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Retinoic Acid Imprints Gut-Homing Specificity on T Cells

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Summary

For a preferential homing of T cells to the gut, expression of the integrin α4β7 and the chemokine receptor CCR9 is essential and is induced by antigenic stimulation with dendritic cells from the gut-associated lymphoid organs. Here, we show that the vitamin A (retinol) metabolite, retinoic acid, enhances the expression of α4β7 and CCR9 on T cells upon activation and imprints them with the gut tropism. Dendritic cells from the gut-associated lymphoid organs produced retinoic acid from retinol. The enhanced α4β7 expression on T cells by antigenic stimulation with these dendritic cells was suppressed by the retinal dehydrogenase inhibitor citral and the retinoic acid receptor antagonist LE135. Accordingly, vitamin A deficiency caused a reduction in α4β7$^+$ memory/activated T cells in lymphoid organs and a depletion of T cells from the intestinal lamina propria. These findings revealed a novel role for retinoic acid in the imprinting of gut-homing specificity on T cells.

Introduction

Persistent diarrhea with infectious diseases is a major cause of the infant mortality in developing areas in the world. It has been demonstrated that vitamin A supplementation significantly reduces the mortality (Sommer et al., 1986). Dietary vitamin A is an essential precursor of tissue retinol, which participates in a variety of biological processes including vision, reproduction, epithelial cell differentiation, bone development, and immunity (Kastner et al., 1995; Napoli, 1999). Vitamin A deficiency causes IFN-γ overproduction and impaired antibody responses. There is a helper T cell imbalance with excess Th1 and insufficient Th2 function (Cantorna et al., 1994). The visual function of vitamin A depends on its metabolite 11-*cis*-retinal, but most of the other vitamin A functions, including immunological functions, depends on retinoic acid (RA), principally all-*trans*-RA and 9-*cis*-RA (Kastner et al., 1995). RA is formed mainly through the intracellular oxidative metabolism of retinol via retinal (Duester, 2000). RA directly and indirectly regulates T cell activities including activation-induced apoptosis and development of Th1 and Th2 (Iwata et al., 1992, 2003; Yang et al., 1993; Racke et al. 1995; Hoag et al., 2002; Stephensen et al., 2002). RAs bind to two families of nuclear receptors, the RA receptor (RAR) isotypes (α, β, and γ) and the retinoid X receptor (RXR) isotypes (α, β, and γ) (Kastner et al., 1995; Mangelsdorf and Evans, 1995). 9-*cis*-RA binds to both RAR and RXR, whereas the major physiologic RA, all-*trans*-RA, binds to RAR and may bind to RXR at high concentrations. These receptors function as ligand-inducible transcription factors mainly as RXR/RAR heterodimers.

Since vitamin A is critical especially for the gut immunity, we considered a possibility that vitamin A might play a role in the specific homing of T cells to the gut. Naive T cells can migrate into any secondary lymphoid organs but usually cannot migrate into nonlymphoid tissues before they are activated with antigen (Butcher et al., 1999; von Andrian and Mackay, 2000; Masopust et al., 2001). Antigen-experienced effector/memory T cells migrate preferentially to tissues that are associated with the secondary lymphoid organs where they first encountered specific antigen (Guy-Grand et al., 1978; Kantele et al., 1999; Campbell and Butcher, 2002). For the preferential homing of T cells to the gut, expression of the gut-homing receptors, namely the integrin α4β7 and the chemokine receptor CCR9, is essential (Hamann et al., 1994; Kantele et al., 1999; Svensson et al., 2002). The α4β7 integrin ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), mediates recruitment of T cells into normal and inflamed intestinal endothelium (Butcher et al., 1999). The CCR9 ligand TECK is expressed by epithelial cells in the small intestine, especially those in the crypt region most closely associated with MAdCAM-1$^+$ vessels (Kunkel et al., 2000; Wurbel et al., 2000). The gut-homing specificity can be imprinted on T cells by antigenic stimulation with dendritic cells (DC) from Peyer's patches (PP) or by stimulation with DC from mesenteric lymph nodes (MLN) in the presence of antigen and adjuvant or anti-CD3 antibody (Mora et al., 2003; Johansson-Lindbom et al., 2003; Stagg et al., 2002). However, the mechanism of the imprinting is unknown. In this study, we found that physiological concentrations of RA, but not retinol or retinal, enhanced the expression of the gut-homing receptors α4β7 and CCR9 in T cells upon activation and that RA imprints T cells with the gut-homing specificity. In vitamin A-deficient mice, α4β7$^+$ memory/activated T cells were markedly reduced in secondary lymphoid organs, and T cells were depleted from the intestinal lamina propria (LP), suggesting that the RA effect is physiological. RA production by DCs from the gut-associated lymphoid organs and binding of RA to RAR in T cells appeared to be involved in the imprinting of T cells with the gut-homing specificity.

