Embryonic macrophages function during early life to determine invariant natural killer T cell levels at barrier surfaces

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It is increasingly recognized that immune development within mucosal tissues is under the control of environmental factors during early life. However, the cellular mechanisms that underlie such temporally and regionally restrictive governance of these processes are unclear. Here, we uncover an extrathymic pathway of immune development within the colon that is controlled by embryonic but not bone marrow–derived macrophages, which determines the ability of these organs to receive invariant natural killer T (iNKT) cells and allow them to establish local residency. Consequently, early-life perturbations of fetal-derived macrophages result in persistent decreases of mucosal iNKT cells and is associated with later-life susceptibility or resistance to iNKT cell–associated mucosal disorders. These studies uncover a host developmental program orchestrated by ontogenically distinct macrophages that is regulated by microbiota, and they reveal an important postnatal function of macrophages that emerge in fetal life.

The initial exposure of the mucosal immune system to microbes during neonatal life plays a critical role in molding the levels of specific immune cell elements and consequently the host’s response to stimuli during later life. An important cell type regulated in this manner is CD1d-restricted iNKT cells, a rare subset of T cells that function in the recognition of self and microbial lipid antigens important to the outcome of infectious, autoimmune and neoplastic disorders in organs where they exist. It is currently thought that the temporal regulation of iNKT cell levels during early life is simply a consequence of microbial exposures prevalent during this time that determine the recruitment of cells from the circulation and their local expansion on entry into tissues. However, it has not been considered that the regulation of iNKT cells during early life may emerge from events associated with host developmental pathways under the subsequent influence of postnatal environmental factors, such as microbes. Therefore, it is interesting that certain barrier surfaces with high concentrations of microbes, such as the colon and dermis, are distinctive in that they are transiently occupied for the first several weeks of life by ontogenically unique macrophages of embryonic origin before weaning. The function of these embryonic macrophages within barrier tissues is unknown but their temporary presence at a time when iNKT cells are developing raises the possibility that they are involved in these processes. In this study, we report that colonic iNKT cell development and residency is dependent on the control of embryonic macrophages during a specific early-life time window.

Results
Colonic iNKT cell residency is established during early life. We performed parabiosis experiments to evaluate iNKT cell residency in the colon. Fifty-six-day-old adult congenic mice bearing the CD45.1 or CD45.2 markers were surgically joined and examined for the proportions of conventional T cell receptor (TCR)αβ+ T and iNKT cells. Appropriate chimerism between CD45.1+ and CD45.2+ iNKT and TCRαβ+ T cells was achieved in the peripheral blood of the parabiotic hosts (Extended Data Fig. 1a). We observed very small numbers of congenic iNKT cells in the spleen (<3%) and colon (<2%) of the parabiotic partners (Fig. 1a and Extended Data Fig. 1b), which was limited in the colon even 8 weeks after surgery (Fig. 1b). In contrast, the TCRαβ+ T cell populations attained higher proportional levels of chimerism in the spleen (approximately 50%) and colon (approximately 15%) 3 weeks after surgery (Fig. 1a and Extended Data Fig. 1b). Therefore, consistent with studies in other organs, iNKT cells exist as a predominantly tissue-resident population in the lamina propria of the adult colon.

This suggested that colonic iNKT cells establish residency earlier in life in a yet-to-be determined time frame. Previous studies have demonstrated that iNKT cells emerge from the thymus around day 5 after birth whereupon they populate peripheral tissues. We observed that iNKT cells begin to appear in the colon during the first 5–6 d after birth and rapidly expand in proportion to TCRαβ+ T cells until approximately 10 d of life (Fig. 1c). This is followed by an intense period of expansion based on the number of iNKT cells detected until approximately 21–28 d after birth when they reach steady-state levels that were maintained into adulthood (Fig. 1d). This delineated a limited time period between 5 and 10 d after birth when iNKT cells preferentially emerge in the colon relative to TCRαβ+ T cells. To confirm and more precisely define this time period, we performed adoptive transfer of adult CD45.1+ thymic T cells in 4-day-old CD45.2+ hosts and collected the colon and spleen at day 11 (Fig. 1e). We observed that congenic iNKT cells...
Fig. 1 | Colonic iNKT cells emerge and expand during early life before establishing residency at steady state. a–d, Circulatory exchange of CD45.1 (black) or CD45.2 (gray) TCRαβ+ T (CD45+ CD3ε+ TCRβ+) and iNKT (CD45+ CD3ε+ TCRβ+ CD1d tetramer+) cells in the spleen and colon of surgically joined CD45.1 (left) and CD45.2 (right) congenic animals (n=2) determined by flow cytometry, 3 weeks (a) and 8 weeks (b) after surgery. Circles are representative of average cell frequency. iNKT cell percentage (c) and absolute counts (d) in the colon over time are shown. Each dot is representative of an individual mouse; the line is representative of the sample means. e, Schematic of the adoptive transfer strategy. f, Adoptive transfer of CD45.1 adult thymic cells into a 4-day-old CD45.2 (n=6) host followed by quantitative analyses of splenic and colonic CD45.1 (black) or CD45.2 (gray) TCRαβ+ T and iNKT cells by flow cytometry on day 11. Left, representative plots. Right, circles are representative of average cell frequency. g, Schematic of adoptive transfer strategy. h, Adoptive transfer of CD45.1 adult thymic cells into a 49-day-old CD45.2 host (n=6) followed by quantitative analyses of splenic and colonic CD45.1 (black) or CD45.2 (gray) TCRαβ+ T and iNKT cells by flow cytometry on day 56. Left, representative plots. Right, circles are representative of average cell frequency. f, h, Data were pooled from two experiments. SSC, side scatter.
were highly enriched in the colon (approximately 71%) compared to the spleen (approximately 11%) of the CD45.2+ hosts at day 11 after birth while the TCRαβ+ T cells populations exhibited limited levels of chimerism in the colon and spleen (approximately 7–8%) (Fig. 1f). Moreover, we observed that the CD45.1+ iNKT cells but not TCRαβ+ T cells that entered the colon during early life persisted long term in the CD45.2+ adult hosts colon (Extended Data Fig. 1c,d). This suggested that thymic iNKT cells display a strong predisposition to engraft in the colon but not the spleen compared to conventional T cells during this early period of life and establish residency. In contrast, adoptive transfer of CD45.1+ thymic T cells into adult CD45.2+ hosts (Fig. 1g) resulted in limited entry of iNKT and TCRαβ+ T cells in the colon and spleen (<3%) (Fig. 1h). This indicates that the colon is uniquely permissive to the local establishment of thymically derived iNKT cells during early, but not later, life resulting in the establishment of long-term residency.

Macrophages regulate the abundance of early-life colonic iNKT cells. We considered whether macrophages were involved in the processes associated with establishing iNKT cell residency during early life in the colon given that embryonic macrophages transiently occupy this tissue niche for the time when iNKT cell tissue establishment occurs14,15. To do so, we used a transgenic mouse model (MM<sup>DTR</sup>) in which the combined expression of Lys2 (or Lys) and Csf1r allows the precise expression of diphtheria toxin receptor (DTR) at the surface of macrophages in vivo. Injection of diphtheria toxin into MM<sup>DTR</sup> mice (Lys<sup>Cre</sup>−/−, Csf1<sup>fl/fl</sup>−/−) results in specific cell death of monocytes and macrophages independent of their ontogeny compared to littermate controls (Lys<sup>Cre</sup>−/−, Csf1<sup>fl/fl</sup>−/−). We performed four sets of diphtheria toxin injections beginning at day 8 until day 14 after birth (Fig. 2a). This regimen led to substantial depletion of macrophages in MM<sup>DTR</sup> mice compared to littermate controls in the lamina propria of the colon, skin and spleen (Fig. 2b and Extended Data Fig. 2a,b). Remarkably, the depletion of macrophages during this critical developmental period was associated with a notable decrease in the numbers of iNKT cells in all three of these organs (Fig. 2c–f and Extended Data Fig. 2c) and in the small intestine and lung (Extended Data Fig. 2d,e) but without modification of splenic or colonic TCR<sup>αβ</sup>+ T cell abundance (Fig. 2c–f). These studies suggest that macrophage depletion specifically affects iNKT cell abundance. Indeed, we observed no diphtheria toxin-associated depletion of other cell types, including B cells, neutrophils, dendritic cells and eosinophils (Extended Data Fig. 3a) or mucosa-associated invariant T cells in the colon (Extended Data Fig. 3b). Furthermore, at two weeks of age in the absence of diphtheria toxin injection, the levels of macrophages (Extended Data Fig. 3c) and TCR<sup>αβ</sup>+ T and iNKT cells (Extended Data Fig. 3d) were the same in MM<sup>DTR</sup> and control littermate mice, suggesting that the effects observed were dependent on diphtheria toxin alone and thus macrophages. To confirm these results, we utilized Cx3cr1<sup>DTR</sup> mice as a model for macrophage depletion. Two injections of diphtheria toxin at days 8 and 10 after birth depleted colonic and splenic macrophages in Cx3cr1<sup>DTR</sup> mice compared to littermate controls lacking the expression of the DTR locus (Extended Data Fig. 4a,b). In accordance with our observations in MM<sup>DTR</sup> mice, the depletion of macrophages by this approach was associated with decreased abundance of iNKT cells but not TCRαβ+ T cells in the colon and spleen of Cx3cr1<sup>DTR</sup> mice but not in control mice (Extended Data Fig. 4c,d). In a third approach, we administered a monoclonal antibody, AF598, that binds to CSF-1R and is known to deplete macrophages in vivo<sup>16,17</sup>, between days 4 and 10 of life (Extended Data Fig. 4e). This treatment also resulted in macrophage depletion and a reduction in the numbers of iNKT but not TCRαβ+ T cells in the colon at day 11 after birth compared to isotype control antibody-treated mice (Extended Data Fig. 4f,g). Thus, three distinct experimental approaches revealed that macrophages control the abundance of iNKT cells in the colon, small intestine, lung, skin and spleen in early life.

