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Protective dendritic cell responses against listeriosis induced by the short form of the deubiquitinating enzyme CYLD are inhibited by full-length CYLD

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The deubiquitinating enzyme CYLD is an important tumor suppressor and inhibitor of immune responses. In contrast to full-length CYLD, the immunological function of the naturally occurring short splice variant of CYLD (sCYLD) is insufficiently described. Previously, we showed that DCs, which lack full-length CYLD but express sCYLD, exhibit augmented NF-κB and DC activation. To explore the function of sCYLD in infection, we investigated whether DC-specific sCYLD regulates the pathogenesis of listeriosis. Upon *Listeria monocytogenes* infection of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice, infection of CD8α$^+$ DCs, which are crucial for the establishment of listeriosis in the spleen, was not affected. However, NF-κB activity of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ DCs was increased, while activation of ERK and p38 was normal. In addition, CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ DCs produced more TNF, IL-10, and IL-12 upon infection, which led to enhanced stimulation of IFN-γ-producing NK cells. In addition CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ DCs presented *Listeria* Ag more efficiently to CD8$^+$ T cells resulting in a stronger pathogen-specific CD8$^+$ T-cell proliferation and more IFN-γ production. Collectively, the improved innate and adaptive immunity and survival during listeriosis identify the DC-specific FL-CYLD/sCYLD balance as a potential target to modulate NK-cell and Ag-specific CD8$^+$ T-cell responses.

Keywords: CYLD · Dendritic cell · Infection · *Listeria*

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Introduction

The Gram-positive bacterium *Listeria monocytogenes* (Lm) may cause severe infections including sepsis and meningitis, especially in immunocompromised patients. In addition, fetal listeriosis can

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induce severe multiorgan infection resulting in abortion [1]. These clinical data point to the importance of the immune system for the control of Lm. In fact, experimental studies in mice have revealed that control of this facultative intracellular bacterium depends on the coordinated activity of innate and adaptive immune responses. Interestingly, DCs have opposing functions contributing both to the dissemination of the pathogen as well as to pathogen control. First, Lm infects splenic CD8α$^+$ DCs within a few hours after infection, before the bacteria spread to monocytes, CD8α$^-$ DCs, and macrophages. The critical importance of CD8α$^+$ DCs as an entry port for Lm and establishment of the infection in the spleen was demonstrated by depletion of DCs prior to infection [2] and genetic deletion of CD8α$^+$ DCs in Batf3$^{-/-}$ mice [3]. Both strategies resulted in a strongly reduced bacterial burden in spleen. Second, CD8α$^+$ DCs orchestrate a protective immune response against Lm. They produce IL-12, which stimulates protective IFN-γ production of NK cells. Of note, TNF/inducible nitric oxide producing monocyte-derived DCs (also designated as Tip-DC or inflammatory DCs) are also an important source of IL-12 and contribute to the control of Lm. Furthermore, CD8α$^+$ DCs are potent APCs, which efficiently prime Lm-specific CD8$^+$ T cells, although other cell types including macrophages can also induce Lm-specific CD8$^+$ T cells [4]. These Lm-specific CD8$^+$ T cells are critical for the clearance of the pathogen from infected organs.

The maturation and Ag-presenting capacity of DCs is greatly influenced by the NF-κB pathway. Inhibition of NF-κB activation in DCs prevents maturation and inhibits the effective priming of T cells [5, 6]. On the contrary, uncontrolled and excessive activation of NF-κB in DCs results in immunopathology as illustrated in mice lacking the ubiquitin-modifying enzyme A20, an important inhibitor of NF-κB [7–9]. In addition to A20, the deubiquitinating enzyme CYLD is an important regulator of NF-κB and MAP kinase activity. CYLD was identified as a tumor suppressor gene mutated in patients with familial cylindromatosis, a disease characterized by the development of benign tumors of the skin appendage [10]. CYLD removes K63-linked polyubiquitin chains from different signaling molecules, which contribute to NF-κB regulation including TRAF2, RIP1, and NEMO, thereby inhibiting activation of NF-κB [11]. In the complete absence of CYLD, the increased activation of NF-κB and MAP kinases results in increased inflammation upon infection with bacteria and viruses [12–15]. With respect to listeriosis, we have shown before that combined FL-CYLD and sCYLD deficiency augmented the NF-κB/IL-6/STAT3 crosstalk and fibrin production thereby protecting from lethal listeriosis [12]. Importantly, the natural occurring short splice variant of CYLD, sCYLD, lacks the TRAF2- and NEMO-binding sites resulting in increased constitutive NF-κB activity. In mice lacking exons 7 and 8 of CYLD (Cyld$^{ex7/8\ fl/fl}$), sCYLD is overexpressed whereas full-length CYLD (FL-CYLD) is absent [16]. Selective expression of Cyld$^{ex7/8}$ in DCs resulted in a hyperactivation of NF-κB upon stimulation with LPS [17]. However in sharp contrast to A20, DC-specific Cyld$^{ex7/8}$ mice did not suffer from spontaneous hyperinflammation.

Here, we show that expression of sCyld is upregulated in DCs upon in vivo infection with Lm. In CD11c-CreCyld$^{ex7/8\ fl/fl}$ mice, the infection of CD8α$^+$ DCs was not altered but their production of IL-12 was increased, which was associated with increased numbers of IFN-γ-producing and cytotoxic NK cells. In addition, the number of IFN-γ-producing and cytotoxic Lm-specific CD8$^+$ T cells was elevated in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. Functionally important, CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice had an improved pathogen control and survival as compared to mice expressing both FL-CYLD and sCYLD.

Results

Improved course of listeriosis in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice

To study whether sCyld of DCs might play a role in listeriosis, we first analyzed the expression of sCYLD and FL-CYLD in CD11c$^+$ and CD11c$^-$ DCs of Cyld$^{ex7/8\ fl/fl}$ and CD11c-Cre-Cyld$^{ex7/8\ fl/fl}$ mice before and after infection with Lm. Western blot (WB) analysis showed that CD11c$^+$ DCs of uninfected Cyld$^{ex7/8\ fl/fl}$ mice predominantly expressed FL-CYLD and only marginally sCLYD (Fig. 1A). However, Lm infection strongly induced sCYLD expression in Cyld$^{ex7/8\ fl/fl}$ mice, whereas expression of FL-CYLD slightly declined. Thus, the ratio of sCYLD/FL-CYLD increased from 0.2 to 0.5 in CD11c$^+$ cells of Cyld$^{ex7/8\ fl/fl}$ mice upon infection. In uninfected and infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice, sCYLD was equally expressed but FL-CYLD was—as expected—absent. In contrast, FL-CYLD was expressed in CD11c$^-$ cells of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice (Supporting Information Fig. 1). These findings illustrate an induction of sCYLD in DC during listeriosis and provide a basis to investigate the functional role of sCYLD.

To study the functional role of sCYLD in DC during listeriosis, CD11c-CreCyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice were i.v. infected with a nonlethal dose (3×10^4 CFU) of Lm. Both control and CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice survived the infection (Fig. 1B). However, the bacterial load was significantly reduced in the spleen, liver, and lung of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice as compared to Cyld$^{ex7/8\ fl/fl}$ mice at days 3 and 6 post infection (p.i.) (Fig. 1C–F). To further analyze whether sCYLD might influence the survival of severe listeriosis, both mouse strains were i.v infected with increased doses of Lm. Upon infection with 1×10^5 CFU of Lm, 67% of Cyld$^{ex7/8\ fl/fl}$ mice succumbed up to day 6 p.i., whereas 100% of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice survived with an improved pathogen control in spleen, liver, and lung but not in the brain of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice (Fig. 1G–K). However upon infection with 1×10^6 Lm, all mice of both mouse strains succumbed without significant differences in CFU of infected organs (Fig. 1L–P). Collectively, these data show that the absence of FL-CYLD in the presence of sCYLD results in an improved course of listeriosis, which is limited by i.v. infection with very high doses of LM.

sCYLD does not improve pathogen control but does enhance activation of CD8α$^+$DCs

Since CD8α$^+$ DCs are the crucial cellular entry port for Lm into the spleen [2, 3], we investigated whether numbers of Lm differed

