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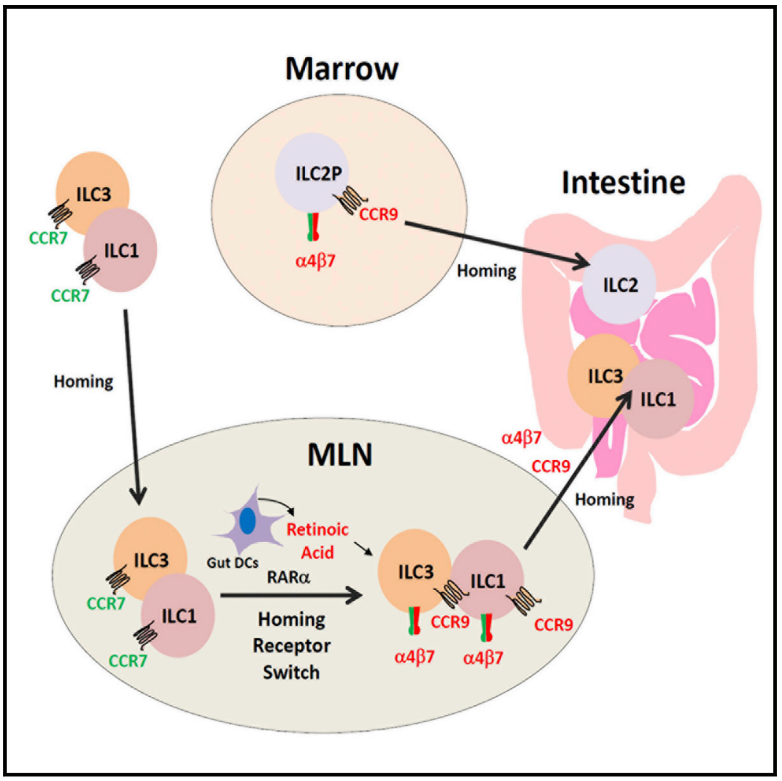
Immunity

Retinoic Acid Differentially Regulates the Migration of Innate Lymphoid Cell Subsets to the Gut

Graphical Abstract



Highlights

- Innate lymphoid cells undergo a homing receptor switch to migrate to the gut
- This process is regulated by the gut-specific tissue factor retinoic acid
- Only ILC1 and ILC3, but not ILC2, undergo the homing receptor switch
- ILC2 acquire gut homing receptors during their development in the bone marrow

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In Brief

The intestine is populated with innate lymphoid cells (ILCs) but how these cells acquire tissue tropism is unclear. Kim and colleagues show that group 1 ILCs (ILC1) and ILC3 undergo retinoic-acid-dependent upregulation of trafficking receptors, whereas ILC2 acquire gut homing receptors during development in the bone marrow.

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Retinoic Acid Differentially Regulates the Migration of Innate Lymphoid Cell Subsets to the Gut

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SUMMARY

Distinct groups of innate lymphoid cells (ILCs) such as ILC1, ILC2, and ILC3 populate the intestine, but how these ILCs develop tissue tropism for this organ is unclear. We report that prior to migration to the intestine ILCs first undergo a "switch" in their expression of homing receptors from lymphoid to gut homing receptors. This process is regulated by mucosal dendritic cells and the gut-specific tissue factor retinoic acid (RA). This change in homing receptors is required for long-term population and effector function of ILCs in the intestine. Only ILC1 and ILC3, but not ILC2, undergo the RA-dependent homing receptor switch in gut-associated lymphoid tissues. In contrast, ILC2 acquire gut homing receptors in a largely RA-independent manner during their development in the bone marrow and can migrate directly to the intestine. Thus, distinct programs regulate the migration of ILC subsets to the intestine for regulation of innate immunity.

INTRODUCTION

Innate lymphoid cells (ILCs) do not have antigen receptors but produce effector cytokines in a manner reminiscent of T cells (Spits et al., 2013; Tait Wojno and Artis, 2012; Walker et al., 2013). ILCs play important roles in host defense against pathogens and can mediate tissue inflammation. Three groups of ILCs with distinct characteristics in development and effector function have been identified. Group 1 ILCs (ILC1), including NK cells, are induced by the transcription factor T-bet (Tbx21) and produce interferon-γ (IFN-γ) (Bernink et al., 2013; Klose et al., 2013; Powell et al., 2012; Sciumé et al., 2012). ILC2, which produce interleukin-5 (IL-5) and IL-13, require transcription factors RORα, Gata3, and Tcf7 for development (Halim et al., 2012; Hoyler et al., 2012; Klein Wolterink et al., 2013; Liang et al., 2012; Mjösberg et al., 2012; Mjösberg et al., 2011; Moro et al., 2010; Neill et al., 2010; Nussbaum et al., 2013; Price et al., 2010; Yang et al., 2011). ILC3 require RORγt and Gata3 for development and are composed of IL-17- and IL-22-producing lymphoid tissue-inducers, IL-17- IL-22- and IFN-γ-producing ILCs, and IL-22-producing natural cytotoxicity triggering receptor $(NCR)^+$ ILCs (Luci et al., 2009; Possot et al., 2011; Satoh-Takayama et al., 2008; Serafini et al., 2014; Sonnenberg et al., 2011; Takatori et al., 2009; Vonarbourg et al., 2010). ILC3 also produce GM-CSF for immune regulation (Mortha et al., 2014). The importance of ILCs in mounting innate immunity and regulating inflammatory responses in the intestine and other mucosal tissues is now widely accepted.

Migration of lymphocytes is regulated by chemokines and integrins (Agace, 2008; Gorfu et al., 2009; Mora and von Andrian, 2008; Sheridan and Lefrançois, 2011). Naive T cells utilize CCR7 and CD62L to migrate to secondary lymphoid tissues. Upon antigen priming, T cells undergo homing receptor switches in a tissue-specific manner. Gut-homing T cells downregulate CCR7 and CD62L but upregulate the chemokine receptor CCR9 and the integrin α4β7 (Iwata et al., 2004; Kang et al., 2007). $CCR9^+\alpha4\beta7^+$ cells migrate to the small intestine (SI), while $CCR9^{-/dim}\alpha4\beta7^+$ cells migrate into the colon. Acquisition of these receptors occurs in gut-associated lymphoid tissues such as mesenteric lymph node (MLN) and Peyer's patches (PP). CCL25, a chemokine expressed in the SI, activates CCR9 for adhesion triggering and chemotaxis (Kunkel et al., 2000; Wurbel et al., 2000; Zabel et al., 1999). α4β7 binds MAdCAM-1, which is widely expressed on gut endothelial cells. It is generally accepted that innate immune cells do not undergo such a sophisticated homing receptor switch for specific migration into a tissue site. For example, NK cells, which arise in the bone marrow (BM), upregulate inflammation-associated homing receptors to directly migrate to peripheral tissues (Morris and Ley, 2004; Walzer and Vivier, 2011). It has been reported that certain subsets of ILCs are characterized by their expression of several trafficking receptors such as CCR6, CCR7, CCR9, CXCR6, and/or α4β7 (Bouskra et al., 2008; Klose et al., 2013; Possot et al., 2011; Satoh-Takayama et al., 2014). However, how ILC subsets migrate and populate the intestine remains largely unknown.

We report here that ILCs undergo homing receptor switches in a shared yet subset-specific manner. ILC1 and ILC3 undergo a homing receptor switch, which is regulated by retinoic acid (RA) in the periphery, whereas ILC2 are developmentally programmed to upregulate gut homing receptors in BM and directly migrate to the intestine. Hence, ILC have sophisticated and

