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Supplementary Materials for

Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis

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This PDF file includes:

Materials and Methods
Figs. S1 to S9
References

Material and Methods

Mice

C57Bl/6, B6.129P2(Cg)-*Rorc*^{tm2Litt}/J (Rorc$^{+/EGFP}$), B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J (Myd88$^{flox/flox}$), B6.129S1-*Csf2rb2*^{tm1Cgb} *Csf2rb1*^{tm1Clsc}/J (*Csf2r*$^{-/-}$*),* B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J (LysM^{Cre}), B6N.129(Cg)-*Foxp3*^{tm3Ayr}/J (Foxp3-GFP), B6.129S7-Il1r1-tm1Imx/J (*Il1r1*$^{-/-}$) and B6.129P2-Il18r1-tm1Aki/J (*Il18*$^{-/-}$) mice were purchased from Jackson Laboratory. B6.129S7-*Rag1*^{tm1Mom}Tg(TcraTcrb)425Cbn (OTII *Rag1*$^{-/-}$) mice were purchased from Taconic. Spleen and Lymph nodes of OTII *Rag2*$^{-/-}$ CD45.1 mice were provided by Dr. Yasmine Belkaid (NIH, Bethesda). *Rorc*$^{Tg-Cre}$ generated as described (*51*) were provided by Dr. Dan Littmann (Skirball Insititute, NY). *Csf2*$^{-/-}$ mice were a kind gift from Dr. Markus G. Manz (University Hospital, Zurich, Switzerland). Unless otherwise stated, mice were used at 6-12 weeks of age. Experiments were carried out using age and gender matched groups. All animal procedures performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Mount Sinai School of Medicine.

Antibiotic treatment

Groups of mice were treated with Ampicillin (1g/L), Streptomycin (1g/L), Metronidazol (0.5g/ml) and Vancomycin (1g/L) ad libidum for 3-4 weeks via the drinking water. Water containing antibiotic was exchanged every three days.

Isolation of lamina propria lymphocytes

Lamina propria lymphocytes were isolated as described (*37*). Briefly, the intestines devoid of Peyer's patches were incubated in EDTA supplemented Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}(Gibco) for 15-20 min at 37°C with mild agitation. The epithelial cell layer was removed by vortexing. Remaining sheets of lamina propria were digested in collagenase (Sigma), DNaseI (Sigma) and Dispase (BD Biosciences). The cell were resuspended in

5ml of 40% Percoll (GE Healthcare) and overlaid onto 5ml of 80% Percoll in a 15ml tube. Lymphocytes were collected at the interphase of the Percoll gradient, washed once and resuspended in media.

Isolation of Isolated Lymphoid Follicles

Intestines from *Rorc*$^{+/EGFP}$ mice were isolated by removing the epithelial cell layer as describe above. Lamina propria sheets were pinned into Petri dishes containing ice-cold media. RORγt promoter-driven green fluorescent protein (GFP) expression was visualized using a Stereomicroscope (Olympus MVX10 MacroView microscope). Visualized isolated lymphoid follicles (ILF) were isolated using 1mm or 300μm biopsy puncher (Ted Pella, Inc.). ILF were either placed into Trizol reagent (Ambion) for RNA isolation or into media when used for flow cytometry.

Antibodies

CD3e (clone 145-2C11, Biolegend), CD4 (clone L3T4, Biolegend), V alpha 2 TCR (clone B20.1, eBioscience) CD11b (clone M1/70, Biolegend), CD11c (clone N418, Biolegend), CD45 (clone 30F11, Biolegend), CD45.2 (clone 104), CD45R (clone RA3-6b2, Biolegend), CD64 (clone X54-5/7.1, BD), CD103 (clone 2E7, eBioscience), F4/80 (clone CI: A3-1, Biolegend), I-A/I-E (clone M5/114.15.2, Biolegend), NKp46 (clone 29A1.4, Biolegend), IFN-γ (clone XMG1.2, eBioscience), IL-2 (clone JES6-5H4, eBioscience), IL-10 (clone JES5-16E3, eBioscience), IL-17A (clone eBio17B7, eBioscience), IL-22 (clone AM22.1, Biolegend), GM-CSF (clone MP1-22E9, Biolegend), Foxp3 (clone FJK-16s, eBioscience), RORγt (clones B2D and AFKJS-9, eBioscience), Ki67 (clone M19, Santa Cruz). Secondary antibodies, isotype controls and fluorophore-conjugated Streptavidins were purchased from Biolegend and eBioscience. ALDEFLUOR staining kit (Stem cell technologies) was used according to the manufacturer instructions.

Flow Cytometry

Isolated cells were surface stained in FACS buffer (PBS w/o Ca^{2+} Mg^{2+} supplemented with 2% heat inactivated FBS and 5mM EDTA) for 20-30 min on ice. Multiparameter analysis was performed on a FACS Canto II (BD), LSR II (BD) or Fortessa (BD) and analyzed with FlowJo software (Tree Star). $DAPI^+$ cells and doublets were excluded from all analysis. For the detection of cytokines, cells were cultured in media in the presence of Brefeldin A (10µg/ml) for 4h at 37°C. *Ex vivo* stimulations were carried out in the presence of Brefeldin A, using 10-100ng/ml recombinant IL-1β (Pepro Tech), 10ng/ml IL-23 (Pepro Tech) or phorbol 12-myristate 13-acetate (PMA) (Sigma) and Ionomycin (Sigma) and then fixed using Cytofix/Cytoperm buffer (BD). Staining with anti-IFN-γ, anti-IL-22, anti-IL-17A, were performed in FACS buffer containing 0.5% Saponin (Sigma). For the detection of transcription factors, cells were fixed and stained using the Foxp3-staining kit (eBioscience) according to the manufacturer's instructions. $DAPI^+$ cells and doublet were excluded from all analysis. Dead cells were excluded using LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen, Carlsbad, CA).

Quantitative real-time PCR (Q-PCR).

