Peripheral Lymphoid Volume Expansion and Maintenance Are Controlled by Gut Microbiota via RALDH⁺ Dendritic Cells

Graphical Abstract

Highlights

- Gut microbiota is required for secondary lymphoid organ development
- Gut RALDH⁺ DCs move to peripheral lymph nodes after gut fungal colonization
- These DCs initiate LN structural development via retinoic acid signaling
- Neonatal lymphocytes are imprinted for gut homing by these DCs

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In Brief
Gut microbiota promotes the development of secondary lymphoid organs. Here, Shi and colleagues demonstrate that CD45⁺CD103⁺RALDH⁺ dendritic cells in the gut respond to microbial colonization and migrate to the periphery. These DCs use retinoic acid signaling to initiate the lymph node cellularity increase and volume expansion.
Peripheral Lymphoid Volume Expansion and Maintenance Are Controlled by Gut Microbiota via RALDH⁺ Dendritic Cells

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http://dx.doi.org/10.1016/j.immuni.2016.01.004

SUMMARY

Lymphocyte homing to draining lymph nodes is critical for the initiation of immune responses. Secondary lymphoid organs of germ-free mice are underdeveloped. How gut commensal microbes remotely regulate cellularity and volume of secondary lymphoid organs remains unknown. We report here that, driven by commensal fungi, a wave of CD45⁺CD103⁺ RALDH⁺ cells migrates to the peripheral lymph nodes after birth. The arrival of these cells introduces high amounts of retinoic acid, mediates the neonatal to adult addressin switch on endothelial cells, and directs the homing of lymphocytes to both gut-associated lymphoid tissues and peripheral lymph nodes. In adult mice, a small number of these RALDH⁺ cells might serve to maintain the volume of secondary lymphoid organs. Homing deficiency of these cells was associated with lymph node atrophy in vitamin-A-deficient mice, suggesting a perpetual dependence on retinoic acid signaling for structural and functional maintenance of peripheral immune organs.

INTRODUCTION

In prenatal lymphogenesis, lymph node (LN) anlagen develop from primitive lymph sacs that appear during lymphatic vascularization, upon the arrival of lymphoid tissue inducer (LTI, RORγt, and ID2 dependent) cells (Eberl et al., 2004; Sun et al., 2000; Yokota et al., 1999), namely the CD4⁺CCR6⁺NKp46⁺ subset of group 3 innate lymphoid cells (Spits et al., 2013). LTI cells mediate the initial development of these structures after receiving cues from retinoic acid (RA) released from neuronal termini in the vicinity (van de Pavert et al., 2009), and LTI-cell-autonomous RA is critical for LN development in utero (van de Pavert et al., 2014). After birth, LTI cells are no longer found in LNs (Kim et al., 2003), yet gut-associated lymphoid tissues (GALTs) and peripheral LNs (pLNs) continue to expand in size and cellularity. By the second week after birth, pLNs form adult-like clear demarcation of T cell zones and B cell follicles (Mebius et al., 1996). However, neonatal pLN developments are arrested in germ-free (GF) animals (Bauer et al., 1963; Inagaki et al., 1996). In GALTs of GF mice, Peyer’s patches (PPs), isolated lymphoid follicles, and mesenteric LNs (mLNs) are fewer in number and less cellular; pLNs are relatively structureless. These defects suggest a critical role of commensal microbes in the development of secondary lymphoid organs (SLOs) after birth (Macpherson and Harris, 2004). How the microbiota promotes these changes, particularly at distal LNs, remains unknown.

Blood lymphocytes travel into LNs through high endothelial venules (HEVs) (Masopust and Schenkel, 2013), attracted by addressins such as MAdCAM-1, receptor for α4β7, and pLN addressin PNAδ, receptor for CD62L (Rosen, 2004). In neonatal specific-pathogen-free (SPF) mice, MAdCAM-1 expression is high shortly after birth (Mebius et al., 1996), followed by a switch to a PNAδ-dominated profile over a course of 2–3 weeks (Hemmerich et al., 2001; van Zante et al., 2003), gradually initiating L-selectin-based adult lymphocyte homing. A “non-T-cell” population has been found immediately before B cell follicle formation (Cupedo et al., 2004); the identity of these mysterious cells and how they might contribute to LN structural buildup remain elusive. In steady-state pLN, two reports suggest that semimature chemokine receptor CCR7⁺ (Wendland et al., 2011) and CD11c⁺ (Moussion and Girard, 2011) DCs regularly enter SLOs to promote the development of HEVs and attract lymphocyte homing. Questions remain regarding how much these three types of cells functionally and phenotypically overlap, and more importantly, whether they are associated with gut microbiota in inducing LN development.

In SPF mice, GALTs are modulated by a stream of incoming transcription factor ID2-, IRF8-, Batf3-, IRF4-, and Notch2-dependent RALDH⁺ (retinaldehyde dehydrogenase) CD11b⁺ CD103⁺ DCs from the gut, mainly the lamina propria (LP) (Helft et al., 2010), and a mirroring population exists in humans (Watchmaker et al., 2014). These DCs are phenotypically unique because, in the periphery of mice, such as in the skin, CD103 and CD11b expressions are mutually exclusive (Ginhoux et al., 2014).
Arriving via CCR7 (Jang et al., 2006; Worbs et al., 2006), these cells have been reported to convert CD4+ T cells into inducible regulatory T cells in concert with TGf-β (Coombes et al., 2007), mediate T helper 17 (Th17) cell homeostasis (both positively and negatively) (Cha et al., 2010; Elias et al., 2008; Muchida et al., 2007; Wang et al., 2010), and imprint lymphocytes for gut homing (Iwata et al., 2004; Mora et al., 2003). Therefore, a coherent scheme linking gut microbiota, those seemingly disparate “visitors,” and neonatal LN structural development is highly desired.

We report here that RA-related genes were highly expressed in SPF pLNs, in comparison to expression in GF pLNs. Notably, a group of RALDH+ cells appeared in the pLNs after birth or in GF LNs after co-housing with SPF mice. Transfer of these cells completely restored the structural and cellularity defects and triggered a HEV MAdCAM-1 to PNAd addressin profile change in GF mice. In early neonatal weeks, these cells impinged peripheral lymphocytes for gut homing, in sync with the rapid establishment of GALT. In adult mice, this signal was disrupted in vitamin A (VitA)-restricted mice, linking this regulation to immune dysfunctions associated with VitA deficiency. Our work therefore unveils the path linking gut microbiota to peripheral immune functions and establishes the instrumental role of RA in the birth, development, and health of SLOs.

RESULTS

Germ-free Mice Have Reduced RALDH Activities in Secondary Lymphoid Tissues

To confirm that reduced lymphocyte homing was the cause of pLN defects in GF mice, we labeled 107 SPF total LN lymphocytes for gut homing (Iwata et al., 2004; Mora et al., 2003). Therefore, a coherent scheme linking gut microbiota, those seemingly disparate “visitors,” and neonatal LN structural development is highly desired.