Next, we sought to define whether there were temporal restrictions in the tissue-specific, macrophage-determined control of iNKT cell abundance. We observed that administration of diphtheria toxin to adult MM<sup>DTR</sup> mice every 2 d beginning at day 56 until day 62 of life (Extended Data Fig. 5a) or beginning at day 15 until day 21 after birth (Extended Data Fig. 5f) resulted in macrophage depletion (Extended Data Fig. 5b,c,g) and reduction in iNKT cell numbers in the spleen but not in the colon (Extended Data Fig. 5d,e,h). This indicated that macrophages regulate iNKT cells during the first two weeks of life but not thereafter in the colon. Therefore, we next empirically parsed out the window of time when macrophages were regulating iNKT cells using the MM<sup>DTR</sup> model. By this approach, we discovered that 2 injections of diphtheria toxin at days 8 and 10 (Fig. 2g) was sufficient to cause efficient macrophage depletion in the colon and spleen of MM<sup>DTR</sup> mice compared to littermate controls (Fig. 2h and Extended Data Fig. 2f) together with decreased abundance of iNKT cells but not TCRαβ+ T cells in these organs (Fig. 2j). Conversely, colonic and splenic macrophage depletion between day 12 and day 14 after birth (Fig. 2k,l and Extended Data Fig. 2g), had no effect on colonic iNKT or TCRαβ+ T cell numbers (Fig. 2m,n), which was in contrast to that observed in the spleen where iNKT cell numbers were specifically reduced (Fig. 2o,p). Together, these results show that whereas macrophage control of iNKT cell abundance in lymphoid organs, such as the spleen, occurs without temporal restriction in early and later life, macrophage regulation of iNKT cell abundance in the colon is restricted to the first 11 d of life when iNKT cells first appear relative to TCRαβ+ T cells (Fig. 1c).

Embryonic macrophages regulate early-life iNKT cell abundance. The ability of macrophages to regulate iNKT cells throughout life in the spleen but only during early life in the colon may...
be explained by recent observations suggesting that the developmental origins of macrophages in the colon shifts from an embryonic-derived population present at birth to a predominantly bone marrow–derived population over time\(^2\). This pointed to a role for embryonic macrophages in the effects observed. Therefore, we monitored the kinetics of these populations using surface markers...
Fig. 3 | Embryonic- but not bone marrow-derived macrophages regulate iNKT cell abundance in the colon. a, Percentage of F4/80<sup>hi</sup>/CD11b<sup>lo</sup> and F4/80<sup>lo</sup>/CD11b<sup>hi</sup> macrophages (CD45<sup>+</sup> Lin<sup>−</sup> CD66<sup>+</sup> cells) in the colon over time (days 7, 8 and 10: n = 5; day 15: n = 7; day 21: n = 14). b, Representative plot (left) and absolute count (right) of iNKT and TCR<sub>αβ</sub> T cells in the colon of WT littermates (n = 5) or Ccr<sub>2</sub><sup>−/−</sup> (n = 5) animals at day 12 after birth. c, Representative plot (left) and absolute count (right) of iNKT (CD45<sup>+</sup> CD3<sup>ε+</sup> TCR<sub>β</sub>) and TCR<sub>αβ</sub> T cells in the colon of control littermates (n = 5) or Plvap<sup>−/−</sup> (n = 4) animals at day 12 after birth. d, Schematic of macrophage depletion model. e, Diphtheria toxin administered at day 1 and days 1–2 after birth followed by quantitative analyses on day 10 (H10) of the absolute count of iNKT and TCR<sub>β</sub> T cells in the colon of control littermates (n = 5) or Ccr<sub>2</sub><sup>−/−</sup> (n = 5) animals at day 12 after birth. f, Representative plot (left) and absolute count (right) of F4/80<sup>hi</sup>/CD11b<sup>lo</sup> and F4/80<sup>lo</sup>/CD11b<sup>hi</sup> macrophages in the colon of WT littermates (n = 6) or Plvap<sup>−/−</sup> (n = 4) animals at day 12 after birth. g, Representative plot (left) and absolute count (right) of iNKT and TCR<sub>β</sub> T cells in the colon of WT littermates (n = 6) or Plvap<sup>−/−</sup> (n = 4) animals at day 12 after birth.

b–e, Data are representative of three experiments. f,g Data were pooled from three experiments. Numbers in the representative plots indicate cell frequency. The error bars indicate the s.e.m. Each dot is representative of an individual mouse. P values were calculated by unpaired two-sided Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

Legend:

- F4/80<sup>hi</sup>/CD11b<sup>lo</sup>
- F4/80<sup>lo</sup>/CD11b<sup>hi</sup>
- CD64+ Lin<sup>−</sup>
- Diphtheria toxin
- Tissue collection
- iNKT
- TCR<sub>β</sub>
- TCR<sub>αβ</sub>
- CD3<sup>ε+</sup>
- CD1d tetramer
- CD1<sub>β</sub>
- APC-Cy7
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whose levels differ on macrophages of embryonic (F4/80+CD11b+),

or bone marrow (F4/80−CD11b+) origin.22. Consistent with previous

reports,22, we observed that colon macrophages were phenotypically

of embryonic origin during the first 11 d of life, corresponding to

the permissive window, after which their numbers begin to dimin-

ish as the number of bone marrow–derived macrophages in the

colon concomitantly increases (Fig. 3a). This led us to assess the

relative contributions of embryonic-derived versus bone mar-

row–derived macrophages on the expansion of colon iNKT cells.

We first examined CCR2-deficient (Ccr2−/−) mice in which bone

marrow–derived monocyte recruitment to tissues is impaired.22,23

As expected, we observed a defect in the levels of colonic bone

marrow–derived (F4/80+CD11b+) but not embryonic-derived

(F4/80−CD11b+) macrophages in Ccr2−/− compared to control mice

at day 12 after birth (Fig. 3b). However, this specific depletion in

Ccr2−/− mice had no effect on iNKT and TCRβ+ T cell levels at

day 12 of life (Fig. 3c) or in the adult (Extended Data Fig. 6a), rul-

ing out an important contribution of bone marrow–derived mac-

rophages on iNKT cell expansion in the colon. To evaluate the role

of embryonic-derived macrophages more directly, we performed

diphtheria toxin injection in MMDTR mice between days 1 and 2

after birth (Fig. 3d), which depletes macrophages present at birth

and most likely before the influx of bone marrow–derived popu-

lations in the colon (Fig. 3a). This regimen led to an early-life deple-

tion of macrophages (F4/80+CD11b+) that was compensated at day

ten after birth by an increase in the levels of bone marrow–derived

populations (F4/80−CD11b+; Extended Data Fig. 6b). Importantly,

despite this enrichment in macrophages of bone marrow origin, we

also observed a specific decrease in the numbers of iNKT cells with-

out modification of TCRβ+ T cell levels in the colon (Fig. 3e). This

strongly suggests that embryonic-derived macrophages present at

birth preferentially regulate colon iNKT cell abundance. To inves-

tigate this further, we took advantage of Plvap-deficient (Plvap−/−)

mice that display normal levels of macrophages derived from bone

marrow hematopoiesis but show a defect in embryonic-derived

macrophages due to an inadequacy of macrophage progenitor egress

from the fetal liver.22,23 We observed that Plvap−/− mice compared to

littermate controls exhibited decreased embryonic (F4/80+CD11b+),

but not bone marrow (F4/80−CD11b+) macrophage populations

(Fig. 3f), resulting in a reduction of TCRβ+ T cells and especially

iNKT cell numbers in the colon at day 12 after birth (Fig. 3g).

Taken together, we conclude that embryonic-derived rather than

bone marrow–derived macrophages determine the abundance of

iNKT cells in the lamina propria of the colon during a specific

period of early life.

Microbiota is not necessary for macrophages to regulate

iNKT cells. Previous studies performed in Swiss Webster mice have

shown that iNKT cells are increased in adult germ-free relative to

specific pathogen-free (SPF) mice and that normalization of these

elevated levels of iNKT cells in germ-free mice only occurs if micro-

biota are introduced in early (preweaned) but not later (postweaned)

life.24. In mice on a C57BL/6 background used in the present study,

we also observed elevated colonic iNKT cell levels in adult germ-free

mice compared to SPF mice (Fig. 4a), which was decreased if the

microbiota were reintroduced at birth (germ-free conventionalized

(GFCV)) (Extended Data Fig. 6c). Therefore, it is of interest that

we observed a substantial increase in F4/80+CD11b+ (embryonic)

but not F4/80+CD11b− (bone marrow) macrophages in germ-free

mice compared to SPF mice at day 15 of life, which was normalized

to SPF levels in GFCV mice (Fig. 4b and Extended Data Fig. 6d).

