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The Tumor Suppressor CYLD Controls the Function of Murine Regulatory T Cells

Sonja Reissig,* Nadine Hövelmeyer,* Benno Weigmann,*† Alexei Nikolaev,* Bettina Kalt,* Thomas F. Wunderlich,‡ Matthias Hahn,* Marcus F. Neurath,*† and Ari Waisman*

CYLD was originally identified as a tumor suppressor gene mutated in familial cylindromatosis, an autosomal dominant predisposition to multiple benign neoplasms of the skin known as cylindromas. The CYLD protein is a deubiquitinating enzyme that acts as a negative regulator of NF-κB and JNK signaling through its interaction with NEMO and TNFR-associated factor 2. We have previously described a novel mouse strain that expresses solely and excessively a naturally occurring splice variant of CYLD (CYLDex7/8). In this study, we demonstrate that CYLD plays a critical role in Treg development and function. T cells of CYLDex7/8 mice had a hyperactive phenotype manifested by increased production of inflammatory cytokines and constitutive activation of the NF-κB pathway. Furthermore, the amount of Foxp3+ regulatory T cells in these mice was markedly enhanced in thymus and peripheral organs. Importantly, these regulatory T cells displayed decreased expression levels of CD25 and CTLA-4 associated with impaired suppressive capacity. Hence, our data emphasize an essential role of CYLD in maintaining T cell homeostasis as well as normal T regulatory cell function, thereby controlling abnormal T cell responses. The Journal of Immunology, 2012, 189: 4770–4776.

Regulatory T cells (Tregs) play an important role in the maintenance of immune homeostasis and self-tolerance by suppressing the proliferation and function of autoreactive T cells (1). Two types of CD4+ Tregs exist, as follows: natural Tregs (nTregs), which develop in the thymus during the course of positive and negative selection, and induced Tregs (iTregs), which are derived from naive conventional CD4+ T cells in the periphery following antigenic stimulation under tolerogenic conditions (2). Tregs are characterized by the expression of the high-affinity IL-2 receptor α-chain (CD25) (3) and the unique winged-helix/forkhead transcription factor Foxp3, which is required for Treg development, maintenance, and function (4, 5). In both humans and mice, genetic mutations in the gene encoding Foxp3 result in a profound autoimmune-like lymphoproliferative disease demonstrating the importance of Tregs in peripheral tolerance (6–8). Moreover, it was shown that the NF-κB pathway is crucial for the induction of Foxp3 expression during thymic Treg development (9–12).

CYLD was identified as a tumor suppressor that is mutated in patients with familial cylindromatosis, an autosomal dominant predisposition to multiple tumors of the skin appendages (13). In vitro studies revealed that CYLD is a member of the family of deubiquitinating enzymes with restricted substrate specificity. The deubiquitinating activity of CYLD was shown to downregulate NF-κB signaling by removing activating K63-linked ubiquitin molecules from Bcl-3, TRAF2, TRAF6, and NEMO (IKKγ) (14–16). Independent studies describing CYLD-deficient mice revealed multiple roles for CYLD in immune cell development and homeostasis (17–20). CYLD has also been identified as a positive regulator of proximal TCR signaling in thymocytes (17). By contrast to other studies that used CYLD-deficient mice, we have generated CYLDex7/8 mice that overexpress a naturally occurring CYLD splice variant encoding short CYLD (sCYLD), whereas full-length CYLD (FL-CYLD) is absent (21). As a result, sCYLD lacks exons 7 and 8 of the cyld gene and is devoid of TRAF2 and NEMO binding sites, although it can still interact with Bcl-3. CYLDex7/8 mice display enlarged secondary lymphoid organs due to the accumulation of mature B cells as a result of enhanced survival and activation of NF-κB signaling. Moreover, bone marrow-derived dendritic cells generated from CYLDex7/8 mice show a hyperactive phenotype in vitro and in vivo, correlating with increased nuclear translocation of Bcl-3 (22).