We report here that RA-related genes were highly expressed in SPF pLNs, in comparison to expression in GF pLNs. Notably, a group of RALDH+ cells appeared in the pLNs after birth or in GF LNs after co-housing with SPF mice. Transfer of these cells completely restored the structural and cellularity defects and triggered a HEV MAdCAM-1 to PNAd addressin profile change in GF mice. In early neonatal weeks, these cells impinged peripheral lymphocytes for gut homing, in sync with the rapid establishment of GALT. In adult mice, this signal was disrupted in vitamin A (VitA)-restricted mice, linking this regulation to immune dysfunctions associated with VitA deficiency. Our work therefore unveils the path linking gut microbiota to peripheral immune functions and establishes the instrumental role of RA in the birth, development, and health of SLOs.

We performed the RNA-seq on the Swiss ICR strain of GF and SPF mice to confirm that RA signaling is a common theme in LN development (Rice and O’Brien, 1980). The upregulation of RA-associated transcripts was also seen 1 week after GF to SPF conversion (Figures S2B and S2C, overall and RA-signaling-related heat maps and numerical display, respectively), suggesting that RA signaling was involved in regulating LN structure in outbred mice. The appearance of CD45+ RALDH+ cells was normal in mLNs and PPs from Nkbp1-/-, Myd88-/-, Tlr4-/-, and Il1r1-/- deficient SPF mice (Figure S2D), indicating that these pathways

Associated with adipogenesis and RA signaling (Figure 1C). For example, Fabp5, Plin1, Apoc1, and ApoI7c are involved in lipid transportation, and G0S2, Cidea, CCL2, Areg, and UCP1 are RA transcriptional targets (Balmer and Blomhoff, 2002). GF mice are lipaotropic (Bäckhed et al., 2004; Bäckhed et al., 2007). transplantation of visceral epididymal fat tissue from SPF mice to the lower region of the abdomen of GF mice led to an increase in the size of ipsilateral, but not contralateral or sham-operated, iLNs within 2 weeks (Figure S1E and data not shown). However, we failed to isolate a population of cells from the transplant that supported LN size expansion, implicating the involvement of a soluble effector stored in the graft.

We were able to detect a statistically significant difference in MAdCAM-1 mRNA expression. The abundance of mRNA transcripts of enzymes involved in PNAad expression also did not change (data not shown) (Sperandio, 2006), suggesting post-translational modification. Two groups with the greatest increases in SPF mice were

Immunity 44, 330-342, February 16, 2016 ©2016 Elsevier Inc. 331
were not essential for the appearance of CD45⁺RALDH⁺ cells in neonatal SLOs.

**RALDH⁺ Migratory Cells Restore the Cellularity of pLNs in GF Mice**

Because RALDH⁺ cells are regarded mostly as being unique to the gut (Hall et al., 2011b), their appearance in pLNs suggested a previously unexplored role of these cells in postnatal SLO expansion. CD45⁺RALDH⁺ cells were purified from 2-week-old SPF pLNs, and 2 × 10⁶ cells were i.v. transferred into 5-week-old GF mice, 7 days later, 10⁷ CFSE-labeled total SPF LN cells were similarly injected, and their homing pattern was monitored after 24 hr. Figure 3A shows that more CFSE⁺ cells migrated to iLNs and mLNs in RALDH⁺ recipients than in RALDH⁻/C0 controls.

**Figure 1. Homing of Lymphocytes to pLNs Is Defective in GF Mice**

(A) 10⁷ CFSE-labeled total SPF pLN lymphocytes were isolated from C57BL/6 SPF mice and i.v. injected into GF or SPF recipients. 24 hr later, the arrival of these cells in iLNs, mLNs, and the spleen, and CD4⁺, CD8⁺ and CD19⁺ subpopulations in iLNs, were analyzed by flow cytometry. The right panels show results from multiple mice. Student’s t analysis results for this and all subsequent figures are indicated as follows: ***p < 0.001, **p < 0.01, *p < 0.05. A p value of 0.05 or greater was considered non-significant (N.S.). The data are representative of four independent experiments.

(B) iLNs of GF, SPF co-housed with GF (1 week), and SPF iLNs were stained with DAPI (blue) and either MECA-367 (anti-MAdCAM-1) or MECA-79 (anti-PNAd) (green) antibody and analyzed by confocal imaging. The scale bars in this and all subsequent figures are 50 μm. See Figure S1A for lumen area size and number analyses. Shown is a representative slide from a total of at least 30 sections, and sections were collected throughout entire LNs. Images were processed with ImageJ, and surface areas were calculated by the ruler-transformed pixels.

(C) Total mRNA from pooled pLNs (excluding mLNs) was extracted from 5-week-old C57BL/6 GF and SPF mice and analyzed as detailed in the Experimental Procedures. mRNA ratios between GF and SPF mice that reached statistical significance were displayed by function groups. p and X² values from RNa-seq assay are shown to the right. The heat maps for gene functions associated with adipogenesis and RA signaling are shown, and corresponding data shown as bar graphs are in Figure S1D. See Figure S1B and S1C for the total heat map and other gene function groups. The data are representative of two independent experiments. Also see Figure S1.
The same trend was seen with CD4⁺, CD8⁺, and CD19⁺ lymphocytes (Figure 3A). In addition, iLNs in RALDH⁺ recipients showed mature structures with enlarged B cell follicles, denser T cells, and a clear demarcation between T and B zones (Figure 3B). Quantitatively, the overall size of LNs and areas of definable B and T cell zones were also increased after RALDH⁺ cell infusion (Figure 3B). When bone marrow DCs (BMDCs, RALDH⁺/CD0⁻) were used for comparison, CD45⁺RALDH⁺ cells were still more robust in triggering lymphocyte homing in both 6 and 24 hr (Figure 3C), suggesting that not all DCs were able to efficiently support pLN development. In addition, total HEV area was larger in the RALDH⁺-cell-infused group (Figure 3D).

All RALDH⁺ cells identified in Figure 2B also expressed CD103, CD11c, and MHC class II (Figure S3A). In comparison with CD45⁺RALDH⁻/CD0⁺ cells, CD45⁺RALDH⁺ cells from 2-week-old SPF mice expressed higher CD103, CD11b, CD11c, CXCR5, CD62L, and α4β7 (Figure 3E), resembling the CD103⁺ DC population in the gut. MHC class II, F4/80, complement receptor 1, mannose receptor, CCR7, ICAM-1, VCAM-1, CD11a, CD80, CD86, and CD83 were higher as well, although the difference in expression was minimal for CD127, CD45RB, CD19, and Ly6G (Figure S3B). CD8 expression, however, was much lower in comparison (Figure S3B). No bimodal expression was detected for the tested markers (Figure 3E and Figure S3B). Therefore, CD45⁺RALDH⁺ cells isolated during the initial expansion of LNs appeared to be homogeneous.