Furthermore, administration of AF598 in germ-free or GFCV mice

deplete macrophages between days 4 and 20 after birth (Fig. 4c)

led to efficient macrophage depletion (Fig. 4d,e) and reduced

iNKT cell abundance in both germ-free (Fig. 4f) and GFCV (Fig. 4g)

mice compared to isotype control antibody treatment. These results

demonstrate that microbiota repress colonic macrophage levels dur-

ing early life, which is associated with decreased iNKT cell levels in

the adult relative to that observed in germ-free mice. However,

a microbial signal is not required for macrophages being able to

supervise iNKT cell levels in the colon since depletion of macro-

phages in germ-free animals results in decreased iNKT cells.

Transcriptomes of early-life colonic macrophages and iNKT cells.

To understand the potential mechanisms by which macrophages

may regulate iNKT cells in early life, we performed bulk RNA

sequencing (RNA-seq) of colonic macrophages and iNKT cells. We

investigated the transcriptional signatures of macrophages defined

as CD64+ F4/80+ at days 8 and 14 after birth to identify transcripts

specifically upregulated or downregulated during the time embry-

onic macrophages control iNKT cell levels in early life within the

colon. We observed 325 transcripts with elevated abundance and

378 transcripts with decreased abundance in colonic macrophages

purified at day 8 after birth compared to day 14 after birth (Fig. 5a

and Supplementary Table 1), which suggests a major switch in

macrophage function during this period. Part of the differentially

expressed genes (DEGs) we identified encoded secreted immune

factors such as interleukins and chemokines, which suggests an

important role of macrophages in determining differentiation,

proliferation and/or migration of iNKT cells. Transcripts encoding

CXCL12 (Cxcl12) were enriched at day 8 while transcripts encod-

ing interleukin-12b (Il12b; Il12b) and IL-27 (Il27) were enriched at
day 14 (Fig. 5a). Consistent with a potential role of one or sev-

eral of these immune factors in colonic iNKT cell regulation during

early life, CXCL12 has been shown to regulate iNKT cell migration

in vitro.24 Furthermore, colonic macrophages at day 8 after birth

were highly enriched in transcripts encoding proteins generally

associated with the extracellular matrix (ECM), such as decorin

(Dcn), lumican (Lum), microfibrillar-associated protein 5 (Mfap5),
collagen alpha-2(VI) chain (Col6a2) and proteins associated with
angiogenesis, such as angiopoietin-related protein 1 (Angptl1)
(Fig. 5a). The upregulation of genes associated with ECM formation
suggests that colonic macrophages at day 8 compared to day 14 may

have an important role in the structural development of the colon

that is unique to early life and thus involved in creating a niche con-
ducive to iNKT cell seeding and development. In agreement with

this, Gene Ontology (GO) term analysis showed that ECM orga-
nization was the most significantly enriched biological process
followed by blood vessel development in day 8 macrophages
(Fig. 5b and Supplementary Table 2).

Fig. 4 | Microbiota determines the quantity of but is not necessary for embryonic macrophages to regulate colonic iNKT cell levels. a, iNKT (CD45+CD3ε+ TCRβ+ CD1d tetramer−) absolute counts (left) and cell percentage (right) in the colon of SPF and germ-free mice over time (day 9, 21 and 35; n=5; day 16; n=3; day 28; n=4). b, Absolute count of F4/80+CD11b+ and F4/80−CD11b+ macrophages (CD45+Lin− F4/80+ CD64+) in the colon of SPF (n=4), germ-free (n=5) or GFCV with SPF microbiota before birth (n=5) 5-day-old mice. c, Schematic of macrophage depletion model with AF598 antibody. d-g, Representative plot (left) and absolute count (right) of macrophages (d,e) and iNKT and TCRβ+ T (CD45+ CD3ε+ TCRβ+) cells (f,g) in the colon of 21-day-old (H21) germ-free or GFCV mice treated with AF598 (germ-free n=7, GFCV n=9) or isotype control (germ-free n=6, GFCV n=9) antibody from days 4 to 20 (AF54-20) after birth. b, Data are representative of three experiments. c-g, Data were pooled from two experiments. Numbers in the representative plots indicate cell frequency. The error bars indicate the s.e.m. Each dot is representative of an individual mouse. P values were calculated by unpaired two-sided Student’s t-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

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We also examined macrophages from SPF and germ-free mice at day 9 after birth. We observed 259 transcripts with elevated abundance and 27 transcripts with decreased abundance at day 9 after birth in macrophages from germ-free compared to SPF mice in the colon (Fig. 5c and Supplementary Table 3), which advocate for a specific role of microbiota in regulating macrophages likely...
of embryonic origin during early life when the establishment of iNKT cell residency is taking place. We intersected the transcripts differentially expressed in colonic macrophages at day 8 compared to day 14 after birth (Fig. 5a) with transcripts differentially expressed in germ-free compared to SPF mice at day 9 after birth (Fig. 5c) and found 19 transcripts to be commonly dysregulated in these two datasets, among which 13 were enriched in germ-free versus SPF mice and day 8 versus day 14 after birth (Fig. 5d). These similarities (>2.5%) between DEGs in both datasets predicted a coincidence in their mechanisms of action. Indeed, the 13 genes commonly identified in macrophages as enriched in germ-free versus SPF mice and day 8 versus day 14 after birth included at least 3 transcripts that

Fig. 5 | Macrophage and iNKT cell transcriptional signature during early life. a, Transcriptome analysis of colonic macrophages (CD45<sup>+</sup> F4/80<sup>+</sup> CD64<sup>+</sup> cells) from 8- and 14-day-old SPF animals (n = 4). b, Pathway analysis by GO enrichment of transcripts increased in colonic macrophages at day 8 as compared to day 14 after birth in SPF animals. c, Transcriptome analysis of colonic macrophages from day 9 SPF (n = 2) and germ-free mice (n = 4). d, Intersection of the DEGs identified by the transcriptome analysis of colonic macrophages from 8- and 14-day-old SPF mice and the transcriptome analysis of colonic macrophages from day 9 SPF and germ-free mice. e, Transcriptome analysis of colonic iNKT (CD45<sup>+</sup> CD3ε<sup>+</sup> TCRβ<sup>+</sup> CD1d tetramer<sup>+</sup>) cells from 14-day-old (n = 3) and 56-day-old (n = 4) mice raised under SPF conditions. f, Pathway analysis by GO enrichment of transcripts differentially expressed in colon iNKT cell populations at day 14 as compared to the adult in SPF mice. Transcripts differentially expressed between groups were identified by DESeq2 analyses (log2 fold change > 1, \( P_{adj} < 0.05 \), red). \( P \) values for the pathway analysis were calculated based on the cumulative hypergeometric distribution according to Metascape.
encoded proteins associated with ECM organization (Dcn, Lum, Mfap5), suggesting that early-life macrophages may participate in this process under the influence of the microbiota.

Interestingly, some of the genes we identified as differentially expressed in sorted macrophages during early life and participating in ECM organization (Dcn, Lum, Mfap5, Col6a2) or angiogenesis (Angptl1, Cxcl12) are more generally associated with non-hematopoietic cells. This makes it possible that their enrichment in macrophages during early life was derived from recent phagocytosis of neighboring non-hematopoietic cells or doublets due to strong anatomical association. However, we observed a notable decrease in the expression of these genes in the colon after specific macrophage depletion at day 8 (Extended Data Fig. 6e). This supports the notion that early-life embryonic macrophages may be another source of these transcripts. We did not observe differences in CXCL16 or CD1d expression by transcriptional analysis of macrophages or by quantitative PCR of the whole colon after macrophage depletion during early life (Extended Data Fig. 6e), which suggests alternative cellular origins of these factors in the regulation of iNKT cells, such as the intestinal epithelium as previously shown. Thus, our studies suggest that the control of iNKT cell levels by early-life embryonic macrophages was most likely derived from a multifactorial process involving their role in sculpting the structure of the colon to create the proper niche for iNKT cell establishment and providing factors that affect iNKT cell expansion.
To further investigate this, we next turned our attention to the transcriptional profiles of iNKT cells during early (day 14) and later (day 56) life by RNA-seq and identified 61 differentially expressed transcripts (Fig. 5e and Supplementary Table 4). Of these, 32 transcripts exhibited elevated abundance in the adult relative to day 14 of life; 29 transcripts were uniquely increased in neonatal iNKT cells. From the GO term analysis, early-life iNKT cells were especially enriched in pathways associated with cell division (Fig. 5f and Supplementary Table 5). This supports the notion that iNKT cells adopt a distinct transcriptional program during early compared to adult life, which is consistent with cells in the process of establishing residency and points toward a potential role for proliferation of iNKT cells during this time.

Macrophages regulate iNKT cell proliferation extrathymically. We explored the capacity of iNKT cells to proliferate during the critical period of the first 11 d of life when iNKT cells are regulated by macrophages in the colon. Diphtheria toxin was administered on days 5 and 7 to MM<sup>DTR</sup> and littermate control mice and the proliferation of colonic iNKT cells was assessed by measuring Ki67 expression, a proliferation marker, on day 8 after birth (Fig. 6a). We observed reduced Ki67 expression in iNKT cells from mice depleted of macrophages compared to littermate controls (Fig. 6b and Extended Data Fig. 7a). Conversely, administration of diphtheria toxin to MM<sup>DTR</sup> mice every 2 d beginning at day 56 until day 62 of life (Fig. 6c) reduced Ki67 expression in splenic (Extended Data Fig. 7b) but not colonic (Fig. 6d and Extended Data Fig. 7c) iNKT cells compared to littermate controls. This suggests that embryonic macrophages can regulate colonic iNKT cell proliferation in their local environment during early life.