In this study, we show that overexpression of sCYLD leads to hyperactivity of T cells manifested by increased production of inflammatory cytokines and constitutive activation of the NF-κB pathway. Interestingly, the NF-κB coactivator Bcl-3 was highly upregulated in T cells of CYLDex7/8 mice. Furthermore, CYLDex7/8 mice exhibited increased numbers of Foxp3+ Tregs in thymus, spleen, and lymph nodes (LNs). Noteworthy, most of the Tregs in CYLDex7/8 mice were negative for the expression of CD25 and displayed low expression levels of CTLA-4. Impaired suppressive capacity of CYLDex7/8 Tregs correlated with decreased expression of CTLA-4, which goes in line with reports showing that CTLA-4 plays a key role in the suppressive function of Foxp3+ Tregs (23–25).
The analysis of CD4+ and CD8+ subpopulations in the periphery required a pivotal role for CYLD in maintaining Treg development and function, most likely through modulating NF-κB signaling as well as CD25 and CTLA-4 expression.

Materials and Methods

Mice

CYLDex7/8 mice were generated, as described previously (21). Rag1−/−, CYLDex7/8, CYLD−/−, and CYLD−/−mice (29) were bred in the animal facility at the University of Mainz. All animal experiments were in accordance with the guidelines of the central animal facility institution (ZVTE, University of Mainz).

RNA analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer’s instruction. Quantitative real-time PCR was performed using primers from Qiagen, as described on their homepage: http://www.qiagen.com/products/pcr/quantitect/primerassays.aspx.

Flow cytometry

Single-cell suspensions were prepared from different organs. RBCs in cell suspensions from LNs and spleen were lysed with Tris-ammonium chloride (pH 7.2). Cells were incubated with combinations of Abs to cell surface determinants. All Abs were purchased from BD Biosciences. All samples were acquired on a FACSVerse II (BD Biosciences), FACSCalibur (BD Biosciences), or FACSscan (BD Biosciences), and results were analyzed with CellQuest (BD Biosciences) or FlowJo software. Absolute numbers of thymocyte, LN, and splenocyte subpopulations were calculated based on their percentage and total number.

Cell purification

Cells from spleen and LNs were purified using CD4+ or CD8+ magnetic beads (Miltenyi Biotec), according to the manufacturer’s instruction. Purity as determined by flow cytometry was >95%.

Immunostaining of purified T cells

Cytospins of purified T cells were fixed in acetone for 10 min at −20°C and stained for Bcl-3 (Santa Cruz Biotechnology), according to standard methods, using polyclonal rabbit IgG Bcl-3 Ab. All incubation steps of the staining procedure were performed at room temperature.

Cytokine quantification

MACS-purified CD4+ T cells were cultured with or without anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), and ConA (5 μg/ml). Supernatants of CD4+ T cells were analyzed for cytokines by FlowCytomix assays, according to the manufacturer’s protocol (Bender MedSystems).

FlowCytomix

Multiple cytokine and chemokine levels were detected using FlowCytomix technology (Bender MedSystems), according to the manufacturer’s instructions.

Western blot

To prepare whole-cell lysates, total thymocytes or MACS-purified CD4+ T cells were lysed in 1% Triton X-100 with protease inhibitors. Protein lysates (30 μg) were separated by 10% SDS-PAGE electrophoresis and subsequently transferred to polyvinylidene difluoride membranes. Protein blots were probed with Abs to IκBα, p100 (Cell Signaling), RelB, CYLD (Santa Cruz Biotechnology), and Abs to tubulin (Cell Signaling) and actin (Santa Cruz Biotechnology) as internal loading controls.

Intracellular staining

Cells were stimulated in culture medium containing PMA (50 ng/ml) and ionomycin (500 ng/ml), in the presence of brefeldin A (1 μg/ml) at 37°C and 5% CO2 for 4 h. After staining of surface markers, cells were fixed and permeabilized (Cytofix/Cytperm and Perm/Wash buffer from BD Biosciences), followed by staining with Abs to mouse Foxp3, IFN-γ, and IL-17A (BD Biosciences) and flow cytometric analysis (FACSscan; BD Biosciences).