Results

Enhanced Expression of the Gut-Homing Receptor α4β7 Integrin by RA

We first determined the effect of RA on the expression of gut-homing receptors on T cells in the absence of

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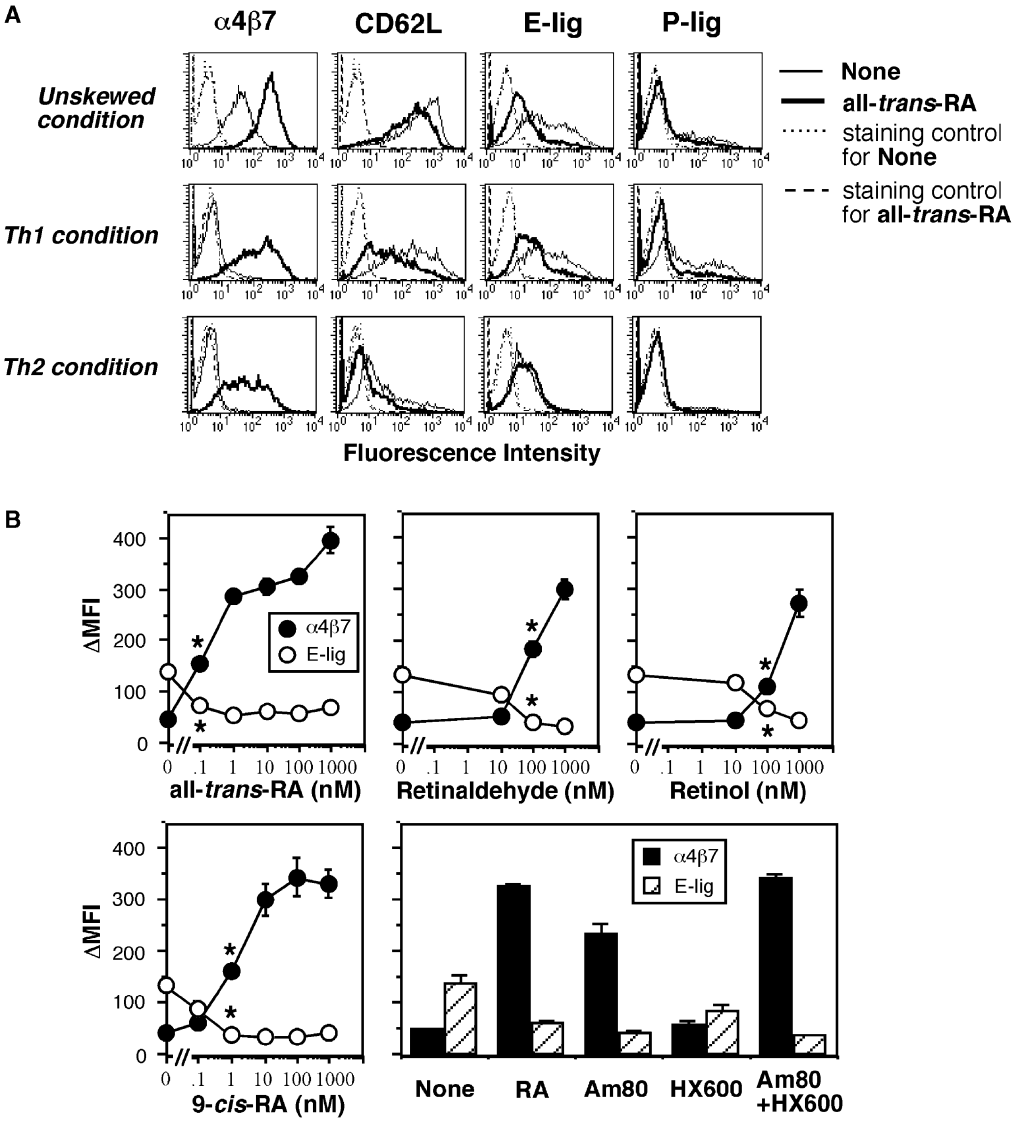

Figure 1. RA and an RAR Agonist Enhance the Expression of the Gut-Homing Receptor $\alpha4\beta7$ Integrin on $CD4^+$ T Cells upon Activation

