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Myeloid cells, innate lymphoid cells, and the cytokines they secrete cooperate to maintain immune tolerance in the gut.

Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis

Arthur Mortha, Aleksey Chudnovskiy, Daigo Hashimoto, Milena Bogunovic, Sean P. Spencer, Yasmine Belkaid, Miriam Merad*

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Introduction: The gastrointestinal tract is colonized by an extraordinarily large number of commensal microbes and is constantly exposed to ingested antigens and potential pathogens. Regulation of intestinal tolerance thus represents the main task of the immune system of the gut mucosa. Accumulated evidence suggests that gut commensals contribute to the maintenance of intestinal homeostasis, partly through their ability to control the differentiation of effector T lymphocytes in the mucosa and to modulate inflammatory responses through the induction of regulatory T cells (T_{reg}) and interleukin-10 (IL-10) production. Tissue-resident mononuclear phagocytes (MNPs), including macrophages (MPs) and dendritic cells (DCs), are specifically equipped to detect a wide range of microbial signals and to capture, process, and present extracellular antigenic material to T lymphocytes. MNPs have been shown to contribute to the maintenance of intestinal immune tolerance through the induction or expansion of T_{reg} in the intestine. Despite their key role in microbial sensing and immune tolerance, the cellular and molecular cues that translate microbial signals into immunoregulatory MNPs in the intestine are not completely understood.

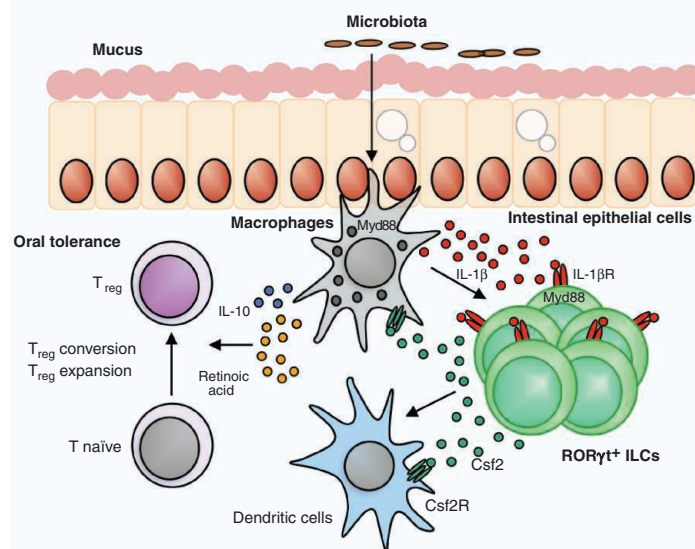
Rationale: The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), recently renamed Csf2, is a key determinant of myeloid lineage differentiation and is required for the optimal function of tissue MNPs. Recent results from our laboratory revealed that although Csf2-deficient mice have normal numbers of lymphoid tissue-resident DCs, they display significantly reduced numbers of steady-state nonlymphoid tissue-resident DCs in the small intestine, including the lamina propria CD103⁺CD11b⁺ DC subset implicated in the induction of lamina propria T_{reg} . These results prompted us to further explore the contribution of Csf2 to intestinal immune homeostasis in vivo. We used detailed profiling

and functional immune assays of the MNP and lymphocyte compartment in the gut, as well as genetically engineered mice that lack Csf2 or the transducer Myd88 specifically in MNPs or lymphocytes, to explore the role of MNPs in the maintenance of immune homeostasis in the gut.

Results: Our results revealed a crosstalk between IL-1 β -secreting MPs and Csf2-producing ROR γ t⁺ type 3 innate lymphoid cells (ILC3) in the intestinal mucosa. Microbiota-driven IL-1 β production by MPs promoted the release of Csf2 by ILC3, which in turn controlled DCs and MPs to maintain colonic T_{reg} homeostasis. Ablation of Csf2 reduced DC and MP numbers and impaired

their ability to produce regulatory factors such as retinoic acid (RA) and IL-10, leading to disrupted T_{reg} homeostasis in the large intestine. Conversely, administration of Csf2 cytokine increased T_{reg} frequency in the gut. Most notably, cell type-specific ablation of IL-1 receptor (IL-1R)-dependent signaling in ROR γ t⁺ ILC3 abrogated oral tolerance to dietary antigens and compromised intestinal immune homeostasis in vivo. Although the reduction in T_{reg} numbers was mostly observed in the large intestine, adoptive transfer studies in Csf2^{-/-} mice revealed impaired T_{reg} differentiation both in the small and large intestine, suggesting that Csf2-dependent MNP immunoregulatory functions control T_{reg} induction in both tissues.

Conclusion: This study established the commensal-driven MNP-ILC3-Csf2 axis as a key regulator of intestinal T cell homeostasis in the mouse intestine. Disturbance of this axis radically altered MNP



ILC3 translate microbial cues into immunoregulatory signals in the intestine. Microbial cues sensed by intestinal MPs lead to IL-1 β release. IL-1 β engages IL-1 receptor on ILC3, promoting Csf2 release. ILC3-derived Csf2 triggers DC and MP production of regulatory molecules (i.e., retinoic acid and IL-10), which in turn promotes the induction and expansion of T_{reg} . Csf2-primed DCs and MPs promote T_{reg} homeostasis locally and in mesenteric lymph nodes.

effector function, resulting in impaired oral tolerance to dietary antigens. These results represent an important advance in our understanding of how commensal microbes can regulate host intestinal immunity and may inform the design of novel immunotherapies for patients with inflammatory intestinal diseases with impaired GM-CSF function.

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Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis

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The intestinal microbiota and tissue-resident myeloid cells promote immune responses that maintain intestinal homeostasis in the host. However, the cellular cues that translate microbial signals into intestinal homeostasis remain unclear. Here, we show that deficient granulocyte-macrophage colony-stimulating factor (GM-CSF) production altered mononuclear phagocyte effector functions and led to reduced regulatory T cell (T_{reg}) numbers and impaired oral tolerance. We observed that $ROR\gamma^t$ innate lymphoid cells (ILCs) are the primary source of GM-CSF in the gut and that ILC-driven GM-CSF production was dependent on the ability of macrophages to sense microbial signals and produce interleukin-1 β . Our findings reveal that commensal microbes promote a crosstalk between innate myeloid and lymphoid cells that leads to immune homeostasis in the intestine.

The gastrointestinal tract is colonized by an extraordinarily large number of commensal microbes and is constantly exposed to ingested antigens and potential pathogens. Regulation of intestinal tolerance thus represents the main task of the immune system of the gut mucosa. Defective immune tolerance in the gut is associated with the onset of inflammatory bowel diseases (IBD), a severe intestinal pathology that results from a dysregulated immune response to commensal microbes leading to chronic intestinal inflammation (1, 2). Accumulated evidence suggests that gut commensals contribute to the maintenance of intestinal homeostasis, partly through their ability to control the differentiation of effector T lymphocytes in the mucosa (3, 4) and to modulate inflammatory responses through the induction of T_{reg} s and interleukin-10 (IL-10) production (4–6).