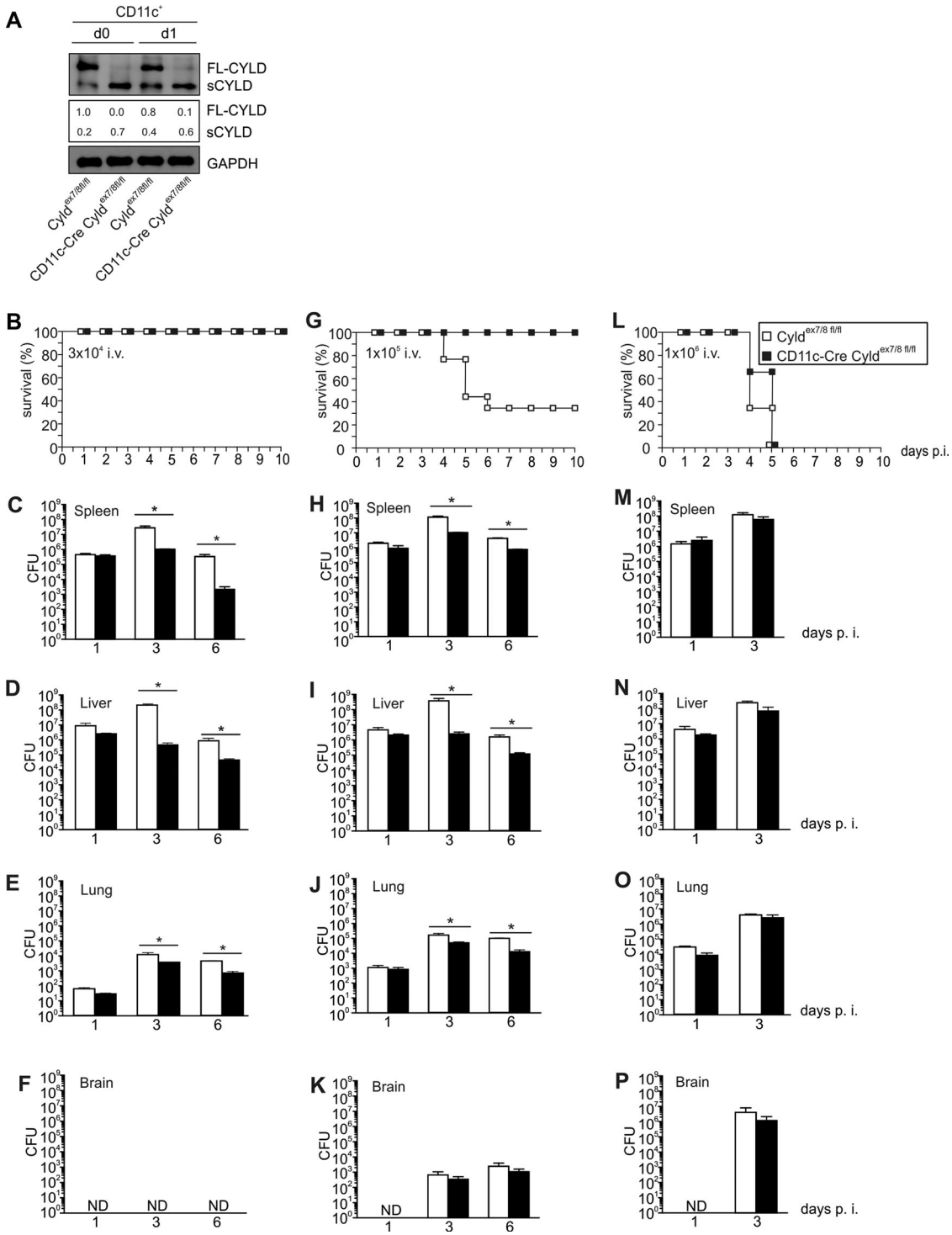

Figure 1. Improved course of listeriosis in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. (A–Q) CYLD $^{ex7/8\ fl/fl}$ and CD11c-Cre CYLD$^{ex7/8\ fl/fl}$ mice were i.v. infected with (A–F) 3×10^4, (G–K) 1×10^5, and (L–Q) 1×10^6 Lm. (A) FL-CYLD and sCYLD proteins were analyzed by Western blot (WB) in FACS-sorted CD45$^+$ CD11c$^+$ cells of both uninfected and Lm-infected CYLD$^{ex7/8\ fl/fl}$ and CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice ($n = 3$ per group). Cells were sorted 24 h p.i. GAPDH was used as loading control. Quantification of FL-CYLD and sCYLD was performed from WB data by densitometry. One of two independent experiments with similar results is shown. (B, G, L) Survival rates of infected mice were monitored until day 10 p.i. In (B), one of three and in (G, M) one of two independent experiments each performed with nine mice per group is shown (*$p < 0.005$, t-test). (C–F, H–K, M–P) CFUs were determined in the spleen (C, H, M), liver (D, I, N), lung (E, J, O), and brain (F, K, P) of Lm-infected Cyld$^{ex7/8\ fl/fl}$ and CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice at the indicated time point p.i. (*$p < 0.05$, t-test). ND: nondetectable. Data are shown as mean + SD of nine mice per experimental group and time point pooled from two independent experiments.

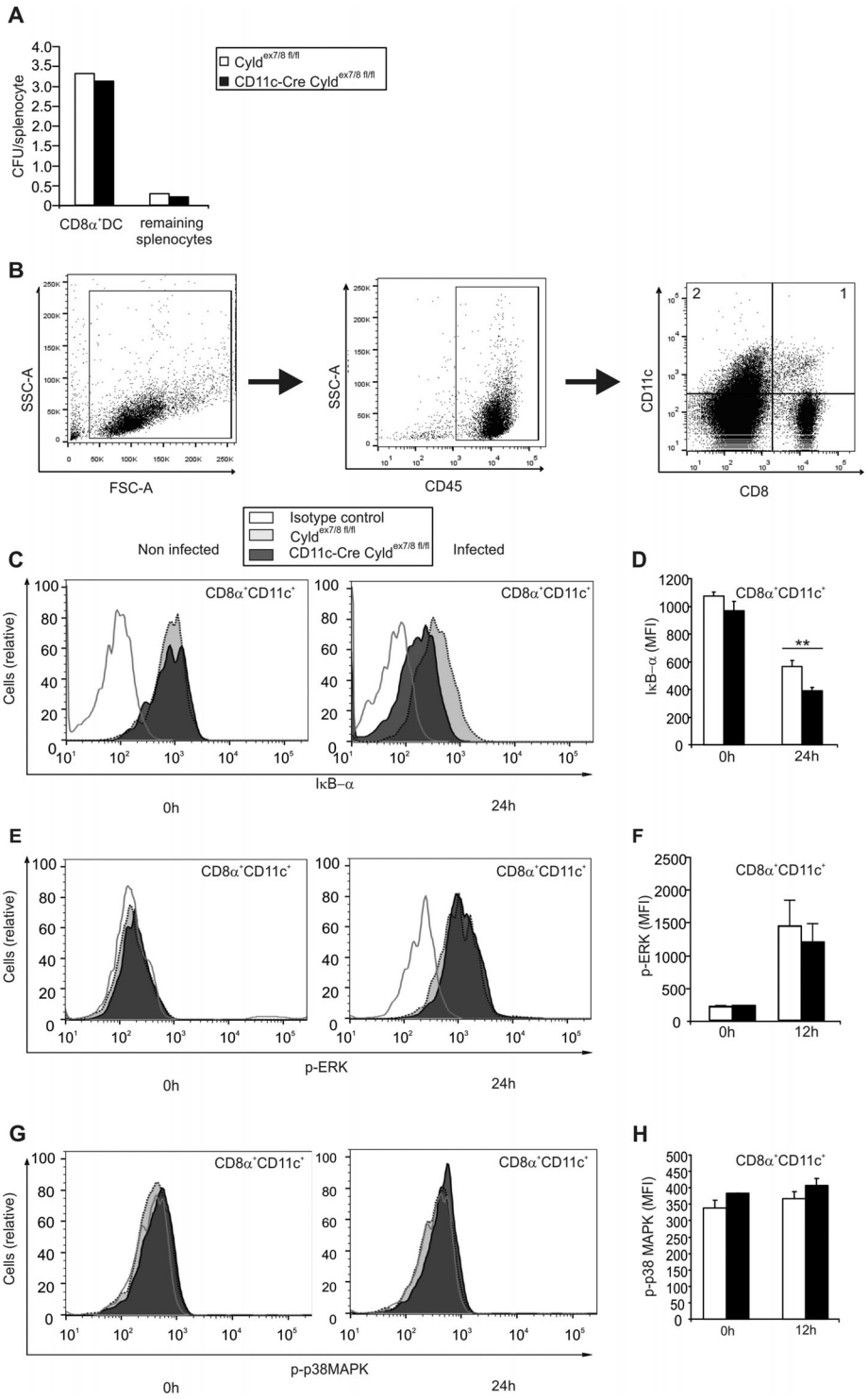

Figure 2. Normal infection but augmented NF-κB activation of CD8α$^+$ DCs from Cyld$^{ex7/8\ fl/fl}$ mice. CYLD$^{ex7/8\ fl/fl}$ and CD11c-Cre CYLD$^{ex7/8\ fl/fl}$ mice were i.v. infected with Lm. (A) Sixteen hours p.i., CFUs were determined in FACS-sorted CD8α$^+$ DCs and the remaining splenocytes from uninfected and Lm-infected mice ($n = 5$). (B) Gating strategy used to identify CD45$^+$ CD11c$^+$ CD8α$^+$ and CD45$^+$ CD11c$^+$ CD8α$^-$ splenocytes. (C, E, G) Histogram overlays show data for CD45$^+$ CD11c$^+$ CD8α$^+$ cells of uninfected (0 h p.i.) and infected (24 h p.i.) mice. The intracellular MFI of IκBα (C), p-ERK (E), and p-p38 (G) is shown. Staining with isotype control Abs is depicted as open histograms; specific staining for CYLD$^{ex7/8\ fl/fl}$ mice is shown in light gray-filled histogram and for CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ in dark gray-filled histogram. Representative data of individual mice from one of two experiments, each performed with five mice per experimental group are shown. (D, F, H) MFI of IκBα (D), p-ERK (F), and p-p38 (H) of uninfected (0 h p.i.) and Lm-infected (24 h p.i.) CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice are shown as mean ± SD of five mice from one experiment representative of two experiments performed (**$p < 0.01$, t-test).

in splenic CD8α$^+$ DC in Cyld$^{ex7/8\ fl/fl}$ and CD11c-CreCyld$^{ex7/8\ fl/fl}$ mice 16 h p.i. Numbers of Lm recovered from FACS-isolated CD45$^+$ CD11c$^+$ CD8α$^+$ DCs did not differ between both mouse strains (Fig. 2A). In addition, the number of Lm was identical in the remaining CD45$^+$ splenocytes (Fig. 2A). Thus, the FL-CYLD/sCYLD balance does not influence the predominant infection of CD8α$^+$ DC in the early stage of listeriosis.

To determine the impact of sCYLD on the activation of NF-κB, ERK, and p38 in CD45$^+$ CD11c$^+$ CD8α$^+$ and CD45$^+$ CD11c$^+$ CD8α$^-$ DC (Fig. 2H, Supporting Information Fig. 2A–F), we performed an ex vivo analysis of these signaling pathways by flow cytometry. Of note, numbers of CD8α$^+$ DCs were too low for WB analysis. CD45$^+$ splenocytes were gated for CD11c and CD8α (Fig. 2B). Lm infection induced a reduction of IκBα in

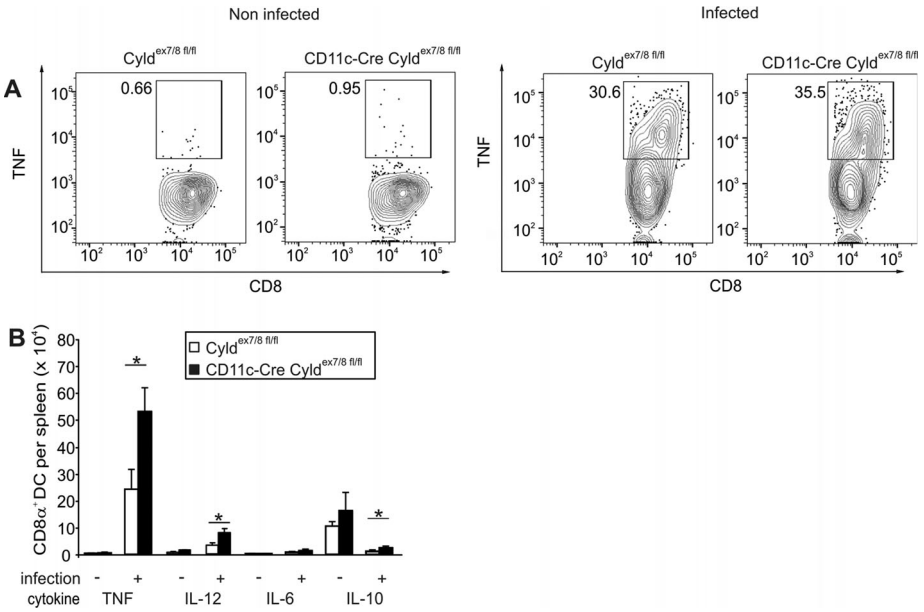

Figure 3. Enhanced cytokine production by CD8α$^+$ DCs of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. CD11c-Cre CYLD$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice were infected with Lm. (A) Twenty-four hours p.i., CD45$^+$ CD11c$^+$ CD8α$^+$ DCs of uninfected and Lm-infected mice were isolated by flow cytometry and TNF production evaluated. Representative data from one of six mice per group are shown. (B) Total numbers of cytokine-producing CD8α$^+$ DCs per spleen of uninfected and Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice are shown as mean + SD of six mice per group from one experiment representative of two performed (*p < 0.05, t-test).