Conventional reverse transcription, using the Sprint PowerScript reverse transcriptase (Clontech) was performed in accordance with the manufacturers' instructions. Q-PCR was performed with SYBR GREEN on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The PCR protocol consisted of one cycle at 95°C (10 min) followed by 40 cycles of 95°C (15 s) and 58°C (1 min). Expression of hypoxanthine-guanine phosphoribosyltransferase (Hprt) was used as a standard. The average threshold cycle number (CRtR) for each tested mRNA was used to quantify the relative expression of each gene: 2^[Ct(Hprt)-Ct(gene)]. Primers are listed below:

Csf2(Fwd)_GCATGTAGAGGCCATCAAAGA
Csf2(Rev)_CGGGTCTGCACACATGTTA
Il1b(Fwd)_TCTTCTTTGGGTATTGCTTGG

Il1b(Rev)_TGTAATGAAAGACGGCACACC
Il10(Fwd)_ CAGAGCCACATGCTCCTAGA
Il10(Rev)_ GTCCAGCTGGTCCTTTGTTT
Hprt(Fwd)_CCTGGTTCATCATCGCTAATC
Hprt(Rev)_TCCTCCTCAGACCGCTTTT
Aldh1a1(Fwd)_CTCCTCTCACGGCTCTTCA
Aldh1a1(Rev)_AATGTTTACCACGCCAGGAG
Aldh1a2(Fwd)_ CATGGTATCCTCCGCAATG
Aldh1a2(Rev)_ GCGCATTTAAGGCATTGTAAC

In vitro stimulation of peritoneal Macrophages

Peritoneal macrophages (pMP) were isolated as described in (*59*). For the rescue of *Il10* and *Aldh1a1* expression, *Csf2*$^{-/-}$ pMP were treated with Csf1 (10ng/ml) or stimulated with Csf2 (50ng/ml) for 12h. For the rescue of IL-10 protein release and ALDH activity, *Csf2*$^{-/-}$ pMP were treated with Csf1 (10ng/ml) or stimulated with Csf2 (50ng/ml) for 5 days.

ELISA

IL-1β, IL-10 and TGF-β secretion was measured in the cell culture supernatants of FACS-purified DC subsets and macrophages using IL-1β, IL-10 and TGF-β ELISA kits according to the manufacturer's protocol.

Induction of regulatory T cells

Naïve T cells ($CD3^+$ $CD4^+$ $CD44^-$ Foxp3-GFP^-) were sorted from the spleens of Foxp3-GFP mice and cultured in the presence of purified DC (pooled CD103+ DC, DP DC, $CD11b^+$ DC subsets) or $MHCII^+CD11c^+CD11b^+F4/80^+$ macrophages isolated from age and sex-matched C57Bl/6 or Csf2$^{-/-}$ mice at a 1:5 or 1:10 APC/T cell ratio. All cultures contained murine TGF-β (5ng/ml) (Peprotech) and purified anti-CD3e (clone 145-2C11, eBioscience). Indicated cultures were supplemented with recombinant murine Csf2 (10ng/ml) (Peprotech), 4-diethly-aminobenzaldehyde (DEAB) (7,5µM) (Stem cell technologies), anti-IL-10 mAb

(10μg/ml) (BioXell) or retinoic acid (RA) (100nM) (Sigma). Foxp3 (GFP) expression on gated $CD4^+$ live cells was analyzed by flow cytometry at day 5 of culture.

Bone marrow chimeric mice

Bone marrow chimeras were generated as previously described (*49*). Mice were analyzed 12 weeks after injection of 1x 10^6 total bone marrow cells.

Csf2 overexpression

B16 melanoma cells overexpressing Csf2 ($B16^{Csf2}$) were kindly provided by Dr. Glen Dranoff (Dana Farber Cancer Institute). B16 or $B16^{Csf2}$ cells were cultured to 80% confluence and 1x10^5 cells were injected into the back skin of $Csf2^{-/-}$ mice. Mice were monitored daily. Animals were analyzed 10-14 days after injection.

In vivo modulation of Tregs using RA or RA blockade

Briefly, naïve mice were injected with either vehicle or all-trans retinoic acid (RA) or the retinoid acid receptor antagonist LE540 as described (*60*). RA was purchased from Sigma (USA), dissolved in 1:1 DMSO+soybean oil (3 mg/ml, for i.p. injections), stored at - 20°C and protected from light and used at 300μg/mouse. LE540 (Wako, Japan) was dissolved in 1:1 DMSO+soybean oil (0.5 mg/ml), stored at -20°C and protected from light and used at 100μg/mouse. Every second day, in an interval of two weeks,

Adoptive transfer

ILC3 were sorted from the small intestine of either $Csf2^{+/+}$ or $Csf2^{-/-}$ mice. 2-3x10^4 cells were adoptively transferred into $Rag2^{-/-}Il2rg^{-/-}$ mice via retro orbital injection as described in (*34*). Two weeks after ILC3 transfer, recipient mice were injected with FACS-purified 0.5x10^6 OT-II cells. Treg conversion was assessed using the oral tolerance model described below.

Oral tolerance

Induction of oral tolerance was performed as described in (*18*). Briefly, $0.5x10^6$ cells $OTII^+$ naïve T cells were purified from the spleen and lymph nodes of *RAG2*$^{-/-}$ $OT\text{-}II^+$ mice and adoptively transferred into each recipient mouse on day 0. Mice were provided 1% Ovalbumin (Sigma) ad libidum for 5 days. Ovalbumin containing water was changed every other day. Treg conversion of naïve OT-II cells was analyzed in the small and large intestine on day 6.

Induction of DTH responses

DTH experiments were performed as previously described (*7*). Mice were fed ad-libidum with water supplemented with 1 mg/ml Ovalbumin (Ova) for 7 days. Four days later, mice were immunized subcutaneously and injected with 300 µg Ova in 200 µl PBS/CFA emulsion (Sigma). Two weeks after immunization, mice were re-challenged subcutaneously and injected with 50 µg Ova in 20 µl PBS into the right ear pinna while 20 µl PBS without Ova were injected into the left ear pinna as a negative control. Ova-specific ear swelling was measured using a caliper, 48h after challenge.