Tissue-resident mononuclear phagocytes (MNP)s are equipped to detect a wide range of microbial signals and to capture and process extracellular antigens, including commensal microbial antigens in the form of peptide–major histocompatibility complexes (MHCs) that can be recognized by T lymphocytes (7). Mucosal tissue-resident MNPs consist of two main cell populations, macrophages (MPs) and dendritic cells (DCs) (8). Tissue-resident macrophages are characterized as

MHCII⁺CD11c⁺CD103⁺CD11b⁺CX3CR1⁺F4/80⁺CD64⁺ cells, whereas tissue-resident DCs are characterized as MHCII⁺CD11c⁺CX3CR1^{int/–}F4/80[–]CD64[–] (fig. S1). DCs can further be subdivided into CD103⁺CD11b[–] (CD103⁺ DCs), CD103⁺CD11b⁺ (double-positive or DP DCs), CD103[–]CD11b⁺ (CD11b⁺ DCs), and CD103[–]CD11b⁺CD64⁺F4/80⁺ (MP) subsets (9–12) (fig. S1). Both DCs and macrophages have been shown to contribute to the maintenance of intestinal immune tolerance through the induction or expansion of T_{reg} s in the intestine (13–19). Despite their key role in microbial sensing and immune tolerance, the cellular and molecular cues that translate microbial signals into immunoregulatory MNPs in the intestine remain poorly understood.

The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), recently renamed colony-stimulating factor 2 (Csf2), is a key determinant of myeloid lineage differentiation and is required for the optimal function of tissue MNPs, including macrophages and DCs, thereby promoting host protection against environmental pathogens and vaccine responses (20, 21). Despite the key role of Csf2 in promoting MNP survival, differentiation, and function, previous studies reported that mice lacking Csf2 or its receptor displayed only minor impairment in the development of spleen and lymph node DCs (22). Subsequent studies showing that Csf2 expression is increased in inflamed mice and that adoptively transferred monocytes generate DCs in the inflamed spleen but not in the steady-state spleen suggested that Csf2 is a major proinflammatory cytokine that controls the differentiation of inflammatory but not steady-state DCs in vivo (23, 24). These results are consistent with the contribution of Csf2 to the pathophysiology of numerous inflammatory and autoimmune diseases (25–27).

In contrast, we recently observed that although Csf2-deficient mice have normal numbers of lymphoid tissue-resident DCs, they display a significant

reduction in steady-state nonlymphoid tissue-resident DCs, including the CD103⁺CD11b⁺ DC subset found in the small intestine lamina propria (11, 28), which have been implicated in the induction of lamina propria T_{reg} s (14, 15). These results prompted us to further explore the contribution of Csf2 to intestinal immune homeostasis in vivo.

Regulation of Gut DC, Macrophage, and T_{reg} Cell Homeostasis by Csf2

We characterized the mucosal T cell compartment in Csf2-deficient mice ($Csf2^{-/-}$) in the steady state. Surprisingly, we observed a significant reduction in the frequency, number, and proliferation of CD45⁺TCR β ⁺CD4⁺Foxp3⁺ T_{reg} s in the colon of $Csf2^{-/-}$ mice compared to littermate controls (Fig. 1A and fig. S2A). The reduced T_{reg} number was specific to the colon and was not observed in the small intestine of $Csf2^{-/-}$ mice. The reduction in the number of colonic T_{reg} s was associated with a significant reduction in the frequency and number of IL-10[–] and IL-2–producing T cells, along with a significant increase in the number of colonic interferon- γ (IFN- γ)–producing T cells, whereas IL-17–producing T cells were unaffected in 6-week-old $Csf2^{-/-}$ mice compared to wild-type mice (Fig. 1B and fig. S2B). Histological analysis of colonic sections from Csf2-deficient animals did not reveal overt inflammatory infiltrates in the lamina propria (fig. S2C).

Because Csf2 plays a critical role in the differentiation and function of tissue MNPs, we hypothesized that the alterations in T helper cell subsets observed in the colon of $Csf2^{-/-}$ animals might be due to defects in mucosal MNPs. Accordingly, we found reduced numbers of colonic DCs and macrophages in the absence of Csf2 (Fig. 1, C and D), thus establishing an important role for Csf2 in the homeostasis of the colonic MNP pool. DCs and macrophages have been reported to generate Foxp3⁺ T_{reg} s, via the production of the regulatory mediators retinoic acid (RA) and IL-10 in the presence of transforming growth factor- β (TGF- β). Thus, we analyzed the capacity of DCs and macrophages to produce these regulatory mediators in the absence of Csf2. We observed a significant reduction in the activity of the RA-generating enzyme retinaldehyde dehydrogenase (ALDH) throughout all colonic DC subsets and macrophages in $Csf2^{-/-}$ mice (Fig. 1E and fig. S2D) associated with reduced expression of *Aldh1* transcripts (fig. S2, E and F). Absence of Csf2 was also associated with a significant reduction in the release of TGF- β by colonic CD103⁺CD11b⁺ DCs and with reduced IL-10 secretion by macrophages (Fig. 1, F and G), which extends previous observations showing that Csf2 controls IL-10 and TGF- β release by peritoneal macrophages upon uptake of apoptotic cells (29). Notably, expression of *Aldh1a2* and *Il10*, and release of IL-10 were restored in $Csf2^{-/-}$ macrophages upon addition of exogenous Csf2 (fig. S2, G and H).

These findings suggest that the absence of Csf2 results in a reduction in the number, frequency,

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and function of DCs and macrophages in the colon. We thus sought to determine whether the alterations in T helper cell subsets observed in *Csf2*^{-/-} animals was due to impaired MNP function. Accordingly, we found that colonic macrophages and DCs isolated from *Csf2*^{-/-} mice were compromised in their ability to drive T_{reg} differentiation ex vivo compared to their *Csf2*^{+/+} counterparts

(Fig. 1H). This was reversed upon addition of exogenous Csf2 (Fig. 1H), suggesting that the reduced T_{reg} pool observed in *Csf2*^{-/-} mice was a consequence of impaired mucosal MNP function and not only reduced MNP numbers. Critically, administration of B16 melanoma cells that overexpressed Csf2 to *Csf2*^{-/-} mice restored MNP numbers (fig. S3A) and increased T_{reg} frequency (Fig. 1I), while

reducing the number of IFN γ -producing intestinal T cells to levels comparable to those of untreated C57Bl/6 mice (fig. S3B), consistent with the ability of exogenous Csf2 to restore the immunoregulatory potential of *Csf2*^{-/-} macrophages and DCs ex vivo (Fig. 1H). Together, these data establish Csf2 as a master regulator of MNP immunoregulatory function in the steady-state colon tissue.