both CD45$^+$ CD11c$^+$ CD8α$^+$ and CD8α$^-$ cells (Fig. 2C and D, Supporting Information Fig. 1A and B) of both strains of mice. However, IκBα degradation was significantly more pronounced in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. Activation of ERK was equally strong in both cell types of control and CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice (Fig. 2E and F; Supporting Information Fig. 2C and D), whereas infection induced no p38 phosphorylation (Fig. 2G and H; Supporting Information Fig. 2E and F). These data indicate that (i) listeriosis induced a stronger degradation of IκBα indicating enhanced activation of NF-κB in DCs expressing only sCYLD, (ii) that ERK and p38 activation were independent of the FL-CYLD/sCYLD balance, and (iii) that activation of these pathways was equal in CD8α$^+$ and CD8α$^-$ DCs. To further analyze whether sCYLD regulates the ubiquitination of STAT3 similar to FL-CYLD [12], STAT3 was immunoprecipitated from CD11c$^+$ DCs of Cyld$^{ex7/8\ fl/fl}$, CD11c-Cre-Cyld$^{ex7/8\ fl/fl}$, and conventional Cyld$^{-/-}$ mice, which lack both sCYLD and FL-CYLD, before and after infection with Lm and the K63 ubiquitination of STAT3 was analyzed by WB. As illustrated in Supporting Information Fig. 4, upon Lm infection, the amount of K63-polyubiquitinated STAT3 proteins was augmented in Cyld$^{-/-}$ DCs as compared to Cyld$^{ex7/8\ fl/fl}$ and CD11c-Cre-Cyld$^{ex7/8\ fl/fl}$ DCs, which showed equally reduced K63-polyubiquitinated STAT3. These data suggest that sCYLD, unlike FL-CYLD does not influence the K63-polyubiquitination of STAT3.

Increased cytokine production by CD8α$^+$ DCs in CD11c-CreCyld$^{ex7/8\ fl/fl}$ mice

Infection of DCs by Lm leads to their maturation, activation, and subsequent cytokine production [18]. To address the role of sCYLD on DC cytokine production in listeriosis, the intracellular expression of TNF, IL-6, IL-12, and IL-10 was assessed by flow cytometry (Fig. 3A and B; Supporting Information Fig. 3A–C). CD8α$^+$ DC from uninfected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and control mice produced very low levels of TNF, IL-6, IL-12, and IL-10 (Fig. 3B). Upon Lm infection, expression levels of all of these cytokines increased in both strains of mice (Fig. 3A and B; Supporting Information Fig. 3A–C). However, the percentages and absolute numbers of TNF-, IL-12-, and IL-10-producing CD8α$^+$DC cells were significantly higher in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ animals (Fig. 3A and B; Supporting Information Fig. 3A–C).

Increased leukocyte numbers in the spleen of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice

To determine whether sCYLD affects leukocyte numbers in the spleen during listeriosis, CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice were i.v. infected with 3×10^4 CFU of Lm. The total number of splenocytes was significantly increased in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice at days 3 and 6 p.i. (Fig. 4A). Uninfected CD11c-CreCyld$^{ex7/8\ fl/fl}$ and CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice harbored equal numbers of B cells, CD4 and CD8 T cells, NK cells, macrophages, monocytes, and granulocytes in their spleens (Fig. 4B, $p > 0.05$ for all cell populations). Upon infection, the number of CD8$^+$ T cells was significantly increased in the spleens of CD11c-CreCyld$^{ex7/8\ fl/fl}$ mice at day 3 p.i. In addition to the increase in CD8$^+$ T cells, numbers of total DCs and CD8α$^+$ DC were significantly increased in CD11c-CreCyld$^{ex7/8\ fl/fl}$ as compared to Cyld$^{ex7/8\ fl/fl}$ mice (Fig. 4C). Furthermore, activation of CD8α$^+$ DC from CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice was enhanced as demonstrated by their enhanced expression of MHC class II Ags (Fig. 4D). The increased activation of MHC class II Ags is most probably due to an increased activation of NF-κB (Fig. 2C and D) and increased IFN-γ

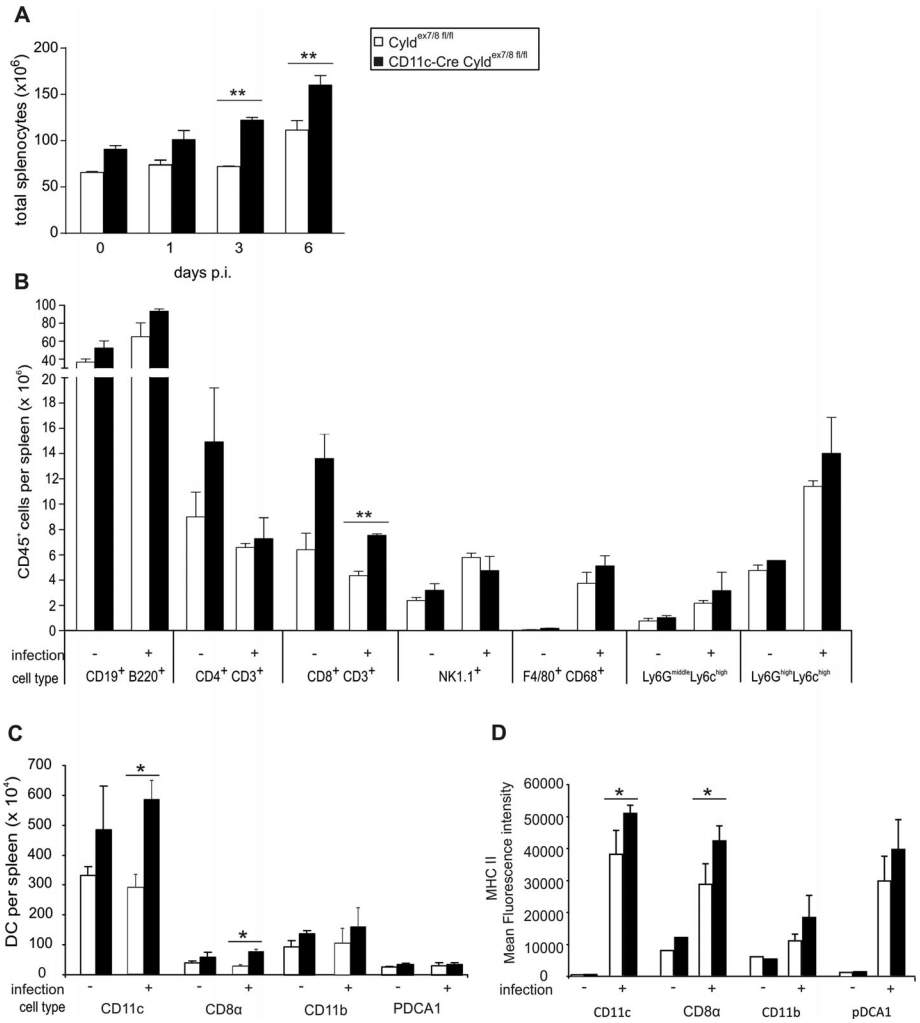

Figure 4. Increased leukocyte numbers in the spleens of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. CD11c-Cre CYLD$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice were infected with Lm. (A) Total numbers of splenocytes in uninfected (day 0) and Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice at days 1, 3, and 6 p.i were determined. Data are shown as mean + SD of $n = 5$ per group and time point from one experiment representative of two performed ($^*p < 0.05$, $^{**}p < 0.01$, *t*-test) (B) The absolute numbers of CD45$^+$ leukocytes, B cells (CD12$^+$ B220$^+$), CD4$^+$ T cells (CD3$^+$ CD4$^+$), CD8$^+$ T cells (CD3$^+$ CD8$^+$), NK cells (NK1.1$^+$ CD3$^-$), macrophages (CD68$^+$ F4/80$^+$), granulocytes (Ly6G^{high} Ly6c^{high}), and inflammatory monocytes (Ly6G^{middle} Ly6c^{high}) were determined by flow cytometry in spleens of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice before and at day 3 p.i. Data are shown as mean + SD of $n = 6$ per group from one experiment representative of two performed ($^*p < 0.05$, $^{**}p < 0.01$, *t*-test). (C) The total number of splenic DCs, conventional CD11b$^+$ CD8α$^-$ CD11c$^+$ DC, CD8α$^+$ DCs (CD11c$^+$ CD11b$^-$ CD8α$^+$), and plasmacytoid DCs (CD11c^{low} PDCA1$^+$) were determined by flow cytometry in uninfected and infected (day 3 p.i.) CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and control mice. Data are shown as mean + SD of $n = 6$ per group from one experiment representative of two performed. (D) The MFI of MHC class I, MHC class II, CD40, CD80, and CD86 was determined by flow cytometry on splenic DCs of uninfected or Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice at day 3 p.i. Data are shown as mean + SD of $n = 6$ from one experiment representative of two performed.

production in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice (Supporting Information Fig. 3D, $^*p < 0.05$).

DCs from CD11c-Cre Cyld$^{ex7/8fl/fl}$ mice cause hyperactivation of NK cells

Since DCs play an important role in the activation of protective NK cells [19], we examined the activation of NK cells by intracellular IFN-γ staining. As shown in Fig. 5A and B, the IFN-γ-producing NK cells were significantly increased in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice as compared to control mice. To further analyze whether NK cells of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice also exhibited an enhanced cytotoxicity, we performed an ex vivo cytotoxicity assay. As shown in Fig. 5C, isolated NK cells of Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice cleared CFSE^{hi} labeled RMA-S cells (TAP-2 deficient cells, with a defect in MHC Class I assembly) better than NK cells of control mice. This indicates that the highly active DCs of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice caused an increased NK-cell cytotoxicity.