Statistical analyses

Student's t test or ONE-way ANOVA Bonferroni's Multiple Comparison Test was used to determine significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

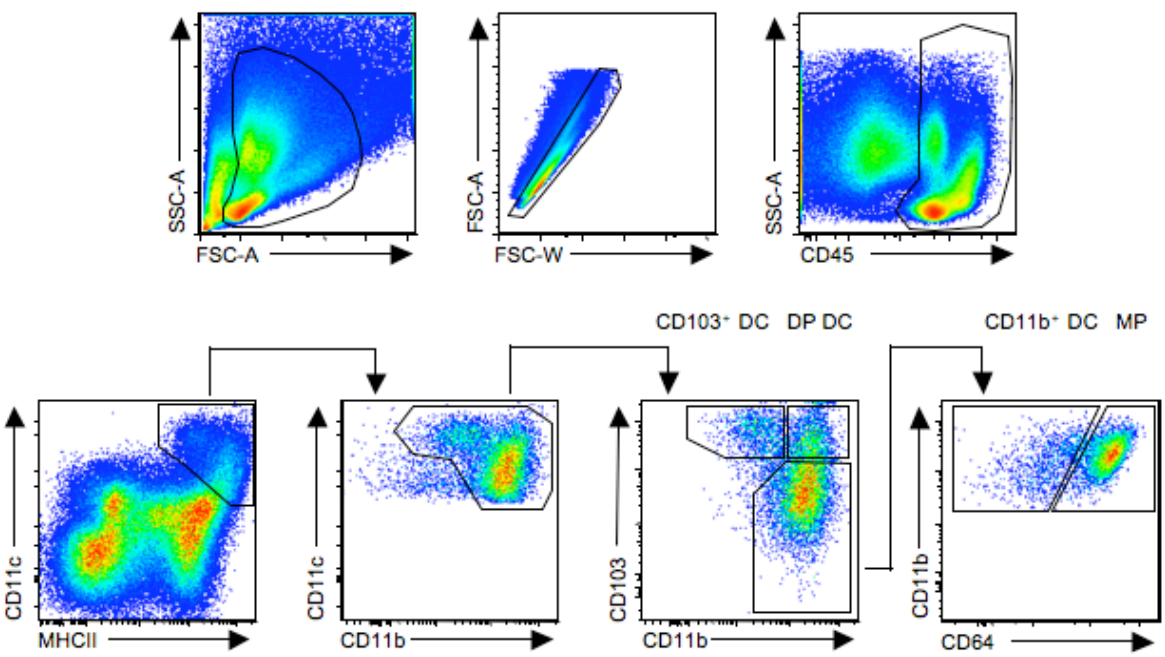

Figure S1. Gating strategy to analyze colonic mononuclear phagocyte subsets.

Colonic lamina propria cells were isolated from conventional C57Bl/6 mice and surface stained with anti-CD45, anti-MHCII, anti-CD11c, anti-CD11b, anti-CD103 and anti-CD64 antibodies. $CD103^+$ DC were identified as $CD45^+MHCII^+CD11c^+CD11b^-CD103^+$, DP DC were identified as $CD45^+MHCII^+CD11c^+CD11b^+CD103^+$, $CD11b^+$ DC were identified as $CD45^+MHCII^+CD11c^+CD11b^+CD103^-CD64^-$ and macrophages were identified as $CD45^+MHCII^+CD11c^+CD11b^+CD103^-CD64^+$.

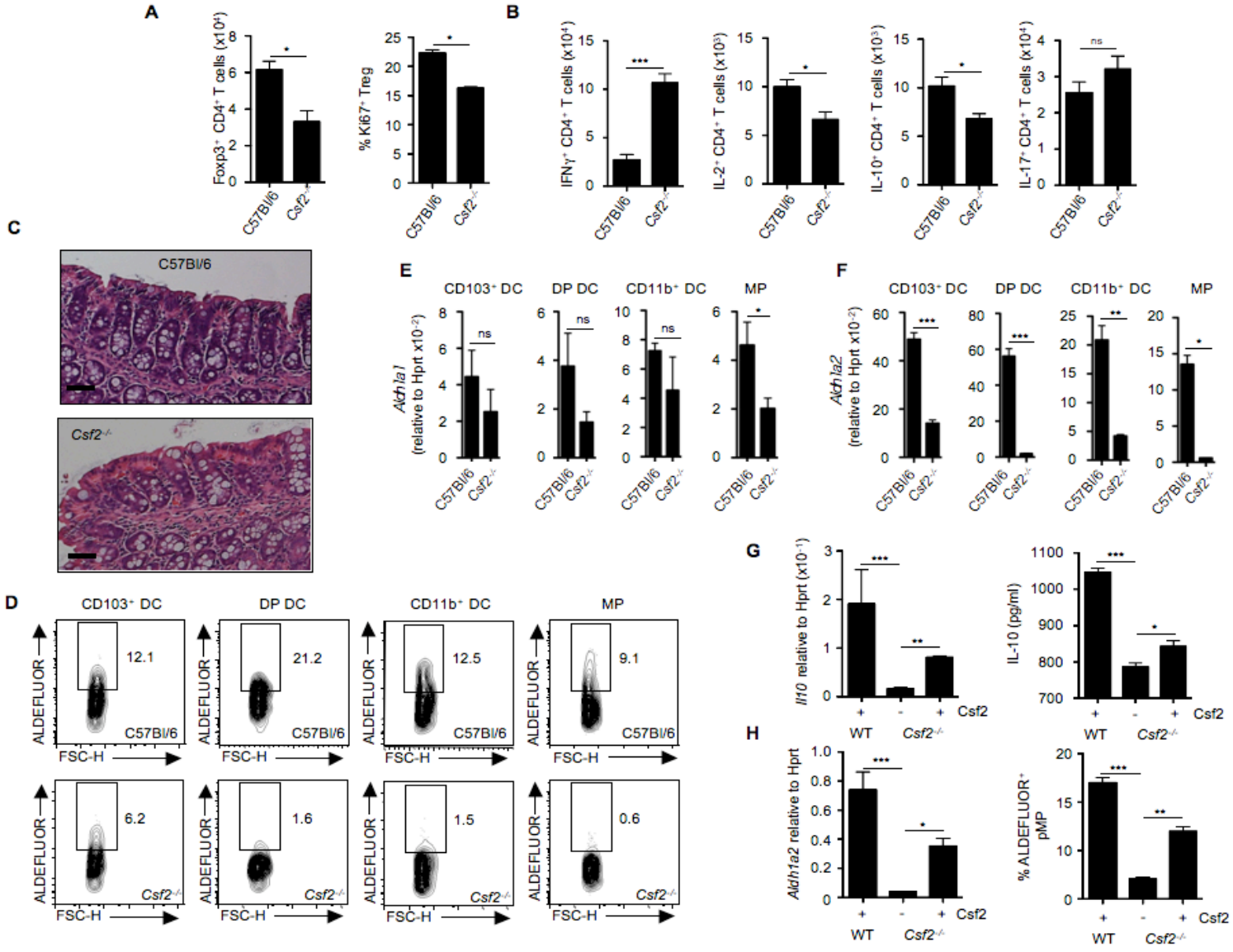

Figure S2. Csf2 controls colonic T cell homeostasis.