Discussed in the context of the gut (Hall et al., 2011b), RALDH⁺ cells have never been reported to travel long distance to initiate pLN development after gut colonization or to regulate mLN development after birth. A comparison of 14 surface markers showed that CD45⁺RALDH⁺ cells in iLNs and mLNs of 2-week-old SPF mice were similar (Figure S3D). In addition, RALDH⁺ cells in 2-week-old iLNs were found to be also positive for CD45, CD103, CD11c, and MHC class II, suggesting the unique role of these cells at the early development of SLOs (Figure S3E).

Gut RALDH⁺ Migratory Cells Are Driven by Commensal Fungi

To verify that neonatal pLN CD45⁺RALDH⁺ cells were indeed CD103⁺CD11b⁺ DCs from the gut, we fasted and orally gavaged 2-week-old SPF mice with FITC-dextran 2,000 kDa. 6 hr later, iLN cells were analyzed by flow cytometry. In contrast to CD103 and CD11b double-negative and single-positive cells, the double-positive population was FITC⁺, suggesting their prior exposure to the label in the gut (Figure 4A). A nearly identical profile was seen in mLNs at this age (Figure 4A). Injection of anti-MAdCAM-1 antibody reduced the number of FITC-labeled double-positive cells in LNs (Figure 4A). These data indicated that the
initial exodus of gut DCs targeted all SLO, and their preference for GALTs was an acquired feature established later. FITC-labeled DCs reached the LNs in 6 hr in neonatal mice. This was most likely due to the higher gastrointestinal permeability, a different gastric emptying time, and greater basal metabolic rates in these young animals (Beach et al., 1982; Catassi et al., 1995; Shearer et al., 1995). Further supporting their similar identity, CD103+ RALDH+ cells purified from 5-week-old LP readily homed to

Figure 3. CD45+RALDH+ Cells are Phenotypically Similar to mLN Migratory DCs

(A) 2 × 10^6 CD45+RALDH+ and CD45+RALDH- (lower RALDH box in Figure 2B) cells were isolated by cell sorting from SPF mice and i.v. injected in containment into tail veins of 5-week-old GF mice. 7 days later, CFSE-labeled 10^7 SPF pLN total lymphocytes were similarly infused into the recipients. 24 hr later, the percentages of CFSE+ cells, as well as their CD4+, CD8+, and CD19+ subsets in iLNs and mLN, were analyzed. The data are representative of four independent experiments.

(B) CD45+RALDH+ or CD45+RALDH- cell-infused GF mice were rested for 7 days and their iLN were stained for anti-B220 and anti-CD3 and analyzed by confocal imaging. SPF iLN was used as the control. Overall surface areas of LN and accumulative T zone defined by dense anti-CD3 stain in one LN are shown in lower panels. For B cell area, a single B cell zone defined by dense anti-B220 stain was counted.

(C) Identical to (A) except that 5 day BMDCs sorted for RALDH- stain were used in place of RALDH+ cells. Overall lymphocyte homing to iLNs and mLNs in 6 and 24 hr is shown.

(D) Individual surface areas of HEV staining per lumen are shown. All images are representative of at least 30 independent photos, and the statistics are shown as the pooled data.

(E) CD45+RALDH+ cells and CD45+RALDH- control from 2-week-old SPF iLNs were gated and further analyzed for CD103, CD11b, CD11c, CXCR5, αβ7, and CD62L. Secondary antibody-only staining of CD45+RALDH+ cells was used as the background control. See Figure S3B for additional markers.

(F) 2 × 10^6 CD45+RALDH+ cells from 2-week-old SPF iLNs were isolated and rested in tissue culture media overnight. Their cytokine secretion was analyzed. Qualitative differences in secretion reaching t test p value < 0.05 are shown. (E) and (F) are representative of three independent experiments. See Figure S3C for the secretion of an additional 22 cytokines that did not reach statistically significant difference (p > 0.05). Also see Figure S3.
Figure 4. RALDH⁺ DC Migration Is Driven by Commensal Fungi

(A) 2-week-old SPF mice were fasted, orally force fed with FITC-dextran 2000 KD, and iLNs and mLN were harvested after 6 hr. Left: the FITC intensity in iLNs was compared in CD103 and CD11b double-negative, double-positive, and single-positive populations (upper panels). All FITC⁺ cells were also analyzed for their CD103 and CD11b expression (lower panels). Right: flow cytometry plots show mLN data. The charts show the percentages of FITC⁺-DP cells in anti-MAdCAM-1-antibody-injected mice in comparison with rat-IgG-injected controls.

(B) 2 × 10⁵ CFSE-labeled CD103⁺CD11b⁺RALDH⁺ cells isolated from adult SPF LPs were i.v. injected into 9-day-old SPF mice. 24 hr later, the presence of these cells in iLNs and mLNs was analyzed by confocal imaging. Right: the cell counts per LN.

(C) Adult SPF mice were treated with AVNM or fluconazole mixed in drinking water for 3 weeks, and the percentages of RALDH⁺CD103⁺ cells in mLNs and PPs were determined by flow cytometry.

(D) Untreated adult SPF mice were given 10⁸ indicated live fungal strains by gavage, and LNs were analyzed as in (C) after 24 hr.

(E) As in (D), 2-week-old SPF mice were used in place of the adult mice.

(F) 8-week-old GF mice were given 10⁸ C. tropicalis gavage, and neo-mi DCs in LNs were analyzed after 48 hr. All data are representative of at least three independent experiments. Also see Figure S4.
9-day-old iLNs (Figure 4B). These long-distance migratory cells were henceforth called neonatal migratory DCs (neo-mi DCs) to functionally distinguish them from the sibling steady-state migration to GALTs in SPF mice.

Neo-mi DCs migrated out of the LP in response to some components in gut microbiota. We first treated adult SPF mice with an anti-bacterial cocktail (ampicillin and vancomycin, neomycin, and metronidazole [AVNM]) or fluconazole (anti-fungal) added to drinking water. Percentages of RALDH+ cells in mLNs and PPs were analyzed after 3 weeks. Fluconazole significantly inhibited their numbers in mLNs and PPs (Figure 4C). Anti-bacterial treatment had an opposite effect (Figure 4C). Candida tropicalis, Saccharomyces cerevisiae, and several strains of Trichosporon are most abundantly found in the gut (Iliev et al., 2012). 4-week-old SPF mice were force fed with those live fungi produced from purified cultures. Candida tropicalis not only enhanced the steady migration of RALDH+ cells to mLNs (Figure 4D), but also revived the neo-mi-DC-like migration in adult mice (Figure 4D). To confirm that C. tropicalis induced neo-mi DC migration, we similarly treated 2-week-old mice. In comparison with PBS control, C. tropicalis significantly increased percentages of neo-mi DCs in iLNs and mLNs (Figure 4E). In GF mice receiving the same gavage in confinement, numbers of neo-mi DCs in iLNs were also significantly higher, mimicking the natural neonatal migration in SPF mice or during GF to SPF conversion (Figure 4F). Therefore, commensal contents, particularly those from C. tropicalis, were capable of triggering neo-mi DC migration to both mLNs and PPs in C57BL/6 mice. Functionally, C. tropicalis might regulate neo-mi DCs either by functionally upregulating RALDH expression in a population of DCs, or driving preexisting CD103+RALDH+ cells to leave the LP and enter SLOs. Figures S4A and S4B show that neither BMDCs nor RALDH+CD11c+ cells from the LP expressed RALDH after being co-cultured with killed C. tropicalis. Conversely, anti-fungi treatment slightly retained neo-mi DCs in the LP, with a corresponding decrease of these cells in iLNs (Figure S4C). After oral gavage of this strain, CD103+RALDH+ cells were reduced in cell number in the LP with an ensuing increase of these cells in iLNs (Figure S4D). Collectively, these data suggest that C. tropicalis most likely triggers migration of neo-mi DCs rather than induced RALDH expression in DCs in the LP.

