<sup>+/+</sup> and CYLD<sup>−/−</sup> mice were cultured in M-CSF media supplemented with the indicated amounts of GST-RANKL. After 7 days, osteoclasts were detected by TRAP staining. Note that this experiment use a longer differentiation time (7 days) than that shown in Fig. 3A in order to detect osteoclasts in the wildtype cell culture at low doses of GST-RANKL. (D) Real-time RT-PCR was performed using RNA isolated from BMDMs or BMDMs cultured in the presence of both M-CSF and 100 ng/ml GST-RANKL for the indicated time. The relative mRNA level of individual genes was expressed as fold of induction compared to NT CYLD<sup>+/+</sup> cells. Data represent mean values of three independent experiments with error bars indicated.
Figure 4-3
Figure 4-4. RANK signaling is normal in BMDMs but aberrant in pre-osteoclasts in the absence of CYLD. (A) BMDMs were cultured in M-CSF-containing media and stimulated with GST-RANKL (100 ng/ml) for the indicated times and subjected to IB assays using phospho-specific (P-) or regular antibodies for the indicated MAPKs or tubulin. (B) BMDMs were stimulated with GST-RANKL for the indicated times as in A, and the nuclear NF-κB DNA binding activity was detected by EMSA. (C) BMDMs were cultured for 2 days in M-CSF medium lacking (NT) or containing the indicated amounts of GST-RANKL. Nuclear extracts were subjected to EMSA to detect the activation of NF-κB or AP-1. An IB of Lamin B was included as a loading control. (D) BMDMs were cultured in M-CSF medium for 2 days in the absence of RANKL (NT) or for the indicated days in the presence of RANKL. Total cell lysates were subjected to IB to detect the indicated proteins.
Figure 4-4
Figure 4-5. Induction of CYLD expression by RANKL but not by LPS or TNF-α. (A) Wildtype BMDMs were cultured in M-CSF-containing medium in the absence of RANKL (NT) or for the indicated times in the presence of GST-RANKL (100 ng/ml). Total cell lysates were subjected to IB to detect CYLD and tubulin. (B) Real-time RT-PCR was performed using RNA isolated from cells as described in A to determine the relative expression of CYLD mRNA. (C) Wildtype BMDMs were cultured in M-CSF media for 1 day either in the absence (NT) or the presence of the indicated inducers. Total cell lysates were subjected to IB to detect the indicated proteins. (D) BMDMs prepared from NIK knockout (−/−) and control (+/+ ) mice were cultured for 1 day in the absence (−) or presence (+) of GST-RANKL (100 ng/ml) followed by IB to detect the expression of CYLD (panel 1) or processing of p100 (panels 2 and 3). A tubulin IB was used as a loading control.
Figure 4-5
**Figure 4-6. CYLD targets TRAF6, which is promoted by p62.** (A) Wildtype and CYLD<sup>−/−</sup> BMDMs were cultured for 2 days in M-CSF media either in the absence (−) or presence (+) of GST-RANKL. TRAF6 was isolated by IP followed by IB to detect ubiquitin-conjugated TRAF6 (upper) or unmodified TRAF6 (lower). In lanes 5 and 6, the RANKL treated cells were chased in RANKL-free media over night before the TRAF6 ubiquitination assay. (B) Wildtype BMDMs were cultured for 2 days in M-CSF media in the absence (−) or presence (+) of GST-RANKL. TRAF6 was isolated by IP followed by detecting the associated CYLD by IB (top panel). The lysates were subjected to IB to monitor the expression of CYLD and TRAF6 (middle and bottom panels). (C) Wildtype BMDMs were cultured for 2 days in M-CSF media in the absence (−) or presence (+) of GST-RANKL. CYLD was isolated by IP followed by detecting the associated p62 by IB (top panel). The cell lysates were subjected to IB to monitor the expression of CYLD and p62 (middle and bottom panels). (D) 293 cells were transfected with CYLD along with either vector control (V), wildtype (WT) p62, or p62<UBA>. CYLD was isolated by IP, and its associated p62 was detected by IB (top panel). Protein expression in cell lysates were monitored by direct IB (middle and bottom panels). (E) 293T cells were transfected with either an empty vector (V) or expression vectors encoding wildtype (WT) p62 or p62<UBA>. Lanes 2-4 were also transfected with CYLD. Endogenous TRAF6 was isolated by IP followed by IB to detect the associated CYLD or the precipitated TRAF6 (top two panels). The cell lysates were subjected to IB to detect the expression of CYLD, EGFP-tagged p62 proteins, and TRAF6 (bottom three panels).
Figure 4-6