Improved MHC class I Ag presentation and CD8$^+$ T-cell priming of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ DCs

To investigate whether the sCYLD influences Lm-specific CD8$^+$ and CD4$^+$ T-cell responses, CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and control mice were infected with OVA-transgenic Lm (LMova). On day 6 p.i., T cells were isolated from spleens of both mouse strains and stimulated either with MHC class I specific (SIINFEKL ($OVA_{257–264}$)) or MHC class II specific listeriolysin $(LLO)_{190-201}$ peptide and the frequency of IFN-γ-producing CD8$^+$ and CD4$^+$ T cells, respectively, was determined by flow cytometry. Upon infection, IFN-γ-producing Lm-specific CD8$^+$ (Fig. 6A) and CD4$^+$ (Fig. 6B) T cells were detectable in both control and CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice but the number of Lm-specific CD8$^+$ and CD4$^+$ T cells was significantly higher in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice.

Since the increased number of Lm-specific T cells could be caused by an increased proliferation of T cells, we further analyzed the effect of FL-CYLD/sCYLD on T-cell proliferation. Therefore, infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and control mice were treated i.p. with BrdU at days 4 and 5 p.i and BrdU incorporation of

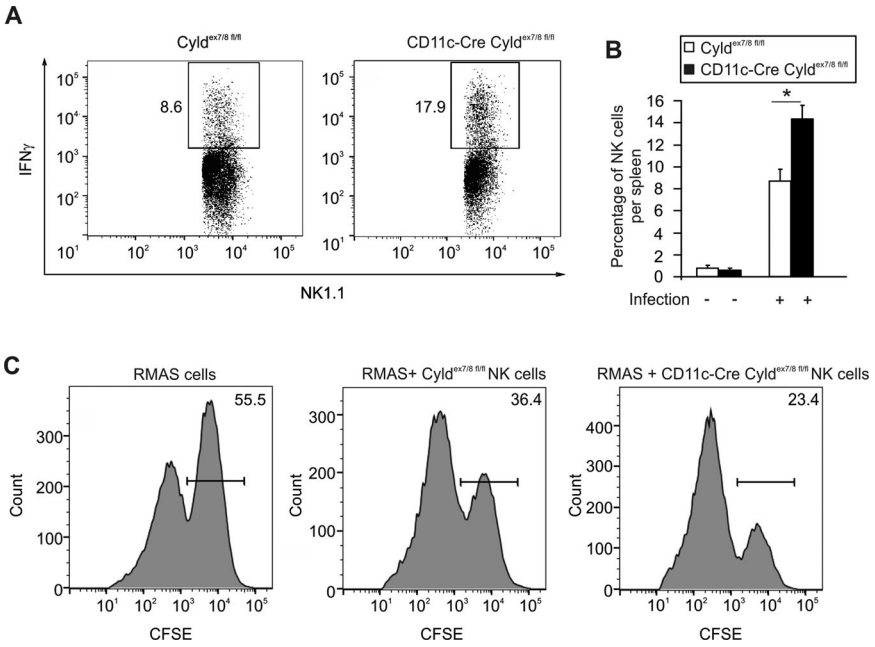

Figure 5. CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ CD8α$^+$ DCs induce stronger activation of NK cells. CD11c-Cre CYLD$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice were infected with Lm. (A, B) At day 3 p.i., the percentage of IFN-γ-producing splenic NK cells from Lm-infected mice were determined by flow cytometry. Data are shown as mean + SD of $n = 6$ per group from one experiment representative of two performed ($^*p < 0.05$, t-test). (C) MACS-isolated NK cells from Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice (day 3 p.i.) were coincubated with CFSE-labeled RMA-S cells. The cytotoxicity of the NK cells was measured after 18 h by flow cytometry analysis of CFSE dye dilution ($^*p < 0.05$, t-test). Data shown are from one of two independent experiments.

CD8$^+$ and CD4$^+$ T cells was determined by flow cytometry 24 h later. CD4$^+$ T cells of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice incorporated only slightly more BrdU than control CD4$^+$ T cells (Fig. 6C and E), whereas CD8$^+$ T cells of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice incorporated more than threefold more BrdU than CD8$^+$ T cells of control mice (Fig. 6D and E). This difference in BrdU positivity was significant for CD8$^+$ T cells ($p < 0.05$) but not for CD4$^+$ T cells (Fig. 6E). To further explore CD8$^+$ T-cell stimulation and priming by DCs, we performed an ex vivo Ag presentation assay with DCs isolated from LMova-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice 24 h p.i. These DCs were incubated with LacZ-inducible B3Z reporter T-cell hybrids, which are specific for OVA$_{257\text{-}264}$ and upregulate β-galactosidase expression upon activation. As shown in Fig. 6F, β-galactosidase expression was significantly higher in CD8$^+$ T-cell hybrids activated by CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ as compared to control DCs. These experiments indicate that the improved Ag presentation by CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ DCs in combination with their increased IL-12 production leads to an augmented priming and proliferation of *Listeria*-specific IFN-γ-producing CD8$^+$ T cells.

Discussion

CD11c$^+$ DCs of uninfected WT mice predominantly expressed FL-CYLD but upon Lm infection FL-CYLD declined and sCYLD was upregulated. The increased ratio of sCYLD/FL-CYLD in DCs of infected mice might be caused by an enhanced splicing of FL-CYLD to sCYLD in activated DCs of infected mice. Importantly, the exclusive expression of the naturally occurring splice variant of CYLD in DCs results in an improved immune response and protection against Lm indicating that the balance of FL-CYLD and sCYLD regulates the infection induced cellular activation of DCs and further identifies sCYLD as an activation-induced antagonist of FL-CYLD. sCYLD lacks the TRAF2- and NEMO-binding sites [20] and, therefore, does not suppress activation of NF-κB. In fact, the degradation of the NF-κB inhibitor IκBα was significantly increased in CD8α$^+$ DCs of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. In contrast, activation of ERK was independent of the differential expression of FL-CYLD and sCYLD in CD8α$^+$ DCs, whereas p38 was not activated in these cells within the first 24 h of infection. In extension of previous studies illustrating that CYLD deubiquitinated STAT3 in Lm-infected hepatocytes [12], we demonstrate that the differential expression of sCYLD and FL-CYLD did not affect the K63-polyubiquitination of STAT3 (Supporting Information Fig. 4). Collectively, these observations identify sCYLD as a specific regulator of NF-κB in CD8α$^+$ DCs of infected mice [17].

Within the first hours of infection, CD8α$^+$ DCs are the major infected cell type and a crucial entry port for Lm in the spleen. Since overexpression of sCLYD did not impact on the number of Lm in CD8α$^+$ DCs within the first hours after infection, these early steps of CD8α$^+$ DC and Lm interaction are independent of the FL-CYLD/sCyld balance.

In addition, CD8α$^+$ DCs play an important role in Ag presentation and initiation of the cellular immune response [4]. Noteworthy, the number and activation of CD8α$^+$ DCs but not of CD11b$^+$ conventional DCs and PDCA1$^+$ plasmacytoid DCs were significantly increased in Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. In line with an important inhibitory function of FL-CYLD in CD8α$^+$ DCs, the exclusive expression of sCYLD resulted in a significant increase of the absolute and relative amount of TNF-, IL-12-, and IL-10-producing CD8α$^+$ DCs in infected mice. Of note, TNF is strongly protective in listeriosis [21] and, therefore, the increased TNF production of CD8α$^+$ DCs might contribute to the improved pathogen control in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. Interestingly, the protective function of sCYLD was limited by the dose of infection (Fig. 1). In contrast to other gene-deficient mice, including the

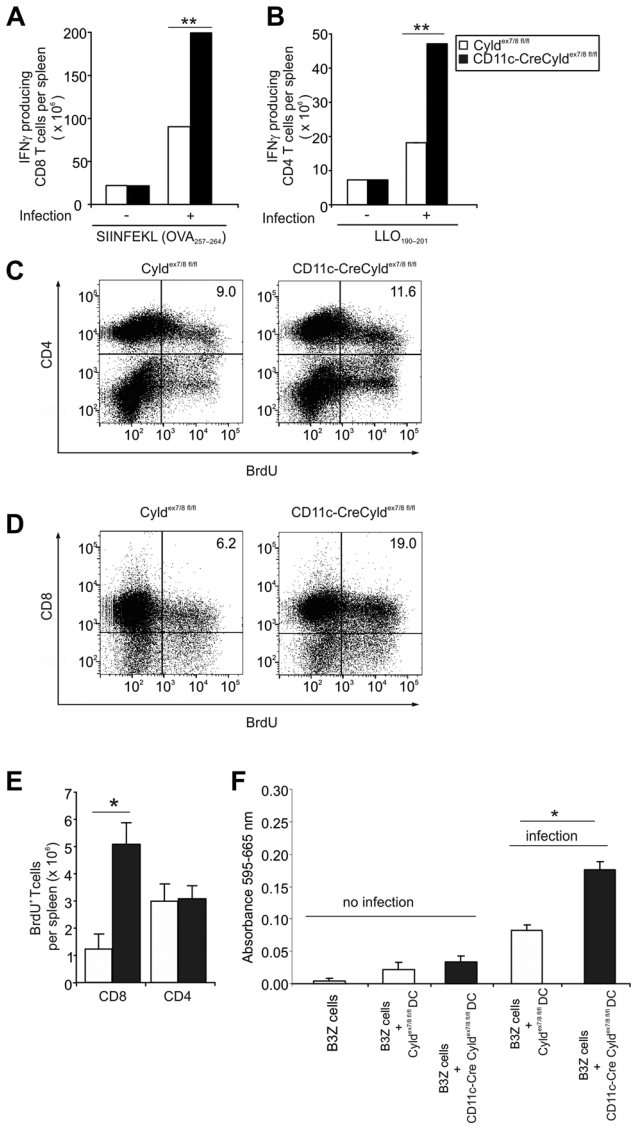

Figure 6. Increased frequency and proliferation of Lm-specific T cells and improved MHC class I Ag presentation of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ DCs. CD11c-Cre CYLD$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice were infected with Lm. (A, B) Splenocytes were isolated at day 7 p.i. and stimulated with (A) SIINFEKL ($OVA_{257–264}$) and (B) $LLO_{190–201}$ peptides. The frequencies of total and *Listeria*-specific, IFN-γ-producing (A) CD8$^+$ and (B) CD4$^+$ T cells were determined by flow cytometry. Data are shown as mean + SD of $n = 6$ per group, from one experiment representative of two performed; $^{**}p < 0.01$, *t*-test. (C, D) At day 6 p.i., BrdU incorporation of (C) CD4$^+$ and (D) CD8$^+$ T cells was measured by flow cytometry in Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice, which were i.p. treated with BrdU at days 4 and 5 p.i. Data shown are representative of six mice per group. (E) The total number of BrdU$^+$ CD8$^+$, and CD4$^+$ T cells are shown as mean ± SD of $n = 6$ per group, from one of two independent experiments; $^*p < 0.05$, *t*-test. (F) CD11c$^+$ DCs were isolated by MACS from spleens of LMova-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$, respectively, 24 h p.i. DCs from five mice per group were cocultivated with B3Z T cells for 6 h. Absorbance was measured at 595 nm and the values were subtracted from absorbance at 665 nm, after addition of β-galactosidase substrate CPRG. Data show the mean ± SD of DCs isolated independently from five mice per group, one experiment representative of two performed ($^*p < 0.05$, *t*-test).

mice lacking the type I IFN receptor [22], CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice conferred a dose-dependent protection.