Naive $CD4^+$ T cells were stimulated for 2 days with antibodies to CD3 and CD28 and expanded for 2 days under the unskewed condition (A and B) or the Th1- or Th2-skewed condition (A). (A) The cells cultured with or without 10 nM all-*trans*-RA were analyzed for $\alpha4\beta7$, CD62L, E-lig, and P-lig expression by flow cytometry. (B) Effects of various doses of all-*trans*-RA, all-*trans*-retinal, all-*trans*-retinol, 9-*cis*-RA, and effects of 100 nM of the RARα (and β) agonist Am80 and the RXR pan-agonist HX600 on the expression of $\alpha4\beta7$ and E-lig. The results are expressed as ΔMFI $\pm$ SD in triplicate cultures. Effects of selected concentrations of retinoids were examined for the statistical significance by performing separate experiments ($n = 5$ per group). Asterisks indicate that the effect of retinoid at the indicated concentration is statistically significant with the Mann-Whitney test ($p < 0.01$ versus the corresponding values of control cultures without RA addition). Data are representative of at least three independent experiments.

other cell types in vitro. Naive CD4-expressing ($CD4^+$) T cells were isolated from DO11.10 T cell receptor (TCR)-transgenic mice with Rag-$2^{-/-}$ background to avoid contamination with antigen-experienced T cells. The naive T cells were stimulated with plate bound antibodies to CD3 and CD28 and were expanded with IL-2 (unskewed condition). The addition of 10 nM all-*trans*-RA, which is the major physiologic RA, enhanced the expression of the gut-homing receptor $\alpha4\beta7$ integrin on these cells and suppressed that of E-selectin ligands (E-lig) (Figure 1A). The effect of RA was significant when it was added within 2 days from the start of the culture (data not shown). RA was also effective under the skewed conditions to induce Th1 or Th2 ("Th1 condition" or "Th2 condition," respectively). The expression of $\alpha4\beta7$ on Th1 and Th2 was low, but RA markedly enhanced it (Figure 1A). The expression of E-lig and P-selectin ligands (P-lig) on Th1 was suppressed by RA, and Th2 cells that were induced with or without RA expressed low levels of E-lig and P-lig (Figure 1A). The expression of CD62L (L-selectin), which participates in the homing of naive and central memory T cells to secondary lymphoid organs (Butcher et al., 1999; Sallusto et al., 1999; Weninger et al., 2001), was moderately suppressed. RA also enhanced $\alpha4\beta7$

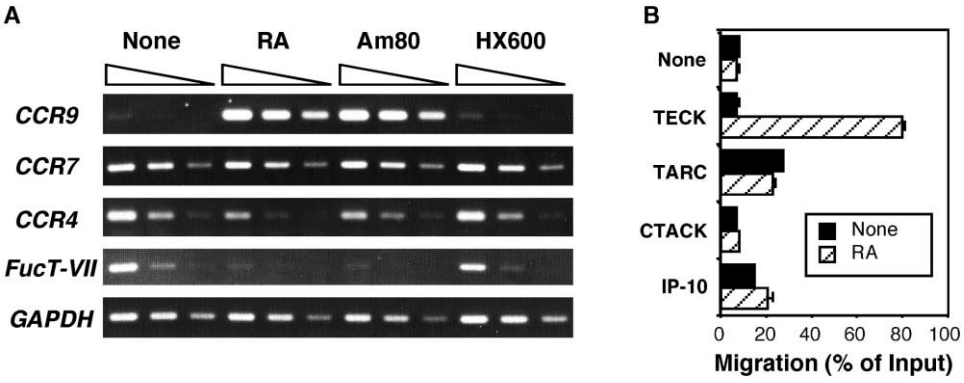

Figure 2. RA Enhances *CCR9* Expression and Chemotaxis to TECK

Naive CD4$^+$ T cells were stimulated as in Figure 1 under the unskewed condition. (A) RT-PCR of CCR9, CCR7, CCR4, FucT-VII, and GAPDH mRNA from the cells cultured with 10 nM all-*trans*-RA, 100 nM Am80, or 100 nM HX600. (B) Naive CD4$^+$ T cells cultured with or without 10 nM all-*trans*-RA were assessed for chemotactic activities toward optimal concentrations of chemokines in Transwells. Deviation bars represent SD in triplicate cultures. Data are representative of at least three independent experiments.

expression and suppressed E-lig on CD8-expressing (CD8$^+$) T cells upon activation (data not shown).