Engagement of RANK by RANKL induces TRAF6 ubiquitination and activation of downstream signaling molecules, leading to induction of genes involved in osteoclastogenesis. The RANK signaling also results in upregulation of CYLD as well as p62. CYLD targets TRAF6 via the assistance of p62, thereby negatively regulating TRAF6 ubiquitination and RANK signaling.
Figure 4-7
Supplementary Figure 4-1. Effect of CYLD deficiency on osteoblastic differentiation and serum osteocalcin. (A) Bone marrow cells (5 X 10^3/well in 6-well plates) were cultured in MEMa medium supplemented with 10% FBS, 1% penicillin/streptomycin, b-glycerophosphate (10mM) and L-ascorbic acid phosphate (50mg/ml) for 21 days, then fixed with 4% paraformaldehyde, and stained by the von Kossa method to detect mineralized bone nodule formation. Cells in the upper three wells were from CYLD^{+/+} mice, and cells in the bottom three wells were from CYLD^{--} mice. (B) Sera were collected from CYLD^{++} and CYLD^{+-} mice (3 of each genotype) and subjected to ELISA to measure osteocalcin concentration.
Supplementary Figure 4-2. Inhibition of osteoclast differentiation by overexpressed CYLD. Wildtype bone marrow derived macrophages were infected with a retroviral vector carrying a GFP marker gene, pCLXSN(GFP), or the same vector encoding CYLD, pCLXSN(GFP)-CYLD. Infected cells were cultured for 4 days in DMEM media supplemented with either MCSF or MCSF plus RANKL and subjected to fluorescence microscopy. CYLD-infected cells form substantially fewer and smaller osteoclasts (arrowheads).
Supplementary Figure 4-3. Surface expression of RANK and M-CSF receptor (M-CSFR) on osteoclast precursors. Bone marrow derived macrophages prepared from CYLD<sup>−/−</sup> and CYLD<sup>+/−</sup> mice were either unstained (background) or stained with PE-conjugated anti-mouse RANK or PE-conjugated anti-mouse M-CSFR and subjected to flow cytometry analysis.
Supplementary Figure 4-4. Inhibition of RANK-induced TRAF6 ubiquitination by transfected CYLD. 293 cells were transfected with HA-tagged ubiquitin along with the indicated expression vectors. Endogenous TRAF6 was isolated by IP using anti-TRAF6, and its ubiquitination was detected by IB using HRP-conjugated anti-HA. Expression of CYLD, a catalytically inactive CYLD mutant (1-932), and RANK were monitored by IB.
Supplementary Figure 4-5. CYLD interacts with TRAF6. 293 cells were transfected with (+) or without (−) the indicated expression vectors. Endogenous CYLD complex was isolated by IP, and the precipitated CYLD and CYLD-associated TRAF6 was detected by IB using anti-CYLD and anti-FLAG, respectively (top two panels). Expression of TRAF6 in the lysates was analyzed by direct IB (bottom panel).
Supplementary Figure 4-6. CYLD does not regulate the p62/TRAFl6 association or activation of PKCζ. (A) 293 cells were transfected with the indicated expression vectors. TRAF6 complexes were isolated by IP using anti-TRAFl6, and the associated p62 was detected by IB using anti-FLAG (panel 1). The levels of precipitated TRAF6 and p62 and CYLD in the lysates were monitored by IB. (B) BM6M derived from CYLD+/- or CYLD−/− mice were cultured for 2 days either in the presence (+) or absence (−) of GST-RANKL. Phosphorylated and total PKCζ were detected by IB using the indicated antibodies.
CHAPTER V