Treg in vitro suppression assay

IMDM culture medium (Life Technologies, Long Island, NY) was supplemented with 5% (v/v) FCS (decocomplemented), 1 mM sodium pyruvate, 2 mM l-glutamine, 1X nonessential amino acids, 0.1 mM 2-ME, and 10 mM HEPEs (Life Technologies). A total of 2 × 104 conventional CD4+ CD25− T cells was stimulated in the presence or absence of different numbers of freshly isolated CD4+CD25+ T cells. Cells were plated in 96-well round-bottom microplates in a total volume of 0.2 ml complete IMDM. Mitomycin C-treated (60 μg/ml/107 cells for 30 min) A20 B tumor cells (2 × 105/well) as accessory cells and anti-CD3 mAbs were used as a stimulus. After 96 h, [3H]thymidine was added to the cultures (0.5 μCi/well), and [3H]thymidine uptake was assessed by β-scintillation counting after additional 18 h.

Treg in vivo suppression assay

A total of 5 × 105 MACS-purified naive CD4+CD25− T cells from wild-type (WT) mice was injected i.p. in 6- to 8-wk-old Rag1−/− mice alone or with equal numbers of WT or CYLDex7/8 MACS-purified CD4+CD25− Tregs. After the cell transfer, Rag1−/− recipients were weekly weighed and monitored by miniendoscopy.

In vivo high-resolution miniendoscopy analysis of the colon

For monitoring of colitis activity, a high-resolution video endoscopic system (Carl Storz) was used. To determine colitis activity, mice were anesthetized by injecting a mixture of ketamine (Ketavest 100 mg/ml−1; Pfizer) and xylazine (Rompun 2%; Bayer Healthcare) i.p. and monitored by miniendoscopy at indicated time points. Endoscopic scoring of five parameters (translucency, granularity, fibrin, vascularity, and stool) was performed.

Histological analysis

Colonic cross sections were stained with H&E. For immunohistochemistry, colon samples were isolated from colitic mice at indicated time points. Immunofluorescence of cryosections was performed using the TSA Cy3 system (PerkinElmer) and a fluorescence microscope (IX70; Olympus) using primary Abs against Foxp3. In brief, cryosections were fixed in ice-cold acetone for 10 min, followed by sequential incubation with methanol, avidin/biotin (Vector Laboratories), and protein-blocking reagent (Dako) to eliminate unspecific background staining. Slides were then incubated overnight with primary Ab specific for the respective Ag. Subsequently, the slides were incubated for 30 min at room temperature with biotinylated secondary Abs (Dianova). All samples were finally treated with streptavidin-HRP and stained with tyramide (Cy3), according to the manufacturer’s instructions (Perkin Elmer). Before examination, nuclei were counterstained with Hoechst 3342 (Invitrogen).

Statistical analysis

Values are presented as the means ± SEM or SD, with the number of independent experiments. Statistical differences were determined using Student t test.

Results

Overexpression of sCYLD inhibits T cell development

CYLD was found to positively regulate TCR signaling of thymocytes by physically interacting with active Lck and promoting its recruitment to ZAP70 (17). To examine whether inactivation of FL-CYLD while retaining sCYLD influences T cell development, we analyzed thymi of CYLDex7/8, CYLDko (29), and WT mice. We detected a reduction in the cellularity of CYLDex7/8 thymi when compared with control mice, whereas no alteration was observed in CYLDko mice (data not shown). This reduced cellularity was not a result of early events in T cell development, as we detected no bias in this process by analyzing the surface expression of CD25 and CD44 in CD4+CD8−CD19−thymocytes (Fig. 1A). In contrast, we detected a significant reduction of CD4+ and CD8+ single-positive (SP) thymocytes in CYLDex7/8 mice compared with control and CYLDko animals (Fig. 1A, 1B).

The analysis of CD4+ and CD8+ subpopulations in the peripheral lymphoid organs revealed further functions of sCYLD in T cell homeostasis. As shown in Fig. 1C and 1D, the percentage as well as the total cell number of splenic CD4+ and CD8+ T cells were significantly reduced in CYLDex7/8 compared with CYLDko and control mice correlating with the absolute reduction of thymic progenitors. The analysis of LNs of CYLDex7/8 mice yielded similar findings (data not shown). Thus, loss of FL-CYLD, ac-
accompanied by the overexpression of sCYLD, results in a strong reduction of the T cell compartment.

