Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity

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Notes

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Figure 1 | Maternal RA controls LTi differentiation. a, Enteric fetal ILC\textsubscript{4\neg} and LTi4 cells. E12.5, n = 8; E13.5, n = 3; E14.5, n = 6; E15.5, n = 3. Left: Sca1-GFP lymph nodes. Right: E15.5 gut cells. LTi4, 124, NATURE | VOL 508 | 3 APRIL 2014

b, Percentage of enteric fetal ILC\textsubscript{4\neg} and LTi4 cells. E12.5, n = 3; E13.5, n = 6; E14.5, n = 3; E15.5, n = 3. Left: Sca1-GFP lymph nodes. Right: females received BMS493. E17.5 SLOs. g, Brachial (Bra) and inguinal (Ing) lymph nodes; n = 6. h, Arrowheads indicate Peyer’s patch. i, Peyer’s patch number per intestine: DMSO, n = 8; BMS, n = 22. Area: DMSO, n = 30; BMS, n = 25. Scale bars: 50 μm (c), 200 μm (g, h right), 1 mm (h, left). Error bars show s.e. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.

Figure 2 | Cell-autonomous RA controls LTi cells and SLO development. a, RT–PCR of enteric cells. Data represent three independent experiments. b, DMSO, BMS493 or RA stimulation. Data represent three independent experiments. c, E15.5 enteric ILC subsets. d, LTin, n = 4; ILC\textsubscript{4\neg}, n = 5; LTi4, n = 5. e, E15.5 ILC\textsubscript{4\neg} cells cultured for 6 days. f, ILC\textsubscript{4\neg} cell cultures at day 6; n = 5. g, Immunostaining of E15.5 embryos and intestines. Developing brachial (Bra) and inguinal (Ing) lymph nodes and Peyer’s patch are shown. CD4, red; VCAM1, grey. Scale bars: 200 μm. h, SLO size; n = 6. Error bars show s.e. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant. ND, not detected.
haematopoietic cell-autonomous impairment of RA responses resulted in severely diminished fetal lymph node size and reduced number of minute Peyer’s patches (Fig. 2g, h and Extended Data Fig. 3h). Our data indicate that LTi cell differentiation is controlled by cell-autonomous RA signalling in developing SLOs.

Previous reports have identified key LTi cell regulators. Mice mutant for the transcription factors ID2 and RORγt lack LTi cells and do not develop SLOs. Runx1, Tmx and Notch1 were also implicated in LTi cell maturation. We found that whereas most LTi-related genes were normally expressed in RarHom and RarHet ILC4neg and LTi4 cells, Runx1 was increased and Rorgt was reduced (Fig. 3a and Extended Data Fig. 4a–d). Expression of pro-inflammatory genes was also reduced in RarHom and RarHet ILC4neg and LTi4 cells (Fig. 3a and Extended Data Fig. 4b–d). The marked reduction of Rorgt expression suggested that RA could provide ILC4neg cells with signals leading to Rorgt regulation. Accordingly, RA stimulation of ILC4neg cells resulted in Rorgt upregulation whereas most other transcription factors were unperturbed, notably Runx1 (Fig. 3b). In agreement, BMS493 inhibited RA-induced Rorgt expression, and efficient block of Rorgt by digoxin prevented RA-induced differentiation of ILC4neg cells into LTi4 cells, while cell viability was unaffected (Fig. 3c and Extended Data Fig. 5a–c).

To test whether RA-induced LTi maturation requires RORγt, viability was unaffected (Fig. 3c and Extended Data Fig. 5a–c). To test further whether RA-induced LTi maturation requires Rorgt, we determined if differentiation of RAR dominant-negative ILC4neg cells is restored by enforced Rorgt expression. Retroviral transduction of Rorgt revealed that RAR dominant-negative ILC4neg cells restored high levels of pro-inflammatory genes and reacquired their potential to differentiate towards LTi4 cells (Fig. 3d–f). Further evidence that RA can directly regulate Rorgt expression was provided by computational analysis of potential RARE sites and chromatin immunoprecipitation (ChIP) with pan-RAR and RXR antibodies. RA stimulation resulted in increased binding of RAR and RXR upstream and within the Rorgt locus (Fig. 3g, h and Extended Data Table 1). To analyse the role of these sites we introduced the RARE C (−5,478 Rorgt transcription start site (TSS)), E (−1,800 Rorgt TSS) and G (−1,619 Rorgt TSS) half-sites in a luciferase reporter vector. Mutations in these sites resulted in significant reduction in the regulatory function of these elements as measured by luciferase activity (Fig. 3i). Thus, cell-autonomous RA signalling provides LTi cells with critical differentiation signals via direct regulation of Rorgt.