OVERVIEW AND DISCUSSION
5.1 Overview of Major Findings

The primary goal of my thesis research is to understand the physiological function of deubiquitinating enzyme CYLD and elucidate potential mechanisms by which CYLD participates in signal transduction. To address these questions, I have employed both the CYLD knockout mouse model and different approaches to demonstrate the function of CYLD in regulating lymphocyte activation and osteoclastogenesis. Additionally, my thesis research has also led to the discovery of important signaling mechanisms of CYLD. The major novel findings are summarized as follows.

1. Loss of CYLD results in reduced peripheral T cells, however, CYLD\textsuperscript{−/−} T cells are hyperresponsive to TCR stimulation. The hyperactivated T cells in CYLD\textsuperscript{−/−} mice mediate autoimmune intestinal inflammation resembling human inflammatory bowel disease. Mechanistically, loss of CYLD causes constitutive activation of JNK and NF-κB, which is mediated by upstream kinase TAK1. CYLD physically interacts with and deubiquitinates TAK1, thereby inhibiting TAK1 kinase activity.

2. CYLD is a critical regulator of B cell homeostasis in the periphery but largely dispensable for B cell development in bone marrow. CYLD\textsuperscript{−/−} mice develop B cell hyperplasia, with dramatically enlarged mesenteric lymph nodes and increased marginal zone B cells in spleen. Consistently, CYLD\textsuperscript{−/−} mice display increased antibody production upon immunization. NF-κB is constitutively activated in CYLD\textsuperscript{−/−} B cells.
3. CYLD plays an indispensable role in maintaining normal processes of osteoclastogenesis and bone homeostasis. CYLD$^{-/-}$ mice spontaneously develop osteoporosis. Consistently, the development of bone-resorbing osteoclasts is significantly enhanced both in vivo and in vitro in the absence of CYLD. Function of CYLD in osteoclastogenesis involves in its deubiquitination and inactivation of TRAF6, a key signaling component of RANK pathway. CYLD protein level is low in macrophages, the osteoclast precursor cells, but is significantly induced upon RANKL stimulation. The increase of CYLD protein level correlates with the observation that CYLD function in RANK signaling only exists in long-term but not short-term stimulation, suggesting the protein level is an important regulatory mechanism for CYLD function.

4. CYLD is a DUB that functions to prevent spontaneous accumulation of ubiquitin chains to its targets under resting states. This function of CYLD in turn is crucial for controlling the inducible nature of IKK/NF-κB factors. Loss of CYLD in different cells, particularly T and B cells, causes chronic activation of IKK and NF-κB even in the absence of stimuli. Since chronic activation of NF-κB is associated with inflammation and cancers, these findings have important implications in the tumor suppressor and anti-inflammatory functions of CYLD. Our findings also distinguish CYLD from other known NF-κB negative regulators, such as the DUB A20, which function to terminate the inducible activation of NF-κB (17, 21, 109).
5.2 Discussion and Future Directions

The findings summarized above established CYLD as a critical negative regulator of both lymphocyte activation and osteoclastogenesis, and shed light on the signaling mechanisms of CYLD function. However, there are several questions that are not completely understood. These issues will be discussed in this section.