Because overexpression of sCYLD in the absence of FL-CYLD affected the T cell development in CYLDex7/8 mice, we further investigated the expression level of sCYLD in WT and CYLDex7/8 thymocytes. Interestingly, sCYLD was highly upregulated in double-positive (DP) as well as CD4 SP WT thymocytes (S1A), suggesting a regulatory role for sCYLD during the transition from the DP to SP stage. Moreover, we could demonstrate by Western blot analysis that sCYLD is expressed in WT peripheral CD4+ T cells, whereas it is strongly overexpressed in CYLDex7/8 CD4+ T cells (S1B).

Hyperactive phenotype of CYLDex7/8 T cells

Next, we investigated whether CYLD is involved in T cell differentiation. By comparing LNs and spleens of CYLDex7/8 mice with those of controls, we observed a large increase in the proportion of CD44hiCD62Llo effector/memory CD4+ T cells from CYLDex7/8 mice (Fig. 2A, Supplemental Fig. 2A). This implies that a majority of T cells in CYLDex7/8 mice are activated in the absence of external stimuli.

Once activated, CD4+ T cells can differentiate into Th1 and Th17 cells, which are characterized by the production of IFN-γ and IL-17A, respectively. As shown in Fig. 2B and Supplemental Fig. 2B, CYLDex7/8 mice exhibited a high proportion of Th17 cells compared with WT or CYLDko mice. Both CYLDex7/8 and CYLDko mice displayed elevated levels of Th1 cells as compared with WT mice (Fig. 2B). We then measured whether this increase in inflammatory T cells was accompanied by augmented cytokine secretion. As depicted in Fig. 2C, CD4+ T cells isolated from CYLDex7/8 mice produced increased levels of IL-17A, IL-6, IL-10, and IFN-γ upon stimulation compared with WT CD4+ T cells. These results indicate that CD4+ T cells isolated from mice lacking FL-CYLD while overexpressing sCYLD are hyperactive, whereas, in the complete absence of CYLD, the inflammatory phenotype is more moderate and confined to IFN-γ production.

Increased frequency of Tregs in CYLDex7/8 mice

Tregs are essential for the maintenance of immunological self-tolerance and immune homeostasis by suppressing the activation and expansion of potential self-reactive T cells (30). They are
characterized by the constitutive expression of CD25 and Foxp3. As shown in Fig. 3A, thymi of CYLD\textsuperscript{ex7/8} mice displayed an increased frequency of Tregs compared with control mice. Similarly, we found a profound increase of this population also in the LNs and spleen, which was not observed in CYLD\textsuperscript{ko} mice (Fig. 3A, middle and lower panels). Of note, most of the Foxp3\textsuperscript{3} Tregs in thymus, LNs, and spleens of CYLD\textsuperscript{ex7/8} mice were negative for the expression of the surface marker CD25. These results suggest a pivotal role for sCYLD in the development as well as the differentiation of naive CD4\textsuperscript{+} T cells into Tregs.