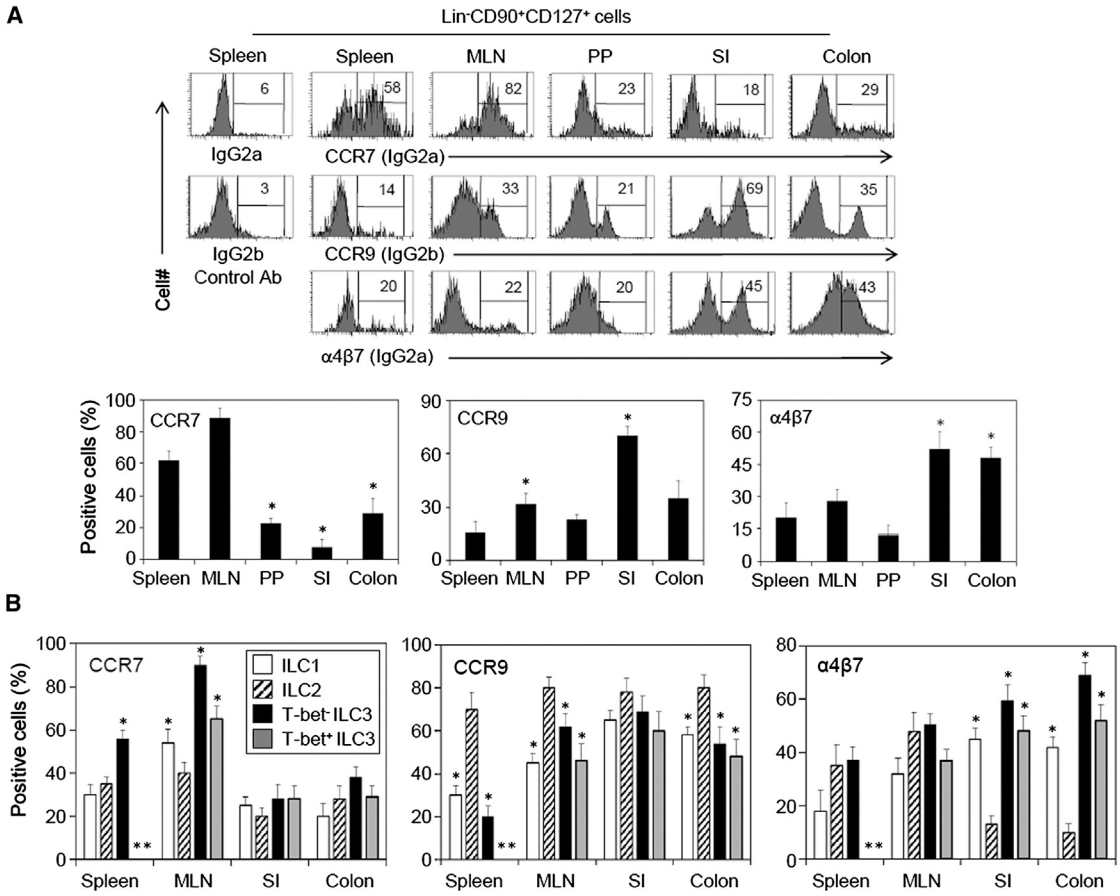

Figure 1. Homing Receptor Expression by ILCs in Lymphoid and Intestinal Tissues

(A) Expression of CCR7, CCR9, and α4β7 by ILCs in secondary lymphoid tissues and the intestine. Total ILCs (Lin$^-$CD90$^+$CD127$^+$) in the indicated organs and intestinal lamina propria of C57BL/6 mice (n = 10) were examined for expression of CCR7, CCR9, and α4β7. Isotype (IgG2a/b) antibodies were used for negative control staining.

(B) Expression of homing receptors by ILC subsets in the spleen, MLN, SI, and colon. Data from *Rag1*$^{-/-}$ or C57BL/6 mice were essentially identical and combined (n = 15). Gating strategies for ILC1 (Lin$^-$CD127$^+$CD90$^+$Eomes$^-$RORγt$^-$T-bet$^+$), ILC2 (Lin$^-$NK1.1$^-$CD127$^+$CD90$^+$Sca-1$^+$KLRG-1$^+$GATA-3$^+$), and ILC3 (T-bet$^-$ or T-bet$^+$ Lin$^-$CD127$^+$CD90$^+$RORγt$^+$GATA-3$^-$) subsets are shown in Figure S1. The "Lin" antibody mixture refers to the "common lineage markers" described in the Experimental Procedures section. *Significant differences ($p < 0.05$) from spleen (A) or ILC2 (B). **The spleen T-bet$^+$ ILC3 population was very small (~0.1% of Lin$^-$CD127$^+$CD90$^+$ cells) and not shown. All error bars represent SEM.

heterogeneous migration programs, which are important to maintain the numbers and activities of different ILC subsets in the gut.

RESULTS

ILCs Display Subset- and Tissue-Specific Expression of Homing Receptors

Tissue-specific expression of homing receptors and their ligands is important for lymphocyte migration. To gain insights into tissue-specific expression of homing receptors by ILCs, we examined the expression of CCR7, CCR9, and α4β7 by T, B, NK, myeloid, and other lineage-excluded (Lin$^-$) ILCs in various tissues. CCR7 was highly expressed by the Lin$^-$CD90$^+$CD127$^+$ ILCs in spleen and mesenteric lymph node (MLN), whereas CCR9 was highly expressed by the ILCs in SI (Figure 1A). Many CCR7$^+$ ILCs were detected in lymphoid tissues such as PP and colon. Many CCR9$^+$ ILCs were detected in SI. MLN and colon also had some CCR9$^+$ ILCs. α4β7 was highly expressed by both SI and colon ILCs. These data indicate that ILCs express homing receptors in a tissue-specific manner. Because ILCs are subdivided into ILC1, ILC2, and T-bet$^-$ or T-bet$^+$ ILC3 populations, we also examined homing receptor expression by these subsets individually (Figure S1). CCR7 is highly expressed by most T-bet$^-$ ILC3 and many T-bet$^+$ ILC3 and ILC1 in MLN (Figure 1B). Many CCR7$^+$ T-bet$^-$ ILC3 were found in MLN and spleen. Many CCR9$^+$ T-bet$^-$ ILC3 were found in SI and MLN. Many α4β7$^+$ T-bet$^-$ ILC3 were found in the intestine. Overall, T-bet$^+$ ILC3 were similar to T-bet$^-$ ILC3 and ILC1 (Figure 1B). The expression of CCR7 by ILC2 was low in all of the organs. CCR9 expression was uniformly high on ILC2 of all organs (Figure 1B). Significant numbers of ILC2 in spleen and MLN but not in the intestine expressed α4β7. Thus, the ILC subsets have similarities and differences in homing receptor expression. Similar results were observed in *Rag1*$^{-/-}$ and WT C57BL/6 mice (not shown).

Homing-Receptor-Deficient Mice Have Abnormal Numbers of ILC Subsets in a Receptor- and Tissue-Specific Manner

Because ILCs expressed tissue and subset-dependent homing receptors, we next examined the numbers and frequencies of ILCs in lymphoid tissues and the intestine of distinct homing receptor-deficient mice. ILC frequencies and numbers were greatly decreased in the MLN of *Ccr7*$^{-/-}$ mice (Figure 2A), as well as in the SI and colon although to a lesser degree. ILC numbers and frequencies were decreased specifically in the SI of *Ccr9*$^{-/-}$ mice (Figure 2A). ILCs were decreased in both the SI and colon of *Itgb7*$^{-/-}$ mice. In contrast, ILCs were compensatorily increased in the lymphoid tissues of *Ccr9*$^{-/-}$ or *Itgb7*$^{-/-}$ mice (Figure 2A).

When ILC subsets were examined (Figures 2B and S2), a clear difference was observed between the tissue distribution of ILC2 and other ILC subsets. ILC2 were decreased in the SI of *Ccr9*$^{-/-}$ mice, but not in other homing receptors-deficient mice analyzed. In contrast, ILC1 and ILC3 were decreased in the MLN of *Ccr7*$^{-/-}$ mice, SI of *Ccr9*$^{-/-}$ mice, and colon of *Itgb7*$^{-/-}$ mice. ILC1 and ILC3 were somewhat different from each other in their reliance on homing receptors in that ILC3 numbers in the SI and colon were more affected by Itg-β7 deficiency than their ILC1 counterparts. Laser scanning confocal microscopy confirmed that CD90^{+} CD3^{-} ILCs were reduced in frequency in the SI villi of CCR9- or Itg-β7-deficient mice (Figure 2C). Many intraepithelial ILC1 expressed CCR9 but few expressed α4β7 and CD103 (Figure S3A). In CCR9-deficient mice, ILC1 numbers were moderately decreased in the intraepithelial compartment of the SI, but not in the colon (Figure S3B). Taken together, the results indicate that the three homing receptors analyzed variably affect the normal population of ILC subsets in lymphoid tissues or in the intestine.

RA Induces CCR9 and α4β7 Expression on ILC1 and ILC3 But Does Not Alter ILC2 CCR9 Expression

Because RA is known to induce gut homing receptors in T cells and B cells (Iwata et al., 2004; Kang et al., 2007; Mora et al., 2006; Wang et al., 2010), we tested whether RA also induces the expression of CCR9 and α4β7 in ILCs. Spleen Lin^{-} cells were cultured with RA for 4 days before assessing homing receptor expression on ILCs. RA efficiently induced CCR9 and α4β7 expression on ILCs while reducing CCR7 expression (Figure 3A). Co-staining of CCR7 and gut homing receptors revealed that RA induces a switch from CCR7^{+} CCR9^{-} α4β7^{-} to CCR7^{-} CCR9^{+} and/or α4β7^{+} expression in CD90^{+}CD127^{+} ILCs (Figure 3B). Because the composition of ILC subsets changes after exposure to RA (Spencer et al., 2014), the induction of gut homing receptors by RA was also examined on individual ILC subsets. Induction of gut homing receptors was detected on ILC1 and ILC3, but not on ILC2 in response to RA (Figure 3C). The transcription factors RARα, RARβ, and RARγ are expressed by ILCs (Mielke et al., 2013). In our study, RARα was most highly expressed by ILCs (data not shown). AM580, an RARα agonist, had clear effects on inducing gut homing receptors in ILC3 (Figure 3D) and ILC1 (data not shown). LE540 (a pan-RAR antagonist) and Ro41-5253 (an RARα antagonist) further decreased the basal expression levels of CCR9 and α4β7 on cultured ILC3 (Figure 3D) and ILC1 (data not shown). Consistent with CCR9 expression, the chemotactic response of ILC3 and ILC1 to the CCR9 ligand CCL25 was increased after culture with RA, but was suppressed by Ro41-5253 to the *Ccr9*$^{-/-}$ level (Figure 3E). ILC2 migrated to CCL25 efficiently, and this was not further regulated by exogenous RA or Ro41-5253. Overall, the homing receptor expression and chemotactic responses of ILC subsets indicate there is a clear difference between ILC1, ILC3, and ILC2.