5.2.1 Physiological targets of CYLD

A major challenge in the studies of DUBs is the identification of their physiological targets. Although previous in vitro studies suggested several CYLD targets, including TRAF2, TRAF6, and IKKγ, it has remained unclear whether the ubiquitination of these proteins is indeed under the control of CYLD in vivo. The CYLD−/− mouse model provides a powerful system for the characterization of the in vivo targets of CYLD. Interestingly, I have shown that the chronic activation of NF-κB in CYLD-deficient T cells is associated with accumulation of ubiquitinated Tak1, a key ubiquitin-dependent kinase that mediates activation of both IKK and JNK. Consistent with this finding, loss of CYLD causes chronic activation of Tak1 as well as its downstream targets, IKK and JNK, in T cells. After extensive analyses, we have not detected upregulated ubiquitination of TRAF2, TRAF6, or IKKγ in the CYLD−/− T cells. Although these negative results could be due to the technical difficulty of detecting endogenous protein ubiquitination, my finding suggests Tak1 to be a physiological target of CYLD in T cells. Indeed, CYLD physically interacts with Tak1 and also deubiquitinates Tak1 under transfection conditions.
My thesis research also suggests that CYLD may target distinct proteins in different cell types (summarized in Figure 5-1). For example, the CYLD target in B cells is unlikely to be TAK1, because TAK1 is required for JNK but not NF-κB activation in B cells and CYLD−/− B cells display enhanced ERK and NF-κB activation. The elevated ERK activity suggests that the CYLD target in B cells is more upstream towards the proximal steps of BCR signaling. One interesting candidate is the tyrosine kinase Lyn, which negatively regulates ERK activation in B cells. This possibility is further suggested by the finding that CYLD targets the Lyn homologue, Lck, in thymocytes (166). However, this possibility is yet to be examined.

5.2.2 CYLD function involves adaptors

Another intriguing finding in my thesis research is the requirement of an adaptor protein, p62, for CYLD/TRAF6 interaction. Previous studies have shown that CYLD only weakly binds to TRAF6 under overexpression conditions and these two proteins do not associate under endogenous conditions (30, 102, 226). My data also demonstrate the inability of CYLD to bind TRAF6. Interestingly, TRAF6 and CYLD exist in a stable complex in pre-osteoclasts, in which CYLD deubiquitinates TRAF6 and negatively regulates RANK-induced signaling. It turns out that the TRAF6/CYLD association in pre-osteoclasts is mediated by the adaptor protein p62. This finding provides the first example that the function of CYLD requires adaptor proteins. Future studies will examine whether the function of CYLD towards other target proteins also involves adaptors.
5.2.3 CYLD protein level is an important regulatory mechanism for its function

Since CYLD is a constitutive DUB, the abundance of CYLD protein in the cells likely plays an important role for its function. This idea is supported by the studies reported in this thesis. We have found that the functionality of CYLD varies among cell types. Disruption of the CYLD gene significantly alters lymphocyte antigen receptor signaling but has little effect on innate immune receptor signaling in macrophages. This phenomenon remained as a puzzle until we compared the expression level of CYLD in T cells, B cells and macrophages. Indeed, CYLD is abundantly expressed in B and T lymphocytes, whereas it is barely detectable in macrophages. A more direct evidence for the importance of CYLD expression level in its function is provided by my studies on osteoclast differentiation. Short-term stimulation with RANKL in macrophages, the osteoclast precursors, yields no difference in signaling. Long-term RANKL stimulation induces CYLD gene transcription, and the CYLD<sup>−/−</sup> cells display significantly increased signaling intensity, leading to hyper osteoclast development. Consistently, overexpression of CYLD in osteoclast precursor cells effectively inhibits osteoclast development, indicating that the induction of CYLD is an important regulatory mechanism for its biological function. We have shown that CYLD gene induction requires both canonical and non-canonical NF-κB pathways, but how the two branches of NF-κB signaling coordinate to induce CYLD transcription is not well understood and will be examined in the future.