RALDH+ Cells Induce pLN Expansion and Neonatal to Adult Addressin Switch

As γδ T expression on peripheral lymphocytes was reduced in GF mice (Figure S5A), MadCAM-1 expression alone was clearly not sufficient to permit their homing to pLN. CD103+ cells appeared in iLNs of Rag1−/− mice (Figure S5B), suggesting they were functionally upstream to the lymphocyte homing and the establishment of pLN volume. This raised the possibility that MadCAM-1 mediated the initial homing of neo-mi DCs and the latter subsequently attracted bona fide lymphocyte accumulation in pLNs. Indeed, neo-mi DCs readily homed to pLNs of 2-week-old SPF mice, but not as efficiently to those of 6-week-old mice (Figure 5A). Infusion of MadCAM-1 antibody blocked this migration (Figure 5A, right panel, and Figure S5C). In 1-week-old SPF mice, the relative percentage of these cells in iLNs was low, followed by a rise by week 2 (Figure 5B). Numbers of these cells became lower after 2 weeks, retaining only 0.33% ± 0.07% at week 4 (Figure 5B, one-way ANOVA p < 0.001). In mLNs, the downward trend was similar, although the percentages were higher at both 2 and 4 weeks (Figure 5B).

The arrival of these cells was associated with a dramatically altered appearance of HEVs in pLNs. 6 days after an infusion of neo-mi DCs, both iLNs and mLNs of GF mice displayed robust HEV portals, coated by thick layers of PNAd, while being...
surrounded by CCL21-expressing cells (Figure 5C) (Wendland et al., 2011). None of these features were present in the pLNs of CD45 + RALDH / C0 recipients (Figure 5C). Injection of anti-MAdCAM-1 antibody greatly reduced the number of HEVs in iLNs, in comparison with those in rat immunoglobulin G (IgG) controls (Figure S5D), suggesting that CD103 + RALDH + cells were important in pLN expansion.

Neonatal RALDH+ Cells Imprint Peripheral Lymphocytes for Gut Homing

Although RALDH+ cells are mostly found in the gut, in pLNs, a small percentage (about 10%) of CD103+CD8α+ DCs express RALDH under steady state (Guillaums et al., 2010; Molenaar et al., 2011). This raised the question as to why LP-originated neo-mi DCs were necessary in the neonatal expansion of pLNs. In the digestive tract, an abundance of food-derived VitA induces RALDH2 expression in DCs, creating a self-amplification loop and promoting a unique homing pattern toward the gut via upregulation of α4β7 and CCR9 in transiting lymphocytes (Bauer et al., 1963). In 2-week-old SPF mice, the overall percentage of α4β7+ cells in pLNs was higher than that in 6-week-old mice (Figure 6A). CCR9+ cells showed a similar trend in aLNs and iLNs, but not in mLNs (Figure 6A). Overall, the data suggested that neo-mi DCs might redirect lymphocytes passing through pLNs to the gut in the early weeks after birth. Indeed, co-culture of neo-mi DCs from 2-week-old iLNs and aLNs with OT-II CD4+ T cells induced higher α4β7 and CCR9 expression on the latter (both p < 0.001), similar to that of mLN counterparts (Figure 6B). For verification, a homing index assay was performed (Mora et al., 2003): OT-II T cells were co-cultured with neo-mi DCs from 2-week-old SPF pLNs in the presence of OVA (chicken ovalbumin), labeled with CFSE. 2×10⁶ cells from this mixture were then injected in combination with an equal number of DDAO-labeled CD4+ T cells isolated from SPF iLN into 2-week-old SPF mice. The homing index was determined by the CSFE:DDAO ratios in iLNs, mLNs, and the spleen.

(D) Similar to (C), untreated total pLN cells from 2- and 6-week-old SPF mice were used in place of OT-II T cells. Homing index was determined as in (C). All panels are representative of at least three independent experiments.

Figure 6. Neo-mi DCs Imprint Lymphocytes for Gut Homing

(A) α4β7+ and CCR9+ cells among the total cells from aLNs, iLNs, mLNs, and the spleen of 2- and 6-week-old SPF mice were analyzed by flow cytometry. (B) Top: 5×10⁵ neo-mi DCs were co-cultured with purified CD4+ iLN cells (1:4) of OT-II mice. 7 days later the presence of α4β7 and CCR9 was determined by flow cytometry. Identical numbers of CD45+RALDH+ cells from the same isolation and mLN CD45+RALDH+ cells were used as controls. Bottom: percentages of α4β7high and CCR9high cells from multiple mice are shown. (C) CD4+ cells isolated from OT-II iLN were cultured with neo-mi DCs in the presence of 200 μg/ml OVA. These T cells were labeled with CFSE. 2×10⁶ cells from this mixture were then injected in combination with an equal number of DDAO-labeled CD4+ T cells isolated from SPF iLN into 2-week-old SPF mice. The homing index was determined by the CSFE:DDAO ratios in iLNs, mLNs, and the spleen.
CD103+CD11b+RALDH+ cells (Figure 6C). In a similar homing index assay, pLN lymphocytes from 2-week-old mice migrated efficiently to PPs and the LP, and less so to iLNs and aLNs of 5-week-old SPF mice (Figure 6D). However, pLN lymphocytes from 6-week-old mice had lost this ability, and instead showed a better migration to pLNs than to GALTs (Figure 6D). Therefore, our data imply that the presence of neo-mi DCs in the pLNs of neonatal mice contributed to the imperative buildup of GALT.