Therefore, we directly addressed whether macrophages affect iNKT cells proximally at the level of the tissue. We first performed adoptive transfer of enriched CD45.1<sup>+</sup> thymic T cells in a CD45.2<sup>+</sup> MM<sup>DTR</sup> and control host at day 4 after birth and administered diphtheria toxin at days 8 and 10 after birth (Fig. 6e). This treatment resulted in macrophage depletion (Fig. 2f and Extended Data Fig. 2f) together with reduced levels of CD45.1<sup>+</sup> iNKT but not TCR<sup>αβ</sup><sup>+</sup> T cells in the colon (Fig. 6f and Extended Data Fig. 7d) and spleen (Fig. 6g and Extended Data Fig. 7e) of recipient MM<sup>DTR</sup> or control mice compared to similarly treated littermate controls. Similarly, we performed adoptive transfer of enriched CD45.1<sup>+</sup> thymic T cells in a CD45.2<sup>+</sup> MM<sup>DTR</sup> or control host at day 3 and administered diphtheria toxin from day 3 to day 7 (Extended Data Fig. 7f). We observed reduced Ki67 expression from CD45.1<sup>+</sup> iNKT but not TCR<sup>αβ</sup><sup>+</sup> T cells in the colon of recipient MM<sup>DTR</sup> mice compared to littermate controls at day 8 after birth (Extended Data Fig. 7g). Therefore, early-life embryonic macrophages can regulate extrathymic proliferation of iNKT<sup>+</sup> cells. Furthermore, we investigated the capacity of macrophages to regulate iNKT cell levels locally and cultured spleen explants from adult MM<sup>DTR</sup> mice with or without the application of diphtheria toxin for 48 h ex vivo and assessed the levels of iNKT and TCR<sup>αβ</sup><sup>+</sup> T cells (Extended Data Fig. 8a). Diphtheria toxin treatment of the explants depleted macrophages (Extended Data Fig. 8b) in association with a decrease in iNKT but not TCR<sup>αβ</sup><sup>+</sup> T cells (Extended Data Fig. 8c). Therefore, we conclude that embryonic macrophages can regulate iNKT cell proliferation independently of the thymus and locally within the tissues during early life.

Early-life macrophages determine iNKT cell-related disease outcome. We investigated whether the consequences of embryonic macrophage deletion during early life had a durable impact that affected the host in later life. Therefore, we first examined whether macrophage deletion in early life caused persistent defects to occur in iNKT cells. Diphtheria toxin was administered to MM<sup>DTR</sup> mice and littermate controls every 2 d beginning on day 8 and until day 14 of life; the numbers of cells in the colon, skin and spleen were assessed in the adult on day 49 (Fig. 7a). By five weeks after treatment (day 49), although the quantities of colonic (Fig. 7b and Extended Data Fig. 8d) or skin (Extended Data Fig. 8e) macrophages recovered to normal levels in MM<sup>DTR</sup> and control mice, which is consistent with the influx of bone marrow-derived macrophages, the abundance of iNKT cells at these barrier sites were substantially depressed in the MM<sup>DTR</sup> versus control mice (Fig. 7c and Extended Data Fig. 8f,g). However, the abundance of iNKT cells in the spleen was similar in diphtheria toxin-treated MM<sup>DTR</sup> and littermate control mice (Fig. 7d and Extended Data Fig. 8h). Therefore, the effects on iNKT cell levels in the colon on early-life depletion of macrophages was durable and extended into later life.

We next examined whether the function of iNKT cells was also altered in later life on early-life elimination of macrophages. We first examined iNKT cell differentiation. iNKT cells can be subdivided into three main differentiated subsets in tissues: NKT1; NKT2; and NKT17 (ref. 28). Although we observed similar proportions of CD45.1<sup>+</sup> iNKT cell subsets in the colon of the MM<sup>DTR</sup> CD45.2<sup>+</sup> recipients at day 8 of life after having received diphtheria toxin treatment from days 3–7 mice (Extended Data Fig. 7h), we found that iNKT cells that survived macrophage deletion during early life exhibited a decreased proportion of NKT17 cells and a relative increase in NKT1 iNKT cell subsets in the colon but not the spleen in later life (Extended Data Fig. 9a,b). However, iNKT cells that remained within the colon in later life after early-life depletion of macrophages in MM<sup>DTR</sup> mice exhibited similar levels of proliferation and activation relative to that observed in littermate controls based on Ki67 or CD69 expression, respectively (Extended Data Fig. 9c–h). These results demonstrate that early-life embryonic macrophages in the colon provide cues that determine the state of iNKT cell differentiation but not the proliferation or activation state in later life.

Next, we sought to determine if the persistently repressed levels of iNKT cells in adult life due to early-life depletion of macrophages would alter iNKT cell-mediated inflammatory responses in later life. Therefore, 49-day-old MM<sup>DTR</sup> mice that had been treated with diphtheria toxin between days 8 and 14 of life (Extended Data Fig. 9i) were exposed to α-galactosylceramide (αGal), the prototypical antigen for stimulation of iNKT cells. Although NKT1 subsets were relatively enriched as observed in Extended Data Fig. 9a, we observed that interferon-γ (IFN-γ) production in the adult colon was lower in mice depleted of macrophages during early life (Extended Data Fig. 9j) despite the fact that the levels of macrophages had recovered (Fig. 7b). These results are consistent with a persistent reduction in iNKT cells.

In view of this, we examined the effects of early-life macrophage depletion on the susceptibility of adult mice to iNKT cell-dependent diseases. Oxazalone-induced colitis is an experimental model of intestinal inflammation that is highly dependent on CD1d-restricted iNKT cells<sup>29</sup>. Diphtheria toxin was administered between day 8 and day 14 of life to MM<sup>DTR</sup> and littermate control mice; mice were exposed on day 49 to oxazolone, a prototypical compound used in oxazalone-induced colitis (Fig. 7e).<sup>23</sup> In association with persistent reductions in colonic iNKT cells, MM<sup>DTR</sup> mice depleted of macrophages in early life were protected from oxazolone-induced colitis in later life as shown by significantly diminished weight loss (Fig. 7f), mortality (Fig. 7g) and pathology (Fig. 7h) relative to control mice. Since oxazolone-induced colitis is known to depend on iNKT cells<sup>28</sup>, these observations are consistent with the protection from colitis observed. Conversely, when MM<sup>DTR</sup> and littermate control mice were treated with diphtheria toxin between day 8 and day 14 after birth and orally inoculated on day 49 with a modified form of <i>Listeria monocytogenes</i> able to infect mice (Fig. 7i), the number of<i>L. monocytogenes</i> colony forming units (CFUs) recovered from the colon and spleen were substantially greater in MM<sup>DTR</sup> compared to littermate control mice (Fig. 7j). In association
Fig. 7 | Early-life embryonic macrophages set mucosal iNKT cell levels in the adult and determine later life sensitivity or resistance to enteric diseases models. a, Schematic of macrophage depletion model. b–d, Diphtheria toxin administered from days 8 to 14 (DT8–14) after birth followed by quantitative analyses on day 49 (H49) of the absolute count of macrophages (CD45+ Lin− F4/80− CD64+ cells) in the colon (b) and the absolute count of iNKT (CD45+ CD3ε+ TCRβ+ CD1d tetramer+) and TCRβ+ T (CD45+ CD3ε+ TCRβ+) cells in the colon (c) and spleen (d) of control littermates (LysCre−/− (n = 12) or MMΔTR (n = 12) animals. e, Schematic of macrophage depletion model and oxazole experimental colitis. f–h, Diphtheria toxin administered from days 8 to 14 after birth followed by intrarectal injection of oxazolone or ethanol on day 49 and evaluation of colitis severity of control littermates LysCre−/− or MMΔTR mice 3 d after infection (H52). i, j, Listeria infection. a–j, Data were pooled from two experiments. Absolute counts were determined by flow cytometry. The error bars indicate the s.e.m. Each dot is representative of an individual mouse. g, P values were calculated by log-rank test. An unpaired two-sided Student’s t-test was used elsewhere in the figure. *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion

In this study, we demonstrated that embryonic macrophages that are transiently present and enriched in early life within barrier tissues, such as the colon, regulate the establishment of iNKT cell residency at these sites. In fact, iNKT cells can only establish a foothold in barrier sites, such as the lamina propria of the colon, during early life if embryonic macrophages are present but not if they are absent. This macrophage-mediated process is under the control of the microbiota, which determines the quantities and function of embryonic

with increased levels of Il12p40 and Il12p80 messenger RNA expression in the colon (Extended Data Fig. 9k,l). L. monocytogenes infection in Cd1d-deficient mice, which lack iNKT cells, exhibited similar findings in the colon and spleen11 suggesting that the decreased colonic iNKT cell levels observed in MMΔTR mice may result in increased susceptibility to such an infection. Together, these results reveal that the embryonic macrophage-mediated control of mucosal iNKT cell proliferation during early life is associated with durable functional consequences.
Articles

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5. Elahi, S. et al. Immunosuppressive CD71

3. Shaw, S. Y., Blanchard, J. F. & Bernstein, C. N. Association between the use of

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s41590-021-00934-0.

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received later where they determine the quantities of iNKT cells that the animal posses-

Consequently, early-life perturbations of colonic macro-

phages of embryonic origin are associated with differential host

susceptibility to iNKT cell-dependent responses to enteropathogens

and environmental stimuli that induce inflammation that models

inflammatory bowel disease. Together, our results show that onto-

genically distinct macrophage subsets uniquely control the host

developmental programs associated with the immune system dur-

ing restricted periods of life within body surfaces highly exposed to

microbes, as observed in mucosal tissues. Furthermore, they speci-

cify demonstrate that early-life control of colonic iNKT cell lev-

els and function represents a host developmental and extrathymic

process that likely begins during fetal life and is under the postnatal

control of the microbiota.