Production of IL-12 by CD8α$^+$ DCs is important for the activation of NK cells and the induction of Lm-specific T cells [4]. Although the numbers of NK cells did not differ significantly between control and CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice, activation of NK cells was greatly enhanced as indicated by increased IFN-γ production. The activated NK cells also displayed an enhanced cytotoxicity as illustrated by an enhanced ex vivo cytotoxicity against RMA-S cells. Thus, the two major protective functions of NK cells in listeriosis, that is, IFN-γ production and cytotoxicity, were enhanced in mice with overexpression of sCYLD in DCs.

Listeriosis induces an apoptosis of T cells in the spleen early after infection [23]. In agreement, numbers of CD4$^+$ and CD8$^+$ T cells decreased in both strains of mice upon infection indicating that CYLD is not involved in Lm-induced T-cell apoptosis. However, a more detailed analysis of the ensuing Lm-specific T-cell response revealed that CD8$^+$ T cells proliferated significantly stronger in infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice resulting in significantly increased numbers of splenic CD8$^+$ T cells in CD11-Cre CYLD$^{ex7/8\ fl/fl}$ mice. In contrast, proliferation and absolute numbers of CD4$^+$ T cells were not significantly increased illustrating that the overexpression of sCYLD and absence of FL-CYLD in CYLD CD8α$^+$ DCs predominantly regulated Lm-specific CD8$^+$ T-cell responses. With respect to T-cell function, the number of IFN-γ-producing Lm-specific CD8$^+$ and—to a lesser extent—CD4$^+$ T cells was augmented in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice. The assumption that DCs lacking FL-CYLD while overexpressing sCYLD induce stronger activation of CD8$^+$ T cells was formally proven by the stronger activation of an OVA-specific CD8 T-cell hybridoma stimulated with CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ DCs isolated from Lm-OVA-infected mice.

In conclusion, expression of sCYLD in the absence of FL-CYLD in DCs leads to enhanced activation of CD8α$^+$ DCs, which stimulate both protective innate immune response via the activation of NK cells as well as adaptive immune response by enhancing pathogen-specific CD8$^+$ T cells. Functionally important, the improved immune response against Lm protected CD11c-Cre mice significantly from lethal listeriosis. These findings indicate that modulation of CYLD expression in DCs offers a therapeutic strategy to enhance DC-induced NK and CD8$^+$ T-cell responses.

Material and methods

Ethics statement

All animal experiments were performed in compliance with the German animal protection law in a protocol approved by the Landesverwaltungsamt Sachsen-Anhalt (file number: 203.h-42502-2-901, University of Magdeburg).

Animals

C57BL/6 CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and C57BL/6 Cyld$^{-/-}$ mice have been described before [16, 24]. Age- and sex-matched C57BL/6 CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and C57BL/6 Cyld$^{ex7/8\ fl/fl}$ mice were used for the experiments. All animals were kept under conventional conditions in an isolation facility throughout the experiments.

Bacterial infection of mice

Lm (EGD strain) and recombinant OVA-expressing LM (LMova) were grown in tryptose soy broth and aliquots of log-phase cultures were stored at –80°C. Fresh log-phase cultures were prepared from frozen stocks and 1×10^5 or 3×10^4 Lm diluted in 200 μL sterile pyrogen-free PBS (pH 7.4) were used for i.v. infection. In each experiment, the bacterial dose used for infection was controlled by plating an inoculum on tryptose soy agar and counting colonies after incubation at 37°C for 24 h.

CFU

To determine CFUs in Lm-infected mice, spleens were dissected and homogenized with sterile tissue grinders. Tenfold serial dilutions of the homogenates were plated on brain heart infusion agar. Bacterial colonies were counted microscopically after incubation at 37°C for 24 and 48 h. To determine CFUs in CD8α$^+$ DCs, splenocytes were isolated from CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice 24 h p.i., and stained for CD45, CD11c, and CD8α. Thereafter, cells were sorted with a FACS VERSE (BD Biosciences, Heidelberg, Germany) into a CD45$^+$ CD11c$^+$ CD8α$^+$ DC fraction and the remaining CD45$^+$ cell fraction. The sorted cells were collected in Hanks BSS with 3% FCS and counted. After centrifugation (3000 rpm), cells were lysed in 0.1% Triton X and aliquots of serial dilutions of the cell suspension were plated onto brain heart infusion agar. Bacterial colonies were counted microscopically after incubation at 37°C for 24 and 48 h, and the number of Lm per cell was calculated.

Isolation of leukocytes from spleen

Splenic leukocytes were isolated from sacrificed mice by passing spleens through a 70 μm cell strainer (BD Biosciences), and erythrocytes were lysed with ammonium chloride.

Western blot

FACS-sorted CD45$^+$ CD11c$^+$ and CD45$^+$ CD11c$^-$ cells were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 100 mM NaCl, 1% Triton-X-100, 10% glycerol, 10 mM KH_2PO_4, 0.5% Na-deoxycholate, 1 mM PSMF, 1 mM NaF, 1 mM $Na_4O_7P_2$, 1 mM Na_3VO_4, and aprotinin, leupeptin, pepstatin (all reagents from Sigma, Taufkirchen, Germany; 1 μg/mL each). Equal amounts of proteins were separated on 10% SDS polyacrylamide gels, transferred to polyvinylidene fluoride membranes followed by incubation with α-CYLD (previously generated by us [15] and GAPDH (Cell Signalling Technology, Danvers, MA). Blots were developed using an ECL Plus kit (GE Healthcare, Freiburg, Germany). WB images were captured using the Intas Chemo Cam Luminescent Image Analysis system (INTAS Science Imaging Instruments, Göttingen, Germany). For quantitation of protein intensities by densitometry, WB images were analyzed with the LabImage 1D software (Kapelan Bio-Imaging Solutions, Leipzig, Germany).

Immunoprecipitation

FACS-sorted CD45$^+$ CD11c$^+$ and CD45$^+$ CD11c$^-$ cells were lysed on ice as described before. In a preclearing phase, Sepharose G beads (GE Healthcare Europe GmbH, Munich, Germany) were incubated for 30 min with cell lysates under continuous shaking at 4°C. The beads were removed by centrifugation and equal amounts of lysates were incubated with anti-STAT3 Ab at 4°C overnight. The immune complex was captured by incubating with Sepharose G beads overnight at 4°C. The beads were then washed three times with PBS by centrifugation. The pellet containing Sepharose G immune complexes was suspended in buffer 2 (SDS, 1 M pH 6.8 Tris, glycerol; 2-ME) and boiled at 100°C for 5 min. After centrifugation, the supernatant was used to detect STAT3 and K63 ubiquitin (Cell Signalling Technology).

Flow cytometry

Leukocytes isolated from the spleen were analyzed by flow cytometry on a FACS Canto II with FACS Diva software (both from BD Biosciences). Cells were stained with anti-CD45 in combination with anti-CD4 and anti-CD3 for CD4$^+$ T cells, anti-CD8 and anti-CD3 for CD8$^+$ T cells, anti-NK1.1 for NK cells, F4/80 and CD11b for macrophages, and CD11c for total DCs. DC subpopulations were differentiated by staining for CD11b, CD8α, PDCA1. Inflammatory monocytes were identified as CD11b$^+$, Ly6G^{middle}, and Ly6C^{high}, and granulocytes as CD11b$^+$ Ly6G^{high}, and Ly6c^{high}. Control staining was performed with isotype-matched control Abs. All Abs were obtained from BD Biosciences. For intracellular staining of cytokines, splenocytes were stimulated with 50 ng/mL PMA, 500 ng/mL ionomycin, and 1 μL/mL Golgi-Plug (containing brefeldin A) in RPMI-1640 at 37°C for 4 h according to cytokine stimulation protocols by BD. After stimulation, cells were stained for NK cells and DC subtypes as described above. Thereafter, cells were fixed with Cytofix/Cytoperm, permeabilized with Perm Wash (BD), and stained with intracellular Abs against IL-12, TNF-α, IL-6, IL-10 for DCs or IFN-γ for NK cells. With respect to CD4$^+$ and CD8$^+$ T cells, splenocytes of LMova-infected mice were restimulated with $LLO_{190\text{-}201}$ (10^{-6} M) and $OVA_{257\text{-}264}$ (10^{-8} M). Isotype-matched control Abs were used.

Intracellular expression of IκBα, p-ERK, and p-p38 by DCs

Splenocytes were stained for CD45, CD11c, and CD8, fixed, permeabilized, and stained intracellularly for IκBα according to the

manufacturer's protocol (Cell Signalling Technology). p-ERK and p-p38 expression levels were measured using the BD Phosflow protocol (BD Biosciences). Isotype-matched control Abs were used.

BrdU incorporation assay

BrdU (1.5 mg/mouse) was administered i.p. to mice at days 4 and 5 p.i. The mice were sacrificed 24 h later and BrdU incorporation of $CD8^+$ and $CD4^+$ T cells was determined by flow cytometry using the APC BrdU flowkit (BD Biosciences).

Ex vivo NK-cell cytotoxicity assay

RMA-S cells were labeled with either 5 μM CFSE ($CFSE^{high}$ cells) or 0.25 μM CFSE ($CFSE^{low}$ cells) in RPMI medium (5 min on ice) and washed twice. $CFSE^{high}$ and $CFSE^{low}$ cells at a ratio of 1:1 were incubated with MACS-purified NK cells isolated from Lm-infected CD11c-Cre $Cyld^{ex7/8\ fl/fl}$ and $Cyld^{ex7/8\ fl/fl}$ mice at day 3 p.i. $CFSE^{high}$ cells were quantified by flow cytometry 24 h post incubation.