(**A**) Bar graphs show absolute numbers of colonic Tregs and percentages of Ki67$^+$ colonic Foxp3$^+$ Tregs in C57Bl/6 or *Csf2*$^{-/-}$ mice. Data is shown as mean ±SD (n= 3 independent experiment with 3 mice per group). (**B**) Bar graphs show absolute numbers of colonic IFNγ$^+$, IL-2$^+$, IL-10$^+$ and IL-17$^+$ T cells in C57Bl/6 or *Csf2*$^{-/-}$ mice. Prior to staining, cells were stimulated *ex vivo* for 4h in the presence of PMA/Ionomycin and Brefeldin A. Data is shown as mean ±SD (*n* = 3 experiments with 3 mice per group). (**C**) Microphotographic pictures of H+E stained sections of colonic tissue from C57Bl/6 or *Csf2*$^{-/-}$ mice. Black scale bar = 100µm (**D**) Contour plots show percentages of ALDEFLUOR$^+$ DC subsets and

macrophages isolated from colonic tissue of C57Bl/6 or *Csf2*$^{-/-}$ mice. Quantitative RT-PCR for (**E**) *Aldh1a1* and (**F**) *Aldh1a2* in FACS-purified mononuclear phagocytic populations isolated from C57Bl/6 or *Csf2*$^{-/-}$ mice (*n* = 2 experiments with 3 mice per group). Peritoneal macrophages (pMP) were isolated from C57Bl/6 and *Csf2*$^{-/-}$ mice and incubated in the presence or absence of Csf2. Bar graphs (left) show *Il10 and Aldh1a2* mRNA expression in pMP 12h post-stimulation using quantitative RT-PCR. Bar graph (top right) show released IL-10 protein, measured by ELISA after 5 days of culture. Bar graphs show (bottom left) quantitative RT-PCR analysis for *Aldh1a2* (bottom right) shows percentages of ALDEFLUOR^{+} pMP after Csf2 stimulation. Data is shown as mean ±SD (*n* ≥ 3 individual experiments with 2-3 mice per group). Statistical significance is indicated by *p<0.05, **p< 0.01, *** p< 0.001

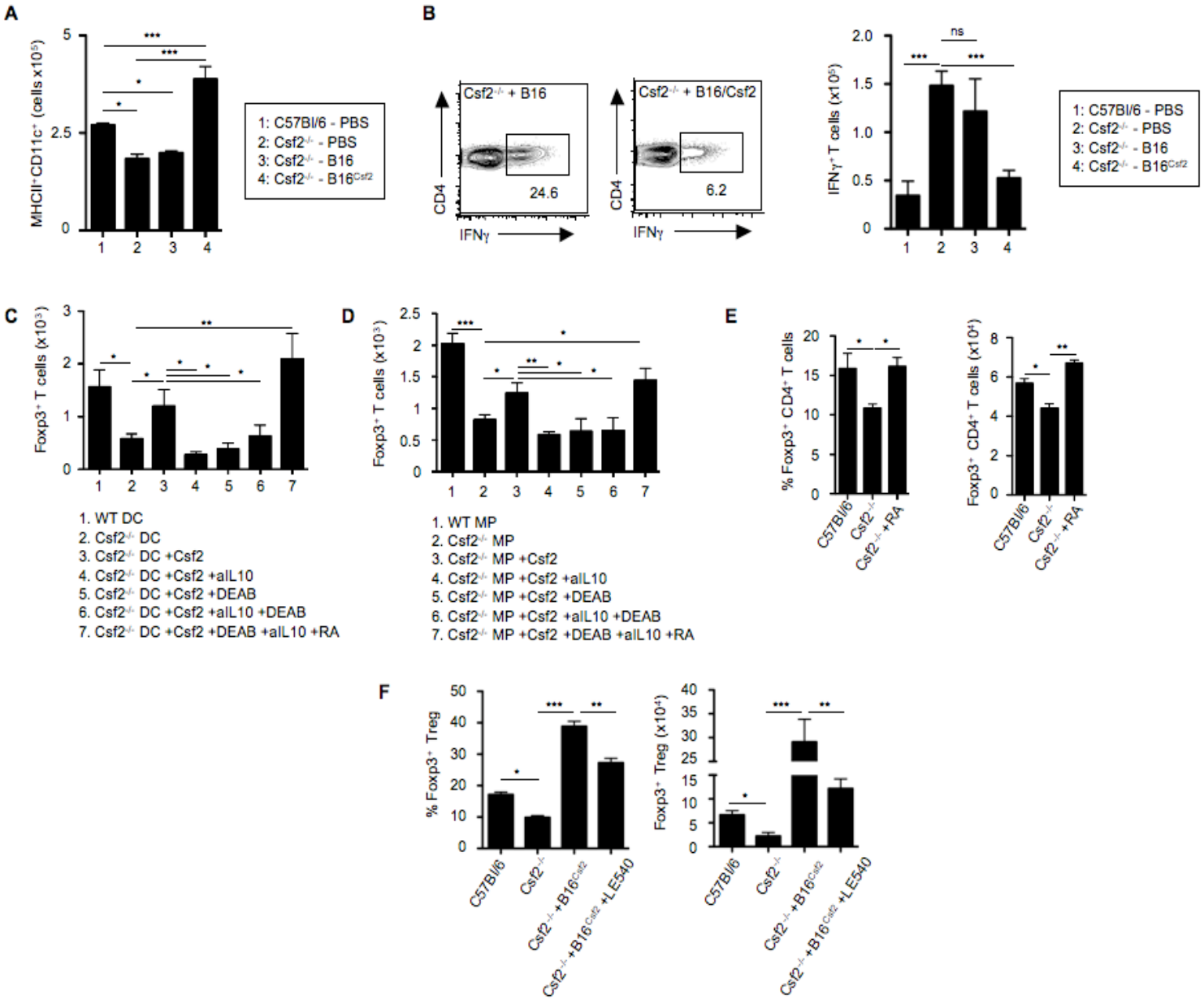

Figure S3. Csf2 promotes regulatory functions of DC and macrophage partly through modulation of RA production.