Online content

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summaries, source data, extended data, supplementary informa-

tion, acknowledgements, peer review information; details of

author contributions and competing interests; and statements of

data and code availability are available at https://doi.org/10.1038/ s41590-021-00934-0.

Received: 27 April 2020; Accepted: 16 April 2021;

Published online: 26 May 2021

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Methods

Mice. Mice (C57BL/6j background) were housed in a specific pathogen-free barrier facility at Harvard Medical School under a controlled 12h/12 h light–dark cycle (light at 7:00) at room temperature (22 ± 2 °C) and 60 ± 5% humidity. Wild-type (WT) and Rosa26Cre (Cre expression is driven by Lys2, stock no. 004781), Cx3cr1Cre+ + (Cre expression is driven by Cxcr1, stock no. 005254), Cx3cr1Cre+−; DTR+− (DTR expression is driven by Cyp11a1 but is induced by a loxp-flanked Stop element, stock no. 204046) and Rosa26+− (DTR expression is driven by Rosa26 but induced by a loxp-Stop element, stock no. 007900) were purchased from The Jackson Laboratory. MM mice (LysCre−; Cx3cr1Cre+−) and control littermates (LysCre−; Cx3cr1Cre−; Rosa26+−) were generated by the schedule shown in the manuscript. Date of birth was considered as day 1 after birth. The ASF98 antibody was provided by F. Ginhoux (SigN) and rat IgG2a isotype control antibody was purchased from Bio X Cell (catalog no. BE0089).

To isolate splenic and thymic cells, tissue was massed by plunger on 70-μm cell strainers, centrifuged at 1,500 r.p.m. for 5 min, resuspended in ammonium-chloride-potassium lysing buffer ( Gibco) for 2 min and washed in FACs buffer. For skin cell isolation, mouse ears were dissociated using the Multi Tissue Dissociation Kit 1 in combination with the gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer’s instructions. For adoptive transfer, CD45.1+ thymic T cells were enriched using the Pan T Cell Isolation Kit II (Miltenyi Biotec) and injected intraperitoneally at around 106 thymic T cells by the schedule shown in the manuscript. Date of birth was considered as day 1 after birth.

Flow cytometry and antibodies. For the flow cytometry analysis, isolated cells were washed, incubated with CD16/CD32 to block the Fc receptors and then stained. iNKT cells were identified by flow cytometry as CD45+ CD3ε+, CD1d tetramer and positive and unloaded tetramer negative cells (Extended Data Fig. 10a). Macrophages were identified by flow cytometry as CD45, CD11b, CD11c, F4/80, CD64+ and lineage negative (Lin: Ly6G, CD3ε, NK1.1, CD19, SigleCf) cells (Extended Data Fig. 10b). F4/80+CD11b+ and F4/80+CD11b− macrophages were identified during early life based on F4/80 and CD11b expression in adult mice (see Supplementary Data 10b). Flow cytometry was performed with a BD FACSARIA II cytometer located at the Dana-Farber Cancer Institute or a CytoFLEX cytometer and data were analyzed with the FACSDiva v6.1 and FlowJo software v10.5.0. Cell sorting was performed with a BD FACSAria II.

The following antibodies were used: PE and APC-labeled BR577-loaded (1:1,000 dilution); PE and APC-labeled unloaded CD1d (1:1,000 dilution); and PE-labeled MR1/OP-RU (1:200 dilution). Tetramers were provided by the National Institutes of Health Tetramer Core Facility. CD16/12 (101302, 1:100 dilution), FITC-labeled CD16/12 (1D4.1, 10:1 dilution), APC-labeled CD64 (139306, 1:300 dilution), CD3e (100312, 1:100 dilution), CD45.1 (101714, 1:100 dilution), PE-labeled Ki67 (652403, 1:50 dilution), APC-Cy7-labeled Ly6G (127623, 1:300 dilution), CD3e (100222, 1:100 dilution) and CD19 (115529, 1:300 dilution), PE-Cy7-labeled CD69 (104512, 1:100 dilution) antibodies were obtained from Biolegend. APC-Cy7-labeled TCRγ (560065, 1:200 dilution), FITC-labeled CD11b (557396, 1:200 dilution), PE-labeled SiglecF (552126, 1:200 dilution), BV605-labeled CD11c (117333, 1:200 dilution), PE-CF594-labeled PLZF (565758, 1:50 dilution), BV786-labeled CD45 (364225, 1:400) and CD45D (563866, 1:100 dilution) antibodies were obtained from BD Biosciences. The PE-Cy7-labeled F4/80 (25-4081-82, 1:200 dilution) antibody was purified from Invitrogen. APECS green (80-1168-05, 1:50 dilution) was obtained from eBiosciences. SYTOX Blue Dead Cell Stain (1:1,000 dilution; Thermo Fisher Scientific) was used to identify and exclude dead cells from the analysis. For Kit6 intracellular staining, LIVE/DEAD Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific) was used to identify and exclude dead cells from the analysis and the Cytofix/Cytoperm kit (BD Biosciences) was used to fix and permeabilize the cells. For ROY1T and PLZF intracellular staining, the Foxp3/Transcription Factor Staining Buffer Set (eBiosciences) was used to fix and permeabilize the cells.

Protein analysis. Seven-week-old mice were injected with α-galactosylceramide (KRN7000) (1 μg by weight of mice). Colon was collected 16 h after injection and homogenized in PBS with lysing matrix E tubes and FastPrep-24 homogenizer (MP Biomedicals). Homogenates were analyzed for the cytokine IFN-γ by ELISA (BD).

Quantitative polymerase chain reaction assays. RNA samples were prepared using a RNeasy Mini Kit and complementary DNA was synthesized using the Omniscript RT Kit (all Qiagen). PCR with reverse transcription was performed using a SYBR Green I Master Mix (Roche) and a CFX96 Real-Time System (Bio-Rad Laboratories). Values were normalized to the expression of β-actin for each sample. The following primers were used: Cxcl12-5'-TGGATCATGACAGTAGAAACA-3' and-5'TTCTCCACCGGCTCAATAC-3'; Lym-5'-CCTTCTGCTTGGCATGTA-3' and-5'GGGGGAGTACATTCTGGT-3'; Dcn-5'-CCCTCTGCGGACATCTTGGG-3' and-5'TCGAAAGTACACTGCGATCGG-3'; Mip5-5'-GCTCTGCGAACATCATCC-3' and-5'CCAGATTAGGGTCGTCTGTGAAT-3'; Col6a2-5'-AAAGCCCTTGGTCATTCCC-3' and-5'CCCTCCTCGACCACTCAGT-3'; Angpt1-5'-GGAAATGCTGCTGTAGCAGAGA-3' and-5'TATGTCGCTCCTCCTCCTT-3'; Cxcl16-5'-CGATACCCGAGGATCTTCTT-3' and-5'TCTGCGCTCCTCGTCCCTGAT-3'; Mfap5-5'-GCTCTGCGAACATCATCC-3' and-5'CCAGATTAGGGTCGTCTGTGAAT-3'; Il12p40-5'-TCGGAAGATGACACTGGCATCGG-3' and-5'CTATGTCGCCCTCCTCCCCCTT-3'; Cxcl15-5'-TCGGAAGATGACACTGGCATCGG-3' and-5'CTATGTCGCCCTCCTCCCCCTT-3'; Sprr1c1-5'-TCTGCGCTCCTCGTCCCTGAT-3' and-5'GGCGACGATCAGCCTTTTTC-3'; and-5'ACAGCTGTGTTCGCTGCGAGT-3'; Ifnγ-5'-TCGCAAGACAGAGGAAAAAGG-3' and-5'CCACCCGGAATCCAGGAGGA-3'; Il1b2p40-5'-CCCTCGACCTCCTGCGGATGC-3' and-5'TCTCGCCTCGTCCCTCAC-3'; β-actin-5'-GATGCTCCCCGGCGGTAGTAT-3' and-5'GGGGTACCTCAGGGTGACA-3'.

Library construction, sequencing and differential gene expression analyses. Between 300 and 3,000 sorted cells were collected directly in RLT Plus Buffer (Qiagen) and RNA was isolated according to the manufacturer’s instructions. Library construction was performed using the SMART-seq v4 Ultra Low Input RNA Kit (Takara Bio) according to the manufacturer’s instructions. Single-end (50 base pairs) sequencing was performed using the Illumina NextSeq 500 sequencing platform. Library construction and sequencing were conducted at the Molecular Biology Core Facilities of the Dana-Farber Cancer Institute.

Transcripts in each cell population were selected with the threshold of log2 fold change > 1 and P adj < 0.05 and then subjected to GO pathway analysis using the Metascape website (http://metascape.org/gp/index.html#/main/step1). Differentially expressed transcripts in each cell population were selected with the threshold of log2 fold change > 1 and P adj < 0.05 and then subjected to GO pathway analysis using the Metascape website (http://metascape.org/gp/index.html#/main/step1).
were evaluated every morning after the day of intrarectal injection (day 0). For colitis scoring, tissues were collected 3 d after intrarectal injection and embedded in paraffin, stained with hematoxylin and eosin and examined by a pathologist (J. Glickman) in a blinded fashion for evidence of colitis according to five established criteria: mononuclear inflammation, crypt hyperplasia, epithelial injury, neutrophilic inflammation and hypervascularization grading on 4-point scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe).