To examine whether the T cell phenotype in CYLD\textsuperscript{ex7/8} mice is T cell intrinsic or secondary due to defects in other cell types, we crossed mice harboring the conditional allele of CYLD (CYLD\textsuperscript{b} mice) to CD4-Cre mice (31), leading to Cre-mediated excision of CYLD-exon 7 solely in T cells (CYLD\textsuperscript{CD4}). We detected an increase in the CD4\textsuperscript{+} Foxp3\textsuperscript{3} population in LNs of CYLD\textsuperscript{CD4} mice, similar to that in CYLD\textsuperscript{ex7/8} mice (Fig. 3B). When we compared the expression of relevant cell surface molecules on Tregs from CYLD\textsuperscript{ex7/8}, CYLD\textsuperscript{CD4}, and WT mice, we detected a decreased expression of CD25 in CYLD\textsuperscript{ex7/8} as well as CYLD\textsuperscript{CD4} Tregs compared with WT (Fig. 3B). Furthermore, Tregs from both CYLD\textsuperscript{ex7/8} and CYLD\textsuperscript{CD4} mice showed decreased expression of the IL-2R\textbeta chain (CD122) (Fig. 3B). Previously, it has been reported that the expression of CD44 is essential for the regulatory function of Foxp3\textsuperscript{3} Tregs, as Tregs isolated from CD44 knockout mice displayed impaired regulatory function accompanied by decreased production of anti-inflammatory cytokines (32). As shown in the histogram of Fig. 3B, CYLD\textsuperscript{ex7/8} and CYLD\textsuperscript{CD4} Tregs expressed substantially lower levels of CD44 compared with WT. Moreover, the expression of the coinhibitory molecule CTLA-4, which plays a key role in the suppressive function of Foxp3\textsuperscript{3} Tregs, was also downregulated in CYLD\textsuperscript{ex7/8} and CYLD\textsuperscript{CD4} Tregs (Fig. 3B). However, Tregs from CYLD\textsuperscript{ex7/8} and CYLD\textsuperscript{CD4} mice displayed higher expression of the integrin CD103 (Fig. 3B) and the thymus (Supplemental Fig. 3B) of CYLD\textsuperscript{ex7/8} and CYLD\textsuperscript{CD4} mice. Next, we examined whether the increased number of Tregs is due to an increased amount of nTregs or iTregs. Therefore, we analyzed the expression of Helios in Foxp3\textsuperscript{3} T cells from LNs and spleen. This allowed us to distinguish between thymus-derived nTregs (Foxp3\textsuperscript{3}Helios\textsuperscript{+}) and iTregs (Foxp3\textsuperscript{3}Helios\textsuperscript{-}) in CYLD\textsuperscript{ex7/8} T cells compared with controls (33). The data revealed an increased amount of nTregs and iTregs when sCYLD is overexpressed (Supplemental Fig. 3C). Thus, overexpression of sCYLD in T cells positively regulates the development of thymus-derived nTregs as well as the induction of iTregs, whereas it downregulates important Treg markers such as CD25, CD122, CD44, and CTLA-4.

**FIGURE 3.** Increased amounts of Tregs in CYLD\textsuperscript{ex7/8} mice. (A) Flow cytometry of intracellular Foxp3 and surface CD25 expression on thymocytes, LN cells, and splenocytes. Cells are gated on live CD4\textsuperscript{+} SP T cells. (B) Flow cytometry of LN CD4\textsuperscript{+} T cells from 8-wk-old WT, CYLD\textsuperscript{ex7/8}, and CYLD\textsuperscript{CD4} mice. Data represent expression of Foxp3, CD25, CD122, CD44, CTLA-4, and CD103 on CD4\textsuperscript{+} T cells. Numbers in quadrants indicate the percentage of cells in the designed gate. Histograms are gated on total Foxp3\textsuperscript{3} T cells. For (A) and (B), data are representative of four experiments with three mice of each genotype.

*Because CYLD\textsuperscript{ex7/8} Tregs downregulate the expression of CD44 and CTLA-4, which are essential for the suppressive capacity of Tregs, we tested the suppressive function of CYLD mutant Tregs in vivo. For this purpose, we used an adoptive transfer model of colitis. In this model, Tregs have been shown to prevent intestinal inflammation caused by the adoptive transfer of naive CD4\textsuperscript{+}CD25\textsuperscript{-} T cells into immunodeficient Rag1\textsuperscript{-/-} mice (34). To this end, we transferred Tregs in equal numbers along with CD4\textsuperscript{+}CD25\textsuperscript{-} T cells isolated from WT mice into Rag1\textsuperscript{-/-} recipients. Rag1\textsuperscript{-/-} mice receiving WT CD4\textsuperscript{+}CD25\textsuperscript{-} together with WT Tregs displayed no signs of colitis as determined by minendoscopy (Fig. 4A), body weight measurement (Fig. 4B), and histological analysis of the colon (Fig. 4C). Importantly, transferred Tregs from CYLD\textsuperscript{ex7/8} mice were not able to suppress the inflammatory activity of the cotransferred WT CD4\textsuperscript{+}CD25\textsuperscript{-} T cells, because the Rag1\textsuperscript{-/-} recipients displayed the same inflammatory colitis phenotype as the control group, which received only WT CD4\textsuperscript{+}CD25\textsuperscript{-} T cells (Fig. 4A–C). We could further show in an in vitro suppression assay that...*
CYLDex7/8 mice were absent in the colon, perhaps an indication mice. Furthermore, and in contrast to WT Tregs, Tregs from the experiments in which these cells were taken from WT mice alone (control) or together with CD4+CD25+ T cells of WT or CYLDex7/8 mice. (A) Rag1−/− recipients were examined for signs of colitis (translucency, fibrin, granularity, stool, vascularity) by miniendoscopy at the indicated time points. (B) Graph displays the body weight of Rag1−/− recipients at indicated time points (mean ± SEM). (C) Histological analysis of colonic inflammation by H&E staining of colonic cryosections 4 wk after adoptive cell transfer. (D) Flow cytometry of intracellular Foxp3 expression in CD4+ T cells of mesenteric LNs. Cells are gated on CD4+ lymphocytes. (E) Colonic cryosections were immunostained with Ab for Foxp3 (red). Nuclei were counterstained with Hoechst 3342 (blue). Magnification was used as indicated. Data are representative for n = 5 mice of each group.