**RALDH+ Cell Migration is Reduced in VitA-Deficiency-Associated Immune Malfunction**

VitA deficiency is associated with a number of infections, reduced mucosal immune activation, limited T helper-1 (Th1) cell response, and one to two million yearly mortalities worldwide (Humphrey et al., 1992). Given that a fraction of neo-mi DCs remained in pLNs (Figure 5B), it was conceivable that a long-term maintenance function was fulfilled by those residual cells that could be impacted by VitA restriction. Because Aldh1a2−/− mice do not survive, we instead injected 5-week-old SPF mice with DEAB. Although the total body weight of the treated mice was no different from that of the untreated mice (Figure S6A), DEAB significantly reduced the number of cells in iLNs and mLNs (Figure 7A). Anti-CD3 and anti-B220 staining of LNs from DEAB-treated mice showed no distinguishable B cell zones and reduced cellularity (Figure 7B). These results suggested that RA was a tonic signal in adult LN maintenance. We fed SPF breeder mice with a VitA-deficient (VAD) diet from the 9th day into gestation, and detected a reduction in the percentage of the neo-mi DC population in 2-week-old LNs (Figure 7C). This was accompanied by a reduction of lymphocyte numbers in iLNs and mLNs in 5-week-old mice weaned onto a VAD diet (Figure 7D). The same pattern was seen for CD4+, CD8+, and CD19+ cells (Figure 7D). VitA restriction severely disrupted the pLN architecture (Figure 7E) and inhibited the expression of PNAd in iLNs (Figure 7F) in 5-week-old mice, providing the mechanistic base for the reduced lymphocyte afflux. The total lymphocytes, and their CD4+, CD8+, and CD19+ subsets isolated from VitA-restricted mice, traveled to SPF pLNs, mLNs, and the spleen with either similar, or, in most cases, increased efficiency to PPs and the LP, and less so to iLNs and aLNs of 6-week-old mice. The migration process takes advantage of default neonatal addressin expression in pLNs to guide neo-mi DCs for their exodus from the LP to the periphery. The gut-microbiota-driven migration most likely remains in adult mice at reduced intensity; however, the importance of this lingering patronage to pLNs becomes evident under VitA restriction: failure to deliver a tonic RA signal causes severe structural defects in distal SLs.

Upon arrival at pLNs, RALDH+ DCs imprint peripheral lymphocytes for their homing to the gut, which seems logical given that the microbial burden increases exponentially in the gastrointestinal tract at this moment, in contrast to the rest of the body (Adlerberth and Wold, 2009). In addition, they alter pLN-targeting characteristics from a MAdCAM-1-mediated to a PNAd-mediated homing profile, and paradoxically reduce the attraction of neo-mi DCs to pLNs. Without the arrival of neo-mi DCs, as in GF mice, the MAdCAM-1 to PNAd switch is halted, arresting neonatal pLN development.

Gut microbiota changes the overall cytokine production in the host and provides additional stimulatory factors such as peptidoglycan (Galani et al., 2012). The role of RA in pLN development, however, might be the most prominent. In addition to altering lymphocyte homing markers in co-culture assay, infusion of neo-mi DCs in GF mice can completely restore the pLN structures; inhibition of RALDH or VitA deficiency result in pLN atrophy. These findings are consistent with the regulatory functions of RALDH+ cells in SLO development, at least in neonatal mice, although gut microbiota might modulate phagocyte activities in the periphery in adult mice (Galani et al., 2012). On the other hand, it is important to note that, although a small percentage of skin-originated CD103+ DCs are also RALDH+, they are relatively fewer in number (Guilliams et al., 2010). Nonetheless, our results do not rule out other factors potentially participating in SLO development.

The decisive role of gut fungi, particularly C. tropicalis, in driving neo-mi DC migration was notable. In a dextran sodium sulfate (DSS)-induced colitis model, Clec7a−/− mice develop a more severe pathology, suggesting that fungal components mediate a suppressive effect on inflammation (Iliev et al., 2012). MGL1, mannose binding lectin (MBL), and SIGNR1, all involved in fungal cell wall component recognition, also demonstrate a similar suppressive function in the mouse model or human ulcerative colitis (Eriksson et al., 2013; Rector et al., 2001; Saba et al., 2009). Therefore, emerging evidence points to a definitive connection between commensal fungi and immune regulation. Given that neo-mi DC migration is possibly a tonic signal in pLN maintenance, it is likely that we will identify additional regulatory functions of gut fungi in modulating peripheral immune responses in the future. Although the role of gut commensal fungi in neonatal LN development is observed in our setting, C. tropicalis might be merely a representative strain in the B6 mouse model. In humans, this strain is uncommon in the gut microbiota (Hoffmann et al., 2013). The key to establishing causality in the future is to identify the biochemical products that drive the neo-mi DCs’ migration.

It has been reported that RA signaling is critical for the development of gut CD103+CD11b+ cells, in line with our findings (Klebanoff et al., 2013), and this suggests that, in VAD animals, the lack of CD103+CD11b+ cells rather than their defect in migration underlies the pathology. It should be noted that limited VitA availability could affect other cellular functions that are essential to
Figure 7. VAD Causes a Reduced Homing of neo-mi DCs into pLNs and a Lack of Maintenance of pLN Structures

(A) Single-cell suspensions of iLNs and mLNs from DEAB-treated 5-week-old SPF mice were counted for total cell numbers.
(B) The same LN in (A) was stained for anti-CD3 and anti-B220. Overall surface area of the LN and the accumulative T zone, defined by dense anti-CD3 stain, in one LN are shown in the lower panels. For B cell area, a single B cell zone defined by dense anti-B220 stain was counted.
(C) iLNs, mLNs, and PPs from 2-week-old VAD and control-fed mice were analyzed by flow cytometry for the presence of neo-mi DCs.
(D) CFSE-labeled 10^7 total lymphocytes were i.v. injected into VAD and control-fed mice, and the presence of these cells and their CD4^+, CD8^+, and CD19^+ subsets in iLNs, mLNs, and the spleen was analyzed by flow cytometry after 24 hr.
(E) Similar to (B), iLNs of VAD and control-mice were stained with anti-CD3 and anti-B220 and analyzed by confocal imaging. Area analysis as in (B) is shown in the lower panels.
(F) iLNs of VAD and control-fed mice were stained for PNAd expression. Scale bar, 100 μm. Right: the cell counts per LN. All data are representative of at least three independent experiments. Also see Figure S6.
plN maintenance, a possibility that we did not explore. On the other hand, in several reports, VitA supplement did not improve overall human survival in developing countries (Benn et al., 2005; Lund et al., 2014) and sometimes is associated with atopy in some populations (Aage et al., 2015). The extent to which neo-mi DCs are associated VitA-related human health issues awaits further analyses.

A revelation of our work is that all LNs (mLNs and pLNs), at the moment of birth, are similar in attracting RALDH+ DCs, and these cells mediate the MadCAM-1 to PNAd addressin switch. By draining the gut, mLNs in adult mice continue, by default, to receive large numbers of CD103+ RALDH+ DCs. The latter serves as the basis for the reported, RA-mediated functions, such as regulatory T cell induction, imprinting lymphocytes for gut homing, and IgA production (Cassani et al., 2012). In contrast to pLNs, it is possible that additional factors present in GALT, such as tumor necrosis factor alpha (TNF-α), IL-1, lymphotixin, and LPS, help maintain the MadCAM-1 expression to continue to attract RALDH+ DCs (Cuff et al., 1998; Sikorski et al., 1993).