A major cell source of RA in the intestine is dendritic cells (DCs) (Coombes et al., 2007; Iwata et al., 2004). DCs express retinal aldehyde dehydrogenases (RALDHs), which convert retinaldehyde to RA. We compared the activity of MLN and spleen DCs in regulating the homing receptor switch in ILCs. MLN DCs were more efficient than splenic DCs in downregulating CCR7 and upregulating CCR9 and α4β7 on ILC3 and ILC1 (Figure 4A,B). The RARα antagonist Ro41-5253 blocked the CCR7→CCR9 and α4β7 switch induced by MLN DCs, suggesting a role for RARα (Figure 4A). Moreover, DEAB (an RALDH inhibitor that blocks de novo production of RA from retinaldehyde) effectively suppressed the switch in ILC3 (Figure 4A) and ILC1 (Figure 4B). These results indicate that the RA derived from mucosal DCs can induce the homing receptor switch in ILC3 and ILC1.

Vitamin A Deficiency Leads to Gut Homing-Receptor Deficiency in ILC1 and ILC3, but Not ILC2

The positive role of RA in regulating the gut homing-receptor expression in ILCs prompted us to examine the homing receptor phenotype of ILCs in vitamin A-deficient (VAD) *Rag1*$^{-/-}$ mice. These mice were generated by feeding mice with VAD or normal (VAN) diet containing a standard level (2,500 IU/Kg) of retinol for 11–12 weeks. ILC3 and ILC1 in the SI of VAD mice had decreased levels of CCR9 and α4β7 expression. However, their expression of CCR7 was somewhat increased compared to their counterparts in VAN mice (Figure 4C). In contrast, the homing-receptor phenotype of ILC2 was unaffected by vitamin A deficiency (Figure 4C). In addition to gut homing receptors, total ILC numbers were decreased by 2–3 times in the SI and colon of VAD mice (data not shown). Our confocal imaging analysis revealed that ILCs numbers were reduced in the intestinal lamina propria of VAD *Rag1*$^{-/-}$ mice (Figure 4D).

BM ILC2 Progenitors Express Gut Homing Receptors in a RA-Independent Manner and Can Directly Migrate to the Intestine

It has been reported that ILC2 progenitors (ILC2Ps) express gut homing receptors (Hoyler et al., 2012). To ask whether expression of gut homing receptor by ILC2P in BM requires vitamin A metabolites, we examined gut homing receptor expression by ILC2P in the BM of VAD mice. Gut homing-receptor expression by BM ILC2P was not affected in VAD mice (Figure 5A), suggesting that the gut homing receptors of ILC2P are not regulated by vitamin A, unlike ILC3 and ILC1. We transferred BM cells into CD45.1 congenic mice for a short-term migration assay to determine whether these Lin^{-}CD127^{+}Sca-1^{hi}CD25^{+}KLRG-1^{-} ILC2P and related KLRG-1^{+} cells (Figure S4A) can migrate to the

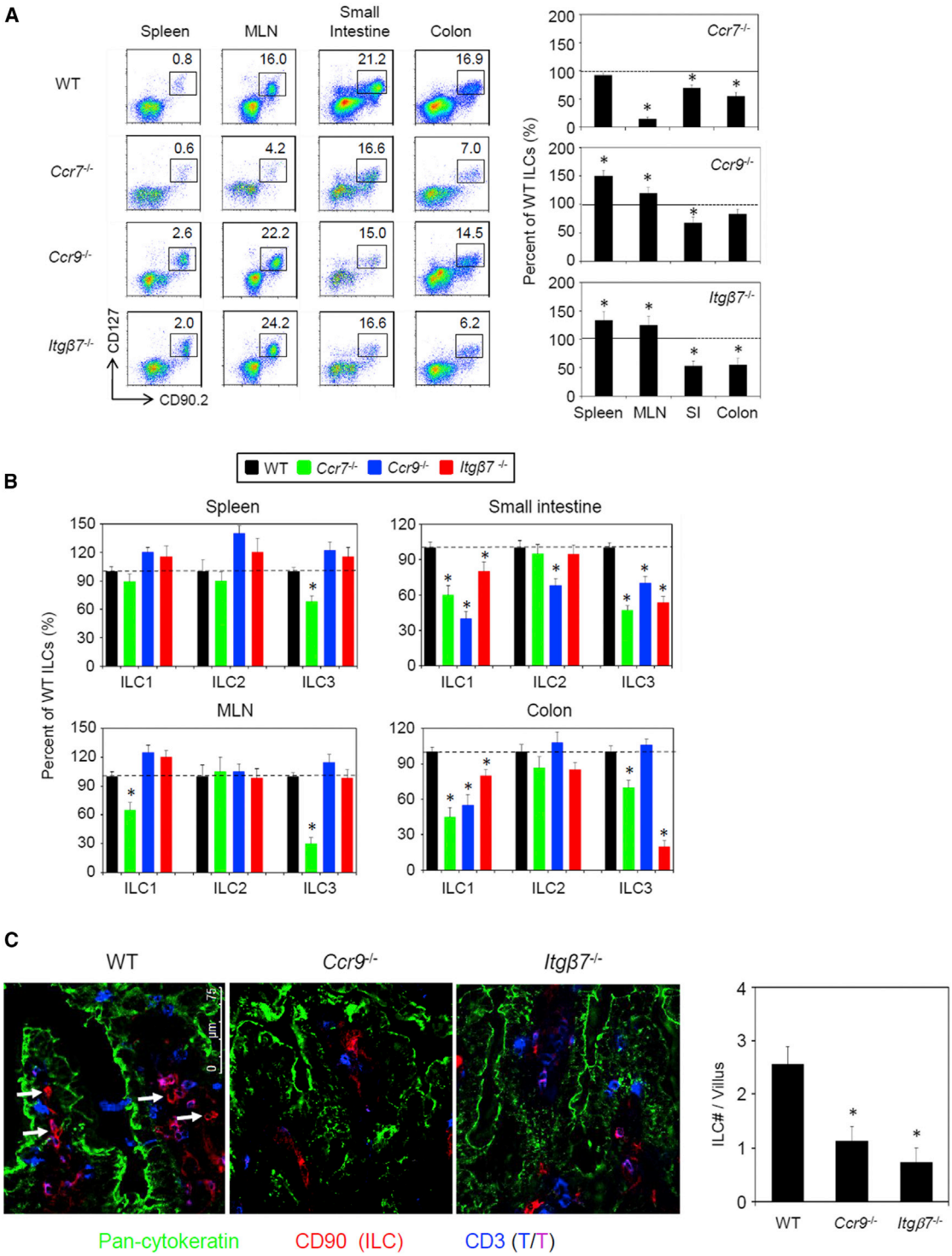

Figure 2. CCR7, CCR9, and Itg-β7 Are Required for Normal Numbers of ILCs in a Tissue-Specific Manner

(A) Frequencies of Lin$^-$CD90$^+$CD127$^+$ total ILCs in lymphoid tissues and the intestine of WT, *Ccr7*$^{-/-}$, *Ccr9*$^{-/-}$, or *Itgb7*$^{-/-}$ mice.

(B) Frequencies of ILC subsets (ILC1, ILC2, and ILC3) in lymphoid tissues and the intestine of WT, *Ccr7*$^{-/-}$, *Ccr9*$^{-/-}$, or *Itgb7*$^{-/-}$ mice. (C) CD3$^-$CD90$^+$ ILCs in the SI of WT, *Ccr9*$^{-/-}$, or *Itgb7*$^{-/-}$ mice were imaged by laser scanning confocal microscopy. Average numbers of CD3$^-$CD90$^+$ ILCs per villus and section are shown in the graph. The dot plots are shown for the Lin$^-$ cell gate, and the graphs are relative numbers of ILCs in *Ccr7*$^{-/-}$, *Ccr9*$^{-/-}$, or *Itgb7*$^{-/-}$ mice compared to WT mice (% of WT mice). The "Lin" mixture refers to the "common lineage markers." Pooled data obtained from at least three different experiments (n = 15) are shown. *Significant differences ($p < 0.05$) from WT. All error bars represent SEM.