B3Z T-cell hybridoma assay

The B3Z T-cell hybridoma, which upon recognition of K^b-SIINFEKL by the T-cell receptor induces the expression of *lacZ* [25], was a gift from N. Shastri. B3Z T cells (1×10^5/well) were incubated with MACS-isolated $CD11c^+$ DCs from Lm-ova-infected CD11c-Cre $Cyld^{ex7/8\ fl/fl}$ and $Cyld^{ex7/8\ fl/fl}$ (24h p.i.). β-Galactosidase activity of B3Z T was determined by addition of 0.15 M chlorophenol red β-D-galactopyranoside (Boehringer Mannheim, Mannheim, Germany), 1 mM $MgCl_2$, and 0.125% Nonidet P-40 (Calbiochem, San Diego, CA) in PBS at 37°C for 6 h. The reaction was stopped with 300 mM glycine and 15 mM Na_2EDTA, and $OD_{595\text{-}665}$ was determined.

Statistical analysis

Statistical significance was calculated using the two-tailed Student's *t*-test or nonparametric Mann–Whitney rank sum test. All experiments were performed at least twice. *p* Values of ≤0.05 were considered significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **FL-CYLD**: full-length CYLD · **Lm**: *Listeria monocytogenes* · **LLO**: listeriolysin · **p.i.**: post infection · **sCYLD**: short CYLD · **WB**: Western blot

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Supporting Information

for

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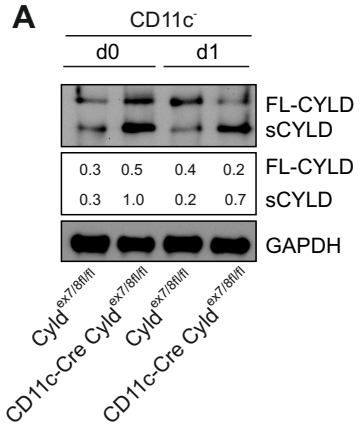
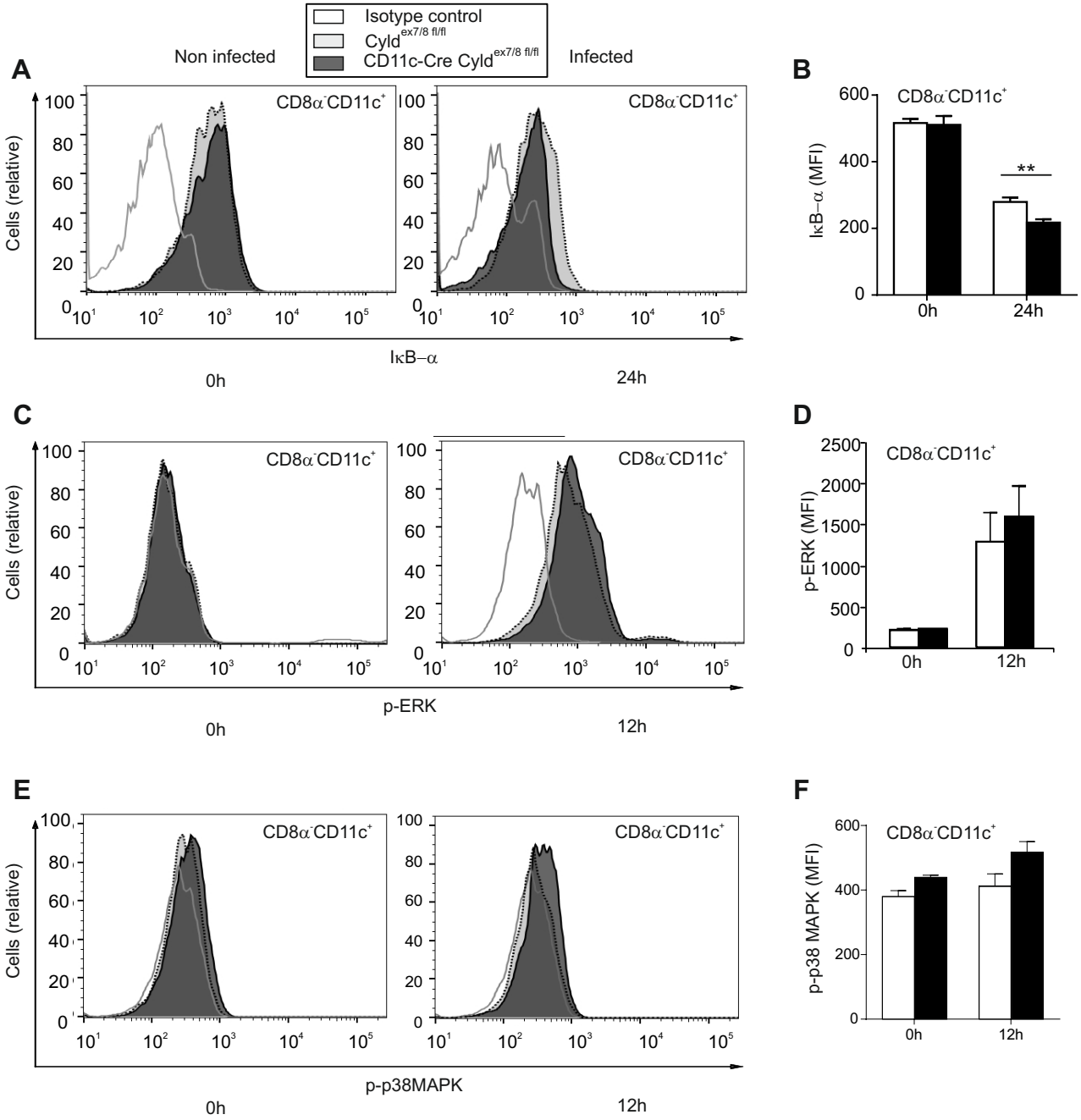
Protective dendritic cell responses against listeriosis induced by the short form of the deubiquitinating enzyme CYLD are inhibited by full-length CYLD

Supporting Information 1



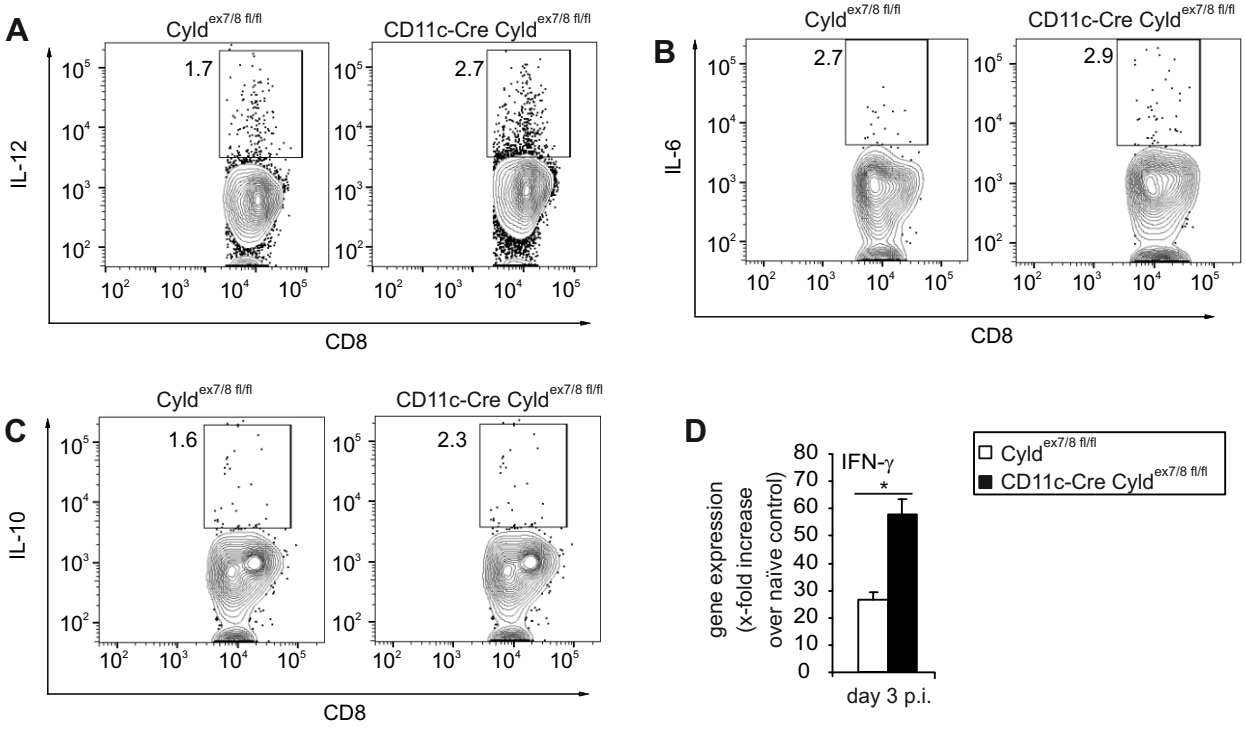
Supporting Information 1. Specific deletion of FL-CYLD in CD11c+ cells of CD11c-Cre Cyld$^{ex7/8\,fl/fl}$ mice FL-CYLD and sCYLD proteins were analyzed by WB in FACS-sorted CD45^{+} CD11c^{-} cells of both uninfected and Lm-infected Cyld$^{ex7/8fl/fl}$ and CD11c-Cre Cyld$^{ex7/8fl/fl}$ mice (n = 3 per group). Cells were sorted 24 h p.i (A). GAPDH was used as loading control. Quantification of FL- and sCYLD was performed from WB data by densitometry. Data show results of one of two independent experiments with similar results.

Supporting Infromation 2



Supporting Information 2. Normal activation of p-ERK and p-p38 in CD8α$^-$ DCs from Cyld$^{ex7/8\ fl/fl}$ mice. Histogram overlays show data for CD45$^+$ CD11c$^+$ CD8a$^-$ cells of uninfected (0 h p.i.) and infected (24 h p.i.) mice. The intracellular mean fluorescence intensity (MFI) of IκBα (A), p-ERK (C) and p-p38 (E) is shown. Staining with isotype control antibodies is depicted as open histograms; specific staining for Cyld$^{ex7/8\ fl/fl}$ mice is shown in light grey and for CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ in dark grey. Representative data of individual mice from one of two experiments with five mice per experimental group are shown. (B, D, F)) MFI of IκBα (B), p-ERK (D) and p-p38 (F) of uninfected (0 h p.i.) and Lm-infected (24 h p.i.) CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice (** $p < 0.01$). Data show the mean $\pm$ SD of five mice from one of two experiments.