All-*trans*-RA significantly enhanced $\alpha4\beta7$ expression and suppressed E-lig expression even at 0.1 nM (Figure 1B). 9-*cis*-RA, another physiologic RA, exerted similar effects at 1 nM or higher concentrations, but the effects of retinal and retinol were significant only at 100 nM or higher concentrations. All-*trans*-RA binds to the RAR isotypes, while 9-*cis*-RA binds to both RAR and the RXR isotypes. Thus, RAR appears to be involved in the effect. However, isomerization of all-*trans*-RA to 9-*cis*-RA might occur within the cells (Urbach and Rando, 1994). We then examined the effect of agonists of RARs and RXRs on $\alpha4\beta7$ expression. Am80 is a potent RARα agonist and is a less potent agonist of RARβ (Kagechika et al., 1988). Am80, but not the RXR pan-agonist HX600 (Umemiya et al., 1997), exerted effects similar to those of RA (Figure 1B). These results indicate that RAs specifically induce or enhance the gut-homing receptor $\alpha4\beta7$ in T cells when they are added during TCR-mediated activation and that RAR participates in the effect.

Enhancement of *CCR9* Expression and Chemotaxis to the Chemokine TECK by RA

We then examined the effect of RA on the other gut-homing receptor, CCR9. All-*trans*-RA and Am80 induced mRNA expression of the chemokine receptor CCR9 but moderately suppressed that of the skin-homing receptor CCR4 (Figure 2A). They also suppressed the mRNA expression of α(1,3)fucosyltransferase (FucT)-VII, which is required for the biosynthesis of E-lig and P-lig (Maly et al., 1996). On the other hand, *CCR7* expression was not significantly affected. In accordance with the enhanced expression of *CCR9*, the RA treatment induced a strong chemotactic activity in CD4$^+$ T cells specifically toward the CCR9 ligand TECK (CCL25) (Figure 2B), indicating that RA induces the surface expression of CCR9 as well. The RA treatment of CD4$^+$ T cells under the Th1- and Th2-inducing conditions also enhanced the chemotactic activity toward TECK, although spontaneous migration was elevated in the RA-treated Th2 (data not shown). The RA treatment, however, did not significantly affect the chemotactic activity toward TARC in spite of the moderate suppression of *CCR4* expression. These results indicate that RA specifically induces or enhances CCR9 expression for the specific chemotaxis to TECK and that RAR participates in the effect.

Preferential Homing of RA-Treated T Cells to the Small Intestine

We next performed competitive homing experiments. After naive CD4$^+$ T cells were activated and expanded with or without all-*trans*-RA, the cells were stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), a green fluorophor, or tetramethylrhodamine isothiocyanate (TRITC), a red fluorophor, respectively. Equal numbers of cells from the two populations were mixed and adoptively transferred into nontransgenic recipients. The homing profiles of these activated cells were analyzed 16 hr later. Compared with control T cells, the RA-treated T cells homed poorly to peripheral lymph nodes (PLN), including inguinal, axillar, and brachial lymph nodes, but homed efficiently to MLN and PP (Figure 3A). The two populations homed equally into the spleen (SPL). These RA effects were not affected by switching the dyes (data not shown). Histological analyses confirmed the results (Figure 3B). Furthermore, we enumerated the labeled cells in LP in cross-sections of the small intestines from four mice (numbers 1–4). The number of RA-treated cells per section (#1, 5.80 $\pm$ 0.92; #2, 6.10 $\pm$ 0.94; #3, 3.95 $\pm$ 0.92; and #4, 2.05 $\pm$ 0.34; mean $\pm$ SEM) was significantly larger than that of control cells (#1, 0.10 $\pm$ 0.07; #2, 0.35 $\pm$ 0.15; #3, 0.20 $\pm$ 0.12; and #4, 0.25 $\pm$ 0.12) in each mouse with the Mann-Whitney test ($p < 0.0001$), indicating that the RA-treated cells migrate into LP of small intestines much more efficiently than control cells.