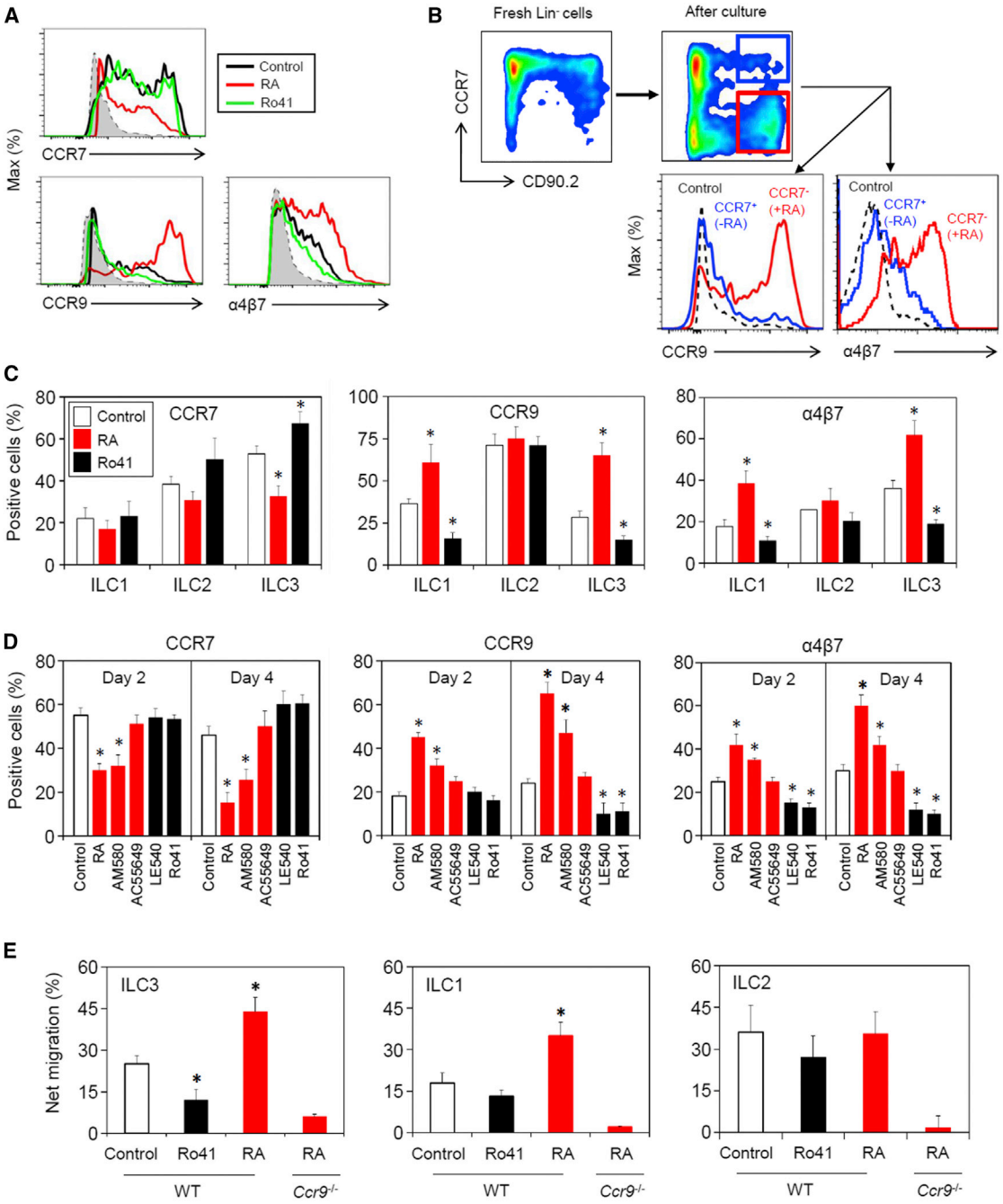

Figure 3. RA Promotes Loss of CCR7 but Gain of CCR9 and α4β7 Expression in ILC3 and ILC1 but Not ILC2

(A) Induction of gut homing receptors and downregulation of CCR7 on ILCs by RA. Expression of homing receptors on Lin$^-$CD90$^+$CD127$^+$ ILCs was assessed after the culture of spleen Lin$^-$ cells for 4 days with RA or Ro41-5253 (an RARα antagonist).

(B) Induction of a homing receptor switch from CCR7 to CCR9 and α4β7 in cultured ILCs by RA.

(C) Impact of RA and Ro41-5253 on homing receptor expression by ILC subsets.

(D) Impact of RAR agonists and antagonists on homing receptor expression by ILC3 in vitro.

(E) Chemotaxis of control, RA, or Ro41-5253-treated ILC subsets to CCL25. Spleen Lin$^-$ (or sorted ILC3 for panel C) cells were cultured with cecal bacterial extract, IL-7, and IL-15 for ILC1; IL-7 for ILC2; cecal bacterial extract, IL-7, and IL-23 for ILC3. The "Lin" mixture refers to the "common lineage markers." Pooled data obtained from at least three different experiments are shown. *Significant differences ($p < 0.05$) from controls. All error bars represent SEM.

intestine. Forty four hr after transfer, ~20% of the BM KLRG-1$^-$ ILC2P and ~30% of KLRG-1$^+$ ILC2 lineage cells were found in the SI and other tissues of the host mice among the tissues examined (Figures 5B and S4B), indicating that BM ILC2 progenitors can migrate to the intestine. They also migrated to MLN and colon but less efficiently to lungs. No significant migration to

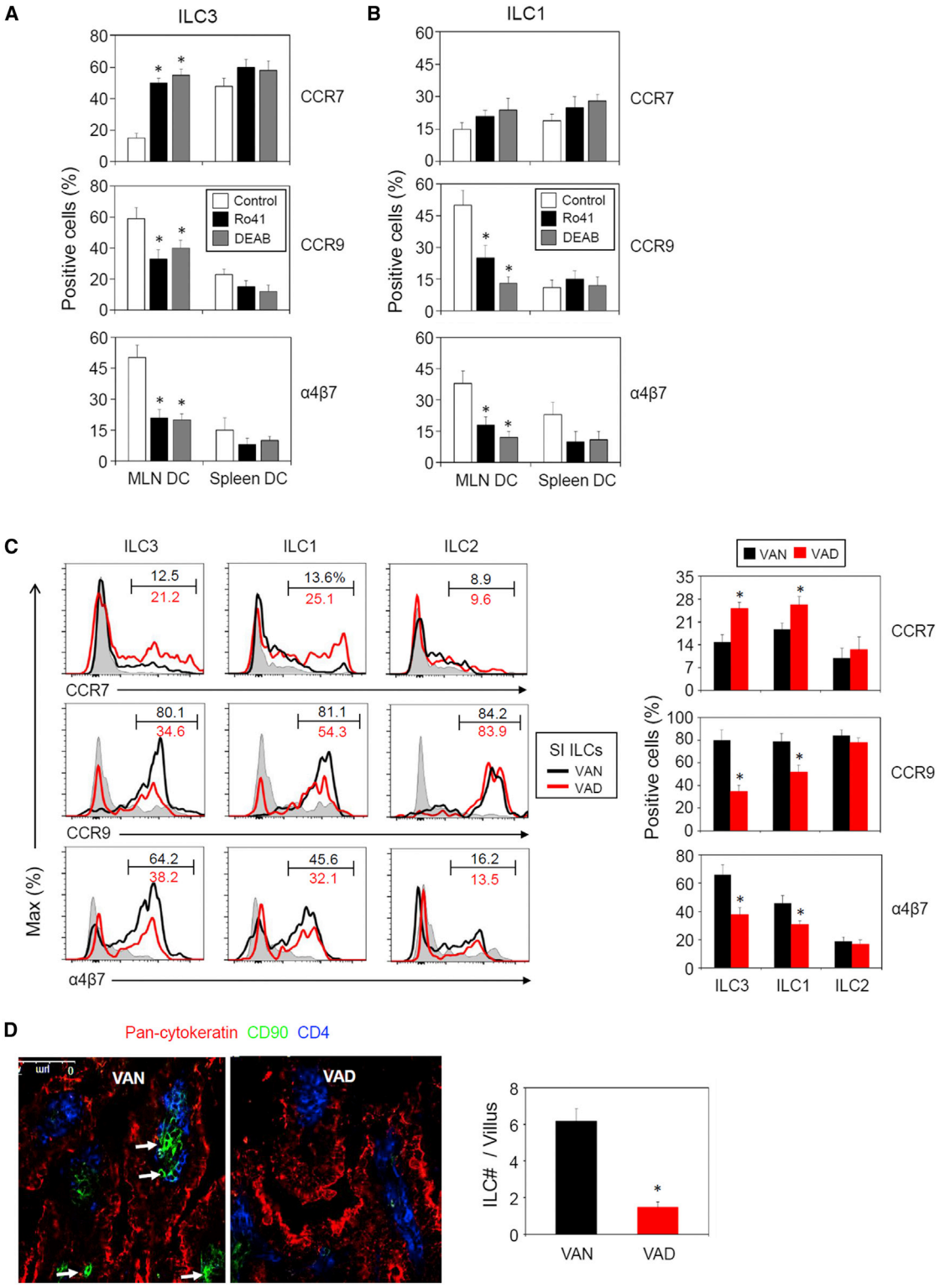

Figure 4. Mucosal DCs and Vitamin A Status Regulate the Expression of Homing Receptors by ILC3 and ILC1

(A) Mucosal DCs induce the homing receptor switch in ILC3.

(B) Mucosal DCs induce the homing receptor switch in ILC1. $CD11c^{+}$ DCs, freshly isolated from spleen or MLN, and spleen Lin^{-} cells were co-cultured (ratio = 1:10, 4 days) for 4 days in the presence of Ro41-5253 or DEAB (an RALDH inhibitor) in the culture conditions described in Figure 3. Ro41-5253 or DEAB was added as indicated.