FIGURE 4. CYLDex7/8 Tregs fail to inhibit colitis in an adoptive transfer model. Conventional CD4+ T cells isolated from WT mice were adoptively transferred into Rag1−/− mice alone (control) or together with CD4+CD25+ T cells of WT or CYLDex7/8 mice. (A) Rag1−/− controls were examined for signs of colitis (translucency, fibrin, granularity, stool, vascularity) by miniendoscopy at the indicated time points. Data are representative for n = 5 mice of each group.

CYLDex7/8 Tregs suppressed to a lower extent the proliferation of conventional CD4+ T cells in contrast to control WT and CYLDKO Tregs (Supplemental Fig. 4).

As CYLDex7/8 Tregs were not able to suppress the inflammatory capacity of the transferred naive CD4+ T cells, we investigated whether the CYLDex7/8 Tregs survived after transfer into Rag1−/− recipients. As shown in Fig. 4D, we detected a drastic reduction in the proportion of Foxp3+ Tregs from the total CD4+ T cells when the Tregs were of CYLDex7/8 origin as compared with the experiments in which these cells were taken from WT mice. Furthermore, and in contrast to WT Tregs, Tregs from CYLDex7/8 mice were absent in the colon, perhaps an indication of a defect in their ability to home to the site of colonic inflammation (Fig. 4E). Collectively, our data demonstrate that overexpression of sCYLD leads to a defect of Tregs to suppress an inflammatory response.

Increased expression of NF-κB signaling molecules in CYLD mutant T cells

In previous reports, it was shown that CYLD acts on different proteins involved in the NF-κB pathway (14–17). To understand the molecular mechanism behind abnormal T cell responses in the absence of FL-CYLD, we investigated the activation status of the transcription factor NF-κB. Similar to our previous results with B cells (21), we detected increased expression of RelB and p100, substrates of the alternative NF-κB pathway, and transcriptional targets of canonical NF-κB, in unstimulated CYLDex7/8 peripheral CD4+ T cells and thymocytes (Fig. 5A). Furthermore, the overexpression of sCYLD enhances the activation of the canonical NF-κB pathway, evidenced by increased IκBα degradation in the cytoplasmic fraction of peripheral CD4+ T cells and thymocytes (Fig. 5B). In addition, we detected increased nuclear translocation of p52 and RelB (Fig. 5C). Importantly, also the amount of nuclear c- Rel was increased in CYLDex7/8 thymocytes. Because nuclear c-Rel was shown to be essential for the development of Tregs, this result might explain the increased amount of Tregs in CYLDex7/8 mice. Moreover, RelB and p100/NF-κB2 mRNA levels were also increased in CYLDex7/8 thymocytes (Fig. 5D) as well as CD4+ and CD8+ peripheral T cells (Fig. 5E, 5F).

Taken together, our results demonstrate that overexpression of sCYLD in the absence of FL-CYLD leads to constitutive NF-κB activation.