Depletion of T Cells from LP of the Small Intestine in Vitamin A-Deficient Mice

If RA is essential for licensing memory/activated T cells to migrate into the nonlymphoid gut tissues, vitamin A deficiency would prohibit this licensing. Thus, we produced vitamin A-deficient mice. Serum retinol levels in

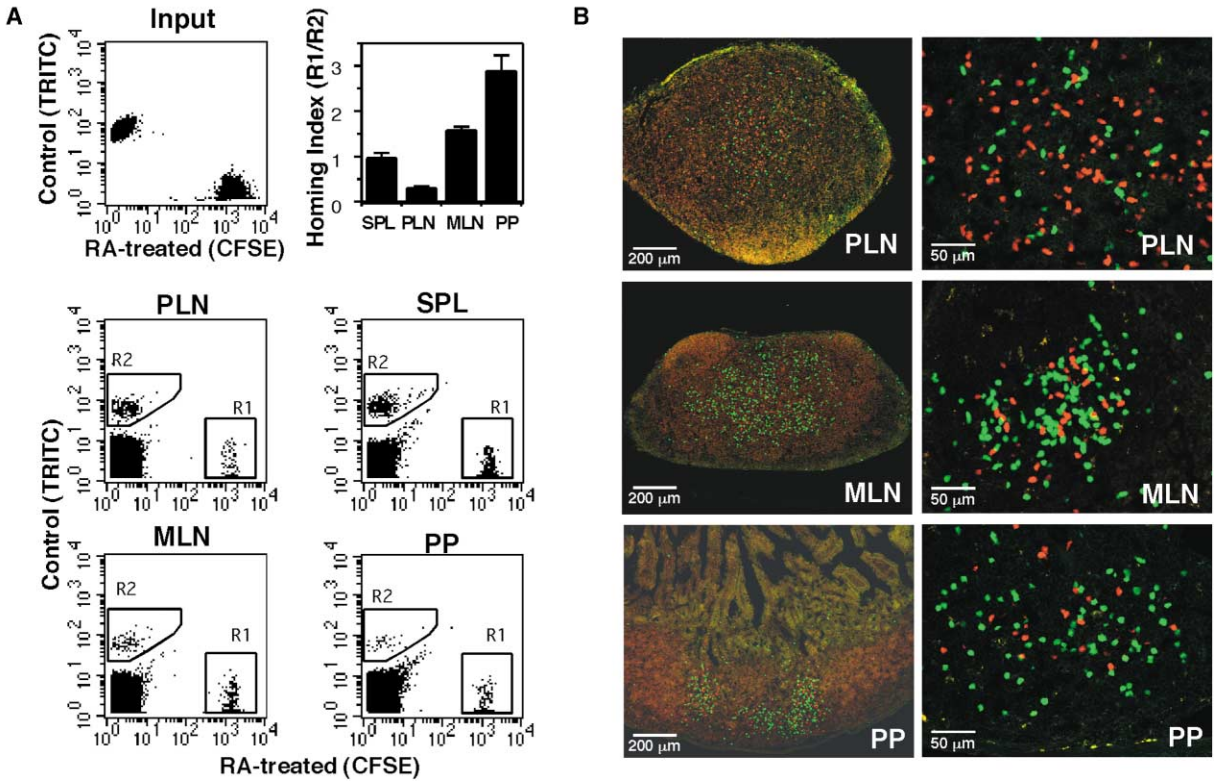

Figure 3. The RA-Treated CD4$^+$ T Cells Preferentially Home to the Small Intestine

Naive CD4$^+$ T cells were activated and expanded with or without 10 nM all-*trans*-RA for 4 days. The RA-treated cells were labeled with CFSE (green), while the cells cultured without RA addition were labeled with TRITC (red). Equal cell numbers of each preparation were mixed and injected into recipient mice. (A) Secondary lymphoid organs were analyzed for the injected T cells, and the homing index (the ratio of [CFSE$^+$ cells] to [TRITC$^+$ cells] in recipient tissues) was determined. (B) Histological analysis of the injected cells in PLN (inguinal LN), MLN, PP, and intestinal villi. A representative result of four independent experiments is shown.

vitamin A-deficient and control mice were estimated to be 0.13 $\pm$ 0.04 μM and 1.08 $\pm$ 0.05 μM, respectively. The vitamin A-deficient or control mice showed no signs of inanition and had equivalent body weights. However, in vitamin A-deficient mice, $\alpha4\beta7^+$CD4$^+$ memory/activated T cells were markedly reduced in their spleens, MLN, and PP, although $\alpha4\beta7^-$CD4$^+$ memory/activated T cells were not reduced in the secondary lymphoid organs (Figure 4B). The naive CD4$^+$ T cells were, however, reduced in SPL and PLN (Figures 4C and 4D). The total cell number of MLN markedly increased in vitamin A-deficient mice with an unknown mechanism. CD4$^+$TCRβ^+ lymphocytes were depleted from LP of the small intestine in vitamin A-deficient mice, but not from their livers or lungs (Figure 4E). Immunohistochemical analyses indicated that both CD4$^+$ cells and CD8$^+$ cells were depleted from LP of their small intestines, while both cell types were present in the T cell areas of PP (Figure 5). The results indicate that vitamin A is physiologically critical for the $\alpha4\beta7$ expression on T cells and the migration of antigen-experienced T cells into the intestinal LP. Since retinol and retinal did not efficiently enhance the expression of the gut-homing receptors (Figure 1B), RA is likely to take charge of the dietary vitamin A to induce the migration of T cells into LP.

