Calcineurin-mediated IL-2 production by CD11c<sup>high</sup>MHCII<sup>+</sup> myeloid cells is crucial for intestinal immune homeostasis

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The intestinal immune system can respond to invading pathogens yet maintain immune tolerance to self-antigens and microbiota. Myeloid cells are central to these processes, but the signaling pathways that underlie tolerance versus inflammation are unclear. Here we show that mice lacking Calcineurin B in CD11c<sup>high</sup>MHCII<sup>+</sup> cells (Cnb1<sup>CD11c</sup> mice) spontaneously develop intestinal inflammation and are susceptible to induced colitis. In these mice, colitis is associated with expansion of T helper type 1 (Th1) and Th17 cell populations and a decrease in the number of FoxP3<sup>+</sup> regulatory T (Treg) cells, and the pathology is linked to the inability of intestinal Cnb1-deficient CD11c<sup>high</sup>MHCII<sup>+</sup> cells to express IL-2. Deleting IL-2 in CD11c<sup>high</sup>MHCII<sup>+</sup> cells induces spontaneous colitis resembling human inflammatory bowel disease. Our findings identify that the calcineurin–NFAT–IL-2 pathway in myeloid cells is a critical regulator of intestinal homeostasis by influencing the balance of inflammatory and regulatory responses in the mouse intestine.
The intestine is a unique and challenging immune microenvironment in which homeostasis requires active tolerance towards self-antigens, dietary-antigens, and commensal microorganisms, balanced with the requirement to detect and respond rapidly to pathogen invasion. Dysregulation of the intestinal immune compartment can result in inflammatory bowel disease (IBD), which affects ~3.5 million people in the US and Europe1.

The mouse intestinal lamina propria (LP) contains distinct subsets of mononuclear phagocytes that maintain tolerance and immune reactivity towards pathogens2,3. These subsets include conventional dendritic cells (DC), monocytes, and tissue-resident macrophages. LP DCs express high levels of CD11c, MHCII, and FMS-like tyrosine kinase 3 (FLT3, also known as CD135) and contribute to initiation of immune responses. Despite the importance of these myeloid cells in supporting intestinal immune homeostasis, we know little about the cellular signaling networks that modulate tolerance-versus-inflammation in the intestine.

The nuclear factor of activated T-cells (NFAT) family of transcription factors is an important mediator of DC function4,5.

**Fig. 1** Calcineurin B and NFAT expression in mouse intestinal myeloid cells. a Relative expression levels of Cnb1, Nfat1, and Nfat2 mRNAs in intestinal CD11c(high)MHCII+ cells (CD11b+ and CD11b−) and MLN CD3+ T cells, assessed by qRT-PCR. Data represent the means ± standard error of three experiments (n ≥ 7 mice/exp). b, c Percentage of NFAT-1+ cells (b) and NFAT-1 protein levels (c) in CD11c(high)MHCII+ cells (CD11b+ and CD11b−) obtained from spleen, MLN, PP, and colonic LP (LP-colon), assessed by flow cytometry. Spleenic CD3+CD4+ T cells are included for comparison. Data represent the means ± standard error of three experiments (n = 4–5 mice/experiment). **P<0.01 versus CD4+ T cells (ANOVA followed by Dunnett’s multiple comparisons test). d Representative images of NFAT-1 labeling in sorted colonic CD11c(high)MHCII+ cells (CD11b+ and CD11b−). Scale bar 5 μm. e NFAT-1 nuclear translocation in CD11c(high)MHCII+ cells (CD11b+ and CD11b−) after 30 min stimulation with thapsigargin (Thap; 200 nM), as assessed by confocal analysis. Data represent the means ± standard error of two experiments (n = 6–10 mice/experiment). Scale bar 5 μm. f NFAT-dependent luciferase activity measured in a DC cell line in response to TLRs (Poly I:C, LPS), dectin-1 ligands (PGG and WGP), and the calcium mobilizer thapsigargin. g Dose-dependent inhibition of NFAT nuclear translocation by cyclosporin A (CsA) and tacrolimus (FK506) in WGP-stimulated D1 cells, as assessed by NFAT-luciferase activity. AU arbitrary units, Ctrl control, Luc luciferase, LP lamina propria, LPS Lipopolysaccharide, MLN mesenteric lymph node, PGG soluble β-(1,3)-glucan, PP Peyer’s Patches, WGP whole glucan particles, Thap thapsigargin.
Initially, NFAT members were thought to be involved solely in the transcriptional activation of T cells, but different NFAT proteins are now known to have a complex influence on host tolerance to self and foreign antigens. Upon calcium influx induced by ligation of pattern recognition receptors, activated NFAT triggers calmodulin-mediated activation of the protein phosphatase calcineurin, which in turn induces NFAT nuclear translocation. NFAT is a master transcription factor that regulates expression of IL-2, an immuno-regulatory cytokine that controls T-cell expansion and differentiation, Treg-cell maintenance, and NK-cell activation. DCs are a source of IL-2 and IL-2 can regulate immune responses in the mouse lung during fungal infection, and promote adaptive immune responses to alum immunization. Whether the calcineurin–NFAT–IL-2 axis in antigen-presenting cells (APC) can contribute to immune homeostasis under basal and inflammatory conditions in other organs, such as the intestine, is unknown.

Here we use mice lacking calcineurin B (encoded by Cnb1) or IL-2 (encoded by Il2) in CD11c<sup>high</sup>MHCII<sup>+</sup> cells, and show that calcineurin–NFAT–IL-2 signaling in intestinal CD11c<sup>high</sup>MHCII<sup>+</sup> APCs prevents spontaneous chronic intestinal inflammation in acute and chronic models of induced colitis. Calcineurin–NFAT signaling in intestinal DCs mediates IL-2 production, which restrains inflammatory T-cell expansion and effector function. Our data shed light on the processes underpinning tolerance versus inflammation in the mouse intestine, and identify the calcineurin–NFAT–IL-2 pathway in intestinal APCs as a potential therapeutic target for IBD.