(**A**) Bar graph show absolute numbers of colonic MHCII^{+}CD11c^{+} mononuclear phagocytes 12-14 days after injection with B16 cells, B16 cells overexpressing Csf2 (B16^{Csf2}) into *Csf2*$^{-/-}$ mice. (**B**) Contour plots show percentages of colonic IFNγ^{+} CD4^{+} T cells in *Csf2*$^{-/-}$ mice, 12-14 days after injection with B16 cells or B16^{Csf2}. Bar graph shows absolute numbers of colonic IFNγ-expressing colonic T

cells in *Csf2*$^{-/-}$ mice 12-14 days after injection with B16 cells or B16^{Csf2}. Staining was performed on cells stimulated in culture for 4h with PMA/Ionomycin in presence of Brefeldin A. (**C**) Bar graphs show absolute numbers of induced Foxp3^{+} T cells generated by FACS-purified colonic *Csf2*$^{-/-}$ DC cocultured with Foxp3^{-} T cells in the presence or absence of Csf2, anti-IL-10 mAb or ALDH blockade using 4-diethyl aminobenzaldehyde (DEAB) in the presence or absence of RA. Data is shown as mean ±SD (*n* = 3 independent experiments). (**D**) Bar graph shows absolute numbers of induced Foxp3^{+} T cells generated by FACS-purified *Csf2*$^{-/-}$ macrophages (MP) *in vitro* under culture similar culture conditions. Data is shown as mean ±SD (*n* = 3 independent experiments). (**E**) Bar graphs show percentages and absolute numbers of CD45^{+}CD3^{+}CD4^{+}Foxp3^{+} colonic Treg in *Csf2*$^{-/-}$ mice, 2 weeks after injection with B16 cells, B16^{Csf2} or B16^{Csf2} injected mice additionally treated with the retinoic acid receptor antagonist LE540 every other day. Data is shown as mean ±SD (*n* = 2 independent experiments with 3 mice per group). (**F**) Bar graphs show percentages and absolute numbers of CD45^{+}CD3^{+}CD4^{+}Foxp3^{+} colonic Treg in C57Bl/6, *Csf2*$^{-/-}$ mice or *Csf2*$^{-/-}$ mice injected with RA every other day for two weeks. Data is shown as mean ±SD (*n* = 2 independent experiments with 3 mice per group). ONE-way ANOVA Bonferroni's Multiple Comparison Test (**A,C-F**) were performed. Statistical significance is indicated by *p<0.05, **p< 0.01, *** p< 0.001

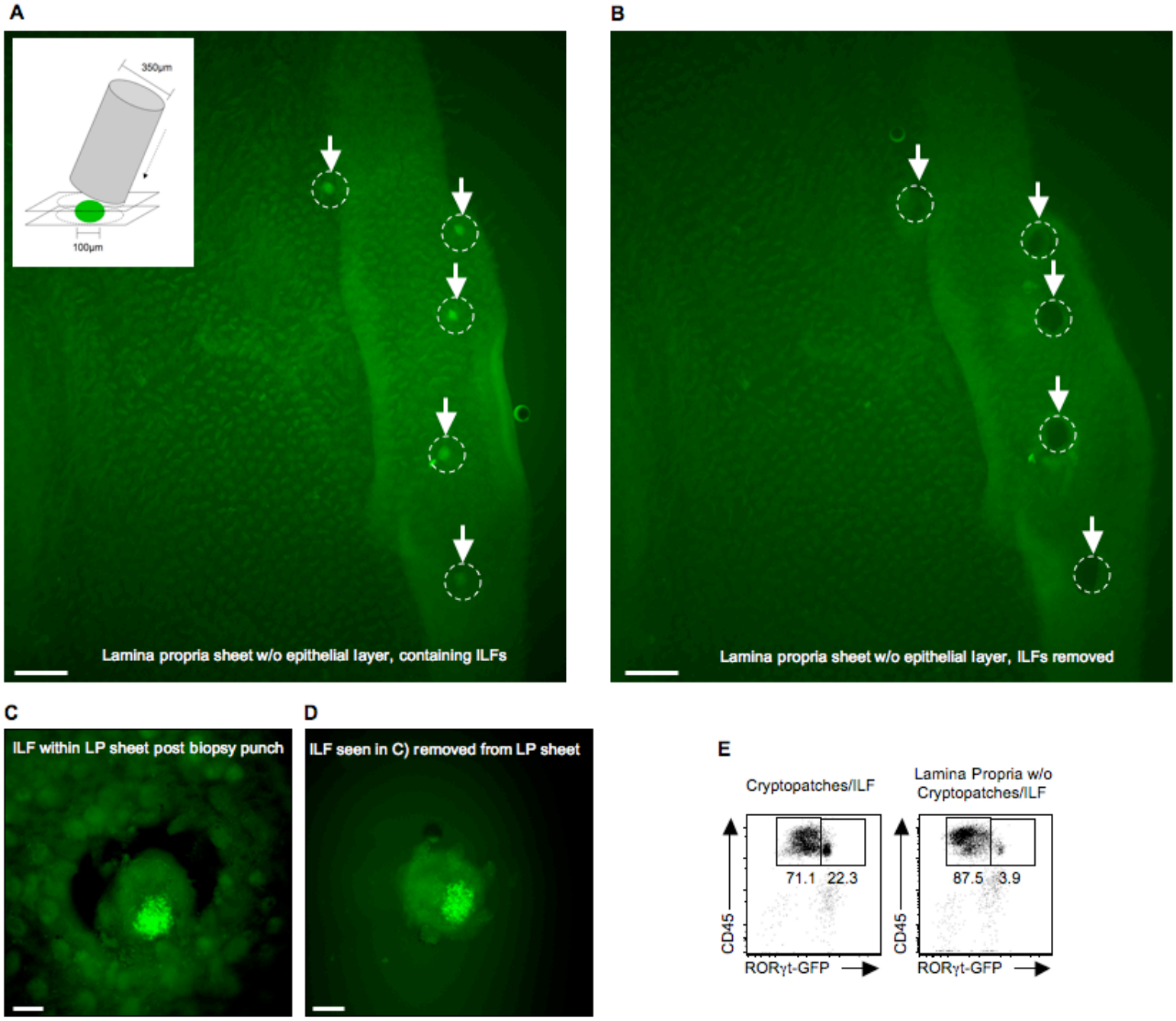

Figure S4. Isolation of ILFs from the small intestinal lamina propria using biopsy punchers.