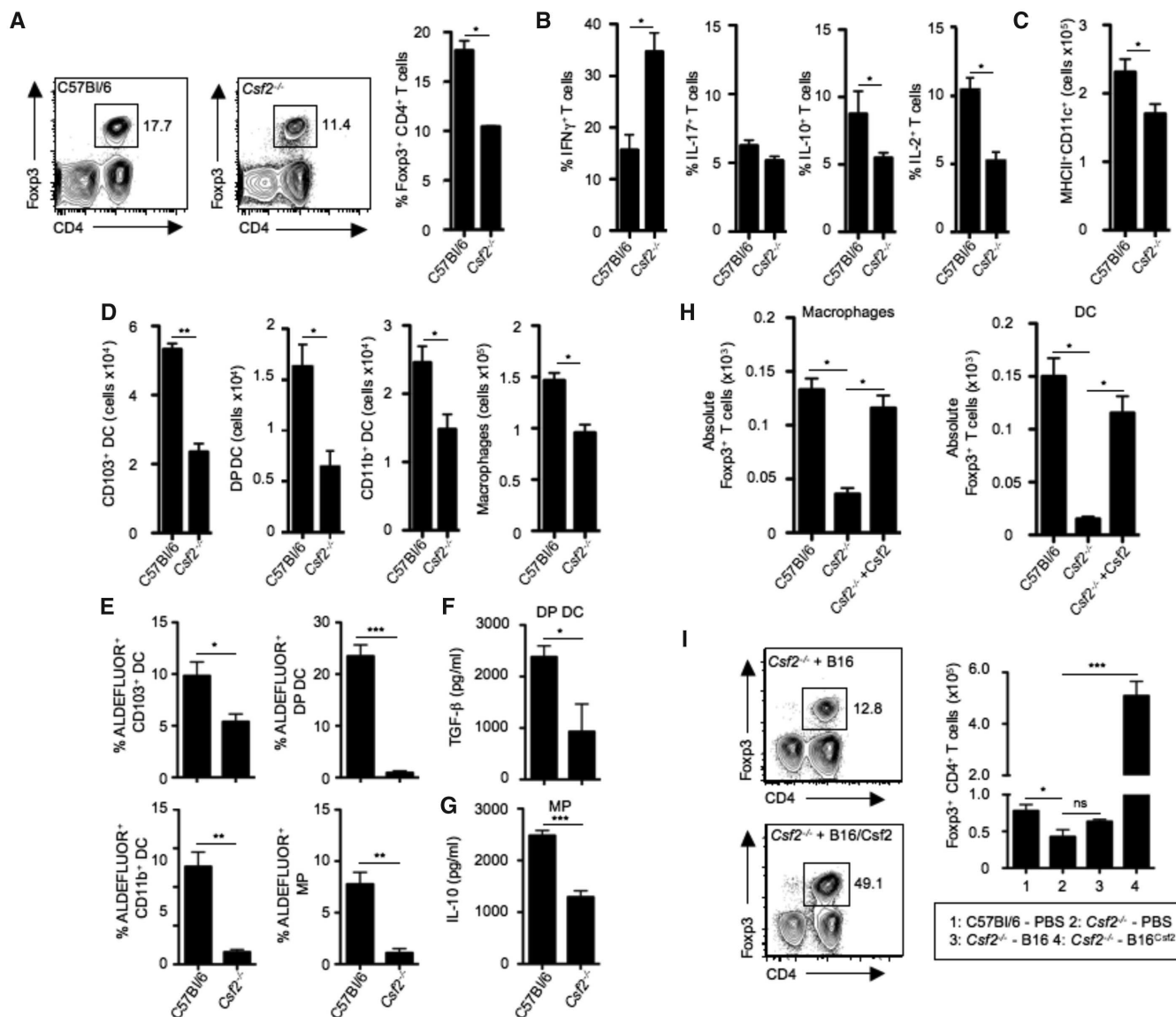


Fig. 1. Csf2 regulates tissue mononuclear phagocyte frequency and effector functions required for T helper cell homeostasis. (A) Contour plots and bar graph show percentages of CD3⁺CD4⁺Foxp3⁺ colonic T_{reg}s among total colonic lamina propria CD45⁺ cells in C57Bl/6 and *Csf2*^{-/-} mice. (B) Bar graphs show percentages of CD3⁺CD4⁺ T cells producing IFN- γ , IL-17, IL-10, or IL-2 among total colonic CD45⁺ cells in C57Bl/6 and *Csf2*^{-/-} mice after 4 hours of stimulation with phorbol 12-myristate 13-acetate (PMA)–ionomycin in the presence of Brefeldin A. (C) Bar graphs show absolute numbers of MHCII⁺CD11c⁺ cells among total colonic CD45⁺ cells in C57Bl/6 and *Csf2*^{-/-} mice. (D) DCs were characterized as CD45⁺MHCII⁺CD11c⁺ and further subdivided into CD103⁺ DCs, CD103⁺CD11b⁺ DCs [double positive (DP) DCs], and CD11b⁺ DCs. Macrophages (MP) were characterized as MHCII⁺CD11c⁺CD11b⁺F4/80⁺CD64⁺ cells. Bar graphs show absolute numbers of CD103⁺ DCs, DP DCs, CD11b⁺ DCs, and MPs in C57Bl/6 and *Csf2*^{-/-} mice. (E) Percentages of ALDEFLUOR⁺ cells among each colonic DC

subset and MPs in C57Bl/6 and *Csf2*^{-/-} mice. (F and G) Bar graphs show enzyme-linked immunosorbent assay (ELISA) measurement of TGF- β production by sorted DP DCs (F) and IL-10 production by sorted MPs (G). (H) Colonic MPs or DCs were FACS-sorted from C57Bl/6 or *Csf2*^{-/-} mice and cocultured with naïve T cells alone or in the presence of exogenous Csf2. Bar graphs show absolute numbers of Foxp3⁺ T_{reg}s. Data are representative of six independent experiments and are shown as mean \pm SD. (I) Contour plots show percentages of CD45⁺CD3⁺CD4⁺Foxp3⁺ colonic T_{reg}s in *Csf2*^{-/-} mice 12 to 14 days after injection with B16 melanoma cells or B16 cells overexpressing Csf2 (B16^{Csf2}). Bar graphs show absolute number of colonic T_{reg}s in the indicated groups. All data (A to H) are shown as mean \pm SD of three independent experiments with at least three mice per experiment. Student's *t* test (A to F) and one-way analysis of variance (ANOVA) Bonferroni's multiple comparison test (H and I) were performed. Statistical significance is indicated by **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant.

Notably, blockade of RA production [with 4-diethyl-aminobenzaldehyde (DEAB)] and/or blockade of IL-10 [with monoclonal antibody (mAb) against IL-10] abrogated the ability of Csf2 to rescue T_{reg} induction in vitro by $Csf2^{-/-}$ MNP, whereas addition of RA rescued T_{reg} induction in these cultures (fig. S3, C and D). Consistent with these findings, injection of the RA receptor antagonist LE540 compromised Csf2-mediated rescue of T_{reg} in $Csf2^{-/-}$ mice in vivo (fig. S3E), and conversely, injection of RA but not IL-10 restored T_{reg} frequency in $Csf2^{-/-}$ mice in vivo (fig. S3F).

Csf2 Is Produced by ROR γ ⁺ ILC3

Our results showing that colonic T_{reg} homeostasis was dependent on Csf2 prompted us to characterize the source of Csf2 in a noninflamed intestine. Previous reports have suggested that Csf2 is primarily produced by radio-resistant epithelial cells (30), including Paneth cells (31) in the gut. Surprisingly, we found that in the large and small

intestine, Csf2 was constitutively produced by tissue-resident CD45⁺ hematopoietic cells expressing the retinoic acid-related orphan receptor γ t (ROR γ t) (Fig. 2A). ROR γ t⁺ T helper 17 (T_H17) cells can produce large amounts of Csf2 in the inflamed brain and intestine (26, 27, 32). Other ROR γ t-dependent cell populations include group 3 innate lymphoid cells (ILC3) composed of lymphoid tissue-inducer (LTi) cells and ROR γ t⁺ ILC expressing the natural killer (NK) cell receptor, Nkp46, recently termed NCR⁺ILC3 cells (33).