**Results**

Calcineurin–NFAT is active in colonic CD11c<sup>high</sup>MHCII<sup>+</sup> cells. mRNA expression analysis of Cnb1 and the major Nfat isoforms in leukocytes Nfat1 and Nfat2 revealed that T cells from mesenteric lymph nodes (MLN) and CD11b<sup>+</sup> and CD11c<sup>high</sup>MHCII<sup>+</sup> cells from colonic lamina propia (LP-colon) expressed Cnb1 and Nfat1 at comparable levels, but Nfat2 was less abundant in both populations compared to T cells (Fig. 1a). At the protein level, NFAT-1 was expressed by ~80% of CD11c<sup>high</sup>MHCII<sup>+</sup> cells abrogates NFAT-1 nuclear translocation. Confocal microscopy analysis of NFAT-1 nuclear translocation in CD11c<sup>high</sup>MHCII<sup>+</sup> cells (CD11b<sup>+</sup> and CD11b<sup>–</sup>) from the MLN of Cnb1<sup>CD11c−/−</sup> and Cnb1<sup>fl/fl</sup> mice after thapsigargin stimulation for 30 min. Data represent the means ± standard error of two experiments (n = 3 mice/experiment). **P < 0.01. Scale bar 10 μm. Representative flow cytometric analysis of intestinal LP mononuclear myeloid CD11c<sup>+</sup> cells evaluated by CD11c, CD103, CD11b, and CD64 expression. Four myeloid cell subsets were identified: three DC populations, CD11c<sup>high</sup>CD103<sup>−</sup>, CD11c<sup>high</sup>CD103<sup>+</sup>, CD11c<sup>low</sup>CD103<sup>−</sup>CD11b<sup>+</sup>, CD11c<sup>low</sup>CD103<sup>−</sup>CD11b<sup>+</sup> and one CD11c<sup>high</sup>CD103<sup>−</sup>CD11b<sup>+</sup> macrophage population. The macrophage population was identified based on the expression of CD64 within the CD11c<sup>low</sup>CD103<sup>−</sup>CD11b<sup>+</sup> population (CD11c<sup>low</sup>CD64<sup>+</sup>). The frequency of the four different myeloid subsets in LP-colon (left) and LP-SI (right) of Cnb1<sup>CD11c−/−</sup> and Cnb1<sup>fl/fl</sup> mice is shown. Data represent the means ± standard error of three experiments (n = 2-3 mice/group per experiment, aged 8-12 weeks). LP lamina propria, SI small intestine
confirmed by confocal microscopy (Fig. 1d) and its translocation to the nucleus was observed in response to the calcium mobilizer thapsigargin (Fig. 1e). Using a mouse DC line (D1) stably expressing an NFAT-luciferase reporter, we found that whole-glucan particles (WGP; particulated dectin-1 agonist) and thapsigargin could robustly activate NFAT; lipopolysaccharide (LPS) and soluble β-(1,3)-glucan PGG activated NFAT to a lesser extent.
(Fig. 1f). Finally, inhibition of calcineurin signaling with cyclosporin A or tacrolimus (FK506) effectively suppressed WGP-induced NFAT-luciferase activity (Fig. 1g). These data indicate that the calcineurin–NFAT pathway is active under steady-state conditions in colonic CD11\(^{\text{high}}\)MHCII\(^{+}\) cells, and can respond to calcium flux and TLR/\text{dectin-1} ligands.

Calcinieurin B in myeloid cells maintains intestinal homeostasis. We next asked whether the calcineurin–NFAT pathway has a functional role in intestinal immune homeostasis. We generated Cnb1\(^{+/+}\)CD11c mice in which calcineurin B expression is lost in cells expressing CD11c at high level, by crossing Cnb1\(^{+/+}\) with CD11c-specific Cre-mice.\(^{15}\) Cnb1 mRNA was significantly diminished in LP-colon of CD11\(^{\text{high}}\)MHCII\(^{+}\)CD11b\(^{+}\) and CD11\(^{\text{high}}\)MHCII\(^{+}\)CD11b\(^{+}\) myeloid cells (mostly belonging to the DC pool) of Cnb1\(^{CD11c}\) mice, compared to CD11\(^{\text{low}}\)CD11b\(^{+}\)CD64\(^{+}\) cells (mostly macrophages) (Fig. 2a). Cnb1 deletion prevented thapsigargin-driven NFAT-1 nuclear translocation in CD11b\(^{+}\) and CD11b\(^{+}\) CD11\(^{\text{high}}\)MHCII\(^{+}\) cell populations in the MLN (Fig. 2b). However, calcineurin–NFAT-1 deficiency in CD11\(^{\text{high}}\)MHCII\(^{+}\) cells did not affect the relative abundance of these myeloid populations in LP-colon of Cnb1\(^{CD11c}\) mice (Fig. 2c), or the expression of maturation markers of CD11\(^{\text{high}}\)MHCII\(^{+}\) myeloid cells in LP-colon and LP-small intestine (LP-SI) (Supplementary Fig. 1). Calcineurin B expression remained intact in BK, and mast cells, and naive, memory CD4\(^{+}\) T cells and Treg cells from MLN of Cnb1\(^{CD11c}\) mice, which also released normal cytokine levels in vitro (Supplementary Fig. 2a–c).

To confirm that Cnb1 deletion in CD11\(^{\text{high}}\)MHCII\(^{+}\) cells did not impact T-cell function in vivo, we monitored the development of colitis following adoptive transfer of naive CD4\(^{+}\)CD45RB\(^{\text{high}}\)CD25\(^{-}\) T cells from Cnb1\(^{CD11c}\) and Cnb1\(^{+/+}\) mice into immune-deficient Rag2\(^{KO}\) mice.\(^{16}\) A normal course of colitis development was observed in Rag2\(^{KO}\) mice receiving naive CD4\(^{+}\) T cells from Cnb1\(^{CD11c}\) mice (Supplementary Fig. 2d, e). These data indicate that CD11\(^{\text{high}}\)MHCII\(^{+}\) cells are the predominant cell population targeted by the Cnb1 deletion and the abundance and activation status of myeloid cells and effector function of CD4\(^{+}\) T cells in the intestine of Cnb1\(^{CD11c}\) mice remain unaffected.

Having confirmed the specificity of our Cnb1 deletion to CD11\(^{\text{high}}\)MHCII\(^{+}\) cells, we asked whether disruption of calcineurin–NFAT signaling in these cells affected intestinal homeostasis in vivo. Macroscopic examination of Cnb1\(^{CD11c}\) mice (aged 10–14 weeks) revealed moderate enlargement of the MLN compared to Cnb1\(^{+/+}\) control mice (Fig. 3a), and spontaneous inflammation of the SI and colon, characterized by an inflammatory infiltrate in the submucosa with marked erosion of the mucosal lining (Fig. 3b). Although Cnb1\(^{CD11c}\) mice did not develop evident symptoms of colitis (such as weight loss, diarrhea or rectal bleeding), they exhibited significantly higher intestinal permeability, titters of fecal lgalA (Fig. 3c), and myeloperoxidase activity in homogenates of the terminal ileum (Fig. 3d) than Cnb1\(^{+/+}\) controls. Increased levels of mucosal TNF and IFN\(\gamma\) were evident in the SI and colon of Cnb1\(^{CD11c}\) mice, compared to Cnb1\(^{+/+}\) controls, while IL-17 levels were comparable (Fig. 3d).