**L. monocytogenes infection.** *L. monocytogenes* infection was performed as described previously. Briefly, 7-week-old C57BL/6 male mice were inoculated by gavage with $3 \times 10^9$ CFUs of a mutated strain of *L. monocytogenes* with high binding affinity to mouse E-cadherin that can infect mice via the oral route. Organs and fecal pellets were collected 72 h after infection and samples were analyzed for CFU enumeration and RNA quantification.

**Statistical analysis.** Results were statistically analyzed in Prism v7 (GraphPad Software). To compare the difference between two separate groups with a single variable, an unpaired t-test was used. Comparisons of mortality were made by analyzing Kaplan–Meier survival curves and the log-rank test was used to assess differences in survival. All P values were two-tailed and statistical significance was accepted at $P < 0.05$.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. Raw FASTQ files and processed reads of the transcriptional analyses can be accessed at the Gene Expression Omnibus under accession no. GSE167975.

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**Acknowledgements**

R.S.B. is supported by National Institutes of Health grant nos. DK044319, DK053656, DK051362, DK088199 and 3P01AI073748 and the Harvard Digestive Diseases Center (no. P30DK034854). T.G. is supported by the Crohn’s and Colitis Foundation of America Research Fellow Award (no. 418509). We thank Blumberg laboratory members for their assistance in manuscript preparation. We thank S. S. Iyer for organizing the RNA sequencing at the core facility and for helping with the oxazolone colitis. We thank H. Gerke for her assistance in performing the *Plvap* experiments and R. Baron for assistance with the isolation of lung cells.

**Author contributions**

T.G and R.S.B. conceived, designed and interpreted the experiments. T.G., T.Z. and M.P. carried out the experiments. M.S. and P.R. provided the *Plvap*−/− mice. T.G. and R.S.B. wrote the manuscript. All authors were involved in the critical revision of the manuscript for important intellectual content.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41590-021-00934-0.

**Supplementary information**

The online version contains supplementary material available at https://doi.org/10.1038/s41590-021-00934-0.

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**Peer review information** Nature Immunology thanks Gerard Eberl and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available. Zoltan Fehervari was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Extended Data Fig. 1 | **a.** Circulatory exchange of CD45.1 (black) or CD45.2 (grey) TCR-αβ⁺ T (CD45⁺ CD3ε⁺ TCRβ⁺) and iNKT (CD45⁺ CD3ε⁺ TCRβ⁺ CD1d Tetramer⁺) cells in the blood of surgically joined CD45.1 (left) and CD45.2 (right) congenic animals (n = 2) determined by flow cytometry, 3 weeks after surgery. Circles are representative of average cell frequency. **b.** Representative plot of the circulatory exchange of CD45.1 or CD45.2 TCR-αβ⁺ T and iNKT cells in the colon of surgically joined congenic animals (n = 2) 3 weeks after surgery. **c.** Schematic of adoptive transfer strategy. **d.** Adoptive transfer of CD45.1 adult thymic cells into a 4 day old CD45.2 host (n = 1) followed by quantitative analyses of colonic CD45.1 or CD45.2 TCR-αβ⁺ T and iNKT cells by flow cytometry on day 42.
Extended Data Fig. 2 | Diphtheria toxin (DT) administered every two days from day 8 to 14 (DT8-14) after birth followed by quantitative analyses on day 15 (H15) of the absolute count of macrophages (CD45+ Lin− F4/80+ CD64+) in the skin (LysCre−/+: n = 3, MM<sup>DT</sup>TR n = 4) (a) or spleen (LysCre−/+: n = 5, MM<sup>DT</sup>TR n = 5) (b) and the absolute count of iNKT (CD45+ CD3ε+ TCRβ+ CD1d Tetracer+) and TCR-αβ T (CD45+ CD3ε+ TCRβ+) cells in the skin (LysCre−/+: n = 8, MM<sup>DT</sup>TR n = 7) (c) of control littermates LysCre<sup>−/−</sup> or MM<sup>DT</sup>TR animals. DT administered from day 8 to 10 (DT8-10) after birth followed by quantitative analyses on day 11 (H11) of the absolute count of iNKT and TCR-αβ T cells in the small intestine (d) and lung (e) of control littermates LysCre−/− (n = 3) or MM<sup>DT</sup>TR (n = 12) animals. DT administered from day 8 to 10 (DT8-10) after birth followed by quantitative analyses on day 11 (H11) of the absolute count of splenic macrophages (f) of control littermates LysCre<sup>−/−</sup> (n = 5) or MM<sup>DT</sup>TR (n = 3) animals. DT administered from day 12 to 14 (DT12-14) after birth followed by quantitative analyses on day 15 (H15) of the absolute count of splenic macrophages (g) of control littermates LysCre<sup>−/−</sup> (n = 9) or MM<sup>DT</sup>TR (n = 10) animals. Absolute counts were determined by flow cytometry. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Extended Data Fig. 3 | Diphtheria toxin (DT) administered every two days from day 8 to 14 after birth followed by quantitative analyses on day 15 of the absolute count of B cells (CD45^+ CD19^+, LysCre^+/--; n = 5, MM^DTR^ n = 5), neutrophils (CD45^+ Ly6G^+, LysCre^+/--; n = 5, MM^DTR^ n = 4), dendritic cells (DC) (CD45^+, Lin^−, CD11c^+, MHCII^+, CD64^+, LysCre^+/--; n = 5, MM^DTR^ n = 4), eosinophils (CD45^+, SiglecF^+, LysCre^+/--; n = 5, MM^DTR^ n = 4) in the colon of control littermates LysCre^+/− or MM^DTR^ animals (a). DT administered every two days from day 8 to 14 after birth followed by quantitative analyses on day 42 of the absolute count of MAIT cells (CD45^+ CD3ε^+ TCRβ^+ MR1/5-OP-RU T etramer^+) in the colon of control littermates LysCre^+/− (n = 5) or MM^DTR^ (n = 5) animals (b). Absolute count of macrophages (CD45^+ Lin^− F4/80^+/80^+ CD64^+) (c), iNKT (CD45^+ CD3ε^+ TCRβ^+ CD1d Tetramer^+) and TCR-αβ^+ T (CD45^+ CD3ε^+ TCRβ^+) cells (d) in the colon in the absence of DT treatment in control littermates LysCre^+/− (n = 4) and MM^DTR^ (n = 4) animals at 2 weeks old. Absolute counts were determined by flow cytometry. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired two-sided Student’s t-test. ns: not-significant.
Extended Data Fig. 4 | Diphtheria toxin (DT) administered from day 8 to 10 (DT8-10) after birth followed by quantitative analyses on day 11 (H11) of the absolute count of macrophages (CD45+ Lin− F4/80+ CD64+) in the colon (a) or spleen (b) and the absolute count of iNKT (CD45+ CD3ε+ TCRβ+ CD1d Tetramer+) and TCR-αβ+ T (CD45+ CD3ε+ TCRβ+) cells in the colon (c) and spleen (d) of control littermates Cx3cr1+/− (n = 4) or Cx3cr1DTR (n = 7) mice.