Supporting Infromation 3

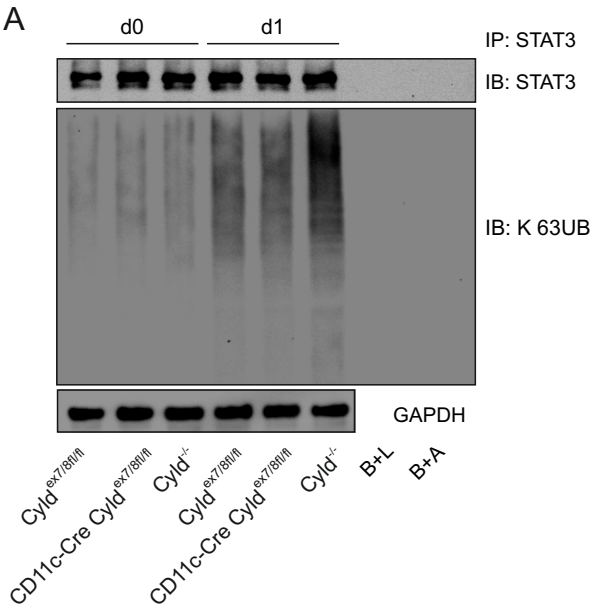


Supporting Information 3 . Enhanced cytokine production by CD8α$^+$ DCs of CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ mice.

Dot plots show the percentage of IL-12 (A) IL-6 (B) and IL-10 (C) production by CD45$^+$ CD11c$^+$ CD8α$^+$ DCs of Lm-infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice 24 h p.i.

Quantitative RT-PCR analysis of splenic IFN-γ (D) mRNA expression showed that IFN-γ mRNA was significantly increased in CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ compared to Cyld$^{ex7/8\ fl/fl}$ mice (* $p < 0.05$). Data show the increase of the respective mRNA expression of Lm-infected over uninfected mice of the same mouse strain. Data represent the mean ± SD of 5 mice

Supporting Information 4



Supporting Information 4. sCYLD does not regulate K63 polyubiquitination of STAT3 in DCs. Protein lysates of uninfected and Lm-infected CD11c^{+} DC from CD11c-Cre Cyld$^{ex7/8\ fl/fl}$, Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{-/-}$ mice were immunoprecipitated with STAT3. Immunoprecipitates were stained for K63-linked ubiquitin and GAPDH. As controls, beads plus lysate (B+L) and beads plus STAT3 antibody (B+A) were also analyzed. Data show equally increased amounts of K63-polyubiquitination of CD11c^{+} DC from Lm infected CD11c-Cre Cyld$^{ex7/8\ fl/fl}$ and Cyld$^{ex7/8\ fl/fl}$ mice. K63 polyubiquitination was augmented in infected Cyld$^{-/-}$ mice lacking both FL-CYLD and sCYLD.

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Peer review correspondence

Protective dendritic cell responses against listeriosis induced by the short form of the deubiquitinating enzyme CYLD are inhibited by full-length CYLD

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Handling Executive Committee member: Prof. Bernard Malissen

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 3 September 2014

Dear Dr. Gopala Krishna,

Manuscript ID eji.201445116 entitled "Full-length CYLD inhibits short CYLD induced protective immune responses of dendritic cells in listeriosis" which you submitted to the European Journal of Immunology has been reviewed.

The comments of the referees are included at the bottom of this letter. Even though ref. # 1 suggested rejection based on the low novelty, the other two referees and the Executive editor would like to see a revised version of your manuscript that takes into account the comments of all the referees.

You should also pay close attention to the editorial comments included below. In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.

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Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Laura Soto Vazquez

On behalf of Prof. Bernard Malissen

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Reviewer: 1

Comments to the Author
The authors investigated the role of the natural occurring short splice variant of CYLD (sCYLD) during infection. In order to do so they infected CD11c-Cre Cyldex7/8 fl/fl mice, which lack full-length CYLD (FL-CYLD) and overexpress the short splice variant with listeria monocytogenes (Lm). Infection of these mice resulted in increased NF-kB activity, increased cytokine production by DCs and subsequently enhanced stimulation of NK cells and beyond a stronger CD8+ T cell proliferation. Collectively the authors showed, that selective expression of sCYLD in DC results in an improved protection against Lm. The paper lacks sufficient novelty.

New / interesting aspects:
The authors mentioned, that the short splice variant is insufficiently described in the literature. But Srokowski et al (cited in this manuscript; ref. 16) showed already that the short splice variant in DCs resulted in an increased NF-kB activation. Furthermore Nishanth et al (cited in this manuscript; ref 11)

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investigated already the combined FL- and sCYLD-deficiency in respect to listeriosis. Therefore the new aspect of this manuscript is the investigation of the role of sCYLD in this particular infection model.

Minor points:

• Page 6, line 22: the word mice is missing

• For both figures (Fig 1C and Fig 2A) CFUs was determined, therefore it would be helpful to label both axes the same way, e.g. both with CFU

• Fig 2 E and G are labeled with 12h but the figure legend says 24h at which time point was the analysis done?

• Fig 3: for a better visualization of the data, it would be helpful to show also FACS blots of uninfected mice. To prevent a too big figure it would be sufficient to show FACS blots of uninfected and infected mice just exemplarily for one of the 4 cytokines, since the data is summarized in Fig 3 E.

• Page 9, line 49: A word is missing “since the increased number of Lm-specific could be caused…”

Reviewer: 2

Comments to the Author

The manuscript „Full-length CYLD inhibits short CYLD induced protective immune responses of dendritic cells in listeriosis“ by Wurm et al. analyses the course of listeriosis in a mouse model lacking the full length form of CYLD in dendritic cells by Cre-mediated recombination (CD11c-Cre Cyldex7/8 fl/fl).

The authors provide evidence that in the absence of FL-CYLD

- the infection of CD8+ DC with listeria is not affected
- the CD11c-Cre Cyldex7/8 fl/fl mice have a higher survival rate than control mice after listeria infection
- NF-kB activation is enhanced in CD8+ DC
- CD11c-Cre Cyldex7/8 fl/fl CD8+ DC produce more TNF, IL-10 and IL-12
- NK cells demonstrate higher IFNγ production and cytotoxic activity
- CD11c-Cre Cyldex7/8 fl/fl CD8+ DC present listeria antigen more efficiently
- CD8+ T cells produce more IFNγ and antigen specific proliferation is increased

The study of Wurm et al. is potentially very interesting for the knowledge on modulation of immune defenses against intracellular pathogens such as listeria, especially that the activation of CD8α+ DC is significantly increased in listeria infected sCYLD expressing mice. However prior to publication some issues should be adequately addressed:

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• Why do infected DC produce more sCYLD than uninfected DC while expression of FL-CYLD appears largely unchanged (Fig. 1A). Is this statement really reflected by the data? The WB band of FL-CYLD at d1 appears reduced as compared to d0.

• Legend Fig. 1A/Fig. 1A: The authors state that WB was performed in FACS sorted CD45+ CD11c+ and CD45+ CD11- cells derived from uninfected and listeria infected CYLDex7/8 fl/fl and CD11c-Cre Cyldex7/8 fl/fl mice. It is assumed the WB of CD11c+ cells is shown and the WB of CD11c- cells are missing? The WB of the CD11- cells should be shown for the control of Cre-deletion specificity

• Fig 1C: listeria counts in other organs such as liver, brain and lungs should be shown to exclude a spleen specific phenotype

• Fig 1D: Infection with higher numbers of listeria (log – titrations) should be performed to address the question how significant the phenotype is, i.e. comparable to IFNAR- knockouts?

• Results, page 7: the conclusion section starting with: “These data indicate that (i) listeriosis …” is inconsistent (missing statement to p38 phosphorylation). The authors should provide direct proof of NF-kB activation or change the statement. The authors should speculate on the mechanism how the absence of FL-CYLD enhances I-κB degradation.

• Fig. 4A/B: At day 3, splenocytes in CD11c-Cre Cyldex7/8 fl/fl mice are increased to approx. 125 x 106 cells whereas Cyldex7/8 fl/fl controls show ca. 80 x 106 cells. The increase of approx. 45 x 106 cells is not explained by the numbers of CD45+ positive cells per spleen as depicted in Fig. 4B. CD4 T cells are the same, CD8 T cell numbers shows an increase of approx. 6 x 106 cells, all the other cell populations are only marginally increased. Which cell types cause the large increase of total splenocytes, do non-immunological cells play a role? Histology should be performed, extra-medullary hemopoesis should be checked

• Fig. 4D: Why is MHC class II expression increased in CD11c-Cre Cyldex7/8 fl/fl DC? Is this due to NF-kB signaling or increased IFNγ-levels. Are there increased IFNγ amounts in spleens or in serum detectable?

• The authors should comment on increased IL-10 production of CD11c-Cre Cyldex7/8 fl/fl DCs since IL-10 has been shown to negatively regulate CD8 T cell functions in listeriosis previously

• The authors should analyze STAT3 K63-ubiquitination in CD11c-Cre Cyldex7/8 fl/fl DC to address the question whether sCLYD influences other signaling pathways (see Nishanth et al, Plos.Pathog. 2013. 9:e1003455

• The CD11c-Cre Cyldex7/8 fl/fl and Cyldex7/8 fl/fl font size in many figure inserts are too small too read

Reviewer: 3

Comments to the Author

In this study, Wurm E et al. investigated the functional role of sCYLD in DC in the pathogenesis of listeriosis. By using CD11c-Cre Cyld ex7/8 fl/fl mice, they showed that sCYLD in DC improved the

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inflammatory responses against Listeria, resulting in activation of NK cell and antigen-specific CD8+ T cell responses. All experiments are well carried out using well-established techniques. The conclusion is fully supported by the convincing data.

There are a few minor comments that need to be addressed.

1. The number of CD8 T cells is not significantly changed after infection in Figure 4B. Interestingly, activation of CD8 T cells is observed in Figure 6. This needs to be discussed.

2. More detailed information about the C57BL/6 CD11c-Cre Cyld ex7/8 fl/fl mice needs to be provided. The previous paper (Srokowski CC et al., Blood 2009) did not provide detailed information of the C57BL/6 CD11c-Cre Cyld ex7/8 fl/fl mice.