Analysis of the immune-cell composition and cytokine profile in the Cnb1\(^{CD11c}\) LP revealed a higher frequency of immune cells (CD4\(^{+}\) and CD4\(^{+}\) T cells), and a higher percentage of antigen-experienced CD4\(^{+}\)CD62L\(^{-}\) CD4\(^{+}\) T cells in the LP-SI and LP-colon compared to Cnb1\(^{+/+}\) controls (Fig. 3e). Cnb1 deficiency in CD11\(^{\text{high}}\)MHCII\(^{+}\) myeloid cells also significantly increased the frequency of IL-17-producing and IFN\(\gamma\)-producing CD4\(^{+}\) T cells infiltrating the LP-SI, and IFN\(\gamma\)-producing CD4\(^{+}\) T cells in the LP-colon (Fig. 3e). No difference in IL-4–producing CD4\(^{+}\) T cells or the CD8\(^{+}\) T-cell phenotype was observed in the LP-colon of Cnb1\(^{CD11c}\) mice compared to Cnb1\(^{+/+}\) controls (Supplementary Fig. 3a). Importantly, Cnb1\(^{CD11c}\) mice exhibited significantly reduced frequencies of FoxP3\(^{+}\) Treg cells in the LP-colon, LP-SI, and MLN, but not in the spleen or thymus (Fig. 3f). Spontaneous chronic inflammation occurred exclusively in the intestine and systemic inflammation, immune phenotype and cytokine release from splenic CD4\(^{+}\) T cells were comparable between Cnb1\(^{CD11c}\) and Cnb1\(^{+/+}\) mice (Supplementary Fig. 3b, c).

These data support that the calcineurin–NFAT axis in CD11\(^{\text{high}}\)MHCII\(^{+}\) cells is critical for CD4\(^{+}\) T-cell homeostasis in the intestine under steady-state conditions.

Cnb1\(^{CD11c}\) mice have unrestrained induced gut inflammation. We next asked how dysregulated T-cell responses would affect the ability of Cnb1\(^{CD11c}\) mice to restrain pathologic mucosal inflammation during acute and chronic models of IBD. Intra-rectal instillation of trinitrobenzene sulfonate (TNBS) rapidly induces intestinal inflammation in mice that recapitulates key features of Crohn’s disease.\(^{17,18}\) Compared to TNBS-treated Cnb1\(^{+/+}\) controls, TNBS-treated Cnb1\(^{CD11c}\) mice exhibited a significant loss in body weight by 6 days post treatment, worse stool consistency (Fig. 4a), and severe ulceration and thickening of the colon wall (Fig. 4b). Using a histopathological scoring approach, we found that Cnb1\(^{CD11c}\) mice exhibited severe leukocyte infiltration that penetrated all colon layers (Fig. 4c) and a significant increase in myeloperoxidase activity, TNF, and IL-17 in colon homogenates (Fig. 4d). Treatment of Cnb1\(^{CD11c}\) mice with naproxen, a non-steroidal anti-inflammatory drug that can exacerbate colitis in susceptible IBD patients, also induced notable colonic mucosal inflammation compared to untreated Cnb1\(^{CD11c}\) mice or naproxen-treated Cnb1\(^{+/+}\) control mice.
To examine the effect of Cnb1LysM depletion in non-DC phagocytes on intestinal inflammation, we generated a Cnb1<sup>LysM<sup>M</sup> mouse model, in which the Cnb1 floxed allele was left-exon deleted by Cre-recombinase in cells expressing LysM<sup>19</sup>. The intestinal phenotype of LP mononuclear cells from Cnb1<sup>LysM<sup>M</sup> mice under steady-state conditions was indistinguishable from Cnb1<sup>β/β</sup> controls with respect to: maintenance of body weight, colon histology, LP-colon immune-cell phenotypes, and levels of pro-inflammatory cytokines (Supplementary Fig. 5a–c). Moreover, upon TNBS treatment, symptoms of acute colitis were significantly less severe in Cnb1<sup>LysM<sup>M</sup> mice than in Cnb1<sup>β/β</sup> controls (Supplementary Fig. 5d–f).

These data highlight that calcineurin B expression in CD11c<sup>high</sup>MHCII<sup>+</sup> cells not only prevents spontaneous colitis under steady-state conditions, but also helps suppress severe intestinal pathology in response to acute and chronic inflammation.

**Cnb1 deletion in non-DC phagocytes reduces colitis severity.**

To examine the effect of Cnb1 depletion in monocytes, macrophages, and granulocytes on intestinal inflammation, we generated a Cnb1<sup>LysM<sup>M</sup> mouse model, in which the Cnb1 floxed allele was left-exon deleted by Cre-recombinase in cells expressing LysM<sup>19</sup>. The intestinal phenotype of LP mononuclear cells from Cnb1<sup>LysM<sup>M</sup> mice under steady-state conditions was indistinguishable from Cnb1<sup>β/β</sup> controls with respect to: maintenance of body weight, colon histology, LP-colon immune-cell phenotypes, and levels of pro-inflammatory cytokines (Supplementary Fig. 5a–c). Moreover, upon TNBS treatment, symptoms of acute colitis were significantly less severe in Cnb1<sup>LysM<sup>M</sup> mice than in Cnb1<sup>β/β</sup> controls (Supplementary Fig. 5d–f).

These data highlight that calcineurin B expression in CD11c<sup>high</sup>MHCII<sup>+</sup>LysM<sup>−</sup> cells, which mainly represent the conventional DCs in the intestine, participates in intestinal homeostasis by restraining pathological inflammation during colitis while calcineurin B expressed in LysM<sup>+</sup> monocytes/macrophages and granulocytes has little effect on intestinal homeostasis during steady-state conditions, but exacerbates intestinal immunopathology during TNBS-induced colitis.

**Calcineurin B partially regulates DC-derived IL-2 production.**

To delineate the mechanistic basis for calcineurin-mediated protection from colitis elicited by CD11c<sup>high</sup>MHCII<sup>+</sup> DCs, we examined a role for calcineurin B in the production of DC cytokines regulating T-helper differentiation. Colonic CD11c<sup>high</sup>MHCII<sup>+</sup>CD11b<sup>+</sup> cells from Cnb1<sup>CD11c<sup>C</sup></sup> mice produced significantly less IL-2 following thapsigargin exposure compared to cells from Cnb1<sup>β/β</sup> mice, while the levels of IL-6, IL-12, IL-23, IL-10, and TGFβ were comparable (Fig. 5a). Similarly, we found that bone marrow (BM)-derived DCs released IL-2 in response to thapsigargin, LPS, and zymosan, as assessed by ELISA and intracellular IL-2 labeling (Fig. 5b, c and Supplementary Fig. 6a), whereas IL-2 production from Cnb1<sup>CD11c<sup>C</sup></sup> DCs, but not IL-12p40 or IL-6, was significantly reduced. Moreover, exposure to NFκB and p38 MAPK inhibitors fully abrogated IL-2 release from Cnb1<sup>CD11c<sup>C</sup></sup> DCs (Supplementary Fig. 6b). These data indicate that IL-2 synthesis in DCs depends on calcineurin–NFAT, NFκB and AP1 signaling.