5.2.4 Functional difference between CYLD and DUB A20

My studies have demonstrated CYLD as a critical negative regulator of NF-κB activation in different cell types. In addition to CYLD, another DUB named A20 has also
been characterized as an important NF-κB suppressor (17). A20 have been reported to function in both T cells and macrophages, similar to CYLD. The two DUBs even share the same targets RIP1 and TRAF6. However, the co-existence of CYLD and A20 in NF-κB pathway suggests their non-overlapping functions. Indeed, CYLD and A20 have distinct roles in NF-κB pathway in terms of receptor specificity and signaling kinetics. A20 inhibits NF-κB downstream of innate immune receptors including TNFR, IL-1R and TLR (21, 109), whereas CYLD mainly functions downstream of lymphocyte antigen receptors and RANK. Loss of A20 results in prolonged NF-κB activation when stimulated with TNF-a, however, CYLDΔ−/− Jurkat T cells display increased signaling magnitude but normal signaling kinetics upon CD3/CD28 stimulation. Another unique feature of CYLD is that it effectively suppresses basal NF-κB activation in lymphocytes. NF-κB is spontaneously activated in CYLDΔ−/− lymphocytes, which is not further increased by stimulation, suggesting CYLD is critical in inhibiting constitutive ubiquitination of NF-κB upstream signaling components thus preventing NF-κB activation in resting stages.

5.2.5 Differential role of CYLD in T cell development and activation

Our studies using CYLDΔ−/− mice demonstrated that loss of CYLD leads to a dramatic decrease in the number of mature T cells in both thymus and peripheral lymphoid organs, suggesting the essential role of CYLD in T cell development. On the other hand, despite the reduced T cell number, peripheral T cells are hyperproliferative upon stimulation via the TCR and CD28 and produce significantly larger amounts of cytokines than control T cells. This finding indicates that CYLD functions as a negative regulator of T cell activation. The functional discrepancy of CYLD in thymocyte development and peripheral activation is not
well understood. We have previously demonstrated that CYLD is required for proximal TCR signaling by positively regulating Lck. However, I also found that CYLD^{−/−} thymocytes display enhanced TAK1 and NF-κB activity, similar to peripheral T cells. This finding raises the question that whether the T cell development defect in CYLD^{−/−} mice is the result of impaired TCR proximal signaling or hyperactivated NF-κB. To examine whether hyperactivated NF-κB in thymocytes causes the thymic defect, CYLD^{−/−} mice will be crossed with nfkb1^{−/−} mice, which lack a major NF-κB family member, p50. FACS analysis will be performed using the double knockout mice to examine whether loss of NF-κB p50 can rescue the T cell development defect due to CYLD deficiency.

5.2.6 Potential mechanisms that CYLD suppresses autoimmunity

The diversity of T cell receptor repertoire is a two-edge sword for host defense. On one hand, it enables T cells to recognize virtually any antigen, on the other hand, it also increases the possibility that T cells recognize self-antigen and attack the host body. In order to prevent autoimmunity, newly generated T cells undergo stringent selection in the thymus to eliminate autoreactive T cells. The fate of immature thymocytes is at least partially determined by their TCR signaling strength delivered by thymic stromal cells bearing self-antigen as TCR ligand. Strong interaction between TCR and self-antigen results in apoptosis of that specific cell. Most autoreactive T cells can be eliminated in the thymus, however, there are still a small portion of them that mature and enter the periphery, potentially due to lack of certain self-antigens in the thymus. Periphery tolerance mechanism and regulatory T cell function suppress autoimmune T cell activation in the periphery.
Intestine is a special region in the periphery in which T cells have access to normal gut flora, which is also considered as self. Improper recognition of normal gut flora and subsequent T cell activation is now considered the major cause of autoimmune intestinal inflammation. Gut regulatory T cells are the major cell type that suppress such abnormal T cell activation and keep the immunological balance in the gut.