Our data indicate that mature LTi cell numbers regulate the size of SLO primordia and may determine lymphoid organ size in adulthood. RarHet adult mice had reduced SLOs and lymphocyte numbers when compared to their wild-type littermate controls (Fig. 4a, b and Extended Data Fig. 6a, b). In agreement, mice that received a vitamin-A-deficient (VAD) diet throughout life had reduced lymphoid organ size when compared to vitamin-A-control mice (VAC) (Fig. 4c). However, because RarHet and VAD lymphocytes are continuously exposed to altered levels of RA signals, it is possible that SLO size might be a consequence of altered lymphocyte pools. To clarify this issue, we provided pregnant mice with a vitamin-A-high (VAH), VAD or VAC diet and switched all diets to the same VAC diet after birth. At 10 weeks of age mice that were exposed to a VAH diet exclusively in utero had larger SLOs, whereas mice exposed to a VAD diet had small SLOs when compared to VAC control mice (Fig. 4d). Notably, provocation of vitamin A diets exclusively after birth no longer controlled SLO size (Extended Data Fig. 6c, d). Additional evidence that RA determines SLO size in early life was provided by transplantation of CD45.1 wild-type bone marrow into lethally irradiated RarHet (WT→RarHet) or wild-type (WT→WT) CD45.2 littermate control hosts at 2 weeks of age (Fig. 4e). Thus, we generated mice that pre- and perinatally received low input of RA signals in haematopoietic cells, but that on transplantation harbour a normal wild-type haematopoietic system. WT→RarHet mice, which received low RA cues in utero, exhibited small SLOs when compared to their WT→WT counterparts at 8 weeks after transplantation (Fig. 4f, g and Extended Data Fig. 6e, f). This phenotype also revealed reduced lymphocyte numbers, albeit normal SLO organization and similar haematopoietic cell reconstitution (Fig. 4h and Extended Data Figs 6f and 7a–d). In agreement, dendritic cells from WT→RarHet or WT→WT chimaeras had similar capacity to activate lymphocytes (Extended Data Fig. 7e, f).

Our data indicate that available RA in utero regulates the size of lymphocyte pools in the offspring, with possible consequences on their adaptive immune responses. To test this hypothesis, WT→RarHet or WT→WT chimaeras were infected intranasally with murid herpesvirus-4, resulting in acute lung infection. Analysis of draining intrathoracic lymph node revealed reduced expansion but normal frequency of CD8+ T cells specific for the viral epitopes ORF61 and ORF75c in WT→RarHet mice (Fig. 4i and Extended Data Fig. 8a–c). Consequently, whereas
Figure 4 | Retinoid levels in utero determine the offspring resistance to infection. a, Adult axillary (Axi), brachial (Bra) and inguinal (Ing) peripheral lymph nodes (PLN). n = 6. b, Peyer’s patch area and follicle number per Peyer’s patch. Wild type (WT), n = 26; RarHet, n = 23. c, Females received diets that were maintained in the offspring. VAC, n = 10; VAH, n = 8; VAD, n = 18. d, Females received variable diets. Their offspring received VAC diet. Lymph node dimension (mm²). Wild type (WT), n = 10; VAH, n = 24; VAH, n = 25. Peyer’s patch: VAC, n = 20; VAH, n = 40; VAD, n = 63. e, Transplantation scheme. f, Chimaeric lymph nodes. Scale bar: 1 mm. g, Dimensions of chimaeric lymph node structure. Scale bar: 200 µm. h, Chimaeric lymph node structure. Scale bar: 200 µm. i, Chimaeras infected with murid herpesvirus-4. Tetramer positive CD8 T cells in intrathoracic lymph node. j, Virus titres (plaque-forming units (p.f.u.) per lung). 7 days post infection (d.p.i.): WT → WT, n = 3; WT → RarHet, n = 3. 10 d.p.i.: WT → WT, n = 4; WT → RarHet, n = 3. 14 d.p.i.: WT → WT, n = 8; WT → RarHet, n = 5. Dashed line indicates the detection limit. Error bars show s.e. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.