LTi cells and NCR⁺ILC3 are highly abundant in the small and large intestine in the steady state (34). To test whether ROR γ t⁺ ILC3 contribute to the steady-state production of Csf2 in the intestine, we measured Csf2 production in $Rorc^{-/-}$ mice, which lack ROR γ t-expressing cells (35). Csf2 expression was reduced in the large and small intestine of $Rorc^{-/-}$ mice and declined to levels almost as low as those found in $Csf2^{-/-}$ animals (Fig. 2B), suggesting that steady-state

production of the Csf2 cytokine in the intestine was predominantly mediated by ROR γ t⁺ cells. Accordingly, we observed that 80% of Csf2-producing cells in the normal small and large intestine resided within the CD3⁺CD45⁺ROR γ t⁺ compartment, consistent with the phenotype of ROR γ t⁺ ILC3 (Fig. 2, A and C). Csf2 was produced by both subsets of ROR γ t⁺ ILC3, including LTi cells and NCR⁺ ILC3, in the small and large intestine but not by Nkp46⁺ROR γ t⁺ NK cells (33) (Fig. 2D). These results are consistent with previous data showing that human ROR γ t-expressing Nkp44⁺ ILC produce Csf2 (36). ROR γ t⁺ ILC3 are reportedly localized within the isolated lymphoid follicles (ILFs) (37, 38), so we used $Rorc^{+/EGFP}$ reporter animals to confirm that Csf2⁺ cells are enriched in ILF throughout the lamina propria (Fig. 2E and fig. S4, A to E). Areas enriched in ROR γ t⁺ cells (ILFs) also contained significantly higher levels of Csf2 transcripts when compared with intestinal epithelial

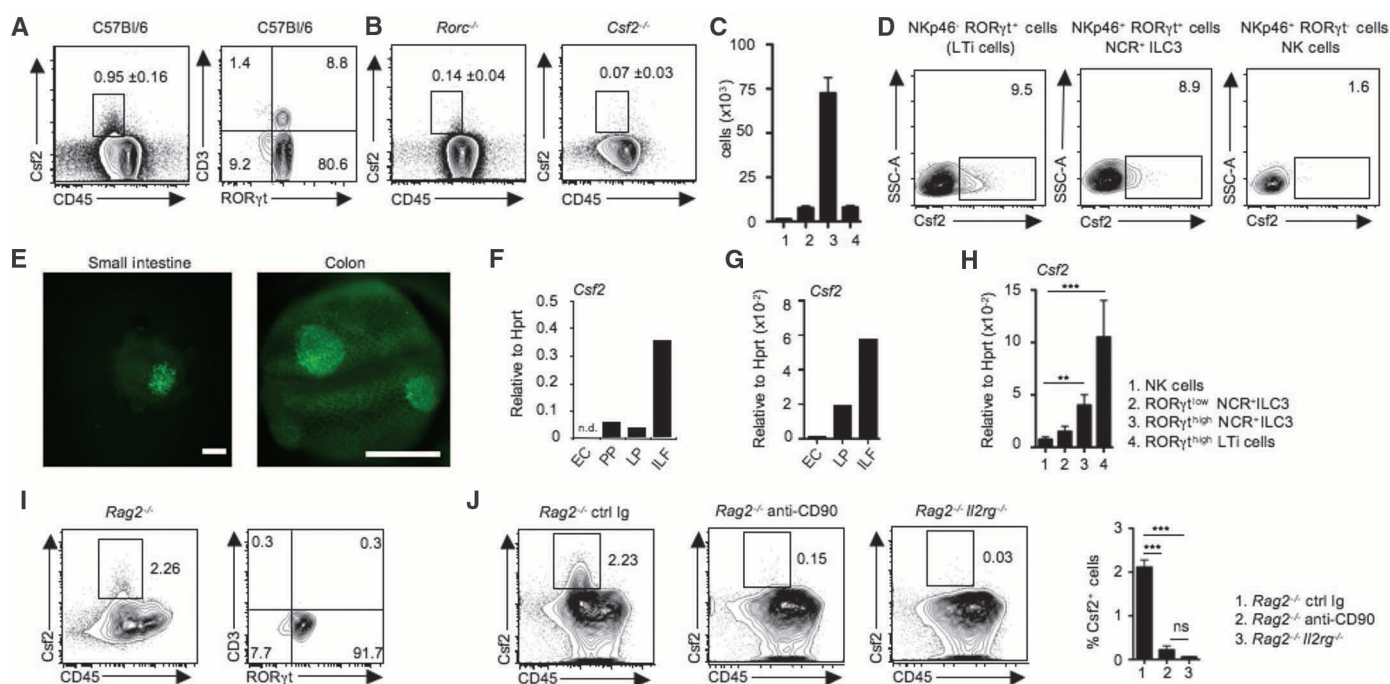


Fig. 2. ROR γ ⁺ ILCs are the major source of Csf2 in the steady-state gut. (A) FACS plots show Csf2 and CD45 expression in lamina propria cells and expression of CD3 and ROR γ t among gated Csf2⁺CD45⁺ lamina propria cells (data shown are representative of 10 independent experiments including at least 5 mice per experiment). Staining was performed on ex vivo isolated cells cultured for 4 hours in the presence of Brefeldin A. (B) FACS plots show percentage Csf2⁺CD45⁺ lamina propria cells in the small intestine of $Rorc^{-/-}$ and $Csf2^{-/-}$ mice. Data are representative of at least three experiments with two mice per group and are shown as mean \pm SD. (C) Bar graph shows absolute numbers of Csf2-producing cells in lamina propria cells. 1: Csf2⁺CD3⁺ROR γ t⁺ T cells; 2: Csf2⁺CD3⁺ROR γ t⁺ T cells; 3: Csf2⁺CD3⁺ROR γ t⁺ ILCs; 4: Csf2⁺CD3⁺ROR γ t⁺ cells. (D) FACS plots show Csf2 expression on gated lamina propria Nkp46⁺ROR γ t⁺ LTi cells, Nkp46⁺ROR γ t⁺ NCR⁺ ILC3, and Nkp46⁺ROR γ t⁺ NK cells. Data are representative of six independent experiments with at least three mice per group. (E) Representative fluorescence stereomicroscopic photographs of live lamina propria biopsy punches obtained from $Rorc^{+/EGFP}$ mice. Image shows clustered enhanced green fluorescent protein (EGFP) (ROR γ t) expression of ILF-residing $Rorc^{+/EGFP}$ cells isolated from small intestine (left; scale bar: 100 μ m) and colon (right; scale bar:

500 μ m). (F and G) Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of Csf2 expression in isolated intestinal epithelial cells (EC), Peyer's patches (PP), lamina propria depleted of ILFs (LP), and ILF from small intestine (F) or colon (G). Data are representative of at least two independent experiments with three mice per group. (H) Quantitative RT-PCR analysis of Csf2 expression in colonic NK cells (1), ROR γ t^{lo} NCR⁺ ILC3 (2), ROR γ t^{high} NCR⁺ ILC3 (3), and ROR γ t^{high} LTi cells (4) isolated from $Rorc^{+/EGFP}$ mice. (I) FACS plots show Csf2 and CD45 staining on total colonic lamina propria cells and expression of CD3 and ROR γ t among Csf2⁺CD45⁺ cells isolated from $Rag2^{-/-}$ mice (data are representative of two independent experiments with three mice per group). (J) FACS plots show Csf2 and CD45 staining on total colonic lamina propria cells isolated from either $Rag2^{-/-}$ mice, $Rag2^{-/-}$ mice injected with depleting anti-CD90 mAb, or $Rag2^{-/-}$ IL2rg^{-/-} mice. Bar graph shows percentages of Csf2⁺ CD45⁺ cells in each group of mice. All Csf2 staining was performed on ex vivo isolated cells cultured for 4 hours in the presence of Brefeldin A. Data are shown as mean \pm SD of two independent experiments with three mice per group. One-way ANOVA Bonferroni's multiple comparison test (H and J) was performed. Statistical significance is indicated by * P < 0.05, ** P < 0.01, and *** P < 0.001.

cells, Peyer's patches, and lamina propria depleted of ILF (Fig. 2, F and G, and fig. S4, A to E). These data identify ROR γ ⁺ ILC3 in ILFs as the main producers of intestinal Csf2 in the steady state.

Analysis of bone marrow chimeric mice that were lethally irradiated and then reconstituted with congenic hematopoietic progenitors revealed that host-derived ROR γ ⁺ ILC3 remained resident in the recipient intestine for several months after lethal body irradiation, consistent with previously published data (39). We observed high levels of Csf2 production in host-derived ROR γ ⁺ ILC3 even 3 months after lethal body irradiation (fig.

S5), suggesting that ROR γ ⁺ ILC3 likely contribute to the steady-state radio-resistant source of Csf2 reported by other investigators (30). Fluorescence-activated cell sorting (FACS) purification of ILC subsets from *Rorc*^{+EGFP} reporter animals further confirmed ROR γ ⁺ ILC3 as major producers of Csf2 (Fig. 2H). Accordingly, although Csf2-producing cells were detectable in high numbers in the small and large intestine of *Rag2*^{-/-} mice, which lack ROR γ -expressing T lymphocytes but not ILCs (Fig. 2I), they were reduced in ILC-deficient *Rag2*^{-/-}*Il2rg*^{-/-} mice and in *Rag2*^{-/-} mice depleted of ILCs with mAb

against CD90 (34) (Fig. 2J). ILC depletion in *Rag2*^{-/-} mice led to impaired RA-generating enzyme activity in colonic DCs and macrophages (fig. S6). Together, these results establish ILCs as a key producer of the myeloid regulatory cytokine Csf2 in the intestine.

Csf2 Production Is Dependent on Microbial Signals

The effector functions of ROR γ ⁺ ILC3, and the development and maturation of ILFs depend on commensal-driven signals (37, 40, 41). We found that Csf2 production was absent in newborns,