We also evaluated the ability of CD11c<sup>high</sup>MHCII<sup>+</sup> cells to produce IL-2 in vivo using an IL-2–GFP reporter mouse<sup>20</sup>. GFP<sup>+</sup> (IL-2<sup>+</sup>) cells among the CD11c<sup>high</sup>MHCII<sup>+</sup> compartment were present in the spleen (~2–4%), MLN (~8–10%), and were absent in CD11b<sup>+</sup> (CD4<sup>+</sup>CD11c<sup>−</sup>) cells belonging to the monocyte/macrophage/granulocyte lineage (Fig. 5d and Supplementary Fig. 7). Intracellular IL-2 labeling of CD11c<sup>high</sup>MHCII<sup>+</sup> cells isolated from spleen and LP-colon showed that up to ~13% of cells produced IL-2 under steady-state conditions (Fig. 5e and Supplementary Fig. 8).

During acute colitis, the proportion of IL-2-producing CD11c<sup>+</sup>MHCII<sup>+</sup> (CD64<sup>+</sup>F4/80<sup>−</sup>) DCs in LP-colon further increased
Fig. 5 Calcineurin-NFAT signaling in CD11c^+MHCII^{high} myeloid cells regulates IL-2 synthesis. a) CD11c^{high}MHCII^{high}CD11b^{-} cells sorted from MLN of Cnb1^{CD11c} and Cnb1^{fl/fl} mice were stimulated with thapsigargin for 16-20 h. Cytokine levels were measured in the culture supernatants using Luminex technology. Data represent the means ± standard error of three experiments (n = 10 mice/group per experiment). *P < 0.05. b) IL-2 production from bone marrow-derived DCs of Il2^{KO}, Cnb1^{CD11c}, and Cnb1^{fl/fl} mice in response to thapsigargin stimulation for 16 h, as assessed by ELISA. Data are presented as the relative percentage of IL-2+ cells and represent the means ± standard error of four experiments (n = 3 mice/group per experiment). *P < 0.05, ***P < 0.001. c) Intracellular IL-2 labeling of colonic CD3^{+}CD11c^{high}MHCII^{+} cells from Il2^{KO}, Cnb1^{fl/fl}, and Cnb1^{CD11c} mice. Data are presented as the relative percentage of IL-2^{+} cells and represent the means ± standard error of five experiments (n = 3 mice/group per experiment). *P < 0.05, ***P < 0.001. d) Percentage of IL-2^{+} cells in intestinal DC (CD45^{+}Lin^{-}CD3^{+}CD11c^{+}MHCII^{+}CD24^{+}CD64^{+}F4/80^{-}) and macrophage (CD45^{+}Lin^{-}CD3^{+}CD11c^{+}MHCII^{+}CD24^{+}CD64^{+}F4/80^{-}) populations in LP-colon and LP-small intestine of Cnb1^{fl/fl} mice at steady state, or during colitis induced in immunocompromised Rag2^{KO} mice by adoptive transfer of naive CD4^{+} T cells (CD45^{+}Lin^{-}CD62L^{+}CD44^{+}CD25^{+}) isolated from the spleens of C57BL/6 mice. Data represent the means ± standard error of three experiments (5-6 mice/group per experiment). *P < 0.05, ***P < 0.001. g) IL-2 expression in CD103^{+}CD11b^{+}MHCII^{+} cells from colonos of Cnb1^{fl/fl} and Cnb1^{CD11c} mice. Representative dot plots of IL-2^{+} labeling in colonic CD3^{+}CD11c^{high}MHCII^{+} cells are shown. Data represent the means ± standard error of three experiments (n = 2-5 mice/group per experiment). *P < 0.05. KO knockout, LP lamina propria, MLN mesenteric lymph node, SI small intestine, Thap thapsigargin, UT untreated, ND not detected.
Fig. 6 Th1/Th17-cell expansion and Treg-cell contraction in the gut of Il2CD11c mice. a Change in body weight (expressed as Δ percentage relative to the mean weight of the control group) in Il2CD11c mice compared to Il2fl/fl mice. Data represent the means ± standard error (n = 20 mice/group). *P < 0.05, **P < 0.01. b Representative images of the MLN (left) and colons (right) from Il2CD11c and Il2fl/fl mice aged 10 weeks old. c H&E staining of colon sections showing massive leukocyte infiltration in Il2CD11c compared to Il2fl/fl mice (×10 magnification, scale bar 0.1 mm). Histological inflammation index of the colon was also evaluated over time. Data represent the means ± standard error (14 mice/group aged 6–10 weeks old, and 6 mice/group aged 14–17 weeks old). ***P < 0.001. d Plasma levels of C-reactive protein (CRP) and IgG1 in the sera of Il2CD11c and Il2fl/fl mice. Data represent the means ± standard error (5–7 mice/group). **P < 0.01. e Immune phenotype of LP-colon mononuclear cells from Il2CD11c and Il2fl/fl mice. Data represent the means ± standard error of the total number of mononuclear cells obtained from the LP, the percentage of total and antigen-experienced CD44hiCD62L– CD4+ T cells, and the proportion of CD4+ T cells producing IL-17, IFNy or IL-4. f Frequency of FoxP3+ Treg cells in thymus, spleen, MLN, and LP-colon isolated from Il2CD11c and Il2fl/fl mice. Data represent the means ± standard error of 3–4 experiments (n = 2–4 mice/group per experiment, aged 6–10 weeks). **P < 0.01, ***P < 0.001. LP lamina propria, MLN mesenteric lymph node.
**Fig. 7** DC-derived IL-2 suppresses pathogenic CD4+ T-cell expansion in the intestine. a Change in body weight and b severity of colitis based on colon length of Il2<sup>CD11c</sup>/ and Il2<sup>fl/fl</sup>, Rag2 knockout (KO) mice (Rag2<sup>Il2</sup>Il2<sup>CD11c</sup> and Rag2<sup>Il2</sup>Il2<sup>fl/fl</sup> mice) adoptively transferred with naïve CD4+ T cells either alone or in combination with Treg cells isolated from spleens of C57BL/6 mice. Representative pictures of colons after adoptive T-cell transfer are shown. Data represent the means ± standard error of two experiments (n = 3–4 mice/group per experiment). **P < 0.01. c Histological inflammatory score based on H&E staining of colon sections (×10 magnification, scale bar 0.5 mm) obtained from Rag2<sup>Il2</sup>Il2<sup>CD11c</sup> and Rag2<sup>Il2</sup>Il2<sup>fl/fl</sup> mice after 110 days of naïve CD4+ T-cell transfer. Data represent the means ± standard error. ***P < 0.001. d, e Phenotypic analysis of colonic lamina propria CD4+ T cells (CD45+ relative %) 40 days after adoptive T-cell transfer. The total number and the relative percentage of total and activated CD4+ T cells (d), as well as the proportions of CD4+ T cells producing IL-17, IFNγ or both (e) are shown. *P < 0.05, **P < 0.01, ***P < 0.001. All data represent the means ± standard error of two experiments (n = 3–4 mice/group per experiment).