e) Schematic of macrophage depletion model with AFS98 antibody. AFS98 or isotype control antibody administered from day 4 to 10 (AFS4-10) after birth followed by quantitative analyses on day 11 (H11) of the absolute count of macrophages (n = 6 per group) (f), and the absolute count of iNKT and TCR-αβ+ T cells (n = 12 per group) (g) in the colon of injected animals. Absolute counts were determined by flow cytometry. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired two-sided Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: not-significant.
Extended Data Fig. 5 | a, Schematic of macrophage depletion model. Diphtheria toxin (DT) administered every two days from day 56 to 62 (DT Adult) after birth followed by quantitative analyses at day 63 of the absolute count of macrophages (CD45+ Lin− F4/80+ CD64+) in the colon (b) and spleen (c), and the absolute count of iNKT (CD45+ CD3ε+ TCRβ+ CD1d Tetramer+) and TCR-αβ+ T (CD45+ CD3ε+ TCRβ+) cells in the colon (d) and spleen (e) of control littermates LysCre−/− (colon n = 10, spleen n = 5) or MMDTR (colon n = 10, spleen n = 5) animals. f, Schematic of macrophage depletion model. DT administered from day 15 to 21 (DT15-21) after birth followed by quantitative analyses on day 22 (H22) of the absolute count of macrophages (g) and the absolute count of iNKT and TCR-αβ+ T cells in the colon (h) of control littermates LysCre−/− (n = 8) or MMDTR (n = 6) animals. Absolute counts were determined by flow cytometry. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired two-sided Student’s t-test. ***P < 0.001, ****P < 0.0001, ns: not-significant.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6  |  **a**, Absolute count of iNKT (CD45+ CD3ε− TCRβ+ CD1d Tetramer+) and TCR-αβ+ T (CD45+ CD3ε− TCRβ+) cells in the colon of wild type (WT, n = 5) littermates or Ccr2−/− (n = 5) animals at day 56 after birth. **b**, Diphtheria Toxin (DT) administered at day 1 and (DT1-2) after birth followed by quantitative analyses on day 10 (H10) of the absolute count of F4/80hi/CD11blo and F4/80lo/CD11bhi macrophages (CD45+ Lin− F4/80+ CD64+) in the colon of control littermates LysCre+/− (n = 8) or MMDTR (n = 4) animals. **c**, Absolute count of iNKT and TCR-αβ+ T cells in the colon of germ-free (GF, n = 12) and GF conventionalized with specific pathogen free (SPF) microbiota prior to birth (GFCV, n = 10) animals at 35 days of life. **d**, Representative plot of F4/80hi/CD11blo and F4/80lo/CD11bhi macrophages in the colon of SPF, GF or GFCV animals at 15 days old. **e**, DT administered from day 5 to 7 after birth followed by the analysis of Cxcl16, Cd1d, Cxcl12, Lum, Dcn, Mfap5, Angptl1 and Col6a2 transcript expression by quantitative polymerase chain reaction in the colon of control littermates LysCre+/− (n = 3) or MMDTR (n = 4) animals. Numbers in the representative plots indicate cell frequency. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired two-sided Student’s t-test. *P < 0.05, **P < 0.01, ****P < 0.0001. ns: not-significant.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | a, Percentage of Ki67 positive TCR-αβ+ T (CD45+ CD3ε+ TCRβ+) and iNKT (CD45+ CD3ε+ TCRβ+ CD1d Tetramer+) cells on day 8 (H8) in the colon of control littermates LysCre+/- (n = 8) or MMDTR (n = 8) animals treated with diphtheria toxin (DT) from day 5 to 7 (DT5-7) after birth. b, DT administered from day 56 to 62 (DT Adult) after birth followed by analyses at day 63 of the Ki67 mean fluorescent intensity (MFI) of TCR-αβ+ T and iNKT cells in the spleen of control littermates LysCre+/- (n = 8) or MMDTR (n = 8) animals. c, Percentage of Ki67 positive TCR-αβ+ T and iNKT cells on day 63 in the colon of control littermates LysCre+/- (n = 8) or MMDTR (n = 8) animals treated DT from day 56 to 62 after birth. Representative plot of TCR-αβ+ T and iNKT cells from 11 day old CD45.2 control littermates LysCre+/- or MMDTR animals adoptively transferred with CD45.1 adult thymic cells at 4 days old and treated with DT from day 8 to 10 (DT8-10) after birth in the colon (d) or spleen (e). f, Schematic of adoptive transfer and macrophage depletion model. g, Adoptive transfer of CD45.1 adult thymic cells into 3 day old CD45.2 control littermates LysCre+/- (n = 3) or MMDTR (n = 5) animals followed by DT administration from day 3 to 7 (DT3-7) after birth and quantitative analyses on day 8 (H8) of the Ki67 MFI (left) and percentage (right) of CD45.1 expressing TCR-αβ+ T and iNKT cells in the colon. Representative plot (left) and cell percentage (right) of iNKT cell subsets (NKT1, NKT2, NKT 17) from 8 day old CD45.2 control littermates LysCre+/- (n = 3) or MMDTR (n = 5) animals adoptively transferred with CD45.1 adult thymic cells at 3 days old and treated with DT from day 3 to 7 (DT3-7) after birth in the colon. SSC-A, side scatter. Numbers in the representative plots indicate cell frequency and were determined by flow cytometry. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired two-sided Student’s t-test. *P < 0.05, **P < 0.01, ****P < 0.0001, ns: not-significant.
Extended Data Fig. 8 | a, Schematic of macrophage depletion model ex vivo. Adult spleen from control littermates LysCre\(^{-/-}\) or MM\(^{DTR}\) animals, digested and cultured for 48 hours with Diphtheria toxin (DT) followed by quantitative analyses of the absolute count of macrophages (CD45\(^{+}\) Lin\(^{-}\) F4/80\(^{+}\), LysCre\(^{-/-}\): n = 4, MM\(^{DTR}\): n = 5) (b), and the absolute count of iNKT (CD45\(^{+}\) CD3\(^{e+}\) TCR\(^{\beta+}\) CD1d Tetramer\(^{+}\)) and TCR-\(\alpha\beta\) T (CD45\(^{+}\) CD3\(^{e+}\) TCR\(^{\beta+}\)) (LysCre\(^{-/-}\): n = 5, MM\(^{DTR}\): n = 5) cells (c). d, Representative plot of macrophages on day 49 in the colon of control littermates LysCre\(^{-/-}\) (n = 4) or MM\(^{DTR}\) (n = 4) animals treated with DT from day 8 to 14 after birth. DT administered from day 8 to 14 (DT8-14) after birth followed by quantitative analyses on day 49 (H49) of the absolute count of macrophages (e), and the absolute count of iNKT and TCR-\(\alpha\beta\) T cells in the skin (f) of control littermates LysCre\(^{-/-}\) or MM\(^{DTR}\) animals. Absolute counts were determined by flow cytometry. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. \(P\) values were calculated by unpaired two-sided Student’s t-test. *\(P < 0.05\), **\(P < 0.001\), ns: not-significant. Representative plot of TCR-\(\alpha\beta\) T and iNKT cells on day 49 in the colon of control littermates LysCre\(^{-/-}\) or MM\(^{DTR}\) animals treated with DT from day 8 to 14 after birth in the colon (g) and spleen (h). Unl, Unloaded. Tet, Tetramer. Numbers in the representative plots indicate cell frequency.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Representative plot (left) and cell percentage (right) of iNKT cell subsets (NKT1, NKT2, NKT17) from 42 day old control littermates LysCre+/− (n = 6) or MMiDTR (n = 4) animals treated with DT from day 8 to 14 (DT8-14) after birth in the colon (a) or spleen (b). Mean fluorescent intensity (MFI) of Ki67, percentage of Ki67+ and CD69+ TCR-αβ+ (CD45+ CD3ε+ TCRβ+) and iNKT (CD45+ CD3ε+ TCRβ+ CD1d Tetramer+) cells on day 42 (H42) in the colon (c,d,g) or spleen (e,f,h) of control littermates LysCre+/− (n = 6) or MMiDTR (n = 4) animals treated with diphtheria toxin (DT) from day 8 to 14 after birth. 1) Schematic of macrophage depletion model and αGalactosylceramide (αGal) treatment. DT administered from day 8 to 14 after birth followed by αGal regimen on day 49 and quantitative analyses 16 hours after, of the IFNγ protein level in the colon of control littermates LysCre+/− (n = 10) or MMiDTR (n = 8) animals by enzyme-linked immunosorbent assay (ELISA) (J). DT administered from day 8 to 14 after birth followed by Listeria monocytogenes administration by oral gavage on day 49 and analyses of Ifnγ (K) or Il12p40 (L) mRNA expression in the colon of control littermates LysCre+/− (n = 6) or MMiDTR (n = 6) animals 3 days after infection by quantitative polymerase chain reaction analysis. Numbers in the representative plots indicate cell frequency and were determined by flow cytometry. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P < 0.05, **P < 0.01, ns: not-significant. Numbers in the representative plots indicate cell frequency.
Extended Data Fig. 10 | a, Gating strategy for iNKT (right panel) and TCR-αβ+ T (middle panel) cells identification by flow cytometry in the colon at day 12 (top) and day 49 (Adult) (bottom) after birth. b, Gating strategy for F4/80hi/CD11blo and F4/80lo/CD11bhi macrophages (right panel) identification by flow cytometry in the colon at day 12 (top) and day 49 (Adult) (bottom) after birth.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- A description of all covariates tested
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- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  
  *Give P values as exact values whenever suitable.*

- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

*Our web collection on statistics for biologists contains articles on many of the points above.*

Software and code

Policy information about availability of computer code

| Data collection | Commercial software: FACSDiva v6.1 |
|-----------------|------------------------------------|
| Data analysis   | The following software were used: Flowjo v10.5.0 for flow data, GraphPad Prism v7 for generating figures and statistical comparisons, Partek flow software v10 to access bioinformatics tools, STAR v2.5.1d for alignment of cDNA sequencing reads, HTSeq v0.11.0 to quantify sequencing reads, R package DESeq2 v1.26.0 to determine differential expression of transcripts, Metascape website for gene ontology analysis, R studio v1.3.1093 for generating volcano plots. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Raw fastq files and processed reads of the transcriptional analyses are accessible in the NIH GEO database: GSE167975.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
Sample sizes were determined based on prior experience with similar experiments in early life [T. Olszak et al., Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. Science (80-. ), 336, 489–493 (2012); D. An et al., Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. Cell. 156, 123–133 (2014)], or based on pilot experiments.

**Data exclusions**
Data exclusion was pre-established: Bulk RNA-sequencing data, genes that count 0 in 4 or more samples of Day8 vs Day14 macrophages; 2 or more samples of SPF vs GF macrophages; 4 or more samples of Day14 vs Day56 INKT cells were not included in DESeq2 analysis and volcano plots due to low expression level.

**Replication**
All attempts at replication were successful. All experiments reported were replicated at least two times with comparable results.

**Randomization**
Mice were allocated randomly into treatment groups

**Blinding**
The mating of the MMDTR and CX3CR1DTR mice was designed so the exact proportion of mice with different genotypes in the same litters would be around equal as per Mendelian's law of segregation of genes and could remain unknown at the time of the analysis. CFU counting, scoring of histological slides were blinded. Adoptive transfer, drug administration, flow cytometry acquisition and analysis were performed blinded to the mouse genotypes. For the rest of the experiments no blinding was deemed necessary as no subjective assessments were included.