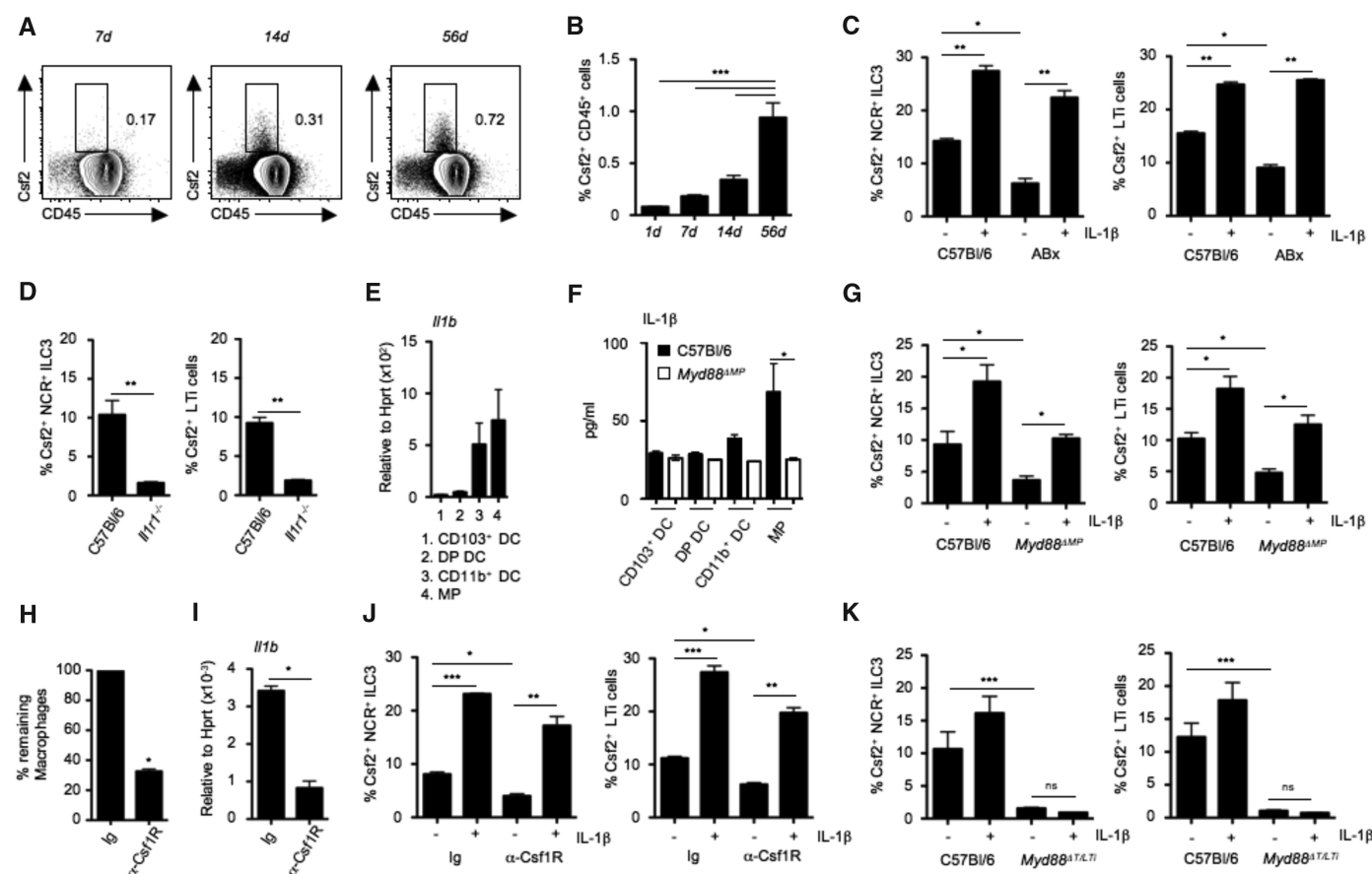


Fig. 3. Microbiota-driven IL-1 β release by intestinal macrophages regulates Csf2 production by ROR γ ⁺ ILC3. (A) FACS plot showing Csf2 expression in whole intestinal lamina propria CD45⁺ cells at the indicated time points after birth. (B) Bar graph shows percentages of Csf2⁺ cells among total lamina propria cells in mice at the indicated time points after birth. Data shown are the results of three independent experiments with at least three mice per group. (C) Percentages of Csf2⁺ cells among gated colonic lamina propria NCR⁺ ILC3 and LTi cells in conventional mice or mice treated with broad-spectrum antibiotics (ABx). Stains were performed on cells cultured for 4 hours with (+) or without (−) IL-1 β in the presence of Brefeldin A. Data are shown as mean \pm SD of three independent experiments with at least three mice per group. (D) Percentages of Csf2⁺ cells among gated colonic lamina propria NCR⁺ ILC3 and LTi cells in *Il1r*^{-/-} or C57Bl/6 mice. Data show the results of two independent experiments with three mice per group. (E) *Il1b* mRNA expression in FACS-purified colonic MNP. Data shown are representative of two independent experiments with pooled cells of three mice. (F) IL-1 β protein production by purified intestinal DC subsets and MPs isolated from the colonic lamina propria of C57Bl/6 and *Myd88* ^{Δ MP} mice, measured by ELISA after 24 hours of culture in complete medium. Data are representative of two independent experiments with pooled cells of three mice. (G) Percent-

ages of Csf2⁺ cells among gated colonic ILC3 in *Myd88* ^{Δ MP} mice. Stains were performed in cells cultured for 4 hours with or without IL-1 β in the presence of Brefeldin A. Data are shown as mean \pm SD of three independent experiments with at least three mice per group. (H) Groups of mice were injected with one injection of anti-Csf1R mAb (3 mg per mouse) or control mAb. Bar graph shows percentages of remaining macrophages in colonic tissue. (I) *Il1b* expression in whole colonic tissue of mice treated with anti-Csf1R mAb or isotype control. Data are shown as mean \pm SD of at least three independent experiments with three mice per group. (J) Percentages of Csf2⁺ cells among gated colonic lamina propria NCR⁺ ILC3 and LTi cells in mice treated with anti-Csf1R mAb or control mAb. Staining was performed on total cells cultured for 4 hours with or without IL-1 β in the presence of Brefeldin A. Data are shown as mean \pm SD of three independent experiments with three mice per group. (K) Csf2 production by colonic lamina propria NCR⁺ ILC3 and LTi cells in *Myd88* ^{Δ TLT} mice measured after 4 hours of culture with or without IL-1 β in the presence of Brefeldin A. Data are shown as mean \pm SD of three independent experiments with at least three mice per group. Student's *t* test (D, H, and I) or one-way ANOVA Bonferroni's multiple comparison test (B, C, F, G, J, and K) were performed. Statistical significance is indicated by **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

slightly increased in 7-day-old mice, and increased substantially from day 14 after birth, concurrent with the increase in numbers and complexity of the intestinal microbial flora at these developmental stages (Fig. 3, A and B). To further investigate the influence of the commensal flora on Csf2 production in the intestine, we treated adult mice with broad-spectrum antibiotics known to strongly reduce the gut microbiota. In accordance with our findings in newborn animals, adult mice treated with broad-spectrum antibiotics displayed reduced Csf2 production in the small and large intestine (Fig. 3C). These results suggest that commensal-driven signals control the steady-state production of Csf2 by ROR γ ⁺ ILC3 in the mouse intestine.

Murine ROR γ ⁺ ILC3 lack Toll-like receptors (TLRs) and cannot directly sense microbial signals in the gut; hence, these cells must rely on other cellular sensors to translate cues from commensal bacteria into effector functions (42). We therefore explored whether cytokines derived from myeloid cells could drive Csf2 production by ROR γ ⁺ ILC3 ex vivo. Among several cytokines

tested, we observed that IL-1 β was a particularly potent inducer of Csf2 production by ROR γ ⁺ ILC3 (Fig. 3C), consistent with the reported role of IL-1 β as a potent driver of ILC function (38). Because the myeloid cytokine IL-23 promotes the production of the cytokine IL-22 by ROR γ ⁺ ILC3, we examined whether IL-23 also promoted Csf2 production by these cells (43). IL-23 was unable to promote Csf2 production by ROR γ ⁺ ILC3 (fig. S7A), whereas it stimulated the release of IL-22 (fig. S7B), as previously reported (43). Furthermore, IL-22 production by ROR γ ⁺ ILC3 was unaffected in *Csf2*^{-/-} mice (fig. S7C). Exposure to IL-1 β rescued Csf2 production by ROR γ ⁺ ILC3 isolated from antibiotic-treated mice (Fig. 3C). Accordingly, LT α and NCR⁺ ILC3 isolated from mice lacking IL-1 receptor 1 (*Il1rl1*^{-/-}) failed to produce Csf2 (Fig. 3D), thereby implicating IL-1 β and IL-1R signaling as key drivers of Csf2 production in the intestine. In contrast, absence of the other IL-1 superfamily member, IL-18, did not compromise intestinal Csf2 production (fig. S7D). Together, these data indicate that IL-1 β —

producing cells that respond to microbial signals control the steady-state production of Csf2 by ROR γ ⁺ ILC3 in the intestine.

Sensing the commensal microflora by the TLR and the activation of the adapter protein Myd88 is critical for maintaining intestinal homeostasis (44) and leads to steady-state IL-1 β production by tissue MNP (45). Tissue-resident macrophages, CD103⁺ DCs, and CD103⁻ DCs arise from different developmental pathways and express distinct pattern recognition receptors (46). We found that intestinal macrophages were the highest producers of *Il1b* and IL-1 β protein, as previously reported (45, 47), suggesting that this population is a key regulator of Csf2 production in the gut (Fig. 3, E and F).

Because microbial signals were required to drive Csf2 production in the intestine, we next examined whether deletion of the TLR-adapter protein Myd88 in phagocytes influenced Csf2 production by ROR γ ⁺ ILC3. Lysozyme M (LysM) is expressed at high levels in macrophages relative to DCs (48), and notably, mice that lack Myd88