**METHODS SUMMARY**

Mice were maintained at Instituto de Medicina Molecular (IMM) or VU University Medical Centre according to national and international guidelines. Bone marrow cells were isolated from 8-week-old C57BL/6 CD45.1 mice and injected intravenously into 8-week-old lethally irradiated C57D2-RosA26-Rara-403 (WT → WT) or wild-type littermate controls (WT → WT). C57BL/6 female mice received either vitamin-A-deficient (VAD), with no vitamin A (vitamin free casein), vitamin-A-high (VAH, 25,000 IU kg⁻¹) or vitamin-A-control (VAC, 4,000 IU kg⁻¹) diets. Retinoic acid was provided to pregnant mice from E10.5 until they were euthanized. Quantitative real-time PCR with reverse transcription (RT–PCR) was performed as previously described. DNA–protein complexes were immunoprecipitated using antibodies against mouse pan-RAR, pan-RXR or control IgG. Computational analysis was performed with TESS. DNA–protein complexes were immunoprecipitated using antibodies against mouse pan-RAR, pan-RXR or control IgG. 

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper. 

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Mice. C57BL6/mice were purchased from Charles River and C57BL6/CD45.1 mice were obtained at IMM. Van-iCre (ref. 17), hCcd2-GFP (ref. 6), Id2-CreERT2 (ref. 31), ROSA26-RARα403loxP (ref. 18), Rorgt-Cre (ref. 32), ROSA26-eYFP (ref. 33), hCcd2-Cre (ref. 17), ROSA26-mGFPTomato (ref. 34), Ly-6A (Scal) GFP (ref. 35), Rorgt-Cre (ref. 19), Id2-GFP (ref. 31), and OT1 Tg Rag1−/− (refs 36, 37) mice were previously described. Tamoxifen (2 mg per female) was injected daily into Id2-CreERT2 pregnant females from E8.5 to E13.5. All animal experiments were approved by national and institutional ethical committees: Direcção Geral de Veterinária and Instituto de Medicina Molecular (IMM) ethical committee and Animal Experimental Committee, VU University (VUMC). Power analysis was performed to estimate the number of experimental mice.

Bone marrow transplantation. Bone marrow cells were isolated from 8-week-old C57BL6/CD45.1 mice and 5 × 10^6 cells were injected intraorbitally into 2-week-old lethally irradiated (1,000 rad) CD45.2 wild-type littermate controls (WT). CD45.2 wild-type littermate controls (WT) and CD45.1 wild-type littermate controls (WT) were purchased to assess the contribution of WT or CD45.1 cells to the hematopoietic system. RA is rapidly degraded under normal light and temperature conditions; thus it is unlikely that bioactive RA is present in normal diets. Therefore, bioactive RA was supplemented food was given from E10.5 until death (Sigma-Aldrich, 250 mg g⁻¹ overnight and the day of vaginal plug detection was marked as E0.5. Retinoic acid (vitamin-A-high (VAH), 25,000 IU kg⁻¹ body weight) was provided to pregnant mice as previously described. In short, mice were mated at 4°C paraformaldehyde and incubated in blocking/permeabilizing buffer (PBS containing 0.0015% Sigma-Aldrich) for 24 h at 37°C and 5% CO₂. Cell suspensions were washed with RPMI (Invitrogen), supplemented with 2% heat-inactivated FCS, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin. For retinoic acid stimulation experiments, all-trans retinoic acid (Sigma-Aldrich) dissolved at 100 μM in 100% ethanol was added at 100 μM as previously described. 24 h incubation at 37°C and 5% CO₂, cells were isolated and analysed by flow cytometry and RT-PCR. Efficiency of RA treatment was assessed by expression of the RA target gene Barh (ref. 16).