3. Figure 2B is not mentioned in text.

First revision – authors' response – 9 January 2015

Reviewer: 1
Comments to the Author
The authors investigated the role of the natural occurring short splice variant of CYLD (sCYLD) during infection. In order to do so they infected CD11c-Cre Cyldex7/8 fl/fl mice, which lack full-length CYLD (FL-CYLD) and overexpress the short splice variant with listeria monocytogenes (Lm). Infection of these mice resulted in increased NF-kB activity, increased cytokine production by DCs and subsequently enhanced stimulation of NK cells and beyond a stronger CD8+ T cell proliferation. Collectively the authors showed, that selective expression of sCYLD in DC results in an improved protection against Lm. The paper lacks sufficient novelty.
New / interesting aspects:
The authors mentioned, that the short splice variant is insufficiently described in the literature. But Srokowski et al (cited in this manuscript; ref. 16) showed already that the short splice variant in DCs resulted in an increased NF-kB activation. Furthermore Nishanth et al (cited in this manuscript; ref 11) investigated already the combined FL- and sCYLD-deficiency in respect to listeriosis. Therefore the new aspect of this manuscript is the investigation of the role of sCYLD in this particular infection model.

The function of sCyld in infectious diseases has not been reported before. In our study, we show for the first time the protective function of sCYLD in an infectious disease model. Furthermore we point out that the selective expression of sCYLD in DCs is sufficient to protect the mice against infection. In addition, we newly uncover the important role of DC-specific sCYLD for the regulation of the protective NK cell and

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pathogen-specific CD8 T cell responses. Our study may further prove to be important for future studies on the cell type-specific function of sCYLD in infectious diseases.

Minor points:

• Page 6, line 22: the word mice is missing

The missing word “mice” has been incorporated into the text on page Page 6, line 9

• For both figures (Fig 1C and Fig 2A) CFUs was determined, therefore it would be helpful to label both axes the same way, e.g. both with CFU

The axis of Fig 2A has been renamed to “CFU”. Now the axes of Fig 1C and Fig 2A read the same.

• Fig 2 E and G are labeled with 12h but the figure legend says 24h at which time point was the analysis done?

The analysis was performed at 24h. The labels of Fig 2 E and G have been corrected to 24h.

• Fig 3: for a better visualization of the data, it would be helpful to show also FACS blots of uninfected mice. To prevent a too big figure it would be sufficient to show FACS blots of uninfected and infected mice just exemplarily for one of the 4 cytokines, since the data is summarized in Fig 3 E.

FACS plots from uninfected mice have been incorporated into Figure 3A.

• Page 9, line 49: A word is missing “since the increased number of Lm-specific could be caused…”

The missing word “T cells” has been incorporated into the text on page Page 10, line 16.

Reviewer: 2

Comments to the Author

• Why do infected DC produce more sCYLD than uninfected DC while expression of FL-CYLD appears largely unchanged (Fig. 1A). Is this statement really reflected by the data? The WB band of FL-CYLD at d1 appears reduced as compared to d0.

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To further address this topic, we performed a quantification of western blots. In control mice, FL-CYLD declined slightly upon infection, whereas sCYLD increased (Fig. 1A). Thus, the ratio of sCYLD/FL-CYLD increased from 0.2 to 0.5 upon infection. These data are described on Page 6, lines 11-15 and discussed on Page 11, lines 12-15.

• Legend Fig. 1A/Fig. 1A: The authors state that WB was performed in FACS sorted CD45+ CD11c+ and CD45+ CD11- cells derived from uninfected and listeria infected CYLDex7/8 fl/fl and CD11c-Cre Cyldex7/8 fl/fl mice. It is assumed the WB of CD11c+ cells is shown and the WB of CD11c- cells are missing? The WB of the CD11- cells should be shown for the control of Cre-deletion specificity

Western blots of the CD11- cells are shown in the new Supplementary Fig. 1A and illustrate that FL-CYLD was not deleted in CD11c- cells of CD11c-Cre Cyldex7/8 fl/fl mice.

• Fig 1C: listeria counts in other organs such as liver, brain and lungs should be shown to exclude a spleen specific phenotype

The listeria counts from liver, brain and lungs have been incorporated into Figure 1C-P. These data show that differences in CFU are not spleen-specific.

• Fig 1D: Infection with higher numbers of listeria (log – titrations) should be performed to address the question how significant the phenotype is, i.e. comparable to IFNAR- knockouts?

We performed additional experiments with different doses of infection: 3x104, 1x105 and 1x106. Unlike IFNAR- knock out mice, CD11c-Cre Cyldex7/8 fl/fl mice succumbed to the high dose infection. The data have been included in Figure 1, described on Page 6, line 23 and Page 7, lines 1-6 and discussed on Page 12, lines 17-19.

• Results, page 7: the conclusion section starting with: “These data indicate that (i) listeriosis …” is inconsistent (missing statement to p38 phosphorylation). The authors should provide direct proof of NF-kB activation or change the statement. The authors should speculate on the mechanism how the absence of FL-CYLD enhances I-κB degradation.

The missing statement on p38 has been incorporated in the results Page 8, line 2. CYLD negatively regulates TRAF2 mediated the activation of IKK (ref.20 of the manuscript) As discussed on page Page 11, lines 18-20, sCYLD lacks the TRAF2 and NEMO binding sites of FL-CYLD. Therefore, the increase of NF-κB activation in CD11c+ cells lacking FL-CYLD but expressing sCYLD is most likely due to the defective removal of K63 Ub chains from TRAF2 and NEMO.

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• Fig. 4A/B: At day 3, splenocytes in CD11c-Cre Cyldex7/8 fl/fl mice are increased to approx. 125 x 106 cells whereas Cyldex7/8 fl/fl controls show ca. 80 x 106 cells. The increase of approx. 45 x 106 cells is not explained by the numbers of CD45+ positive cells per spleen as depicted in Fig. 4B. CD4 T cells are the same, CD8 T cell numbers shows an increase of approx. 6 x 106 cells, all the other cell populations are only marginally increased. Which cell types cause the large increase of total splenocytes, do non-immunological cells play a role? Histology should be performed, extra-medullary hemopoesis should be checked.

The large increase in the splenocyte number is caused due to the B cells. The B cell numbers have been incorporated into the total CD45+ cells. Additionally we have analysed extra-medullary hemopoesis by histology. In both uninfected and infected (day 5 p.i.) mice of both mouse strains, extra-medullary hemopoesis was not detectable. Representative histology of uninfected mice are attached in the document response to reviewer´s comments.doc

• Fig. 4D: Why is MHC class II expression increased in CD11c-Cre Cyldex7/8 fl/fl DC? Is this due to NF-kB signaling or increased IFNγ-levels. Are there increased IFNγ amounts in spleens or in serum detectable?

To address this question, we performed additional quantitative RT-PCRs for IFN-γ from spleens. At day 3 p.i., IFN-γ mRNA was significantly increased in CD11c-Cre Cyldex7/8 fl/fl mice (new Suppl. Fig. 3D). Based on these new data, we suggest that the increased MHC class II expression of sCYLD DCs is most probably a combinatorial effect of both increased IFN-γ expression and NF-κB activation. The new figure has been added (Suppl. Fig. 3 D). These data are described on page 9, lines 12-15.

• The authors should comment on increased IL-10 production of CD11c-Cre Cyldex7/8 fl/fl DCs since IL-10 has been shown to negatively regulate CD8 T cell functions in listeriosis previously

IL-10 expression levels declined in both strains of mice upon infection. Although IL-10 mRNA levels of CD8α+ DCs were significantly increased in infected CD11c-Cre Cyldex7/8 fl/fl mice, the IL-10 mRNA may be too low to substantially modify the ensuing CD8 T cell response. In particular, the increased IL-12 production of CD8α+ DCs of CD11c-Cre Cyldex7/8 fl/fl mice may override the effects of IL-10.

• The authors should analyze STAT3 K63-ubiquitination in CD11c-Cre Cyldex7/8 fl/fl DC to address the question whether sCLYD influences other signaling pathways (see Nishanth et al, Plos.Pathog. 2013. 9:e1003455

Upon Lm infection, K63 polyubiquitination of STAT3 of Cyld−/−, Cyldex7/8 fl/fl and CD11c-Cre-Cyldex7/8 fl/fl DCs was upregulated. However, the amount of K63-polyubiquitinated STAT3 proteins was augmented in DCs from Cyld−/− mice as compared to the DCs from Cyldex7/8 fl/fl and CD11c-Cre-Cyldex7/8 fl/fl mice, both of which showed equally reduced K63-polyubiquitinated STAT3. These data suggest that

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sCYLD, unlike the FL-CYLD does not influence the ubiquitination of STAT3.The new figure has been added (Suppl. Fig. 4 A).The experimental procedure is described on page 16, lines 9-19. The data are described on page 8, lines 4-13 and discussed on page 12, lines 1-4.

• The CD11c-Cre Cyldex7/8 fl/fl and Cyldex7/8 fl/fl font size in many figure inserts are too small too read

The font size in the figure inserts have been increased.

Reviewer: 3
Comments to the Author
There are a few minor comments that need to be addressed.

1. The number of CD8 T cells is not significantly changed after infection in Figure 4B. Interestingly, activation of CD8 T cells is observed in Figure 6. This needs to be discussed.

The number of CD8 T cells declines in both strains of mice upon Lm, which is compatible with the described apoptosis of CD8 T cells early after infection (ref.23 of the manuscript). Of note, the significantly increased number of CD8 T cells in infected CD11c-Cre Cyldex7/8 fl/fl as compared to Cyldex7/8 fl/fl mice is caused by an increased activation and proliferation of CD8 T cells by CD11c-Cre Cyldex7/8 fl/fl DCs. (discussed on page12, lines 8-23 and page13, line 1)

2. More detailed information about the C57BL/6 CD11c-Cre Cyld ex7/8 fl/fl mice needs to be provided. The previous paper (Srokowski CC et al., Blood 2009) did not provide detailed information of the C57BL/6 CD11c-Cre Cyld ex7/8 fl/fl mice.
The citation describing the generation of C57BL/6 CD11c-Cre Cyldex7/8 fl/fl mice in detail has been included (Hövelmeyer et al. J Exp Med 2007; (ref.16 of the manuscript)

3. Figure 2B is not mentioned in text.

Figure 2B is now mentioned in the text (page7, line 18).

Peer review correspondence

European Journal of Immunology

Second Editorial Decision – 3 February 2015

Dear Dr. Gopala Krishna, Dr. Schlüter,

It is a pleasure to provisionally accept your manuscript entitled "Full-length CYLD inhibits short CYLD induced protective immune responses of dendritic cells in listeriosis" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. Bernard Malissen

Dr. Karen Chu
Editorial Office
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