The NF-κB molecule Bcl-3 has previously been shown to interact with both FL-CYLD and sCYLD and thereby modulate NF-κB activity (21, 22, 34). Hence, we examined the expression of Bcl-3 in CYLDex7/8 T cells and detected increased levels of Bcl-3 in thymocytes, CD4+, and CD8+ T cells compared with WT cells (Fig. 5D–F). We could demonstrate that Bcl-3 is located predominantly in the nucleus of unstimulated CYLDex7/8 T cells, whereas in WT T cells Bcl-3 was predisposed in the cytoplasm (Fig. 5G). These findings suggest that overexpression of sCYLD in the absence of FL-CYLD results in enhanced Bcl-3 transcription and nuclear Bcl-3 translocation, which can lead to the activation of NF-κB signaling.

Discussion

CYLD is a member of the family of deubiquitinating enzymes and was shown to be a negative regulator of the NF-κB signaling pathway (14) by interfering with signaling molecules TRAF2, TRAF6, NEMO, Bcl-3, and TAK1 (15, 16, 18, 29). To date, several groups have generated CYLD knockout mouse strains to investigate the function of CYLD in vivo, all of which show marked phenotype differences (17, 20, 29). We identified a naturally occurring splice variant of CYLD (sCYLD), which lacks exons 7 and 8 of the cyld gene encompassing the binding sites for TRAF2 and NEMO (21). In CYLDex7/8 mice, sCYLD is overexpressed, whereas full-length CYLD is absent. In this study, we investigated the role of sCYLD in T cells using CYLDex7/8 mice in comparison with control mice. We provide evidence that sCYLD regulates the activation of NF-κB and thereby influences the regulation and function of Tregs. First, we detected a marked reduction of CD4+ and CD8+ SP thymocytes as well as peripheral T cells in CYLDex7/8 mice. These T cells exhibited a hyperactivated phenotype accompanied by increased activation of the NF-κB pathway. Correlating to this phenotype, we observed increased Bcl-3 expression and nuclear localization of Bcl-3 in CYLDex7/8 T cells, reflecting a T cell state of increased NF-κB activity.

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Previous publications with mice in which an IκB super-repressor has been expressed in T cells have shown that NF-κB is involved in negative selection of thymocytes (35). In these mice, DP thymocytes expressing a nondegradable form of IκBα are partially resistant to apoptosis, suggesting that NF-κB plays a role in the negative selection of thymocytes by exhibiting a proapoptotic function during the selection processes, possibly through upregulating the expression of proapoptotic genes (36). Hence, the stronger activation of the NF-κB pathway in CYLD<sup>ex7/8</sup> thymocytes might explain the reduction of thymocyte turnover during the transition of DP to SP thymocytes. However, the CYLD<sup>ko</sup> mice we analyzed (29) did not show signs of defect in T cell development as their CD4<sup>+</sup> and CD8<sup>+</sup> SP thymocyte numbers were comparable to those of WT mice. NF-κB signaling has also been implicated in the development and regulation of Tregs (10–12, 37–39). Because of the increased frequency of Tregs in thymus, spleen, and LNs of CYLD<sup>ex7/8</sup> mice compared with WT and CYLD<sup>ko</sup> mice. It is important to note that most of the Foxp3<sup>+</sup> Tregs were negative for the surface expression of CD25. In contrast to the CYLD<sup>ko</sup> mice that we analyzed (29), Lee et al. (40) detected in their CYLD<sup>ex7/8</sup> mice isolated in the same experiment. Tubulin-specific Ab was used as loading control. Western blots on cytoplasmic (B) and nuclear extracts (C) using Abs against the indicated proteins. RT-PCR of total RNA from unstimulated total thymocytes (D), splenic CD4<sup>+</sup> (E), and splenic CD8<sup>+</sup> (F) T cells. For (A–E), results are representative of two or three independent experiments. (G) Cytospins were prepared from unstimulated T cells isolated from CYLD<sup>ex7/8</sup> and WT mice and immunohistostained with anti–Bcl-3 (red), and nuclei were counterstained with Hoechst 3342 (blue).

In summary, our results indicate that sCYLD promotes via NF-κB activation thymic-Treg lineage differentiation. Thus, enhanced NF-κB activity in thymocytes might play a crucial role in shifting the ratio of conventional cells and Tregs toward the Tregs. Furthermore, overexpression of sCYLD inhibits the suppressive capacity of Tregs, probably by downregulating the expression of the coinhibitory receptor CTLA-4.
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Disclosures

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