In contrast, in adult mice, additional factors in pLNs, such as T zone fibroblastic reticular cells, provide essential assistance (CCR7 and CCL19) to maintain the peripheral homeostasis under greatly reduced RA signaling (Link et al., 2007), creating sometimes diametrically different immune regulations in pLNs and GALT toward activation and tolerance.

Although several cell types have been proposed to be essential to maintenance of peripheral SLOs, no previous reports have attempted to address the dependence of postnatal pLN development on gut microbiota. Those preceding observations can be appreciated as being in accordance with ours in hindsight. It is very likely these analyses approached a similar question but were not appreciated as being in accordance with ours in hindsight.

microbial Component-Driven RALDH+CD103+ Cell Migration

For depletion of intestinal bacteria or fungi, mice were given an antibiotic cocktail containing ampicillin (1 g/l), vancomycin (500 mg/l), neomycin sulfate (1 g/l), and metronidazole (1 g/l) or flucytosine (0.5 mg/ml) in drinking water for 3 weeks. Fungal strains, Trichosporon asahii, Candida tropicalis, and Saccharomyces cerevisiae were purchased from China General Microbiological Culture Collection Center and were cultured in YM Broth Medium for 48 hr. Live fungi were purified by multiple washes with cold PBS and were delivered as PBS suspension into recipient mice by gavage. 48 hr later, mice were sacrificed and LNs and the LP were removed and single-cell suspension was prepared for flow cytometry staining.

Microbial Component-Driven RALDH+CD103+ Cell Migration

For depletion of intestinal bacteria or fungi, mice were given an antibiotic cocktail containing ampicillin (1 g/l), vancomycin (500 mg/l), neomycin sulfate (1 g/l), and metronidazole (1 g/l) or flucytosine (0.5 mg/ml) in drinking water for 3 weeks. Fungal strains, Trichosporon asahii, Candida tropicalis, and Saccharomyces cerevisiae were purchased from China General Microbiological Culture Collection Center and were cultured in YM Broth Medium for 48 hr. Live fungi were purified by multiple washes with cold PBS and were delivered as PBS suspension into recipient mice by gavage. 48 hr later, mice were sacrificed and LNs and the LP were removed and single-cell suspension was prepared for flow cytometry staining.

ExPERIMENTAL PROCEDURES

Mice

Tg (RARE-Hspa1b/lacZ)2Jlt/J strain was obtained from Jackson Laboratories and maintained as homozygotes to retain the reporter activity. These mice, along with C57BL/6, OT-II, Rag1−/−, Il1r1−/−, Nkb1−/−, Tlr4−/−, Myd88−/−, Ralda1−/−, and Ralda3−/− in C57BL/6 background and ICR (Swiss CD1) mice were bred and housed at Tsinghua University Animal Facilities. C57BL/6 and ICR GF mice were obtained from Shanghai Slac Laboratory Animal, the experimental animal center of the Third Military Medical University and the Institute of Laboratory Animal Sciences, Peking Union Medical College and were maintained in GF isolators, which were ventilated with sterile filtered air under positive pressure. GF mice were fed with autoclaved standard chow and water. For gut flora colonization, GF mice were transferred to regular isolators and co-housed with SPF mice for 1 week. For VAD mice, C57BL/6 pregnant females were given a TD.86143 diet (Harlan Laboratories), starting at 8–9 days of gestation, versus a control VitA-sufficient diet provided by the same manufacturer. The pups were weaned at 3 weeks of age and maintained on the same diet before analysis. The Animal Experiments Committee of Tsinghua University approved all of the experiments reported in this study.
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Supplemental Information

Peripheral Lymphoid Volume Expansion and Maintenance Are Controlled by Gut Microbiota via RALDH⁺ Dendritic Cells

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Supplemental Information for:

Peripheral lymphoid volume expansion and maintenance are controlled by gut microbiota via RALDH+ dendritic cells

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Supplemental Experimental Procedures

Adipose tissue isolation and implanting
Visceral epididymal adipose tissues were surgically removed from 8 week-old SPF C57BL/6 mice and implanted subcutaneously near iLN of 5 week-old GF C57BL/6 mice through standard sterile surgical operation performed in GF isolators. Anesthesia was induced by injection of a mixed zoletil (tiletamine + zolazepam) and xylazine at 50 and 20 mg per kg of body weight, respectively, prior to the surgery.

FITC-dextran gavage
2 week-old SPF C57BL/6 mice were fasted for 4 hrs, orally gavaged with FITC-dextran 2000KD (Sigma) at 0.3 mg per gram of body weight. 6 hrs later, iLN and mLN were collected; collagenase digested to single cells, and stained for CD103 and CD11b before flow cytometry analysis.

Intracellular cytokine stain
SPF C57BL/6 CD4+ T cells were stimulated with plate-bound anti-CD3e (1 µg/ml) + anti-CD28 (0.5ug/ml) antibodies for 3 hrs, followed by monensin + Brefeldin A (eBioscience) for additional 9 hrs. These activated CD4+ T cell were then stained for surface antigen for 30 min in flow cytometry buffer at 4°C. After washing, 1 ml of Permeabilization working solution (eBioscience) was added and the suspension was incubated at 4°C for 30 min. The cells were then pelleted in 100 µl of Permeabilization working solution, and anti IFN-γ and IL-4 antibodies were added for 30 min for cytokine detection by flow cytometry.

Confocal microscopy
Briefly, LN were fixed in 4% PFA overnight at 4°C, washed in PBS, cryo protected in 20% sucrose in PBS at 4°C overnight, embedded in OCT (Sakura) and cryo sectioned into 16 µm thick
sections. The sections were blocked for 1 hr (10% FBS in PBS) at room temperature and incubated with primary antibodies (1:50-100) overnight at 4°C. After washing, secondary antibodies were added for 1 hr at room temperature. Primary antibodies used were: murine PNAd (MECA-79), CCL21 (polyclonal goat IgG), CD3 (17A2), and B220 (RA3-6B2). Secondary antibodies were conjugated with AlexaFluor 488, 555, or 647 (1:200-500). Images were collected using a Zeiss 710 META confocal microscope. All raw images were processed with Image J and HEV lumen areas were calculated by the ruler transformed pixels. Adobe Illustrator was used for figure panel arrangement and labeling.

**Multiple cytokine detection**

Isolated RALDH+ and RALDH- cells were cultured in vitro, supernatants were collected after 24 hrs. Cytokines were measured with a Luminex 100 platform (Luminex) and analyzed with BioManager software (Bio-Rad). The following multiplexing kits were purchased from Millipore: Mouse Cytokine and Chemokine Panel I (MPXMCYTO-70K) containing Eotaxin, G-CSF, GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IFN-γ, IP-10, KC, LIF, LIX, CCL2, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α and VEGF. Kits were run according to the manufacturer’s instructions.