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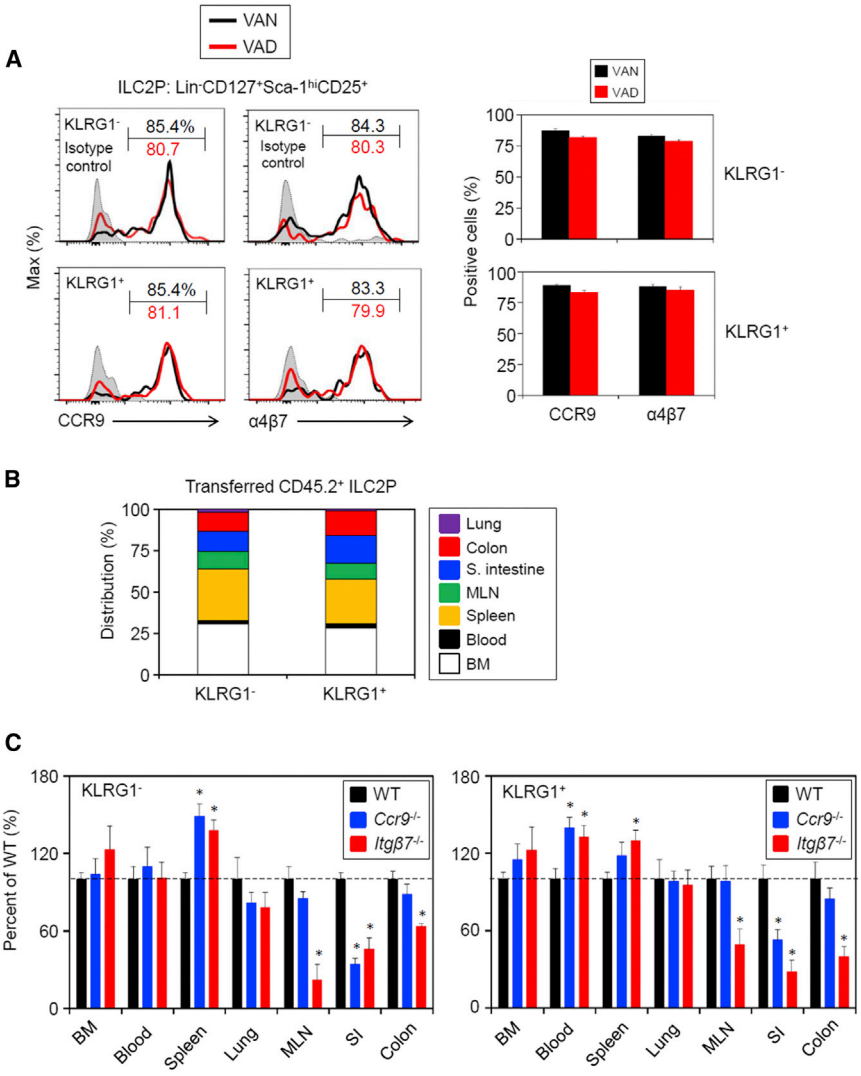

Figure 5. BM ILC2P Can Directly Migrate to the Intestine and Their Expression of Gut Homing Receptors Is Not Affected by Vitamin A Status

(A) Expression of CCR9 and α4β7 by BM ILC2P ($Lin^-CD127^+Sca\text{-}1^{hi}CD25^+KLRG\text{-}1^-$) and more mature ILC2 lineage cells ($Lin^-CD127^+Sca\text{-}1^{hi}CD25^+KLRG\text{-}1^+$) in VAN and VAD mice.

(B) Tissue distribution of BM KLRG-1^- and KLRG-1^+ ILC2 lineage cells 44 hr after cell transfer (i.v.).

(C) In vivo migration of WT versus homing receptor-deficient BM ILC2 lineage cells. Lin^- Ly-6B.2^- BM cells were injected i.v. into host mice and sacrificed 44 hr later in (B) and (C). The "Lin" mixture refers to the "BM lineage markers." *Significant differences ($p < 0.05$) from WT. Pooled data obtained from 3–7 different experiments (n = 3–10) are shown. All error bars represent SEM.

adipose tissues was detected. CCR9 was required for migration of BM KLRG-1^- and KLRG-1^+ ILC2 linage cells to the SI (Figures 5C and S4C). Migration of Itg-β7-deficient ILC2P and ILC2 to MLN, SI and colon was reduced compared to that of WT cells. These results indicate that optimal ILC2 migration to the intestine requires gut homing receptors, and this is programmed in BM rather than regulated by vitamin A status.

The RA-Induced Homing Receptor Switch Is Required for ILC3 Migration to the Intestine

We next asked whether CCR9 and α4β7, induced by RA, play a role in ILC3 migration to the intestine. $CD45.2^+$ $CD90.1^+$ RA-ILC3 and $CD45.2^+$ $CD90.2^+$ control ILC3 were prepared by culturing spleen Lin^- cells in an ILC3-promoting condition with or without RA. These cells were co-injected intravenously (i.v.) into $CD45.1^+$ $CD90.2^+$ mice for a 20 hr homing assay (Figure 6A). The RA-treated ILC3 (RA-ILC3) were highly efficient in migrating to SI and colon, while their numbers were decreased in the blood (Figure 6A).

To more directly assess the role of homing receptors, we prepared *Ccr7*$^{-/-}$, *Ccr9*$^{-/-}$, and *Itgb7*$^{-/-}$ ILC3-enriched cells by culture with RA (WT, *Ccr9*$^{-/-}$, and *Itgb7*$^{-/-}$ RA-ILC3) or without RA (WT and *Ccr7*$^{-/-}$ ILC3) and assessed for their short-term (20 hr) migratory behavior in vivo. *Ccr9*$^{-/-}$ RA-ILC3 were unable to efficiently migrate to SI (Figures 6B and S5). The migration of *Itgb7*$^{-/-}$ RA-ILC3 to both SI and colon was defective. *Ccr7*$^{-/-}$ ILC3 were less efficient than their WT counterparts in migration to MLN (Figures 6B and S5). The numbers of remaining homing receptor-deficient ILC3 in the blood and/or spleen were compensatorily increased (Figure 6B), which is consistent with their defective migration to MLN or intestine.

Homing Receptors Are Required for Long-Term Population of ILC Subsets in Secondary Lymphoid Tissues and the Intestine after BM Transplantation

To further examine the specific roles of homing receptors in long-term population of ILCs in lymphoid tissues and intestine, we transplanted mixed BM cells from WT and homing receptor-deficient mice into lethally irradiated CD45.1 mice, and the numbers of ILC subsets in lymphoid tissues and the intestine were examined 8 weeks later. *Ccr7*$^{-/-}$ ILC3 were inefficient in populating MLN (Figures 6C and S6). *Ccr9*$^{-/-}$ ILC3 failed to

(C) Expression of homing receptors by ILC subsets in the SI of VAN and VAD mice. Mice were fed with vitamin A-deficient (VAD) or normal (VAN) diets for 11–12 weeks before the examination of the ILC subsets.

(D) Confocal imaging of ILCs in SI of VAN and VAD *Rag1*$^{-/-}$ mice. Average numbers of $CD90^+$ ILCs per each villus and section are shown in the graph. Pooled data obtained from at least three different experiments (n = 3 for A,B; n = 8–12 for C; n = 12 for D) are shown. *Significant differences ($p < 0.05$) from control or VAN. All error bars represent SEM.