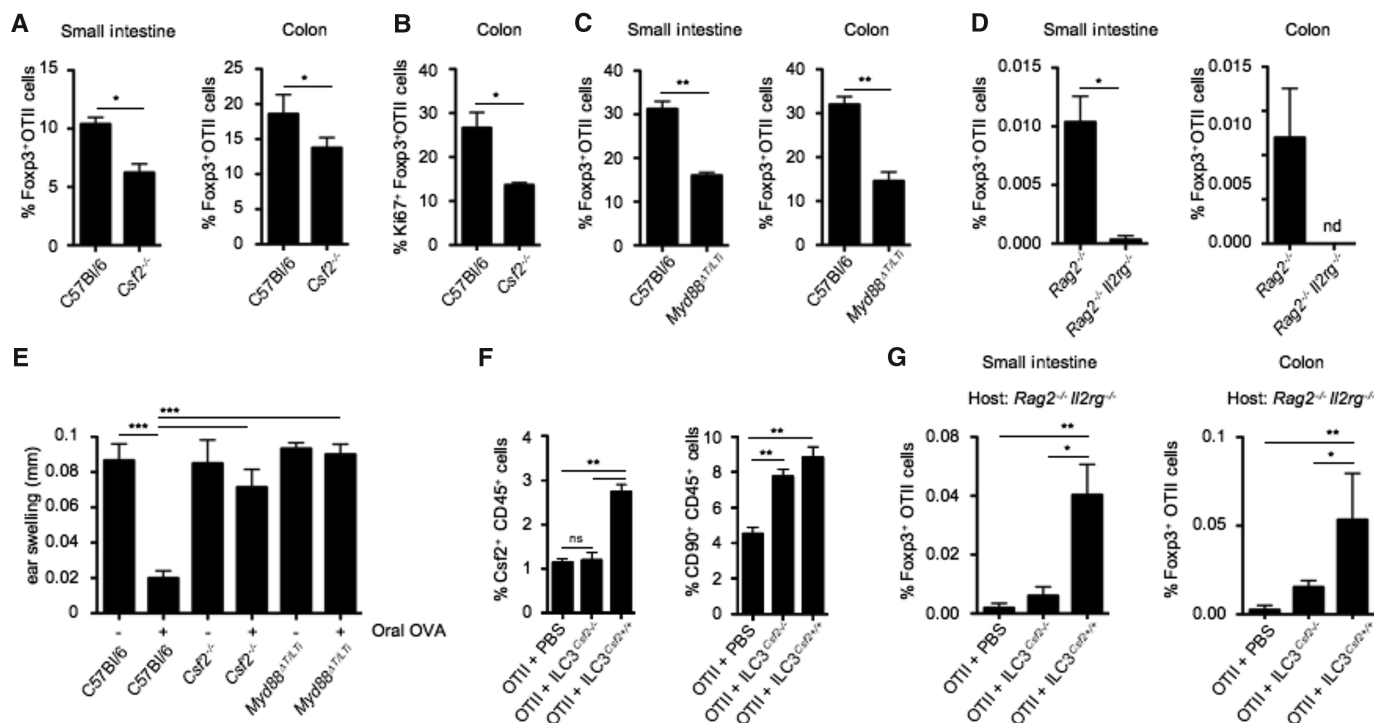


Fig. 4. ILC3-derived Csf2 controls oral tolerance to dietary antigens.

(A) Naïve OTII *Rag2*^{-/-} CD45.1⁺ T cells were adoptively transferred into CD45.2⁺ C57BL/6 and CD45.2⁺ *Csf2*^{-/-} mice. Bar graph shows percentages of small intestinal and colonic OTII-specific Foxp3⁺ T_{regs} after oral feeding with OVA ad libitum. Data are shown as mean \pm SD of three independent experiments with three mice per group. (B) Bar graph shows percentages of Ki67⁺ colonic Foxp3⁺ OTII T cells in C57BL/6 and *Csf2*^{-/-} mice after OVA feeding. Data are shown as mean \pm SD of three independent experiments with three mice per experiment. (C and D) Naïve OTII *Rag2*^{-/-} CD45.1⁺ T cells were adoptively transferred into C57BL/6 and *Myd88* ^{Δ TLT} mice (C), or *Rag2*^{-/-} and *Rag2*^{-/-} *Il2rg*^{-/-} mice (D). Bar graphs show percentages of small intestinal and colonic OTII-specific Foxp3⁺ T_{regs} after OVA feeding. Data are shown as mean \pm SD of three independent experiments with three mice per group. (E) *Csf2*^{-/-}, *Myd88* ^{Δ TLT} and control mice were either fed (+) or not fed with OVA (-) for 7 days to induce oral tolerance. Four days later, fed mice were immunized subcutaneously with

OVA (300 μ g) and complete Freund's adjuvant and rechallenged 14 days later with OVA (50 μ g) into the right ear, as described in the materials and methods. Skin DTH response was determined by ear swelling (mm). Data are shown as mean \pm SD (n = 10 mice) and are representative of two independent experiments. (F and G) Purified wild-type or *Csf2*^{-/-} ILC3 were injected into *Rag2*^{-/-} *Il2rg*^{-/-} hosts, 2 weeks before injection of naïve OTII CD45.1⁺ T cells. Reconstituted hosts were fed with OVA and analyzed 5 days later. (F) Bar graph shows percentages of Csf2⁺ CD45⁺ cells (left) and total CD90⁺ CD45⁺ ILCs (right) in the colonic lamina propria of the indicated host mice. Data are shown as mean \pm SD of two independent experiments with three mice per group. (G) Bar graphs show percentages of small intestinal and colonic OTII-specific Foxp3⁺ T_{regs} after OVA feeding. Data are shown as mean \pm SD of two independent experiments with three mice per group. Student's *t* test (A to D) or one-way ANOVA Bonferroni's multiple comparison test (E to G) were performed. Statistical significance is indicated by **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

specifically in $LysM^+$ cells [$LysM^{Cre}Myd88^{fllox/flox}$ ($Myd88^{\Delta MP}$) mice] exhibited a significant reduction in IL-1 β production in intestinal macrophages, whereas IL-1 β production by intestinal DCs was unaffected (Fig. 3F). The disruption of IL-1 β release by intestinal macrophages in $Myd88^{\Delta MP}$ mice abrogated the production of Csf2 by ROR γ^+ LTi and NCR $^+$ ILC3 in these animals (Fig. 3G), whereas addition of exogenous IL-1 β cytokine rescued Csf2 production by ROR γ^+ ILC3 (Fig. 3G). Consistent with a central role for macrophages in intestinal IL-1 β production, administration of depleting anti-Csf1 receptor monoclonal antibody (anti-Csf1R mAb) depleted tissue macrophages (Fig. 3H), as we previously showed (49), and reduced total *Il1b* expression (Fig. 3I). Accordingly, Csf2 production by intestinal ROR γ^+ ILC3 was significantly reduced after depletion of colonic macrophages (Fig. 3J). IL-1 β cytokine was capable of rescuing Csf2 production by ROR γ^+ ILC3 isolated from mice treated with the anti-Csf1R mAb (Fig. 3J), thus confirming that macrophage-derived IL-1 β is a key driver of Csf2 production by ROR γ^+ ILC3. Myd88 is an essential signal transducer in both the TLR and IL-1R pathway (50). To establish whether IL-1 β and IL-1R signaling are required to promote Csf2 production by ROR γ^+ ILC3, we crossed $Rorc^{Cre}$ mice with $Myd88^{fllox/flox}$ mice to achieve deletion of Myd88 specifically in ROR γ^+ ILC3 and T cells ($Myd88^{\Delta TLTi}$) (51). As expected, we observed a reduction in Csf2 production by ROR γ^+ ILC3 from $Myd88^{\Delta TLTi}$ mice (Fig. 3K). In this case, administration of IL-1 β cytokine failed to rescue Csf2 expression by ROR γ^+ ILC3, suggesting that Myd88 functions downstream of IL-1R in ROR γ^+ ILC3 (Fig. 3K). Taken together, our results suggest that Myd88-dependent sensing of the commensal microflora by intestinal macrophages elicits production of IL-1 β , which in turn activates the IL-1R–Myd88 pathway in ROR γ^+ ILC3 to drive the steady-state production of Csf2. Alteration of the commensal flora using broad-spectrum antibiotics or deletion of macrophages using anti-Csf1R mAb treatment led to impaired RA production in all DC subsets (fig. S8A) and to reduced T_{reg} numbers and proliferation (fig. S8, B and C), confirming the role of tissue macrophages in translating microbial cues into immunoregulatory signals that help promote T_{reg} homeostasis in the steady-state colon.