(Fig. 5f and Supplementary Fig. 9a, b). Moreover, intestinal macrophage population (CD11c<sup>+</sup>MHCII<sup>+</sup>CD64<sup>+</sup>F4/80<sup>+</sup>/−) was negative for IL-2 labeling both during steady-state and colitic conditions (Fig. 5f and Supplementary Fig. 9a, b). IL-2 production by CD11c<sup>+</sup>MHCII<sup>+</sup> DCs, but not macrophages, was also confirmed by qRT-PCR (Supplementary Fig. 9c). These data indicate that IL-2 expression in vivo is high in the intestine and restricted to the CD11c<sup>+</sup>MHCII<sup>+</sup> DC population (predominantly CD103<sup>+</sup> cells) (Fig. 5g and Supplementary Fig. 9).

To examine whether deficient IL-2 expression in CD11c<sup>+</sup>MHCII<sup>+</sup> DCs of Cnb1<sup>−/−</sup> mice was the key factor involved in dysregulation of intestinal immune responses, we administered IL-2-anti-IL-2 complexes (IL-2C), which increase IL-2 bioactivity in vivo, and assessed whether normal intestinal
homeostasis could be restored in these mice. While the proportions of Foxp3+ Treg cells were increased to a similar extent in the spleens of both Cnb1CD11c and Cnb1IL2 fl/fl mice after IL-2C treatment, these cells expanded only in the intestinal mucosa of Cnb1CD11c mice compared to Cnb1IL2 fl/fl controls, suggesting that lack of Cnb1 in CD11c+ cells is partly responsible for altered numbers of Treg cells in these mice (Supplementary Fig. 10a). To confirm that the impairment in DC-derived IL-2 signaling induces intestinal CD4+ T-cell dysregulation observed in Cnb1CD11c mice, we adoptively transferred wild-type or IL2-null DCs into the Cnb1CD11c mice. Wild-type DCs selectively restored the levels of activated CD4+ T cells producing IFNγ in the LP-colon of Cnb1CD11c mice compared to untreated or Cnb1CD11c mice receiving IL2-null DCs (Supplementary Fig. 10b). Moreover, wild-type DC transfer into Cnb1CD11c mice normalized the percentage of Treg cells in MLN, whereas the injection of IL2-null DCs induced an additional reduction of colonic FoxP3+ Treg cells. (Supplementary Fig. 10c). DC IL-2 production prevents severe spontaneous chronic colitis. A role for T-cell-derived IL-2 in regulating T-cell responses and maintaining Treg-cell-mediated self-tolerance is well-established, but a distinct contribution of myeloid IL-2 to intestinal homeostasis in vivo is unknown. To address this issue, we used an IL2CD11c knock-out mouse model3 to monitor the effects of myeloid IL2 deficiency. IL2CD11c mice were initially indistinguishable from IL2 fl/fl mice, but from ~5 weeks of age they exhibited significant weight loss, resulting in ~40–45% lower body mass compared to IL2 fl/fl mice by 22 weeks (Fig. 6a). Enlargement of the MLN and macroscopic thickening and shortening of the colon without splenomegaly was observed in 7–10-week-old IL2CD11c mice compared to IL2 fl/fl mice (Fig. 6b). Histopathological analysis of colons from 8-week-old IL2CD11c mice revealed moderate leukocyte infiltration of the mucosa and sub-mucosa, which affected 50–100% of the colon surface. (Fig. 6c); this pathology increased in severity with age, until systemic inflammation (high serum levels of CRP and IgG1) had developed by 14–16 weeks (Fig. 6d). Consistently, IL2CD11c mice also exhibited substantially higher numbers of CD45+ leukocytes and total, antigen-experienced, and IFNγ-producing or IL-17-producing CD4+ T cells (Fig. 6e), and a markedly smaller population of FoxP3+ Treg cells in colons and the MLN (but not thymus and spleen), compared to IL2 fl/fl mice (Fig. 6f). MLN CD4+ T cells isolated from IL2CD11c

Fig. 8 IL2 or Cnb1 deficiency in DCs causes a dysregulated T-cell response. a, b Naive congenic OTII cells were sorted from spleens of donor mice and transferred intravenously into Rag2KOIl2 fl/fl and Rag2KOIl2CD11c (a) or Cnb1CD11c and Cnb1IL2 fl/fl mice (b). After 24 h, mice were administered OVA protein antigen by daily oral gavage for 5 days. The frequencies of CD4+ T cells expressing FoxP3, IL-17, and IFNγ were examined in the mesenteric lymph node 7 days after adoptive cell transfer. Representative FACS plots of OTII donor CD4+ T cells are shown (left panels). Data represent the means ± standard deviation of two experiments (n = 1–2 mice/group). *P < 0.05 (two-tailed, unpaired Student’s t test)
mice robustly expanded with age (Supplementary Fig. 11a) and produced more IFNγ and IL-17, while IL-2 release was normal when cells were re-stimulated ex vivo with antiCD3/CD28 antibodies (Supplementary Fig. 11b).

To address the contribution of myeloid IL-2 in LP during differentiation of naïve CD4+ T cells in vivo, syngeneic naïve T helper cells from wild-type donor mice were adoptively transferred into Il2fl/fl and Il2fl/+, Rag-deficient mice (Rag2 KO/Ill2fl/+) and Rag2 KO/Ill2fl/+ mice, respectively), alone or in combination with wild-type Treg cells. Loss of myeloid IL-2 in the absence of lymphocytes did not affect the LP composition of colonic immune cells (Supplementary Fig. 12). By contrast, Rag2 KO/Ill2fl/11c mice receiving naïve CD4+ T cells showed an early and severe onset of wasting disease characterized by substantial weight loss (Fig. 7a), shortening of colon (Fig. 7b), mucosal inflammation (Fig. 7c), and an increase in activated CD4+ T cells (Fig. 7d) secreting higher levels of IL-17 and IFNγ (Fig. 7e) compared to Rag2 KO/Ill2fl/+ control mice. Co-transfer of Treg cells prevented colitis by reducing the expansion of activated IFNγ-producing and IL-17-producing CD4+ T cells (Fig. 7a–e).

After the induction of T-cell-mediated experimental colitis, IL-2 loss in CD11chighMHCII+ cells was also associated with increased signs of skin and joint inflammation, which are the most common extra-intestinal manifestations of IBD in humans (Supplementary Fig. 13)21. These results indicate that a deficiency in myeloid IL-2 is necessary and sufficient to trigger spontaneous autoimmune colitis characterized by spontaneous and chronic mucosal damage reminiscent of Crohn’s disease22,23.

Treg cells are essential to intestinal homeostasis as they monitor effector T helper-cell activation24. As we observed a consistent reduction in Treg cells in both Cnb1 KO/Ill2fl/11c and Il2KO/Ill2fl/11c mice in the MLN and LP, but not in the thymus or spleen (Figs. 3f and 6f), we examined whether myeloid IL-2 is important for the induction of antigen-specific Treg cells in the LP. Naïve CD4+ T cells from OTII mice were adoptively transferred into Rag2 KO/Ill2fl/11c and Rag2 KO/Ill2fl/+ mice, and the mice were then fed with ovalbumin (OVA) for 5 days. Conversion of naïve OTII CD4+ T cells into FoxP3+ Treg cells was significantly impaired,
while Th1 differentiation (and to a lesser extent Th17) was significantly enhanced in the MLN of Rag2<sup>−/−</sup>Il2<sup>CD11c</sup> mice compared to controls (Fig. 8a); similar results were obtained in Cnb1<sup>CD11c</sup> mice (Fig. 8b). These data indicate that myeloid IL-2 regulates Treg cell induction, which can directly suppress effector T helper cell expansion and activity.