In vitro stimulation, lymph node embryonic cells were collected as previously described. Tissues were digested with Bazo2 (2.5 mg ml⁻¹, Roche) and DNase (0.1 mg ml⁻¹, Roche) in PBS for 15 min at 37°C while stirring continuously. Cell suspensions were washed with RPMI (Invitrogen), supplemented with 2% heat-inactivated FCS, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin. For retinoic acid stimulation experiments, all-trans retinoic acid (Sigma-Aldrich) dissolved at 100 μM in 100% ethanol was added at 100 μM as previously described. Cell culture and viral transduction. For in vitro stimulation, lymph node embryonic cells were collected as previously described. Tissues were digested with Bazo2 (2.5 mg ml⁻¹, Roche), DNase (0.2 mg ml⁻¹, Roche) and CD45.2 wild-type littermate controls (WT) were purchased to assess the contribution of WT or CD45.1 cells to the hematopoietic system.

For quantitative real-time RTPCR, total RNA was extracted using RNeasy mini kit (Qiagen) or using Trizol (Invitrogen) according to the manufacturer’s protocol. RNA concentration was determined using Nanodrop Spectrophotometer (Nanodrop Technologies). Quantitative real-time RTPCR was performed as previously described. Listed primers are sense and antisense, respectively. When a nested-sense primer was used, this format was [first sense] - [nested antisense].

For immunofluorescence analysis of adult lymph nodes, samples were snap frozen in isopentane pre-cooled in liquid nitrogen and kept at −80°C. Lymph nodes were sectioned (5 μm sections), fixed with 4% paraformaldehyde for 10 min and incubated in blocking/permeabilizing buffer (PBS containing 10% fetal bovine serum, 2% BSA and 0.3% Triton X-100). Samples were incubated with primary and secondary antibodies overnight or for 2 h in PBS containing 10% fetal bovine serum, 2% BSA and 0.3% Triton X-100. Sections were mounted in Mowiol with DAPI (Calbiochem). Images were acquired on Zeiss LSM 710 (Carl Zeiss) using EC Plan-Neofluar 10×/0.30 M27 and objective Plan Apochromat 63×/1.4 oil. Images were processed using Zeiss LSM Image Browser 4.2 software (Carl Zeiss).

Flow cytometry analysis and cell sorting. Embryonic guts and lymph node cells were digested, collected with collagenase (5 mg ml⁻¹, Roche) or Bazo2 (2.5 mg ml⁻¹, Roche) and DNase (1.0 mg ml⁻¹, Roche) in DMEM, 3% FBS for approximately 40 min at 37°C under gentle agitation. Fetal liver, adult spleen and lymph node cell suspensions were obtained using 70 μm strainers. Lineage (Lin) was Tcr119, Cgrf3, Cdx3, Cdx9, NK1.1, CD11c and Gr1. Flow cytometry analysis and cell sorting were performed using FORTESSA, FACScount 1, FACSaria I and II flow cytometers (BD Biosciences), MoFlo XDP or Cyan ADP flow cytometer (Beckman Coulter). Data analysis was done using FlowJo software (Tristar). Sorted populations were ≥95% pure.

Cell culture and viral transduction. For in vitro stimulation, lymph node embryonic cells were collected as previously described Tissues were digested with Bazo2 (2.5 mg ml⁻¹, Roche), DNase (0.2 mg ml⁻¹, Roche) and CD45.2 wild-type littermate controls (WT) were purchased to assess the contribution of WT or CD45.1 cells to the hematopoietic system.
Cells were negatively depleted for TCRβ, CD19, TER19, Mac1, Gr1, CD3c using DynaBeads Biotin Binder kit (Life Technologies) and positively enriched for CD11c using MACS Cell Separation Reagents (Miltenyi Biotech). OTI CD8 T cells were isolated from peripheral lymph nodes of OTI Tg Ragi/+/mice by Thy1.2 positive enrichment using MACS Cell Separation Reagents (Miltenyi Biotech). 2.5×10⁴ dendritic cells pre-loaded with 10⁻³M OVA peptide (Thermo Scientific) were cultured for 3 days with 1.25×10⁵ monoclonal OTI CD8 T cells labelled with 2.5 μM CFSE (BioLegend). Expansion of OTI CD8 T cells was measured by CFSE dilution and proliferative index was calculated using FlowJo (TreeStar, Inc.). Intracellular IFN-γ levels were measured by flow cytometry using IC fixation/permeabilization kit (eBioscience).