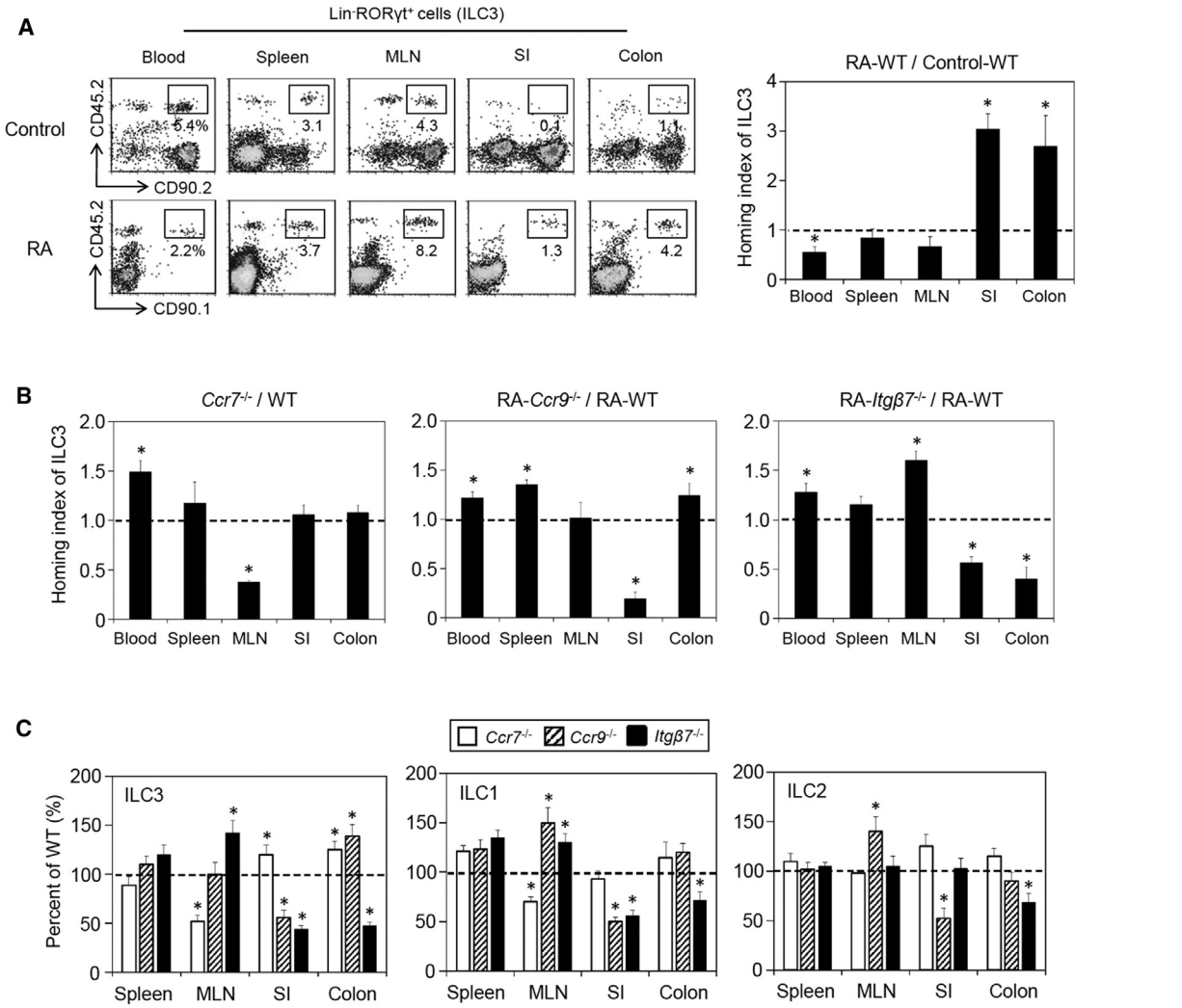

Figure 6. RA-Induced CCR9 and Itg-β7 Are Required for Short-Term Migration of ILC3 and Long-Term Population of ILC Subsets in the Intestine

(A) Short-term (20 hr) homing of control versus RA-treated ILC3 to the intestine and secondary LT.

(B) Short-term homing of WT versus homing receptor-deficient ILC3. Spleen cells, depleted of cells expressing the "splenocyte depletion markers" mentioned in the Experimental Procedures section, were cultured for 4 days in an ILC3 culture condition (commensal bacteria extract, IL-7, and IL-23) for 4 days. The cells, except *Ccr7*$^{-/-}$ and matching WT cells, were treated with RA for the homing experiment.

(C) Impact of homing receptors on long-term population of ILC3, ILC1, and ILC2. A long-term competitive population study of WT and homing receptor-deficient ILC subsets following mixed (1:1) BM transfer from CD45.2^{+}CD90.1^{+} WT and CD45.2^{+}CD90.2^{+} homing receptor-deficient mice. The ILC subsets were examined in the indicated organs 8 weeks after the mixed BM transfer. Pooled data obtained from 2–3 different experiments (n = 7–10) are shown. *Significant differences ($p < 0.05$) from control or between WT and homing receptor-deficient ILCs. All error bars represent SEM.

normally populate the SI. *Itgb7*$^{-/-}$ ILC3 failed to normally populate both SI and colon (Figures 6C and S6). The tissue population of ILC1 was similarly affected by homing-receptor deficiency albeit at a reduced level compared to that of ILC3. CCR9 deficiency decreased ILC2 population in SI, and Itg-β7 deficiency slightly decreased ILC2 population in the colon but not SI. CCR7 deficiency did not have any influence on the population of ILC2 in any tissues (Figure 6C). These results reveal the distinct roles of homing receptors in determining the tissue-specific population of ILC subsets in the intestine and lymphoid tissues.

ILC3 with Their Homing Receptors Switched by RA Can Effectively Restore Defective Mucosal Immunity in Vitamin A-Deficient Hosts

As reported recently (Spencer et al., 2014), VAD *Rag1*$^{-/-}$ mice are more susceptible to *C. rodentium* infection than VAN *Rag1*$^{-/-}$ mice and are deficient in ILC3 in the intestine (data not shown). We tested whether ILC3, isolated from the spleen and cultured in the presence of RA to induce gut homing receptors, can restore the mucosal immune deficiency in VAD *Rag1*$^{-/-}$ mice, and whether the homing receptors play any role in this process. First, we compared the ability of control and RA-treated

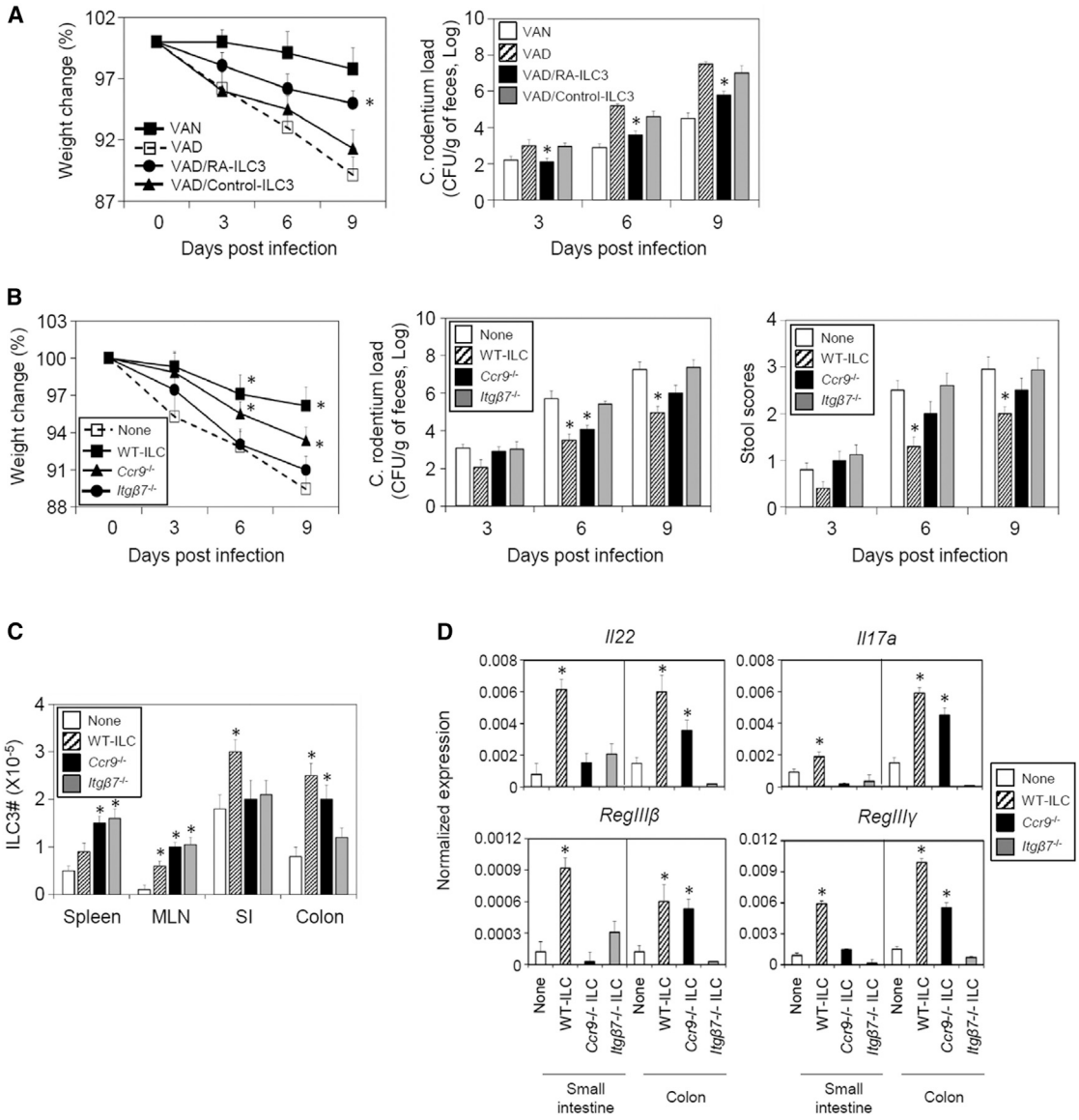

Figure 7. Gut Homing Receptors Are Required for Optimal ILC3 Effector Function during *C. rodentium* Infection

(A) Protective effects of control and RA ILC3 in *C. rodentium*-infected VAD mice. Purified ILCs ($Lin^-NK1.1^-KLRG\text{-}1^-CD90^{hi}$), isolated from WT, *Ccr9*$^{-/-}$, or *Itgβ7*$^{-/-}$ mice, were cultured with RA (10 nM) in an ILC3 culture condition (commensal bacteria extract, IL-7, and IL-23) for 4 days for the cell transfer. Control- and RA-treated ILC3 were transferred into VAD mice and infected with *C. rodentium*. Weight change and *C. rodentium* load were monitored.

(B) Protective effects of WT versus gut homing receptor-deficient RA-ILCs on *C. rodentium* infection. Weight change, pathogen burden, and stool scores were determined.

(C) Tissue distribution of $Lin^-CD90^+CD127^+ROR\gamma t^+$ ILC3 and (D) expression of ILC3 cytokines and antimicrobial peptides following adoptive transfer of WT or gut homing receptor-deficient ILC3 in VAD mice infected with *C. rodentium*. qRT-PCR was performed for panel D. The "Lin" mixture refers to the "splenocyte depletion markers." Pooled data obtained from two different experiments (n = 7–8) are shown. *Significant differences between VAN and VAD or from the control (none) groups. All error bars represent SEM.