(A) Cartoon (top left) displays ILF isolation method from the intestinal lamina propria. The cylinder (grey) represents the tip of a 350µm biopsy puncher used to

excise 100-300μm wide Cryptopatches or ILF (green circle) located within the intestinal lamina propria (stacked squares). Epithelial cells were removed from the small intestinal lamina propria. Fluorescent stereomicroscopic micrographs of living intestinal lamina propria isolated from *Rorc*$^{+/GFP}$ reporter mice shows four ILF (highlighted by white arrows and white dashed circles) enrichment in RORγt-expressing (green) ILC3. (B) The same sheet of lamina propria after isolation of ILF (missing green cells highlighted by white arrows and white dashed circles). (C) Higher magnification of a small intestinal ILF after biopsy punching. (D) The same ILF as in (C) after removal from the remaining intestinal tissue. (E) Representative dot plots show enrichment of RORγt^{+} cells in biopsy punches of Cryptopatches/ILFs and reduced RORγt^{+} cells in lamina propria without Cryptopatches/ILFs respectively. White bars represent 1mm (A and B) or 100μm for C and D. Original magnification 12x (SI). Data are representative of more than 10 individual experiments.

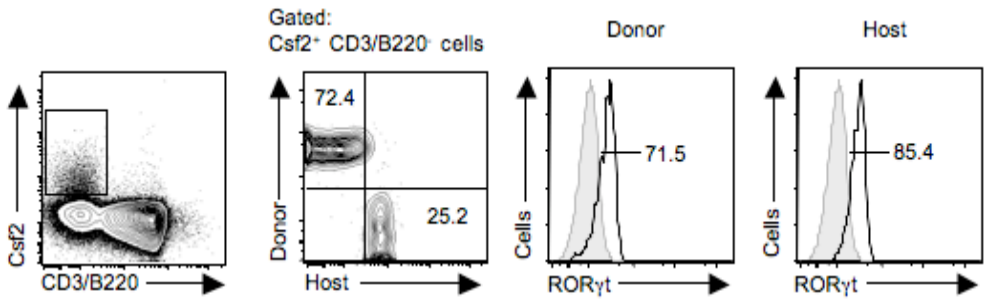

Figure S5. RORγt$^+$ ILC are radio-resistant cells.

CD45.2$^+$ mice (host) were lethally irradiated and reconstituted with CD45.1$^+$ (donor) bone marrow cells. Three months after transplant, flow cytometry analysis of lamina propria cells show that 25% of Csf2$^+$CD3$^-$B220$^-$ ILC are of host origin. RORγt expression was analyzed in host and donor-derived Csf2$^+$CD3$^-$ B220$^-$ ILC (black line open) and compared to control Ig staining (grey line filled). Data shown is representative of 3 independent experiments with 3 mice per group.

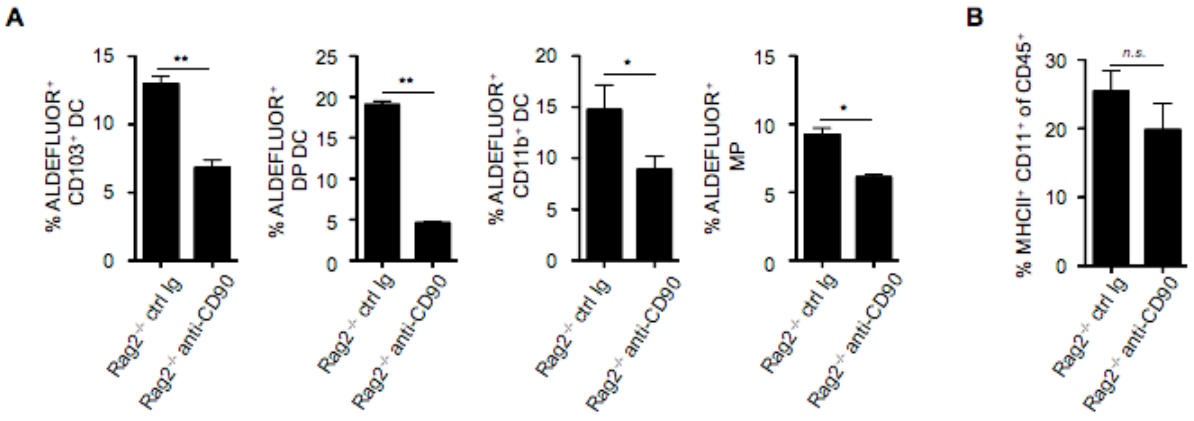

Figure S6. ILC control regulatory functions of mononuclear phagocytes.

(**A**) Bar graphs show percentages of ALDEFLUOR$^+$ cells in the indicated DC and macrophage (MP) subsets in *Rag2*$^{-/-}$ mice injected with control Ig or anti-CD90 antibodies 5 days after injection. (**B**) Bar graph shows percentages of MHCII$^+$CD11c$^+$ MNP among all CD45$^+$ colonic lamina propria cells in the indicated groups of mice. Data shown (**A** and **B**) is representative of 2 independent experiments with 3 mice per group. Data is shown as mean ±SD. Student's t test (**A-B**) was performed. Statistical significance is indicated by *p<0.05, **p< 0.01, *** p< 0.001