**Antibody list.** Cell suspensions were stained for flow cytometry using anti-CD45 (30-F11); anti-CD11c (N418); anti-CD7 (IL-7Rα); A20; anti-Ly-6A/E (Scal); anti-CD45.1 (A20); anti-CD8x (53-67); anti-CD19 (B220); anti-Thy1.2 (53-2.1); anti-NK1.1 (PK136); anti-CD69 (BioLegend); anti-CD4 (BioLegend) and TCRβ (BioLegend) antibodies were purchased from BD Pharmingen. Anti-GEF (A11008) antibody was purchased from Invitrogen. Intracellular staining for flow cytometry was performed using IC fixation/permeabilization kit (eBioscience). Embryo sections for immunofluorescence analysis were stained with Gl1 (anti-CD45.1, MP34 (anti-CD45) and A7R34 (anti-IL-7Rα) provided by S. Nishikawa) purified from hybridoma cell culture supernatants with protein G-Sepharose (Pharmacia). Anti-Ki67 and anti-podoplanin (8.1.1) antibodies were purchased from BD Biosciences and BioLegend, respectively. Embryos were whole-mount-stained using Alexa Fluor 647-conjugated anti-CD4 (YTS191.1) from AbD Serotec; intestines were whole-mount–stained using rat anti-CD106 (VCAM-1, 429 VCAM-A) from BD Biosciences. Adult lymph node sections were stained using rabbit polyclonal anti-denmin and rat monoclonal anti-B220 (RA3-6B2) from Abcam and eBioscience, respectively. Anti-species-specific Alexa Fluor 594, Alexa Fluor 488 or Alexa Fluor 647 were used as secondary antibodies (Invitrogen).

**Statistics.** Variance was analysed using an F-test. Student’s t-test was performed on homoscedastic populations and Student’s t-test with Welch correction was applied on samples with different variances.