**Real time PCR**

RALDH+ and RALDH- cells were flow cytometry sorted from mLN and pLN of 2 week-old SPF mice. 2 x 10^5 cells were used to extract RNA using an E.Z.N.A. MicroElute Total kit (Omega bio-tek) according to the manufacturer’s protocols, and cDNA was synthesized using a PrimeScript cDNA synthesis kit (Takara). Primers for RALDH2 and GAPDH were purchased from OriGene. Real-time PCR was performed with SYBR Green Master Mix (Takara) on a CFX96 realtime PCR detection system (Bio-Rad). The comparative CT method (Delta CT) was used to assess relative changes in mRNA abundance between samples. Expression of transcripts was normalized to GAPDH. Relative expression of RALDH2 transcript in one sample of RALDH- cells (lowest) was set at 1, which served as the base for other relative expressions.

**Co-culture and gut homing assay**

Briefly, flow cytometry-isolated iLN CD11c+RALDH-, iLN CD103+CD11b+RALDH+ (neo-mi DCs), mLN RALDH+CD11b+CD103+ cells from 2 week-old SPF C57BL/6 were loaded with OVA (200 µg/ml) for 4 hrs, then mixed with CD4+OT-II splenocytes (DC/T ratio 1:4) isolated with EasySep mouse CD4+ T cell enrichment kit (StemCell), and co-cultured for 6 days. Expression of CCR9 and α4β7 was analyzed by flow cytometry on cells in the CD4+ gate. 2 x 10^6 activated OT-II splenocytes were labeled with CFSE and mixed with the same number of purified naive SPF C57BL/6 CD4+ T cells labeled with DDAO and injected i.v. into 6 week-old SPF C57BL/6 mice. After 18 hrs, recipient mouse LN and LP were harvested to measure CFSE/DDAO ratios by flow cytometry. The data is expressed as the Homing Index (HI), as previously described (Hall et al., 2011).

**Cell isolation and flow cytometry**
LN single cell suspension was achieved by frosted cover slide grinding. PP were excised from small intestine, digested in 5% FBS RPMI 1640 with 0.1% collagenase I/0.05% dispase (Sigma) for 1 hr at 37°C. The remaining tissue was incubated with 1 mM DTT, 5 mM EDTA, 5% FBS PBS for additional 30 min at 37°C before intraepithelial lymphocyte removal. Residual intestinal tissue was digested for LP cell isolation. RALDH activity in individual cells was measured using an Aldefluor kit according to the manufacturer’s protocol. 7.5 µM of DEAB was added in different tubes at 37°C for 15 min as Aldefluor fluorescent baseline control. For conventional flow cytometry analysis, primary antibodies at 1 µg/ml were used to stained target cells at 4°C for 30 min followed by fluorophore-conjugated secondary antibodies and washed three times with FASC buffer (2% FBS + PBS). Flow cytometry was performed on Accuri C6 (BD Biosciences) and analyzed using FlowJo software (Tree Star). Percentage values were charted with Graphpad Prism.

Adoptive cell transfer
Pooled-single cell suspension of iLN and aLN were either first purified for marker-positive populations as described or directly labeled with 5 µM CFSE for 15 minutes at 37°C. After washing twice with PBS containing 5% FBS, cells at the indicated number per mouse were injected into the tail vein of recipients. Lymphocyte transfer to GF mice was conducted in GF isolator (over the fixed gloves). After the injection suspensions were prepared, they were passed into the isolator through germicidal dip tank filled with 3% Peracetic acid. After tail vein injection, the mice were maintained in GF isolator until analysis. The GF status was confirmed at the time of sacrifice by enlarged cecum, microbial culture and 16s rDNA PCR amplification of feces (not shown). BMDCs were produced from C57BL/6 bone marrow in the presence of 5 ng/ml of GMC-CSF and IL-4 and harvested after a 5 day culture. The cells were stained by Aldefluor and the negative population was sorted for the assay.

Cell sorting
For isolation of pLN (neo-miDCs) and mLN CD103+CD11b+RALDH+ cells and their RALAH- control, 2 week-old C57BL/6 iLN, aLN and mLN were digested in 5% FBS RPMI 1640 with 0.1% collagenase I/0.05% dispase for 1 hr at 37°C. After washing twice with 5% FBS RPMI 1640, single cells were labeled with Aldefluor fluorescent substrate for 15 minutes at 37°C in the provided assay buffer. Aldefluor-labeled cells were incubated with PE or APC-conjugated anti-mouse CD103, CD11b or CD11c antibodies at 4°C to prevent the reduction of Aldefluor fluorescence. Labeled lymphocytes were sorted by Influx cell sorter (BD) at 4°C. Occasionally, mLN or LP from 6 week-old C57BL/6 mice were used for isolation of RALDH+CD11b+CD103+ cell, as indicated. In all experiment, a separate cell suspension was treated with DEAB and used as Aldefluor fluorescent baseline control.

β-galactosidase activity
Isolated RALDH reporter and control CD1 LN were digested into single cells, incubated with DDAOG (10 µM) in Hanks’ buffer for 2 hrs at 37°C. The cells were washed three times with Hanks’ buffer before flow cytometry analysis. Upon excitation, DDAO generates a far-red-shifted fluorescent signal that can be analyzed by Flow cytometry.
cDNA library preparation and Illumina sequencing

Total tissue RNA from pLN of 5 week-old GF or SPF C57BL/6 was extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. Poly (A)⁺ RNA was purified from 5 μg of pooled total RNA using oligo(dT) magnetic beads, sheared into short fragments, and primed for cDNA library synthesis using the TruSeq RNA sample preparation kit per Illumina protocol. After quantitation using a TBS-380 mini-fluorometer (PicoGreen), the samples were clustered (TruSeq paired-end cluster kit, v3-cBot-HS; Illumina) and sequenced on the HiSeq2000 platform (100 bp, TruSeq SBS kit v3-HS 200 cycles; Illumina).

Annotations for the entire data set were obtained with TopHat, and followed by statistical analysis with Cufflink (Cuffdiff module). FKPM values with $X^2$ less than 0.05 were extracted and logarithmized by 2. The heat maps were generated by HemI (Heatmap Illustrator) software.

DEAB treatment and anti-MAdCAM-1 antibody injection

2 week-old SPF C57BL/6 mice were injected i.p. with DEAB (100 μg/ per gram of weight, dissolved in 50 ul DMSO) 3 to 6 times (3 times/week) and the control groups were injected with DMSO alone. For MAdCAM-1 blocking, anti-MAdCAM-1 antibody or rat IgG was injected i.p. into neonatal mice (40 μg/mouse) for four times every other day starting at 6 days of age.