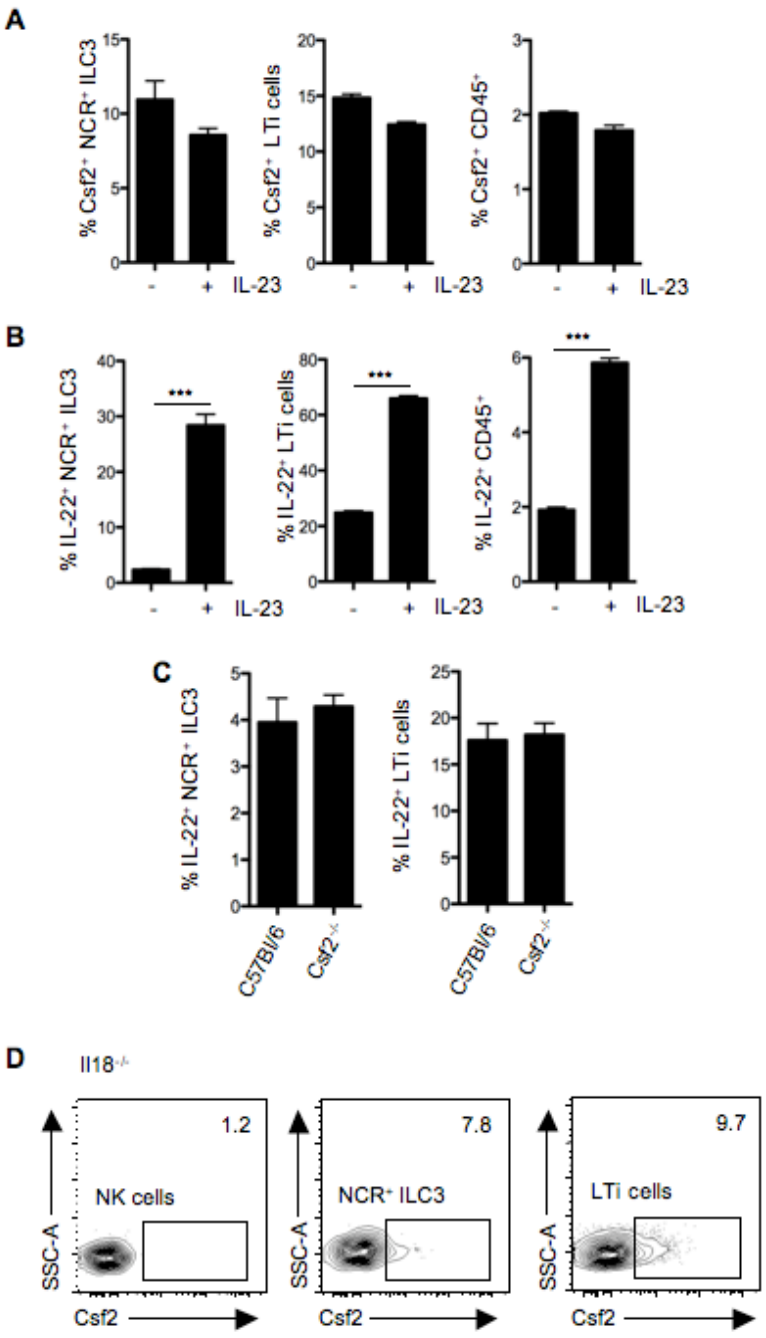

Figure S7. IL-23 stimulation does not increase Csf2 in RORγt$^+$ ILC and Csf2 is dispensable for IL-22 expression.

(A) Percentages of Csf2$^+$ cells in gated colonic NCR$^+$ ILC3, LTi cells and CD45$^+$ cells stimulated *ex vivo* with and without IL-23 for 4h in the presence of Brefeldin A. (B) Percentages of IL-22$^+$ cells in gated colonic NCR$^+$ ILC3, LTi cells and

$CD45^+$ cells stimulated *ex vivo* for 4h with and without IL-23 in the presence of Brefeldin A. (C) Percentages of IL-22^+ cells in gated colonic NCR^+ ILC3 and LTi cells isolated from C57Bl/6 and *Csf2*$^{-/-}$ mice and cultured for 4h in the presence of Brefeldin A. All data are shown as mean ±SD ($n \geq 3$ independent experiments with 3 mice per group). (D) Contour plots show Csf2 expression in NK cells, NCR^+ ILC3 and LTi cells isolated from *Il18*$^{-/-}$ mice. Prior to staining, cells were cultured for 4h in the presence of Brefeldin A. Data shown is representative of 3 independent experiments with 3-5 mice per group. Student's t test (A-C) was performed. Statistical significance is indicated by *p<0.05, **p< 0.01, *** p< 0.001