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Extended Data Figure 1 Fetal ILCs. a, ILC subsets in fetal gut and lymph nodes (LN). b, E15.5 intestines and lymph node cells were purified from Id2<sup>GFP</sup> and wild-type mice. Id2<sup>GFP</sup> and ROR<sup>ct</sup> expression are shown in ILC<sub>4neg</sub> (CD3<sup>−</sup>IL-7R<sup>a</sup>1α4β7<sup>−</sup>IL7Rxα4β7<sup>−</sup>CD11c<sup>−</sup>CD4<sup>−</sup>) and LT<sub>i</sub> (CD3<sup>−</sup>IL-7Rxα4β7<sup>−</sup>CD11c<sup>−</sup>ROR<sup>ct</sup><sup>−</sup>CD4<sup>−</sup>) cells. c, E13.5 and E14.5 Ly6A-GFP anlagen lymph nodes were stained with GFP, IL-7R<sup>a</sup> and Ki67 antibodies and analysed by confocal microscopy. d, E14.5 Rorgt<sup>−/−</sup> mesenteric lymph nodes were stained with podoplanin, IL-7Rx and CD45 antibodies and analysed by confocal microscopy. e, Percentage of E16.5 ILC<sub>4neg</sub> and LT<sub>i</sub> cells gated in CD45<sup>−</sup>CD3<sup>−</sup>CD11c<sup>−</sup> determined by flow cytometry in Rorget<sup>+/−</sup> and Rorget<sup>−/−</sup> intestines. Data are representative of three independent experiments. f, Left: pregnant mice received RAR antagonist BMS493 or the vehicle DMSO from E10.5 until E13.5. The ratio LT<sub>i</sub>/ILC<sub>4neg</sub> cells in the fetal liver was determined at E13.5; n = 8. Right: pregnant mice received BMS493 or the vehicle DMSO. Frequency of colonizing haematopoietic cells was determined in E17.5 intestines by flow cytometry; n = 4. g, Pregnant hCD2-GFP mice were administered BMS493 or DMSO from E10.5 until E13.5. Embryos were analysed at E17.5; n = 13. Arrowheads show anlagen lymph nodes. Scale bars: 50 μm (c, d); 500 μm (g). Error bars show s.e. Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.
Extended Data Figure 2 | Analysis of mouse haematopoietic-cell-specific Cre lines. a, Vav-iCre and hCD2-Cre mice were crossed with ROSA26-Tomato-mGFP mice. Rorgt-Cre and Id2-CreERT2 mice were crossed with ROSA26-eYFP mice. E15.5 intestines were analysed by flow cytometry. Left: results show the percentage of mGFP or eYFP positive cells in gut cell suspensions. Right: percentage of mGFP or eYFP positive cells in non-haematopoietic (CD45$^-$), haematopoietic (CD45$^+$), LTi and LTin cells. Results are representative of three independent experiments. b, Percentage of enteric E15.5 ILC$_{iineg}$ and LT$_i$ cells determined by flow cytometry in wild-type, Rorgt-Cre Rar$^{Het}$ and Rorgt-Cre Rar$^{Hom}$ littermates. c, Frequencies of enteric ILC$_{iineg}$ and LT$_i$ cells in wild-type, Rorgt-Cre Rar$^{Het}$ and Rorgt-Cre Rar$^{Hom}$ littermates. WT, n = 5; Rorgt-Cre Rar$^{Het}$, n = 5; Rorgt-Cre Rar$^{Hom}$, n = 4. d, Peyer’s patch area at 6–7 weeks of age. WT, n = 3; Rorgt-Cre Rar$^{Het}$, n = 4. Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.
Extended Data Figure 3 | Analysis of Vav-iCre/ROSA26-RARγt403 mice. a, E15.5 intestines from wild-type, Vav-iCre RarHet and RarHom mice were analysed by flow cytometry. Representative analysis of six independent experiments is shown. b, E15.5 regions of cervical, brachial and inguinal lymph node from wild-type, Vav-iCre RarHet and RarHom mice were analysed by flow cytometry. Representative analysis of two independent experiments. c, LTin cell percentage and LTin/ILC4neg cell ratios are shown in E15.5 lymph nodes; n = 4. d, E15.5 fetal livers from wild-type and Vav-iCre RarHet mice were analysed by flow cytometry. Results show number of CD45−CD3− and CD45−Lin−IL-7Rα−RORc+ progenitors (n = 3). e, Percentage of CD45−CD3−CD11c−IL-7Rα−RORγt−CD4+ (RORγt+CD4+), CD45−CD3−CD11c−IL-7Rα−RORγt+CD4− (RORγt−CD4−) and CD45−CD3−CD11c−IL-7Rα−RORγt+CD4+ (RORγt+CD4+) cells determined by flow cytometry in RARhet and wild-type littermate controls in E15.5 guts and lymph nodes. f, Frequencies of RORγt−CD4+ and RORγt+CD4+ cells in mice described in e; WT, n = 9; RarHet, n = 3. g, ILC4neg cells were purified from E15.5 wild-type intestines by flow cytometry and cultured for 6 days. LTin cells raised in vitro were purified by flow cytometry and quantitative RT−PCR analysis performed. Results show log2 fold increase in comparison to their cultured ILC4neg cell counterparts. Results were normalized to Hprt1 and Gapdh. h, Left: E15.5 embryos were whole-mount stained for CD4 (red) and imaged by confocal microscopy. Cervical (Cer) lymph node dimensions are shown. WT, n = 5; RarHet, n = 7; RarHom, n = 6. Scale bar: 50 μm. Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant. ND, not detected.
Extended Data Figure 4 | Gene expression patterns in ILC4neg and LTi4 cells. 
a, E15.5 intestines from Rar<sup>Hom</sup> and wild-type littermate controls were brought to suspension and analysed by flow cytometry. Upper panel: ROR<sub>ct</sub> expression. Lower panel: mean fluorescence intensity of ROR<sub>ct</sub> expression in ILC4neg cells; n = 3. 
b, ILC4neg cells were purified from E15.5 Rar<sup>Het</sup> and wild-type littermate control intestines and lymph nodes. Quantitative RT–PCR analysis was performed. Results show log<sub>2</sub> fold increase to wild type. Results were normalized to Hprt1 and Gapdh. Results from three independent measurements are shown. 
c, d, LTi4 cells were purified from E15.5 Rar<sup>Het</sup> (c), Rar<sup>Hom</sup> (d) and wild-type littermate control intestines and lymph nodes. Quantitative RT–PCR analysis was performed. Results show log<sub>2</sub> fold increase to wild type. Results were normalized to Hprt1 and Gapdh. Data from three independent measurements are shown. Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. ND, not detected.
Extended Data Figure 5 | Treatment of ILC4neg and LTi4 cells with digoxin.