Reference

Figure S1: related to Figure 1

A

B

C

D

E

Sham

Transplantation
Figure S1 (related to Figure 1). Adipose tissue regulation and RA signaling are involved
in pLN volume and cellularity development

(A) Related to Figure 1B, the HEV$^+$ area size per lumen and HEV number per
node are shown. (B) The overall heatmap showing the comparison between 5 week-old
C57BL/6 GF and SPF mice. mRNA from pLN (pooled) was extracted and subjected to
RNAseq mRNA transcriptome analysis as indicated in the methods. (C) Additional
functional groups that were different in GF and SPF mice: chemotaxis and metabolism.
(D) The same data used in Figure 1C are displayed in bar graph. (E) The visceral fat
tissues were harvested under sterile conditions and transplanted to inguinal area of GF
mice under GF confinement. Photos were taken 2 weeks after the operation and the
sham-operated site is shown as the control. The arrow indicates the expanded LN. All
operated GF mice were determined to remain GF by enlarged colon, feces culture and
lack of 16S RNA by PCR at the time of photographing.
Figure S2: related to Figure 2
Figure S2 (related to Figure 2). RALDH activities are robust in neonatal iLN and mLN.

(A) Quantitative PCR for RALDH2 in mRNA isolated from RALDH+ cells in iLN and mLN of 2 week-old SPF mice.  (B) The overall heatmap showing the comparisons of mRNA expression levels in pooled pLN from 5 week-old ICR GF and identical GF mice 1 week after cohousing with SPF mice.  (C) Left panel: the heatmap for genes associated with RA signaling, derived from B.  Right panel: the same data used in the left panel are displayed in bar graph.  (D) The presence of CD45+RALDH+ cells in the mLN and PP was not affected by the absence of NFkb (classical), Myd88, TLR4 and IL-1 receptor. The percentage of these cells in 2 week-old Nfkb1−/−, Myd88−/−, Tlr4−/− and Il1r1−/− SPF mice were compared with WT mice.
Figure S3: related to Figure 3
Figure S3 (related to Figure 3). Phenotypical characterization of CD45^RALDH^ cells

(A) FACS analysis of CD45^RALDH^ cells. The top panels show the FSC and SSC gating and the population in the oval in the left panel was analyzed in the right panel. The RALDH^+ box in the top right was stained by three different pairs of antibodies in the bottom panels. (B) Supplemental to Figure 3E, shown are the additional staining for F4/80, complement receptor 1 (CR1), mannose receptor (MR), ICAM-1, VCAM-1, CD11a, CD80, CD86, CD83, CD127, CCR7, CD45RB, CD19, CD8, MHC class II and Ly6G. The lack of heterogeneity in the expression of specific markers in RALDH^− cells was due to the suboptimal gating in our pre-gating to isolate RALDH^+ cells for best analysis of respective markers on these cells. (C) Supplemental to Figure 3F, shown are the additional cytokine comparisons that did not reach statistical significance. (D) CD45^RALDH^+ cells from iLN and mLN of 2 week-old SPF mice were stained for a panel of surface markers to identify any differences in expression. Secondary antibody-only staining of CD45^RALDH^+ cells was used as the background control. (E) Similar to A except that only 2 week-old LN were used and CD11b expression was also analyzed.
Figure S4: related to Figure 4

A

B

C

D

CD103^+RALDH^+ %

CD103^+RALDH^+ %

CD103^+RALDH^+ %

NS

**

0

2

4

6

NS

0

2

4

6

NS

0

2

4

6

NS

0

2

4

6

NS

0

2

4

6

NS

0

2

4

6

NS

0

2

4

6

NS

0

2
Figure S4 (related to Figure 4). CD103⁺RALDH⁺ DCs from the gut are the likely the driving force of pLN development

(A) 10⁵ Purified CD103⁺CD11c⁺RALDH⁻ LP cells were either untreated or incubated with 5 x 10⁵ boiled *C. tropicalis* for 24 hrs. The resulting CD11c⁺/PI⁻ population was stained for RALDH expression. Triplicates were analyzed and no RALDH⁺ cell was found. (B) Similar to A except 10⁶ BMDCs were either untreated or incubated with the same number of boiled *C. tropicalis* for the same duration. Chart graphs to summarize multiple repeats are shown. (C) LP and iLN of control or anti-fungi-treated (for 2 weeks) mice were gated on CD11c⁺ and stained for CD103⁺RALDH⁺ expression. (D) 2 week-old SPF mice were force-fed with PBS or 10⁶ live *C. tropicalis* in PBS suspension. 48 hrs later, the percentage of CD103⁺RALDH⁺ cells in LP and iLN was determined by FACS.
Figure S5: related to Figure 5

A

B

C

D

Figure S5: related to Figure 5

A

B

C

D
Figure S5 (related to Figure 5). Neo-mi DCs imprint pLN lymphocytes for gut homing 

(A) Lymphocytes from iLN of GF and SPF mice were stained for α4β7 expression. The positive percentage values were graphed. (B) The detection of CD103+ cells in iLN of 2 week-old Rag1−/− mice. (C) CFSE-labeled neo-mi DCs were i.v. injected into anti-MAdCAM-1-pretreated or control 2 week-old SPF mice. 24 hrs later, the presence of these cells in iLN was analyzed by FACS. The lower panel shows the result from multiple assays. (D) Related to Figure 5C. Operations were identical to Figure 5A with anti-MAdCAM-1 injection starting from the birth. HEV numbers from 2 week-old iLN were counted. Rat Ig was used as control.
Figure S6: related to Figure 7
Figure S6 (related to Figure 7). Vitamin A deficiency is associated with the lack of
tonal homing of neo-mi DCs to pLN

(A) The body weight of DEAB-treated SPF mice 6 days from the first dose
(one dose every other day) in comparison with the untreated mice. (B) Sizes of iLN 6
days into DEAB treatment; untreated and DMSO-treated are displayed as controls.
(C) CFSE-labeled total lymphocytes from the pooled pLN of VAD and control fed
mice were i.v. injected into 5 week-old SPF mice; their and the CD4+, CD8+, CD19+
subset percentages in iLN, mLN and the spleen were determined after 24 hrs. (D) 5
week-old SPF mice were i.p. injected with DEAB 3 times once every other day, or
received one injection and then rested for two day (to induce a LN structural attrition
as in the 3 injection group, yet allowing RALDH+ cells to recover). The iLN were
isolated and single cell suspension was stimulated with plate-bound anti-CD3. 24 hrs
later, intracellular IFN-γ+ and IL-4+ CD4+ cells were analyzed by FACS and the
percentage values are shown.
Supplemental Data S1. Related to Figure 1. RNAseq results of GF vs SPF C57BL/6 mice.
The total dataset for the RNAseq described in Figure 1, with statistical analyses provided in the table.

Supplemental Data S2. Related to Figure 2. RNAseq results of GF vs SPF ICR mice.
Similar to Supplemental Data S1 with data from ICR mice.