ILC3 to protect VAD *Rag1*$^{-/-}$ mice from *C. rodentium* infection (Figure 7A). Only RA-treated ILC3 were effective in protecting VAD *Rag1*$^{-/-}$ mice. This could be due to the reported property of RA in inducing ILC3 expansion and IL-22 expression (Mielke et al., 2013; Spencer et al., 2014) rather than its effect on ILC3 migration. To sort out this issue, we performed the transfer of WT versus *Ccr9*$^{-/-}$ or *Itgb7*$^{-/-}$ ILC3 into VAD mice. The weight change and stool scores following *C. rodentium* infection indicate that both *Ccr9*$^{-/-}$ and *Itgb7*$^{-/-}$ ILC3 were less effective in protecting VAD *Rag1*$^{-/-}$ mice and clearing *C. rodentium* (Figure 7B). *Ccr9*$^{-/-}$ and *Itgb7*$^{-/-}$ ILC3 were less efficient than their WT counterparts in populating the SI (*Ccr9*$^{-/-}$) or the whole intestine (*Itgb7*$^{-/-}$) of infected VAD *Rag1*$^{-/-}$ mice (Figures 7C and S7). A compensatory increase in ILC3 was observed in the spleen and MLN (Figure 7C). The transfer of WT RA-ILC3 restored the defective mRNA expression of cytokines (*Il17a*

and *Il22*) and anti-microbial peptides (*RegIIIb* and *RegIIIγ*) in the intestine of VAD *Rag1*$^{-/-}$ mice (Figure 7D). In general, *Ccr9*$^{-/-}$ RA-ILC3 were less efficient than WT ILC3 in restoring the expression of these factors in SI, and *Itgb7*$^{-/-}$ RA-ILC3 were defective in inducing the factors in both SI and colon. Taken together, these results indicate the importance of the homing receptor switch regulated by RA for the effector function of ILC3 in the gut. All indicators such as weight change, stool scores, and pathogen burden following the infection indicate that Itg-β7 deficiency had a greater effect than CCR9 deficiency on ILC3 effector function. This might be because CCR9 does not significantly affect ILC3 migration to the colon, which is the major site of infection by *C. rodentium*.

DISCUSSION

We found that ILC subsets actively migrate to the gut and lymphoid tissues, and homing receptors are differentially required for this process. We also observed that ILC1, ILC2, and ILC3 are different from each other in homing receptor expression and in vivo migration behavior. There exists a clear dichotomy in the migration of ILC2 versus ILC1 and ILC3 to the intestine. RA and vitamin A induce a homing receptor switch for the migration of ILC3 and ILC1 to the gut but ILC2 migration to the gut is not regulated by RA. The gut-homing ability of ILC2 is developmentally programmed in BM rather than induced in the periphery. Beyond the homeostatic migration of ILC subsets, gut homing receptors are required to mount a normal ILC3 response to infection.

It has been unclear whether ILCs can specifically migrate to the intestine utilizing homing receptors. We demonstrated that ILCs can migrate into the intestine utilizing the two homing receptors, CCR9 and α4β7, which are also utilized by other immune cells such as T and B cells. α4β7 acts as an adhesion molecule to bind MAdCAM-1, which is widely expressed on intestinal endothelial cells in SI and colon. We found that ILC3 and ILC1 in both SI and colon highly express α4β7 and require α4β7 to migrate into the intestine. CCR9 is activated by the chemokine CCL25, which is specifically expressed in SI. Indeed, SI ILCs most highly express CCR9, and all ILC subsets require CCR9 to migrate into SI. 30%–50% of ILC1 and ILC3 in MLN also express CCR9 and α4β7, but these homing receptors are not required for ILC migration into MLN. This implies that MLN is more a generating site than a destination for CCR9^{+} α4β7^{+} ILCs. Thus, CCR9 is the SI-specific homing receptor, whereas α4β7 is the pan-gut homing receptor required for migration of ILC3 and ILC1.

CCR7 is highly expressed by the majority of ILC3 and ILC1 in MLN. It is also expressed by ~60% T-bet^{-} ILC3, but not many T-bet^{+} ILC3 and ILC1 in the spleen. The CCR7 expression by ILC3 and ILC1 is required for efficient migration of these ILCs to MLN, but not to spleen. A recent report indicates that CCR7 plays a role in ILC3 recruitment from the intestine to MLN (Mackley et al., 2015), which together our results indicate that ILC3 can migrate in both ways between the MLN and intestine. Homing receptor-deficient ILCs are often enriched in the spleen, suggesting that ILC migration to the spleen is probably a passive process and does not involve specific homing receptors. Overall, our data indicate that ILC3 and ILC1 undergo the homing receptor switch to change their tissue tropism. Dendritic cells in gut-lymphoid tissues such as MLN produce RA and upregulate gut homing receptors in ILC3 and ILC1. Thus, certain ILC subsets, such as ILC3 and ILC1, can migrate from one organ to another after the homing receptor switch from CCR7 to CCR9 and α4β7, which is typically conserved among antigen receptor-expressing lymphocytes. We demonstrated that RA is a key factor in upregulating the expression of both CCR9 and α4β7 on ILC1 and ILC3. RA activates RARα, which functions to induce the transcription of *CCR9* and *Itg-α4* genes (Iwata et al., 2004; Kang et al., 2011). The fact that pan-RAR or RARα antagonists downregulate the expression of CCR9 and α4β7 on ILCs supports the idea that ILCs utilize RARs, particularly RARα, for upregulating gut homing receptors and downregulating CCR7.

A question of interest is whether all ILC subsets undergo the homing receptor switch induced by RA. ILC subsets are differentially regulated by RA. RA suppresses ILC2 but increases ILC3 numbers and IL-22 expression (Mielke et al., 2013; Spencer et al., 2014). We found a similar heterogeneity in homing receptor expression. ILC3 were most sensitive to RA in the homing receptor switch from CCR7 to CCR9 and α4β7. ILC1 also responded to RA in the homing receptor switch, but ILC2 did not respond to RA. A rather unexpected observation is that ILC2 express CCR9 in all of the organs that we examined, including spleen, and that even immature BM ILC2P express gut homing receptors (Hoyler et al., 2012). Unlike intestinal ILC3 and ILC1 and their BM counterparts, intestinal ILC2 do not highly express α4β7. A clear difference between ILC2 and ILC1/3 is the site of gut homing-receptor upregulation. This appears to occur in the periphery for ILC1/3 based on their tissue-specific homing receptor profiles. However, the gut homing receptor upregulation for ILC2 occurs in BM. We observed that significant numbers of BM ILC2P (~20%) and ILC2 (~30%) can migrate to SI within 44 hr after cell transfer. Itg-β7 appears to play a role in BM ILC2 migration not only to the intestine but also to MLN, but its function in the long-term population of ILC2 was detected only in the colon. This might be because other trafficking receptors might replace α4β7 in the MLN and small intestine. CCR9 is important for both the migration and population of ILC2 specifically in SI. Overall, the gut-homing machinery of ILC2 is primarily developed early in their development in BM rather than during their maturation in the periphery.

Vitamin A status is a key factor in shifting the ILC distribution in the body. ILC deficiency in the intestine is translated into deficient expression of anti-microbial peptides and IL-22, leading to defective mucosal immunity. In this regard, the decreased immunity to *C. rodentium* infection in VAD mice is, in part, due to reduced numbers of ILC3 in the intestine. We demonstrated that the homing behavior of ILC3 can be re-programmed by RA. Importantly, without the normal expression of gut homing receptors, RA-treated ILC3 were not able to migrate and provide effective immunity to fight pathogens. This underlies the importance of the migration ability of ILCs in determining their effector function. A recent report indicates that CXCR6 plays a role in maintaining a subset of ILC3 in SI lamina propria (Satoh-Takayama et al., 2014). It is likely that additional trafficking receptors, regulated by non-RA signals, might be involved in the recruitment of ILC subsets to specialized sites in the gut at various stages of immune responses.

Our results provide novel insights into the migration programs of major ILC subsets. ILC1 and ILC3 can reprogram their homing receptor expression to migrate into the intestine in lymphoid tissues, a process regulated by RA. This indicates that ILC3 and ILC1 possess sophisticated migration programs that are similar to T and B cells. In contrast, the migration behavior of ILC2 is tied to their development in BM rather than it is acquired through the homing receptor switch in the periphery. In sum, our findings point out that ILC subsets differentially utilize the migration programs of both innate and adaptive immune cells.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments in this study were approved by the Purdue Animal Care and Use Committee. C57BL/6 mice were from Harlan. CD45.1 congenic (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ, Stock# 002014), CD90.1 congenic (B6.PL-*Thy1*$^{a/CyJ}$, Stock# 000406), *Rag1*$^{-/-}$ (B6.129s7-Rag1^{tm1Mom}/J, Stock# 002216), *Ccr7*$^{-/-}$ (B6.129P2(C)-*Ccr7*$^{tm1Rfor/J}$, Stock# 006621), and *Itgb7*$^{-/-}$ (C57BL/6-Itgb7^{tm1Cgn}/J, Stock# 002965) mice were originally from the Jackson Laboratory. *Ccr9*$^{-/-}$ mice in the C57BL/6 background were described previously (Uehara et al., 2002). All strains were kept at Purdue for at least 12 months. VAD and VAN *Rag1*$^{-/-}$ or C57BL/6 mice were prepared by keeping mice on custom research diets (0 and 2,500 IU/kg of retinyl acetate respectively for VAD and VAN, Harlan Teklad diets) immediately following birth and after weaning until they are 11–12 weeks of age.