**DC and T cell IL-2 have distinct functions in gut homeostasis.** To dissect the contributions of myeloid cell-derived IL-2 and T helper cell-derived IL-2 to intestinal immune homeostasis, we compared the systemic and intestinal phenotypes of Il2<sup>CD11c</sup> Il2<sup>CD</sup> (generated by crossing Il2<sup>−/−</sup> and CD4<sup>cre</sup> mice), and Il2<sup>KO</sup> (carrying IL-2 deletion in myeloid and CD4<sup>+</sup> T cells) mice. IL-2 deletion in the CD4<sup>+</sup> T-cell compartment resulted in characteristic IL-2 Deficiency Syndrome originally described in Il2<sup>KO</sup> mice and characterized by severe anemia (Fig. 9a), splenomegaly (Fig. 9b), uncontrolled expansion of splenic T cells associated with reduction of Treg cells and loss of B cells. None of these features were observed in Il2<sup>CD11c</sup> mice (Fig. 9b), indicating that IL-2 Deficiency Syndrome is mostly driven by the loss of “adaptive” IL-2. The intestinal phenotypes of Il2<sup>CD11c</sup> mice were even more divergent from Il2<sup>CD4</sup> mice: although both Il2<sup>CD11c</sup> and Il2<sup>CD4</sup> mice developed colitis at 10–13 weeks, the intestinal pathology was exacerbated in Il2<sup>CD11c</sup> mice compared to Il2<sup>CD4</sup> mice, and more similar to that of Il2<sup>KO</sup> mice (Fig. 9c). A higher increase in total leukocytes, total and activated CD<sup>4</sup><sup>+</sup> T cells, and IL-17-producing CD4<sup>+</sup> T cells was observed in the LP-colon of Il2<sup>CD11c</sup> mice compared to Il2<sup>CD4</sup> mice, and was similar to that seen in Il2<sup>KO</sup> mice, whereas accumulation of CD<sup>8</sup><sup>+</sup> T cells was specific to Il2<sup>KO</sup> mice (Fig. 9d, e). Similar to the LP-colon of Il2<sup>KO</sup> mice, frequencies of Treg cells were reduced in the LP-colon of Il2<sup>CD11c</sup> and to a lesser extent in Il2<sup>CD4</sup> mice compared to Il2<sup>KO</sup> mice (Fig. 9f). These results indicate that innate (myeloid cells) and adaptive (CD4<sup>+</sup> T cells) IL-2 exert both distinct and overlapping functions at the systemic level and in the intestine.

**Discussion**

This study shows that activation of the calcineurin–NFAT pathway in CD11c<sup>high</sup>MHCII<sup>+</sup> cells (representing the intestinal DC pool) prevents spontaneous intestinal inflammation in vivo by orchestrating a balance between inflammatory and regulatory responses in the colonic mucosa. By characterizing mice in which Cnb1 gene was deleted either in CD11c<sup>high</sup>MHCII<sup>+</sup> APCs (mostly representing the DC pool) but not CD64<sup>+</sup> macrophages, or LysM-expressing cells (mainly macrophages, granulocytes, and monocytes), we identified two distinct roles for the calcineurin–NFAT pathway: calcineurin activation has an anti-inflammatory role in macrophages, granulocytes, and monocytes. Accordingly, Cnb1<sup>CD11c</sup> mice developed spontaneous intestinal inflammation, whereas Cnb1<sup>LysM</sup> mice were unaffected by targeted Cnb1 deletion, and were protected from induced colitis. We also identified the importance of calcineurin–NFAT-mediated production of IL-2 by the intestinal DC pool in regulating intestinal homeostasis. The colonic CD103<sup>+</sup> DC subset is the main producer of IL-2, whereas CD64<sup>+</sup>F4/80<sup>−</sup>/− macrophages do not produce this cytokine. Colonic DCs from Cnb1<sup>CD11c</sup> mice still retain the ability to release IL-6, IL-12, IL-23. IL-2 deletion in CD11c<sup>high</sup>MHCII<sup>+</sup> DCs caused more severe, spontaneous intestinal inflammation in Il2<sup>CD11c</sup> mice compared to Cnb1<sup>CD11c</sup> mice, which was characterized by expansion of activated CD4<sup>+</sup> effector T cells and substantial reduction of Treg cells. IL-2 complex treatment and DCs transfer results support that reduced levels of DC-derived IL-2 could be the primary cause triggering the dysregulation of intestinal CD4<sup>+</sup> T cells observed in Cnb1<sup>CD11c</sup> mice.

Spontaneous colitis in Cnb1<sup>CD11c</sup> mice was milder compared to Il2<sup>CD11c</sup> mice, suggesting that other signaling pathways that trigger DC-IL-2 production may exist. Using pharmacological inhibitors, we found that IL-2 release also depends on NFκB in response to LPS, zymosan, and thapsigargin stimulation, and on p38 MAPK in response to zymosan and thapsigargin stimulation. A recent study reported that deletion of TRAF6, a component of the TLR–NFκB pathway, is associated with ~50% reduction of DC-derived IL-2<sup>27</sup>. Our study identifies the calcineurin–NFAT pathway as an additional axis that operates in parallel with the TRAF6–NFκB pathway in myeloid cells to produce IL-2, suggesting that both NFκB and NFAT cooperate to activate IL-2 transcription. Although these pathways contribute to the overall production of IL-2, the nature of the intestinal immune-pathologies observed in the Traf6<sup>−/−</sup> and Cnb1<sup>−/−</sup> mice diverge: loss of Traf6 in DCs causes reduced inflammatory cytokines secretion, including IL-12, whereas Cnb1-deficient DCs release normal levels of inflammatory cytokines. Moreover, partial loss of IL-2 in Traf6-deficient DCs causes spontaneous Th2-mediated enteritis characterized by eosinophilic infiltration and decreased Treg-cell numbers specifically in the small intestine<sup>27</sup>. This phenotype markedly differs from Cnb1<sup>CD11c</sup> mice, as these mice develop a spontaneous form of enteritis in both the small and large intestine resembling Crohn’s disease, and is associated with Th1 and Th17 colitogenic T-cell expansion, mucosal accumulation of mononuclear cells and progressive colon thickening<sup>28</sup>. Through a series of in vivo experiments, our data suggest that the calcineurin–NFAT–IL-2 pathway in myeloid cells is crucial for the induction of Treg cells, which contributes to maintaining immune tolerance in the gut by preventing autoimmunity. Indeed, Il2<sup>CD11c</sup> and lymphopenic Rag2<sup>−/−</sup>Il2<sup>CD11c</sup> mice receiving normal naive T cells developed an exacerbated form of spontaneous colitis associated with extra-intestinal manifestations, including cutaneous and articular inflammatory reactions, which are the foremost comorbidities of IBD<sup>21</sup>