Expression of RA-Producing Enzymes in DCs of the Gut-Associated Lymphoid Organs

It has been shown that antigenic stimulation of CD8$^+$ T cells with DC from PP (PP-DC) induces the gut tropism on them (Mora et al., 2003). DC from MLN (MLN-DC) in the presence of antigen and adjuvant or anti-CD3 antibody has a similar effect on T cells (Stagg et al., 2002; Johansson-Lindbom et al., 2003). Thus, we considered a possibility that PP-DC and MLN-DC might provide RA to T cells during antigen presentation and thereby imprint the gut tropism on them. The main pathway of RA biosynthesis is dependent on the intracellular oxidative metabolism of retinol via retinal (Napoli, 1999; Duester, 2000). The reversible pathway from retinol to retinal is catalyzed by a subfamily of alcohol dehydrogenases (ADH). mRNA of the ADH class III isoenzyme (ADH5) was expressed ubiquitously among DC preparations from all the secondary lymphoid organs that we examined. In addition, PP-DC expressed mRNAs of ADH class I (ADH1) and class II (ADH4) isoenzymes (Figure 6A). The irreversible pathway from retinal to RA is catalyzed by retinal dehydrogenases (RALDH), a subfamily of class I aldehyde dehydrogenases (Duester, 2000). There are at least four isoenzymes identified so far. Among them, expression of three isoenzymes was detected in sec-