Characterization of ILC Subsets and Marrow Progenitors By Flow Cytometry

Cells from intestinal lamina propria, PP, spleen, MLN, and blood were prepared as previously described (Kang et al., 2007). For ILC1 and ILC3, cells were first stained with antibodies to following the common lineage markers: CD3ε (clone 145-2C11), CD5 (53-7.3), CD8α (53-6.7), CD19 (6D5), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), Ter119 (Ter-119), F4/80 (BM8), Gr-1 (RB6-8C5), TCRβ (H57-597), TCRγδ (GL3), CD49b (DX5), and FcεR1α (MAR-1) and other antigens (CD45.2/104, CD90.2/53-2.1, CD90.1/OX-7, and/or CD127/A7R34), and then fixed and permeabilized for further staining for intracellular antigens (T-bet/eBio-4B10, GATA-3/TWAJ, RORγt/AFKJS-9, and/or Eomes/Dan11mag). For ILC2, cells were stained with antibodies to the common lineage markers mentioned above and NK1.1/PK136, CD90.2, CD90.1, CD127, Sca-1/D7, KLRG-1/2F1, and GATA-3. Intraepithelial cells, extracted with HBSS (20 mM EDTA) followed by density-gradient centrifugation on 44%-percoll gradient, were stained with antibodies to the common lineage markers, NKp46/29A1.4, and NK1.1. Most of the antibodies were from BioLegend or eBioscience unless indicated otherwise. Gating strategies for ILC subsets are described in Figure S1. BM cells were isolated from femurs and tibias of *Rag1*$^{-/-}$ or WT and homing receptor-deficient mice. BM cells were stained with antibodies to CD127, Sca-1, CD25, KLRG-1, and BM lineage markers (CD3ε, CD5, CD8α, B220, CD11b, Gr-1, Ter-119, TCRγδ, and NK1.1). BM ILC2P and relatively more mature KLRG-1^{+} ILC2 cells were respectively identified as Lin^{-}CD127^{+}Sca-1^{hi}CD25^{+}KLRG-1^{-} and Lin^{-}CD127^{+}Sca-1^{hi}CD25^{+}KLRG-1^{+} cells. Expression of GATA-3 by these BM ILC2P and ILC2-related cells was verified. The expression of homing receptors by ILCs and ILC2P was determined using antibodies to CCR7 (4B12), CCR9 (242503), α4β7 (DATK32), and CD103 (2E7) (Wang et al., 2010).

Cell Isolation

Lineage-depleted cells (~95% pure) were isolated from the spleen by depleting cells expressing CD3ε, CD5, CD19, B220, CD11b, CD11c, Ter119, F4/80, and Gr-1 (splenocyte depletion markers) using biotin-labeled antibodies and anti-biotin beads (Miltenyi Biotec) for most experiments that did not need highly pure ILCs (Figures 3A–3E, 4Aand 4B, 6A and 6B). ILC3 were FACS-sorted (Lin^{-}NK1.1^{-}KLRG-1^{-}CD90^{hi}CD127^{+}; ~90% pure based on RORγt expression) or MACS-sorted (Lin^{-}NK1.1^{-}KLRG-1^{-}CD90^{hi}; ~87% pure) by depleting cells expressing above mentioned antigens and NK1.1, and then further depleted of KLRG-1^{+} cells using PE-conjugated KLRG-1/anti-PE beads followed by positive selection of CD90^{+} cells using FITC-conjugated CD90.2 antibody/anti-FITC beads. BM cells depleted of cells expressing the BM lineage markers and Ly-6B.2 (7/4) were prepared by MACS and used as the source of BM ILC2P for the homing study.

Cell Culture

The spleen lineage-depleted cells or sorted Lin^{-}NK1.1^{-}KLRG-1^{-}CD90^{hi} CD127^{+} ILC3 were cultured for 4 days in complete RPMI 1640 medium (10% FBS) supplemented with cecal bacterial extract (10 μg/ml), IL-7 (20 ng/ml), and IL-23 (20 ng/ml) for ILC3. Spleen Lin^{-} cells were also cultured with cecal bacterial extract, IL-7 and IL-15 (20 ng/ml) for ILC1 or IL-7 for ILC2. When indicated, RA (10 nM, all-trans RA), Ro41-5253 (500 nM, a RARα antagonist), AM580 (10 nM, RARα agonist), AC55649 (10 nM, a RARβ2 agonist), or LE540 (500 nM, a pan-RAR antagonist), was used. These chemicals were from Sigma-Aldrich or Enzo Life Sciences. Cells were harvested on day 4 and stained for CCR7, CCR9, α4β7, and ILC-associated antigens and transcription factors. For DCs and ILC co-culture, CD11c^{+} cells were isolated from splenocytes and MLN cells with CD11c microbeads (Miltenyi Biotec) (purity > 90%). 5 × 10^{4} spleen and MLN-DCs, pulsed with cecal bacterial extracts (10 μg/ml) for 3 hr, were co-cultured with 5 × 10^{5} spleen Lin^{-} cells in complete RPMI medium containing 10% FBS (non-heat inactivated) for 4 days.

Chemotaxis Assay

Spleen Lin^{-} cells (5 × 10^{5}/well), cultured in the presence or absence of RA (10 nM) or Ro41-5253 (500 nM) for 4 days, were placed in the upper chamber of Transwell inserts (Corning) and allowed to migrate to the lower chamber containing murine CCL25 (3 μg/ml, R&D Systems) for 3 hr. The cells in the lower chambers were collected, stained with antibodies to identify ILC1, ILC2, and ILC3, and counted by a time-based flow cytometry (Kim and Broxmeyer, 1998).

Short-Term Migration of ILCs

Cultured CD45.2^{+}CD90.1^{+} WT and CD45.2^{+}CD90.2^{+} homing receptor-deficient spleen Lin^{-} cells (10-15 million cells each) were co-injected i.v. into CD45.1 mice. The host mice were sacrificed 20 hr later, and indicated organs were harvested. The numbers of injected ILC3 migrated into each organ or in the blood were determined by flow cytometry. The relative homing index for homing receptor-deficient ILCs was calculated as below: Homing index of homing receptor-deficient ILC for organs A = [CD90.2^{+} homing receptor-deficient^{-} ILCs in organ A] / [CD90.1^{+} WT ILCs in organ A] ÷ [CD90.2^{+} homing receptor-deficient ILCs in injected cells] / [CD90.1^{+} WT ILCs in injected cells]. Similar experiments were performed for control versus RA-treated ILCs. For migration of BM KLRG-1$^{-/+}$ ILC2 lineage cells, CD45.2^{+} Lin^{-} BM cells, isolated from WT or homing receptor-deficient BM (~35 million cells/mouse), were singly injected i.v. into CD45.1 mice. The host mice were sacrificed 44 hr later, and indicated organs were harvested and examined by flow cytometry for numbers of transferred ILC2 lineage cells in each organ. Migration efficiency of homing receptor-deficient ILC2P to an organ was determined based on the normalized absolute numbers of transferred ILC2 lineage cells in each organ that were divided by the total numbers of the transferred ILC2 lineage cells in indicated organs.

Confocal Microscopy

Ileum tissues were frozen in Tissue-Tek OCT Compound (Sakura) and 9-μm tissue sections were made using a cryostat (Leica). The sections were fixed in cold acetone, and stained with fluorochrome-conjugated antibodies to pan-cytokeratin (Clone AE1/AE3), CD90.2, and CD3 (Clone 17A2) or CD4. The images were collected with a SP5 II (Leica) confocal microscope.

Long-Term Population of ILCs following Mixed-BM Transplantation

A 1:1 mixture of BM cells (5 × 10^{6} each) from CD45.2^{+}CD90.1^{+} WT mice and CD45.2^{+}CD90.2^{+} homing receptor-deficient mice were co-injected i.v. into irradiated (2 × 500 cGy with a 3 hr interval) CD45.1 congenic mice. The mice were sacrificed 8 weeks later for evaluation of ILC1/2/3 frequencies and numbers in each organ. On average, the marrow reconstitution efficiency in these animals was greater than 95% based on CD45.2 frequency in the spleen.

ILC Transfer and Infection with *C. rodentium*

VAD mice were generated as described before (Kang et al., 2009) and injected i.v. with WT control ILC3, WT RA-ILC3, or homing receptor-deficient RA-ILC3 (~92% pure; ~1 × 10^6 cells/mouse) two times on day −2 and 2 post-infection with *C. rodentium*. Mice were infected with *C. rodentium* (DBS100, 10^9 CFU/mouse) via oral gavage and monitored for weight change, stool consistency, and *C. rodentium* load (Kim et al., 2013). Mice were sacrificed on day 9 post-infection.

qRT-PCR

Quantitative real-time PCR (qRT-PCR) was performed as described before (Kim et al., 2013) to detect mRNA expression for *Il22*, *Il17a*, and anti-microbial peptide genes in SI and proximal colon tissues. The primers used for qRT-PCR are shown in Table S1.

Statistical Analysis

Student's t test (paired, two-tailed) was used to determine significance of the differences between two groups. p values < or = 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.06.009.

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