a, Wild-type ILC4neg cells were FACS purified, starved overnight and stimulated with DMSO, BMS493 (100 nM), RA (100 nM) and RA plus BMS493 (100 nM each) for 16 h. Results show quantitative RT–PCR analysis normalized to Gapdh; n = 3. b, E13.5 lymph node cell suspensions were cultured with vehicle (ethanol), digoxin, digoxin + RA and RA alone for 24 h. Alive/dead cell ratios were determined by flow cytometry and DAPI staining; n = 4. c, ILC4neg cells were isolated from wild-type E15.5 embryos starved overnight and stimulated for 6 h in the presence of RA (100 nM) or DIG (10 μM) + RA (100 nM). Results show quantitative RT–PCR analysis of Rorgt and RORγt downstream targets normalized to Gapdh. Representative of three independent experiments. Error bars show s.e. Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.
Extended Data Figure 6 | Analysis of SLOs from adult mice with variable RA signalling levels. a, Axillary (Axi), brachial (Bra) and inguinal (Ing) lymph nodes from adult Rar<sup>Het</sup> and wild-type littermate controls were analysed. Results show lymph node cell numbers; n = 6. b, Results show Peyer’s patch number per intestine from adult Rar<sup>Het</sup> and wild-type littermate controls; n = 6. c, Six-week-old wild-type females received VAC, VAH or VAD (n = 3) diet for 7 weeks. Axillary (Axi), brachial (Bra), inguinal (Ing), intrathoracic (IntraT) lymph nodes and Peyer’s patches (PP) dimensions were analysed; n = 3. d, Percentage of CD45<sup>+</sup>CD19<sup>+</sup> B cells; CD4<sup>+</sup> and CD8<sup>+</sup> T cells in inguinal lymph nodes; n = 3. e, f, Two-week-old CD45.2 Rar<sup>Het</sup> and wild-type littermate controls were lethally irradiated and transplanted with wild-type CD45.1 bone marrow cells. Chimaeric mice were analysed 8 weeks after reconstitution. e, Results show Peyer’s patch dimensions and follicle number/Peyer’s patch; n = 6. f, Results show number of cells in axillary (Axi), brachial (Bra) inguinal (Ing) and intrathoracic (IntraT) lymph nodes; n = 6. Scale bar: 1 mm. Error bars show s.e. Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.
Extended Data Figure 7 | Analysis of WT→WT and WT→RarHet bone marrow chimaeras. Two-week-old CD45.2 RarHet and wild-type littermate controls were lethally irradiated and transplanted with wild-type CD45.1 bone marrow cells. Chimaeric mice were analysed 8 weeks after reconstitution.

a, Reconstitution of donor CD45.1 cells in WT→WT and WT→RarHet chimaeras in the spleen (n = 4).
b, Reconstitution of donor CD45.1 CD4 and CD8 T cells in WT→WT and WT→RarHet chimaeras in the spleen. WT→WT, n = 4; WT→RarHet, n = 11.
c, Reconstitution of donor CD45.1 CD11c<sup>+</sup>MHCII<sup>+</sup> and CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells in WT→WT and WT→RarHet chimaeras in the spleen; n = 3.
d, Reconstitution of donor CD45.1 CD11c<sup>+</sup>MHCII<sup>+</sup> and CD11b<sup>+</sup>Gr1<sup>+</sup> cells in WT→WT and WT→RarHet chimaeras in intrathoracic lymph nodes; n = 3.
e, Dendritic cells (DCs) were purified from WT→WT and WT→RarHet chimaeras. Dendritic cells were loaded with OVA peptide (10<sup>−5</sup> μM) and co-cultured for 3 days with CFSE-labelled monoclonal OT1 CD8 T cells. OT1 CD8 T-cell proliferation was analysed by CFSE dilution. Proliferation index is shown. WT, n = 4; RarHet, n = 6.
f, Dendritic cells were purified from WT→WT and WT→RarHet chimaeras. Dendritic cells were loaded with OVA peptide (10<sup>−5</sup> μM) and co-cultured for 3 days with OT1 CD8 T cells. Percentage of IFN-γ-producing OT1 CD8 T cells is shown; n = 4. Error bars show s.e.
Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.
Extended Data Figure 8 | Infection of WT→RarHet or WT→WT chimaeras with murid herpesvirus-4. a, Intrathoracic lymph node cellularity in WT→WT and WT→RarHet chimaeras at different days post-infection (d.p.i.). WT, n = 5; RarHet, n = 3. b, Percentage of CD8^+ ORF61+ (left) and ORF75c+ (right) T cells in intrathoracic lymph nodes at different days post-infection (d.p.i.). WT, n = 5; RarHet, n = 3. c, Percentage of donor CD45.1 CD8^+ ORF61+ (left) and CD8^+ ORF75c+ (right) T cells in WT→WT and WT→RarHet chimaeras after infection with murid herpesvirus-4. WT, n = 6; RarHet, n = 12. Error bars show s.e. Two-tailed t-test P values are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant.
Extended Data Figure 9 | Impact of maternal retinoids in ILC3s. Maternal dietary intake of vitamin A is catabolized into bioactive retinoic acid (RA). RA signals control type 3 innate lymphoid cells (ILC3) in the embryo. Fetal ILC3s include ILC3neg and LTi4 cells. LTi4 cells are Id2\(^+\) ROR\(\gamma\)\(^+\), whereas enteric ILC3neg cells contain a minor subset of Id2\(^+\) ROR\(\gamma\)\(^-\) cells (pre-ILCs). RA signalling operates in a cell-autonomous fashion, via direct regulation of Rorgt, programing innate pro-inflammatory cytokines and chemokines and differentiation of LTi4 cells.
### Extended Data Table 1 | Computational analysis of putative RARE sites in the *Rorc* locus