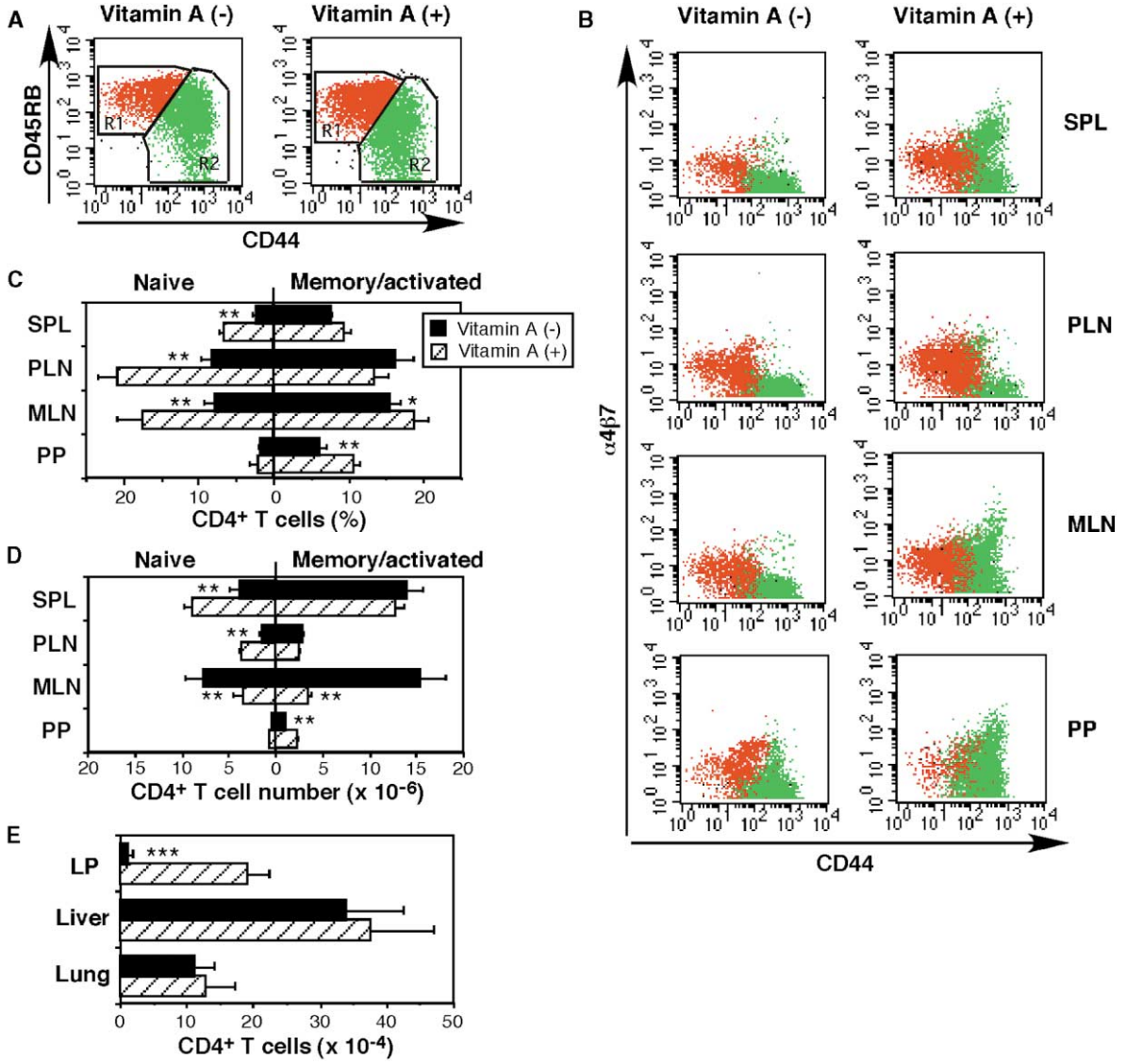

Figure 4. Vitamin A Deficiency Reduces $\alpha4\beta7^+CD4^+$ Memory/Activated T Cells in the Secondary Lymphoid Organs and $CD4^+$ T Cells in LP of the Small Intestine

Isolated $CD4^+$ T cells from (A) MLN and (B) SPL, PLN, MLN, and PP of vitamin A-deficient or control mice were stained with anti-CD44 (FITC), anti-$\alpha4\beta7$ (PE), anti-CD45RB (biotin), and streptavidin-Tri-Color. (A) Naive (red) and memory/activated cells (green) were determined by the expression of CD44 and CD45RB. (B) The expression of $\alpha4\beta7$ in the naive and memory/activated populations was analyzed. (C and D) The proportions of $CD4^+$ naive or memory/activated T cells in single cell suspensions of whole SPL, PLN, MLN, and PP (C) and the total number of $CD4^+$ T cells in the organ(s) per mouse (D) were assessed by cell counting and flow cytometry (mean $\pm$ SD, n = 5 per group). (E) The total number of $CD4^+$ T cells in the LP of the small intestine, liver, or lungs per mouse was assessed (mean $\pm$ SD, n = 5 to 9 per group). Asterisks indicate that the difference is statistically significant with the Mann-Whitney test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the corresponding values of control mice).

ondary lymphoid organs. PP-DC expressed *RALDH1* (*AHD2, Aldh1a1*) mRNA, while MLN-DC expressed *RALDH2* (*Aldh1a2*) mRNA (Figure 6B). *RALDH3* (*Aldh1a3, Aldh-6*) mRNA expression was weakly detected in both MLN and PP. In PLN, the mRNA expression of RALDHs was relatively low, and PLN-DC only weakly express *RALDH2* mRNA (Figure 6B). Immunohistochemical analyses with an anti-RALDH1 antibody indicated that mucosal epithelial cells of the small intestine highly expressed this enzyme and that many $CD11c^+$ DC in the dome areas and some DC in the T cell areas adjacent to the follicles in PP also expressed RALDH1 (Figure 6C). There were some, but not many, $RALDH1^+$ $CD11c^+$ DCs in MLN and few in the axillar LN (data not shown). In SPL, few cells expressed both RALDH1 and CD11c (Figure 6D), and SPL-DC only weakly expressed *RALDH1* mRNA and essentially no *RALDH2* and *RALDH3* mRNA (Figure 6B and data not shown). These