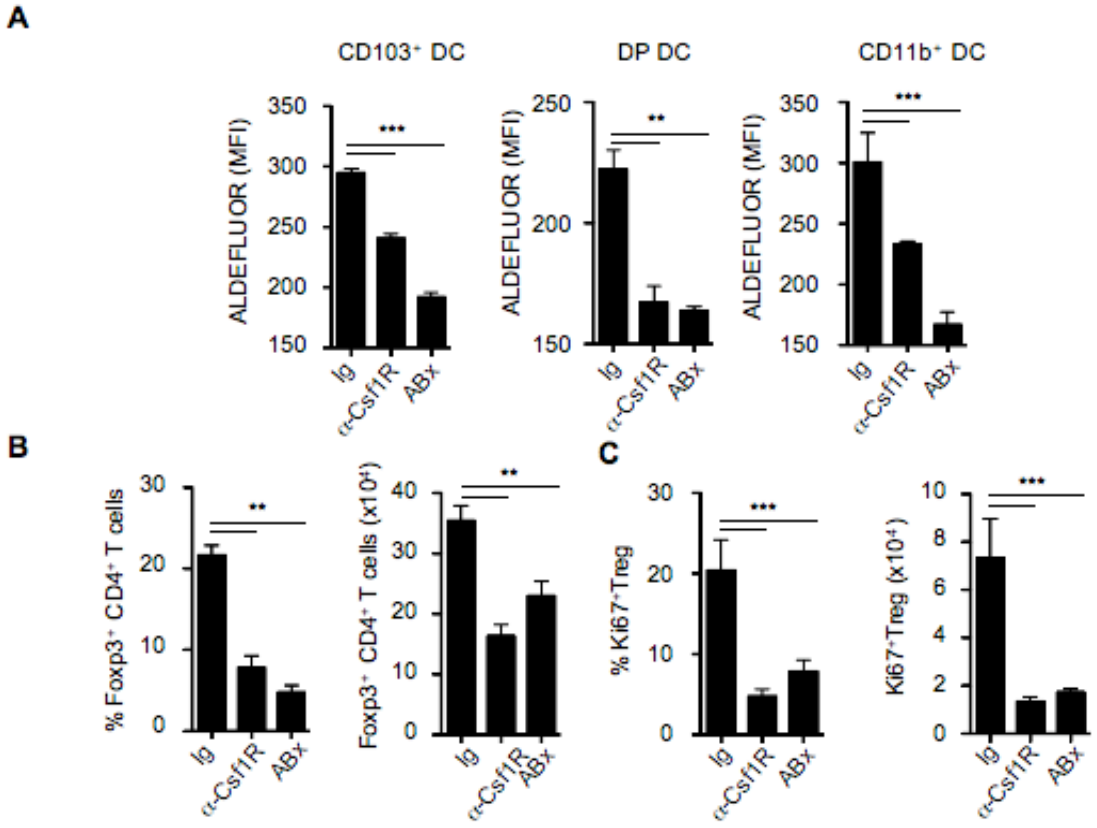

Figure S8. Macrophages and the commensal microflora control regulatory functions of mononuclear phagocytes and colonic Treg homeostasis.

(**A-C**) Bar graphs show ALDEFLUOR intensity (MFI) in the indicated DC subsets and MP (**A**) and relative and absolute numbers of $CD45^+CD3^+CD4^+Foxp3^+$ colonic Tregs (**B**) or $CD45^+CD3^+CD4^+Foxp3^+Ki67^+$ colonic Tregs (**C**) in C57Bl/6 mice injected with control or anti-Csf1R mAb and in mice treated with broad-spectrum antibiotics (ABx). All data are shown as mean ±SD ($n \geq 3$ experiments with at least with 5 mice per group). ONE-way ANOVA Bonferroni's Multiple Comparison Test (**A-C**) was performed. Statistical significance is indicated by *p<0.05, **p< 0.01, *** p< 0.001

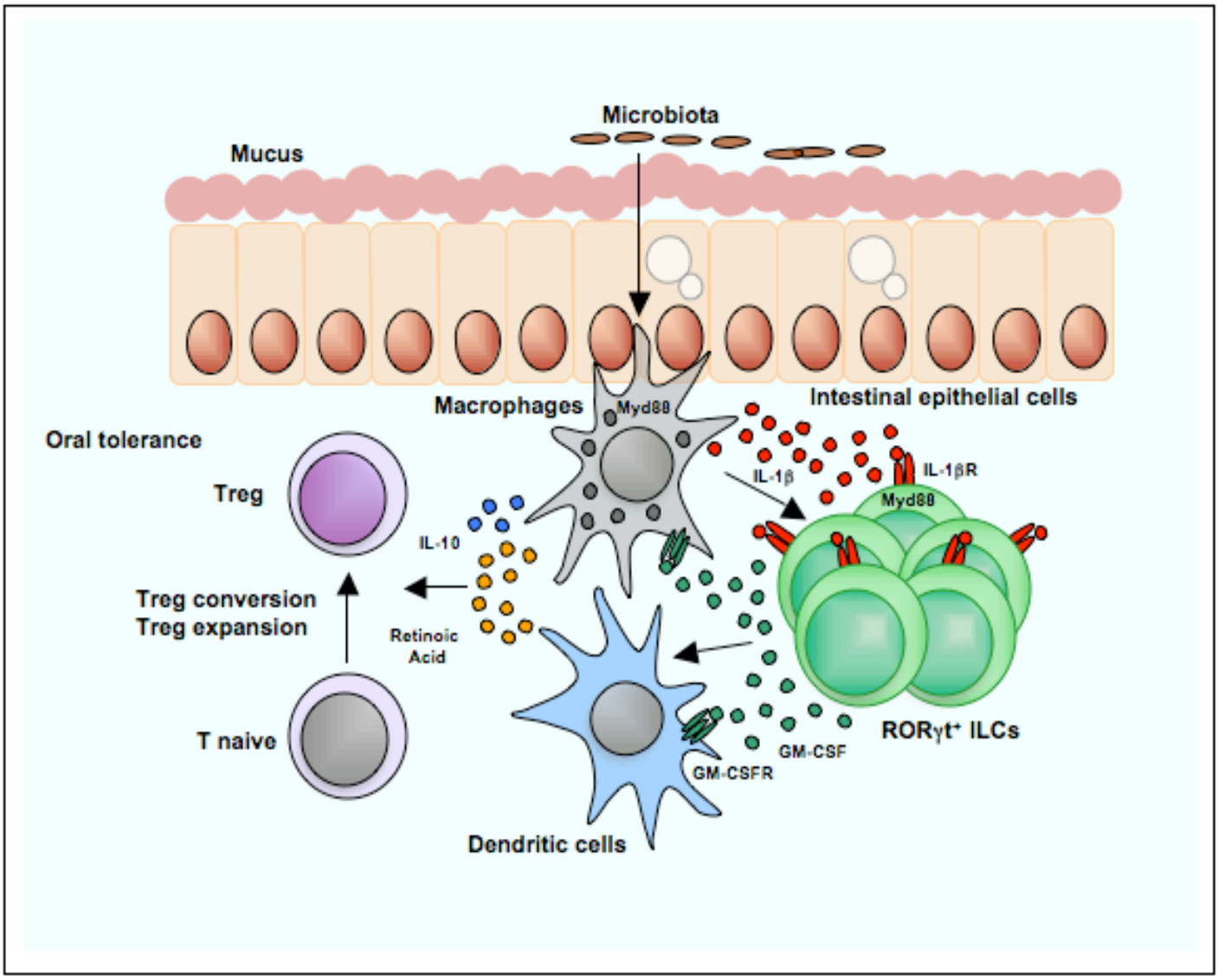

Figure S9. Microbial signals promote a regulatory cross-talk between macrophages and innate lymphocyte cells that contribute to oral tolerance to dietary antigens.

The graphical abstract summarizes our working model, suggesting that microbial signals trigger Myd88-mediated IL-1β release by macrophages (MP). IL-1β engages the IL-1R on RORγt$^+$ ILC3 and induces Myd88-dependent expression of Csf2. ILC3-derived Csf2 triggers the induction of regulatory functions (i.e. RA and

IL-10 production) in DC and MP. IL-10 and RA expression by DC and MP triggers the conversion and expansion of naïve T cells into regulatory T cells, contributing to the maintenance of oral tolerance to dietary antigens.

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