Csf2 Promotes Oral Tolerance to Fed Antigens

Because one of the key functions of intestinal T_{reg} s is the maintenance of oral tolerance to fed antigens, we asked whether deficiency in Csf2 affects de novo generation of intestinal T_{reg} s upon oral administration of ovalbumin (OVA). Conversion and expansion of OVA-specific T_{reg} s in the small and large intestine were impaired in $Csf2^{-/-}$ mice compared to wild-type mice (Fig. 4, A and B). Similar results were obtained when T_{reg} conversion was analyzed in mice selectively lacking Csf2 in ROR γ^+ ILC3 ($Myd88^{\Delta TLTi}$) or in ILC-deficient mice ($Rag2^{-/-}Il2rg^{-/-}$) (Fig. 4, C and D). Consistent with Csf2's key role in oral toler-

ance, OVA feeding of $Csf2^{-/-}$ mice and $Myd88^{\Delta TLTi}$ mice failed to protect the mice from delayed-type hypersensitivity (DTH) reaction upon OVA challenge, whereas control mice were protected (Fig. 4E). Together, these results establish that altered Csf2 production by ILCs impairs the induction of oral tolerance to dietary antigens. The defect in T_{reg} conversion observed in the small intestine of $Csf2^{-/-}$ mice contrasts with the apparent normal total T_{reg} numbers observed in the small bowel of these mice. These results suggest that compensatory mechanisms specific to the small bowel may help restore the number but likely not the repertoire of small intestinal T_{reg} s in $Csf2^{-/-}$ mice.

To establish the direct contribution of Csf2 produced by ILC3 to T_{reg} conversion in vivo, we reconstituted ILC-deficient $Rag2^{-/-}Il2rg^{-/-}$ mice with $Csf2^{-/-}$ or $Csf2^{+/+}$ ILC3. Two weeks later, $Rag2^{-/-}Il2rg^{-/-}$ mice reconstituted with ILCs were adoptively transferred with OVA-specific T cell receptor transgenic OTII cells and fed with OVA for 5 days. $Csf2^{-/-}$ and $Csf2^{+/+}$ ILC3 engrafted with the same efficiency in $Rag2^{-/-}Il2rg^{-/-}$ mice (Fig. 4F), and reconstitution of $Rag2^{-/-}Il2rg^{-/-}$ mice with $Csf2^{+/+}$ ILC3 led to partial recovery of Csf2 production in the intestine (Fig. 4F). Although the rate of T_{reg} conversion was low in all $Rag2^{-/-}Il2rg^{-/-}$ mice due to a defect in lymphoid organ development in these mice, OVA-specific T_{reg} conversion was nonetheless significantly higher in $Rag2^{-/-}Il2rg^{-/-}$ mice reconstituted with $Csf2^{+/+}$ compared to mice reconstituted with $Csf2^{-/-}$ ILC3 (Fig. 4G), further emphasizing the contribution of ILC3-derived Csf2 to the control of oral tolerance to dietary antigens.

Discussion

Previous studies have established the role of microbial commensals that colonize the large bowel to promote the induction of Foxp3 $^+$ T_{reg} differentiation (5). However the cellular cues that promote T_{reg} accumulation in response to gut commensals have only recently started to be unraveled (52–54). Our data identify a mechanism by which the gut microbiota promotes intestinal homeostasis by supporting a crosstalk between IL-1 β -secreting macrophages and Csf2-producing ROR γ^+ ILC3 in the intestinal mucosa. Microbiota-driven IL-1 β production by macrophages promoted the release of Csf2 by ILC3, which in turn acted on DCs and macrophages, allowing for the maintenance of colonic T_{reg} homeostasis (fig. S9). Ablation of Csf2 altered DC and macrophage numbers and impaired their ability to produce regulatory factors such as RA and IL-10, which led to disrupted T_{reg} homeostasis in the large intestine. Conversely, administration of Csf2 cytokine increased T_{reg} frequency in the gut. Most notably, cell type-specific ablation of IL-1-dependent signaling in ROR γ^+ ILC3 abrogated oral tolerance to dietary antigens and compromised intestinal T_{reg} homeostasis in vivo. Although the reduction in total T_{reg} numbers was mostly observed in the large intestine, adoptive transfer studies in $Csf2^{-/-}$ mice revealed impaired T_{reg} differentiation both in the small and large intestine, suggesting that Csf2-

dependent MNP immunoregulatory functions control T_{reg} induction in both tissues

Establishing intestinal tolerance is critical for the prevention of intestinal diseases such as IBD. IBD includes two broad disease classifications known as ulcerative colitis and Crohn's disease, but there is substantial variation in IBD clinicopathology in individual patients; hence, it is likely that numerous subtypes of IBD exist in this group. In a study of more than 300 patients with Crohn's disease, the presence of neutralizing antibodies to Csf2 in the serum correlated with ileal involvement and the development of penetrating pathology, whereas a more recent study identified reduced levels of Csf2 receptor (Csf2R) and impaired receptor activity in a mixed group of IBD patients (55, 56). Previous clinical trials of recombinant Csf2 in IBD have established patient benefit in terms of reduced disease severity and lower burden of corticosteroid use (57). Unpublished results of a larger trial of Csf2 in IBD has since failed to achieve primary clinical end points, but it remains likely that a subset of IBD patients with defective Csf2 production or function could benefit from this therapy.

The uncovered key role for Csf2 in the maintenance of intestinal tolerance is consistent with previous studies showing that absence of Csf2 can also contribute to lupus-like disease, insulinitis, and age-related glucose intolerance (29, 58) and further emphasizes the critical role of tissue-resident phagocytes in the maintenance of tissue integrity.

Our data reveal a mechanism by which the gut commensal flora promotes immune homeostasis in the host. We have identified the commensal-driven MNP-ILC-Csf2 axis as a key regulator of intestinal T cell homeostasis in the mouse intestine. Disturbance of this axis radically altered MNP effector function, resulting in impaired oral tolerance to dietary antigens. These results represent an important advance in our understanding of how commensal microbes can regulate host intestinal immunity and may inform the design of new immunotherapies for the use in patients with subtypes of IBD.

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Microbiota-Dependent Crosstalk Between Macrophages and IL3 Promotes Intestinal Homeostasis

Arthur Mortha, Aleksey Chudnovskiy, Daigo Hashimoto, Milena Bogunovic, Sean P. Spencer, Yasmine Belkaid and Miriam Merad (March 13, 2014)

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Editor's Summary

Gut Immune Tolerance

With the constant assault of food antigens and its billions of resident microbes, the gut is an important site of immune tolerance. By studying specific intestinal immune cell populations in genetically modified mice, **Mortha *et al.*** (10.1126/science.1249288, published online 13 March; see the Perspective by **Aycheh and Jung**) found that gut macrophages produce the cytokine interleukin-1 (IL-1) in response to signals derived from the microbiota. IL-1 acts on type 3 innate lymphoid cells in the intestine, which then produce the cytokine, colony-stimulating factor 2 (Csf2). Csf-2, in turn, induces myeloid cells (including dendritic cells and macrophages) to produce regulatory factors like retinoic acid and interleukin-10, which support the conversion and expansion of regulatory T cells, a population of cells known to be critical for maintaining immune tolerance in the gut.

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