To date, the regulatory and effector functions of the calcineurin–NFAT–IL-2 axis in orchestrating intestinal immune homeostasis has been largely studied in terms of T helper-cell and Treg-cell biology<sup>6</sup>. The current literature supports that IL-2 is pivotal for maintaining tolerance to self and microbial antigens<sup>8,9,18,22</sup>, which is achieved via various mechanisms. First, CD4<sup>+</sup> naive T cells constitutively produce IL-2 in the intestinal mucosa, which induces and maintains the intestinal Treg-cell pool, as these cells are uniquely dependent upon IL-2<sup>29</sup>. Second, IL-2 can directly suppress the differentiation of pathogenic Th17 cells<sup>30</sup>. Although T-cell-derived IL-2 likely has an important role in promoting intestinal tolerance, our data also highlight the requirement for DC-derived IL-2 in this process. Of note, in the absence of ex vivo restimulation, the detected IL-2 signals in intestinal DCs are relatively weak. However, it is important to bear in mind that levels of IL-2 and intensity of the signal cannot be compared to the IL-2 levels in T cells. Like DCs, unstimulated
T cells do have weak IL-2 transcription/production levels, which is dramatically boosted upon stimulation. In this scenario, low level IL-2 production by DCs seems to have an important biological function and can be directly used by naïve CD4+ T cells to differentiate into Treg cells thereby suppressing the differentiation of pathogenic Th17 cells. It is also possible that some regulatory feedback mechanisms might be in place in DCs to limit and fine tune the level of IL-2 expression, as very high levels could disrupt optimal function of these cells, as well as affecting their interaction with other cells such as CD4+ T cells. Using various in vivo models, we have shown that DC-derived IL-2 in the gastrointestinal tract plays a crucial biological role in maintaining the immune homeostasis. Thus, we are confident that the immune phenotype observed in our DC-IL-2-deficient mouse model was mostly due to reduction of IL-2 production by DCs.

We further analyzed the distinct contributions of innate and adaptive IL-2 to intestinal immune homeostasis. Here, we compared the systemic and intestinal phenotype of II2CD11c mice (lacking II2 in the myeloid compartment), II2CD4 mice (lacking II2 in the CD4+ T cells), and II2KO mice (in which both myeloid and lymphoid II2 is absent). II2CD4 mice exhibited IL-2 Deficiency Syndrome characterized by severe anemia, splenomegaly, uncontrolled expansion of T cells associated with a reduction of Treg cells, and loss of B cells. The same phenotypes noted in the II2KO model. Mice deficient in myeloid II2 (II2CD11c) did not develop the same pathology, but instead showed a transmural infiltration of mononuclear cells, without affecting CD8+ T cells compartment involved in erosive mucosal lesion observed in II2KO mice, indicating that lymphoid IL-2 is crucial for immune homeostasis in the peripheral immune tissues. The difference between II2CD4 and II2CD11c is even more striking in the intestine, where loss of myeloid IL-2 resulted in a higher exacerbation of spontaneous colitis and a higher increase in the total number of leukocytes and activated CD4+ T effector cells producing IFNy and IL-17 compared to mice lacking T-cell-produced IL-2. A significant reduction in the frequencies of Treg cells in the LP-colon of both II2CD11c and II2CD4 mice was observed, indicating that both myeloid and adaptive IL-2 are equally important for the induction and/or maintenance of Treg cells in the mouse intestintest20,31. Of note, the aberrant Treg:Th effector-cell ratio that accompanied pathologic inflammation in Cnb1CD11c and II2CD11c mice seems to be restricted to the intestine of these mice, as we did not detect any changes in Treg-cell frequency in the lung or axillary and popliteal lymph nodes, suggesting a fundamental role for the gut microbiota in the activation of this pathway and thus IL-2 production by intestinal myeloid cells.

The results presented here pose interesting questions for future research. It would now be interesting to investigate how DC-derived IL-2 mechanistically regulates the induction of intestinal Treg cells, as well as how it modulates effector T-cell functions in the mouse intestine. Moreover, the requirement of DC-derived IL-2 for the development and maintenance of innate cells, such as innate lymphoid cells, should be examined. Considering the differential effects of calciumneurin signaling in various myeloid cell subsets, it will be important to investigate the effects of calciumneurin inhibitors on different cell subsets in vivo. Calciumneurin inhibitors are already in clinical use and elicit some benefits in patients with selected forms of IBD, including acute severe ulcerative colitis and steroid-refractory IBD. Data remain insufficient to recommend the use of calciumneurin inhibitors (cyclosporin A or FK506) in Crohn’s disease.

In summary, our data support that the calciumneurin–NFAT–IL-2 pathway in intestinal DCs has a central role in eliciting proper T-cell responses in the mouse intestine under both steady-state and inflammatory conditions, by supporting Treg-cell induction and by suppressing excessive expansion of Th1/Th17 cells (directly or indirectly). We propose that therapeutic strategies that selectively boost DC-derived IL-2 production may have a beneficial effect in patients with IBD.

**Methods**

**Mice.** C57BL/6 Pp3yp1tam1Blj (Cnb1fl/fl, Stock No. #6581), B6.Cg-Tg(Itgax-cre)1-MaLe (Itgax-cre), Stock No. #105681, B6.Cg-Il2rtasrtg2R2-Jr-cre (Il2fl/fl, Stock No. #4781), Tg(Cd4-cre)1Cwi/B1a (Il2KO, Stock No. #2522) mice were obtained from The Jackson Laboratory, USA. C57BL/6 and Rag2−/− mice were obtained from the Biological Resource Center, Agency for Science, Technology and Research (A*STAR), Singapore. Il2−/− mice were generated in our laboratory by breeding C57BL/6 Pp3yp1tam1Blj mice (Cnb1fl/fl) with B6.Cg-Tg(Itgax-cre)1-MaLe (Itgax-cre) and B6.Cg-Il2rtasrtg2R2-Jr-cre (Il2fl/fl) mice. Lymph node and spleen cells were isolated by FACS. Naïve T cells (3×10⁵/mouse) were injected intravenously alone or in combination with Treg cells (1×10⁵/mouse) into Rag2–/– recipient mice. Whole gut was collected and processed for immunohistochemical analysis, fluorescence microscopy, and flow cytometry.

**Intestinal permeability assay.** Mice were fasted for 6 and then administered 500 mg/kg FITC-dextran (4 kDa; Sigma-Aldrich) by oral gavage. Blood samples were collected 1 and 3 h later, centrifuged at 12,000 g at 4 °C for 3 min, and then analyzed for FITC-dextran concentration using a fluorescence spectrophotometer (485 nm excitation, 535 nm emission). A standard curve was obtained by diluting FITC-dextran in untreated plasma diluted with PBS (1:3 v/v).