The dual effect of CYLD on T cell development and peripheral activation provides more than one mechanism for suppressing autoimmunity by this protein. One possibility is that positive and negative T cell selection is altered in CYLD−/− thymus, which leads to autoreactive T cells escaping into periphery and causing autoimmunity. To examine T cell selection in the absence of CYLD, two TCR transgenic mouse strains OT-1 and HY, which has been established as mouse models to study positive and negative selection respectively, can be used to cross with CYLD−/− mice. T cell development in double knockout mice can be examined using FACS. Treg is essential for suppressing autoimmunity in the periphery. However, since loss of CYLD causes spontaneous T cell activation, it is necessary to examine whether CYLD−/− T cells still respond to Treg suppression. It would also be interesting to investigate whether loss of CYLD affects Treg suppressive function.

Another intriguing question that arises from the autoimmune phenotype is why gut but not other tissues become the primary inflammation site. It is possible that the existence of autoimmune T cells recognizing gut flora breaks the immunological balance or, alternatively, loss of CYLD increases the gut-homing chemokine receptor CCR9 expression. If the first hypothesis is true, then removing gut flora by housing mice under germ-free conditions is expected to prevent the autoimmune symptom in CYLD−/− mice. The second hypothesis can be easily tested using FACS.
5.2.7 CYLD with Paget’s disease of bone (PDB)

Paget’s disease of bone is a type of osteolytic bone disease due to enlarged and hyperactivated osteoclasts. Mutations in the adaptor protein p62 has been predisposed in PDB. Interestingly, all the p62 mutations identified from PDB patients were located in the C-terminal ubiquitin-binding domain (UBA). Full-length p62 is an adaptor for TRAF6 and is required for RANK signaling, whereas mutations in the UBA domain results in hyper NF-κB activation and enhanced osteoclastogenesis. It is not clear that how UBA domain mutants of P62 cause the aberrant activation of NF-κB. The finding in Chapter 4 suggests that CYLD may be involved in this pathological process. CYLD deubiquitinates TRAF6 and inhibits TRAF6 activation downstream of RANK. Adaptor protein p62 not only facilitates TRAF6 signaling, but also performs negative role by recruiting CYLD to TRAF6. UBA domain mutants of p62 fail to facilitate CYLD/TRAF6 binding, providing a possible mechanism of hyper NF-κB activation due to p62 mutation in the UBA domain.

In the future, I will map the CYLD domains necessary for TRAF6 and p62 interaction and examine the functional importance of this interaction in RANK signaling and osteoclast development. Based on the domain mapping result, I will perform site-directed mutagenesis to create CYLD mutants carrying point mutations in its p62/TRAF6-binding domain and identify those that are either competent or deficient in association with p62 and TRAF6. These mutants will be used to determine whether the binding of CYLD to p62 and TRAF6 is essential for its negative role in regulating osteoclastogenesis. It will also be interesting to examine whether CYLD mutations or aberrant expression exist in PDB patients.
5.2.8 Potential involvement of autoimmunity to bone loss in CYLD^{−/−} mice

IBD is often associated with bone loss observed from human patients, which is at least partially due to inflammation-induced osteoclastogenesis (203, 211), therefore, there is an intriguing possibility that the bone destruction phenotype in CYLD^{−/−} mice is not only due to osteoclast intrinsic defect in the absence of CYLD, but also influenced by the inflammation condition. Interestingly, I have identified marked accumulation of a CD11b+Gr1- myeloid cell population in the bone marrow and spleen of CYLD^{−/−} mice. This CD11b+Gr1- population has been characterized as osteoclast precursors generated under inflammation conditions (257), suggesting the potential involvement of T-cell mediated chronic inflammation in bone loss observed in CYLD^{−/−} mice. Based on these data, I will examine in the future the contribution of immune cells and inflammatory cytokines to the bone erosion in CYLD^{−/−} mice. More specifically, I will examine the effect of lymphocytes and inflammatory cytokines on osteoclast development in CYLD^{−/−} mice by investigating the role of lymphocytes and inflammatory cytokines in the induction of CD11b+Gr1- osteoclast precursor cells and the function of CYLD^{−/−} CD11b+Gr1- osteoclast precursors in naturally occurred and inflammation-induced bone loss.
Figure 5-1
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