| Site ID | Primer Sequences | Putative RARE Sites | Position | TESS Sequence entry | TESS Factor |
|---------|------------------|---------------------|----------|---------------------|-------------|
| A       | TGAAGCAGCTAGTCGACTTCC CAGCTTCCAGCTTGTATTG | GAGGAGCAGGG | -7,837 Rorg TSS | GAGGICAGGG | T00721 RAR-beta T01334 RXR-beta |
| B       | GAAACTTTATCTGGGCTGCTGG TGAATCAGGAAGAGGAGACTGCA | GGGTCAGAGGT | -7,446 Rorg TSS | GGTTCA | T00721 RAR-beta |
| C       | AACCTGGCACCTTCGACCTTAA GAGTGAGGCGGACTTCTCAGA | TGAACCT | -5,478 Rorg TSS | TGAACCT | T00721 RAR-beta |
| D       | GAGGCCCCTCTAGAATCCGCCATT CGCCCTGAAATCTGTCACACC | AGGTCA | -4,690 Rorg TSS | AGGTCA | T00721 RAR-beta |
| E       | CAGAGATGACCTAGTCAGTGGAGACTG GCCCCTGAACCCCTGGAATAC | GGGGCAGGGT | -1,800 Rorg TSS | GGGTCAGGGTT | T00719 RAR-alpha1 T00720 RAR-gamma T00721 RAR-beta T01329 RAR-gamma |
|         | ACCCCCCAAAACCCTTGA | TGACCT | | | |
|         | | | | GGGTCAGGGGT | _00000 RAR-beta |
|         | | | | GGTCAGAAGGTT | _00000 RXR-alpha |
|         | | | | GGTCAGAAGGTT | _00000 RXR-alpha |
|         | | | | GGTCAGAAGGTT | _00000 RXR-alpha |
| F       | ACCACTGAGCCCATCTCCTCACC TTTTGTATGTGGTTCTCTGGG | GGGTCAGGGTT | -1,638 Rorg TSS | GGGTCAGGGTT | T00719 RAR-alpha1 T00720 RAR-gamma T00721 RAR-beta T01329 RAR-gamma |
|         | | | | GGGTCAGGGTT | _00000 RAR-beta |
|         | | | | GGGTCAGGGTT | _00000 RAR-beta |
| G       | GACAATCTCATCGAGGAGGG GGCAACCCATGAGGTATGGTG | TCACCTCT | -1,619 Rorg TSS | TCACCTCT | _00000 RAR-gamma |

Computational analysis was performed using TESS (Transcription Element Search System) (http://www.cbil.upenn.edu/tess), TSS (Rorg), ACGGGCAAGGCTCTCCTCC; TSS (Rorg), AGAAACACTGGGGAGAGCTTGTATTG.