**Inflammatory histological score.** Sections of medial colon and small intestine were fixed in buffered formalin, cut (5 µm sections, ~150 µm between each section, 4–8 per fragment), and then stained with hematoxylin and eosin (H&E). Stained sections were examined and scored in a blinded fashion. The “degree of inflammation” was graded semi-quantitatively from 0 to 4: none, 0; 1, very low level; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, and thickening of the colon wall; and 4, transmural infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall. The “extent of inflammation” was graded from 0 to 4: none, 0; 1, 0–25% of the mucosal surface area; 2, submucosal; 3, mucosal and submucosal; 4, full thickness. Finally, the "involved surface area" was graded from 0 to 4: none, 0; 1, 0–25% of the mucosal surface area; 2, submucosal; 3, mucosal and submucosal; 4, full thickness.
surface; 2, 25–50%; 3, 50–75%; 4, 75–100%. The overall inflammatory score was obtained by summing the “degree” and “extent” scores, and multiplying by the involved surface area (minimum score 0, maximum 32).

Measurement of tissue inflammatory markers. Frozen fragments of small intestine and colon were homogenized using Tissue Protein Extraction Reagent (Thermo Scientific) and the homogenates were used to assess protein concentrations and cytokine levels. Neutrophil infiltration into the colon was monitored by measuring MPO activity using a spectrophotometric assay with triphenyltetrazolium chloride (TMB) as the substrate and myeloperoxidase activity expressed as μM/g of protein.

IL-2-mAb complex treatment. IL-2-mAb complex was prepared by mixing 1 μg of recombinant IL-2 (Miltenyi Biotec) with 5 μg of anti-IL-2 mAb (clone JES6-1A12, eBioscience) in PBS per mouse for i.p. administration 3 per week, every 2 days for 4–5 weeks.

DC culture and stimulation. Bone marrow cells from C57BL/6 CD45.1 or Il2−/− mice were cultured in conditioned media containing recombinant mouse granulocyte macrophages colony-stimulating factor (GM-CSF) or recombinant mouse Flt-3L for 11 days. CD103+ and CD11b+ DCs (CD11c+MHCII+CD45+B220−) were FACS-sorted and adaptively transferred intravenously into 12-week-old Cnb1−/− or Cnb1+CD11c mice at a ratio of 1:6 CD103+CD11b+ (total number of transferred cells: 5 × 105). Two weeks post-transfer, mice were killed and the immune phenotype of mononuclear cells from spleen, SI, and colon was investigated by flow cytometry.

Isolation of LP leukocytes from the colon. Isolated colon was cut longitudinally and divided into segments of 0.5–1 cm in length. The tissue segments were then added to RPMI medium supplemented with 2% FBS, and incubated for 20 min at 37 °C with constant stirring. After incubation, the resultant suspension was passed through a 70 μM sterile strainer and pieces of colon tissue were placed into a 50 ml tube containing 15 ml serum-free RPMI, shaken vigorously for 30 s, and then filtered twice through a 70 μm cell strainer. The remaining fragments of colon were washed in calcium-free and magnesium-free HBSS and treated with 1 ml EDTA in PBS (twice for 20 min) to remove the epithelium. The tissue was then digested with type-IV collagenase (0.8 mg/ml, Sigma-Aldrich) containing DNase and the leukocytes were enriched on a 40:75 Percoll gradient (Pharmacia) and the interface collected after centrifugation at 700 × g for 20 min.

Isolation of LP leukocytes from the small intestine. Isolated SI was flushed with HBSS, the PP was removed, and then cut longitudinally and divided into pieces of 1 cm in length. Epithelial cells were removed by incubation with 2 ml EDTA in HBSS supplemented with 10% FCS for 40 min at 37 °C, followed by vigorous shaking for 20 s. The samples were then collected and incubated with type-VIII collagenase (0.3 mg/ml, Sigma-Aldrich) containing DNase I and the leukocytes were enriched on a 40:75 Percoll gradient (Pharmacia) and the interface collected after centrifugation at 700 × g for 20 min.

Cell culture and stimulation. Splenic DC1 DCs were cultured as described previously33. NFAT-luciferase reporter D1 cells (D1-NFAT-Luc) were generated using the Cignal Lenti NFAT Reporter (luc) kit (Qiagen) as previously described5. Briefly, D1 cells were enriched on a 40:75 Percoll gradient (Pharmacia) and the interface collected after centrifugation at 700 × g for 20 min. Total cellular RNA was extracted using the Arcturus PCR (Invitrogen) were used for reverse transcription. Quantitative real-time PCR was carried out with the following validated SYBR Green primer pairs: Cnb1 forward 5′-TGGTCCGCTGCTTGAAGTTG-3′, reverse 5′-CTGGTT CTTATTGGCTGTTGAC-3′; Nfat1 forward 5′-CGCTGTACAGGCGGGCCCA-3′, reverse 5′-GGCAAGGACTGTGTTGAG-3′; Nfat2 forward 5′-TGCAACCCAATTTCCCTGTTGG-3′, reverse 5′-GGGGTCCGGGAGGGCTGATT-3′; IκB-α forward 5′-CCCCCATGCTCACCCTCT-3′, reverse 5′-CATCCTGATATGAGAAG-3′; GAPDH forward 5′-TGTCCTCCTGACAAATAAG-3′, reverse 5′-TTTAGGCTAATGAGGAGGTC-3′; Amplification was performed on a 7500 Real-Time QPCR system (Applied Biosystems) and the relative gene expression was calculated using the comparative Ct method (2−ΔΔCt).

Statistical analysis. Data are expressed as the means ± standard error. One-way ANOVA was used for comparisons of more than two groups, followed by Tukey’s test for the analysis of naproxen-induced colitis, or by Dunnnett’s multiple comparisons test to assess the levels of Cnb1, Nfat1, and Nfat2 expression in CD11c−expressing cells compared with controls. A two-tailed, unpaired Student’s t test was used to compare two groups of data. P values < 0.05 were considered statistically significant. GraphPad Prism version 6 (GraphPad Software) was used to perform graphics and perform the statistical analyses.
Data availability. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding authors on reasonable request.

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Author contributions
A.M. conceived, designed, and performed the experiments, analyzed primary and aggregated data, and wrote the manuscript; H.J.K., J.F., M.V., and T.S.L. performed some experiments; S.E.D. analyzed data from the IL-2-GFP reporter mice experiments; H.J.K. critically revised the manuscript; J.P.T. performed qRT-PCR experiments and genotyped mice; S.N. performed animal screening; A.B. performed confocal microscopy; B.J. contributed with DC transfer experiments; F.G. provided methodological guidance for identifying myeloid cell subsets; P.R.-C. conceived the research, supervised the project and secured funds; A.Mo. conceived and supervised the project, designed experiments, analyzed aggregated data, secured funds, and wrote the manuscript.

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