Reporting for specific materials, systems, and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | Antibodies           |
|     | Eukaryotic cell lines |
|     | Palaeontology and archaeology |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |
|     | Dual use research of concern |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChIP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging |

#### Antibodies

| Antibodies used |
|-----------------|
| Flow cytometry: |
| - Anti-mouse CD16/32 (Clone 93, BioLegend, catalog number 101302) |
| - Anti-mouse FITC-labeled CD3 (Clone 145-2C11, Biolegend, catalog number 100306) |
| - Anti-mouse FITC-labeled CD45.1 (Clone A20, Biolegend, catalog number 110705) |
| - Anti-mouse APC-labeled CD45.1 (Clone A20, Biolegend, catalog number 110714) |
| - Anti-mouse APC-labeled CD64 (Clone 54-S/7.1, Biolegend, catalog number 139306) |
| - Anti-mouse APC-labeled CD3 (Clone 145-2C11, Biolegend, catalog number 100312) |
| - Anti-mouse PE-labeled K67 (Clone 16A8, Biolegend, catalog number 652403) |
| - Anti-mouse APC-Cy7-labeled Ly6G (Clone 1A8, Biolegend, catalog number 127623) |
| - Anti-mouse APC-Cy7-labeled CD3 (Clone 17A2, Biolegend, catalog number 100222) |
| - Anti-mouse APC-Cy7-labeled NK1.1 (Clone PK13, Biolegend, catalog number 108723) |
| - Anti-mouse APC-Cy7-labeled CD19 (Clone 6D5, Biolegend, catalog number 115529) |
| - Anti-mouse BV605-labeled CD11c (Clone N418, Biolegend, catalog number 117333) |
| - Anti-mouse PE-Cy7-labeled CD69 (Clone H12F3, Biolegend, catalog number 104512) |
| - Anti-mouse APC-Cy7-labeled TCRbeta (Clone HS7-597, BD Biosciences, catalog number 506056) |
| - Anti-mouse FITC-labeled CD11b (Clone M1/70, BD Biosciences, catalog number 557396) |
| - Anti-mouse PE-labeled SiglecF (Clone E50-2440, BD Biosciences, catalog number 552126) |
Validation

AF598 antibody has been validated in previous publications and in this study for its capacity to deplete macrophages and is also commercially available (https://d2c7dqyl445u.cloudfront.net/tds-sheets/BE0213-tds.pdf). All other antibodies used were from commercial sources and have been validated by the vendors: CD3e FITC (https://www.biologend.com/en-us/products/flc-anti-mouse-cd3epsioplon-antibody-23); CD45.1 FITC (https://tonbobio.com/products/flc-anti-mouse-cd45-1-a20); CD11b FITC (https://www.bdbiosciences.com/eu/applications/research/stem-cell-research/mesenchymal-stem-cell-markers-bone-marrow/mouse-negative-markers/fltc-anti-cd11b-m170/p/557936); SiglecF PE (https://www.bdbiosciences.com/eu/applications/research/b-cell-research/surface-markers/mouse/pe-rat-anti-mouse-siglec-f-e50-2440/p/552126); Ki67 PE (https://www.bdbiosciences.com/en-us/products/pe-anti-mouse-ki-67-antibody-8134); CD45.1 Alexa Fluor 700 (https://www.bdbiosciences.com/us/applications/research/stem-cell-research/cancer-research/mouse/alexa-fluor-700-mouse-anti-mouse-cd451-a20/p/561235); CD64 APC (https://www.bdbio.com/en/products/apc-anti-mouse-cd64-fcgammari-antibody-7874); CD30 APC (https://tonbobio.com/products/apc-anti-mouse-cd3-17a2); RORγt APC (https://www.thermofisher.com/antibody/product/ROR-gamma-t-Antibody-clone-BD-Monoclonal/17-6981-82); Ly6G APC-Cy7 (https://www.bdbio.com/en-us/products/apc-cy7-lg-6-antibody-6755); CD3e APC-Cy7 (https://www.bdbio.com/en-us/products/apc-cy7-anti-mouse-cd3-antibody-6068); NK1.1 APC-Cy7 (https://www.bdbio.com/en-us/products/apc-cy7-anti-mouse-rk-1-1-antibody-4002); CD19 APC-Cy7 (https://www.bdbio.com/en-us/products/apc-cy7-anti-mouse-cd19-antibody-3903); TCRO APC-Cy7 (https://www.bdbio.com/us/reagents/research/antibodies-buffers/immunology-reagents/anti-mouse-antibodies/cell-surface-antigens/apc-cy7-hamster-anti-mouse-tcr-chain-hs7-597/p/560656); CD69 PE-Cy7 (https://www.bdbio.com/en-us/products/pe-cy7-anti-mouse-cd69-antibody-3168); F4/80 PE-Cy7 (https://www.thermofisher.com/antibody/product/F4-80-Antibody-cloned-BM8-Monoclonal/25-4801-82); CD11b BV605 (https://www.bdbio.com/en-us/products/brilliant-violet-605-anti-mouse-cd11c-antibody-7865); PLZF PECA594 (https://www.bdbio.com/us/reagents/research/antibodies-buffers/immunology-reagents/anti-human-antibodies/intracellular-antigens/pe-c594-mouse-anti-plzf-r17-809/p/565738); CD45.2 BV786 (https://www.bdbio.com/us/applications/research/stem-cell-research/cancer-research/mouse/bv786-mouse-anti-mouse-cd452-104/p/563686); CD45 BV786 (https://www.bdbio.com/us/reagents/research/antibodies-buffers/immunology-reagents/anti-mouse-antibodies/cell-surface-antigens/bv786-rat-anti-mouse-cd45-30-f11/p/564225). The murine CD11b/ MR1 tetramers loaded or unloaded were validated with a known positive and negative controls in-house.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Mice (C57BL/6j background) were housed in a specific pathogen-free barrier facility at Harvard Medical School under a controlled 12 hours/12 hours light cycle (light at 7am) at room temperature (22 ± 1°C) and 60 ± 5% humidity: Wild type [ref000664], CD45.1 [ref002014], LysCre+/− [ref004781], Cx3cr1Cre+/− [ref025524], Csf1rTR+/+ [ref024046], Rosa26TetR+/+ [ref079900], Ccr2−/− [ref004999] were purchased from the Jackson Laboratory. Pvpav−/− mice were provided by Pia Rantakari and Marko Salmi (University of Turku). Specific Pathogen Free and Germ free mice were provided by the Harvard Digestive Disease Center Gnotobiotic and Microbiology Core. The different ages at the time of collection are indicated in the manuscript. Both male and female mice were included in the manuscript.

Wild animals

Study did not involve wild animals.

Field-collected samples

Study did not involve field collected samples.

Ethics oversight

All procedures were approved by the Harvard Medical Area Standing Committee on Animals.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).

☑ All plots are contour plots with outliers or pseudocolor plots.

☑ A numerical value for number of cells or percentage (with statistics) is provided.
Large or small intestines were collected, fat tissue removed, the intestines cut longitudinally and washed in PBS in order to remove fecal content, cut into 30mm pieces, put into a 50mL falcon tube, shaken in 10mL HBSS (GIBCO) 2mM EDTA at 250 rpm for 15 min at 37°C, rinsed in HBSS at 37°C, shaken in 10mL HBSS 2mM EDTA at 250 rpm for 30 min at 37°C, rinsed in HBSS at 37°C. The epithelial cell fractions were discarded and the lamina propria tissues were digested in 10mL 1mg/mL Collagenase VIII (Sigma C2139) and 10ug/mL Dnase I (DS025) diluted in RPMI (Corning) 10% Fetal Bovine Serum (FBS), 1.5% HEPES (Corning) at 250 rpm for 45 min at 37°C. Lungs were collected, minced into small pieces (1-2 mm) with scissors and digested in 10mL 1mg/mL Collagenase VIII and 10ug/mL Dnase I diluted in RPMI 10% Fetal Bovine Serum, 1.5% HEPES at 250 rpm for 60 min at 37°C. Digestive enzyme activity were stopped by adding 10mL cold FACS buffer (PBS, 2% FBS, 1mM EDTA), digested tissues were filtered with 70um cell strainer, centrifuged at 1500 rpm for 5 min, filtered with 40um cell strainer, centrifuged and stained for flow cytometry analysis. To isolate splenic and thymic cells, tissue was mashed by plunger on 70um cell strainers, centrifuged at 1500 rpm for 5 min, resuspended in ACK lysing buffer (GIBCO) for 2 min and washed in FACS buffer. Mouse ear was dissociated using the Multi Tissue Dissociation kit 1 in combination with the gentleMACS Dissociators (Miltenyi) according to the manufacturer instructions. For adoptive transfer, CD45.1+ thymic T cells were enriched using Pan T cell isolation kit II (Miltenyi).

**Instrument**

BD FACSArria II UV high speed cell sorter. 4 way sorting. 5 lasers. 18 fluorescent parameters.

Cytoflex S cytometer

**Software**

FACSDiva v6.1, FlowJo v10.5.0

**Cell population abundance**

300 to 3000 cells per sorted population were obtained for cell sorting. Sorted cells were rerun on flow cytometer to ensure purity.

**Gating strategy**

Gating strategy is provided in Extended Data. 10 and representative plots are provided for every main experiment. All flow cytometry gating was performed analyzing singlets with FSC-H and FSC-W and live cells. FSC and SSC were used to gate in the lymphocyte population when analyzing INKT T cells and to gate in the leukocyte population for macrophages. INKT cells were identified by flow cytometry as CD45, CD3e, TCRb, CD1d Tetramer, positive and unloaded Tetramer negative cells. Macrophages were identified by flow cytometry as CD45, CD11b, CD11c, F4/80, CD64 positive and lineage (Lin: Ly6G, CD3, NK1.1, CD19, SglecF) negative cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.