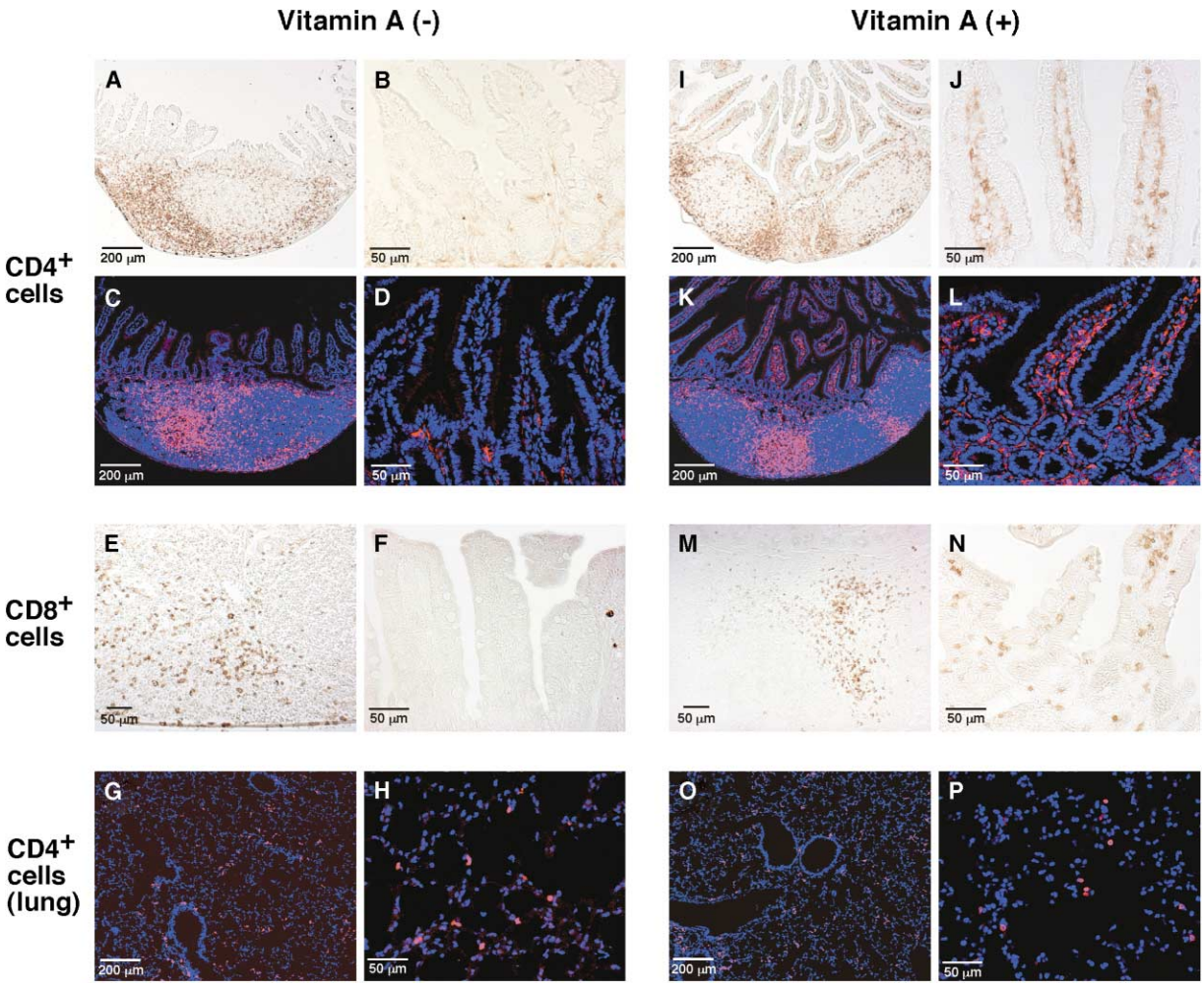

Figure 5. In Vitamin A-Deficient Mice, Both $CD4^+$ Cells and $CD8^+$ Cells Are Depleted from LP in the Small Intestine

Histochemical analysis was performed on PP and villi of the small intestines and on the lung from vitamin A-deficient mice (A–H) and control mice (I–P). $CD4^+$ cells (A–D, G–L, O, and P) and $CD8^+$ cells in PP (E and M) and the intestinal villi (F and N) were visualized with avidin-conjugated peroxidase and its substrate diaminobenzidine (blown) (A, B, E, F, I, J, M, and N) or with streptavidin-conjugated fluorophor Cy3 (red) followed by nuclear staining with TO-PRO-3 (blue) (C, D, G, H, K, L, O, and P). A representative result of three mice from each group is shown.

results suggest that there are potential producers of RA among DCs in MLN and PP, but few in PLN or SPL.

RA Production by MLN-DC and PP-DC and Its Contribution to the $\alpha4\beta7$ Expression on T Cells

To determine if DCs from MLN and PP can actually produce RA during antigen presentation, MLN-DC or PP-DC preparations were cultured with DO11.10 $CD4^+$ T cells and their specific antigen, ovalbumin peptide P323-339. [^{3}H]-all-*trans*-retinol was included in the culture, and the production of [^{3}H]-all-*trans*-RA after culture was assessed by fractionation of the cell extracts with an HPLC system. A peak corresponding to all-*trans*-RA was detected in fractions of the MLN-DC culture or the PP-DC culture, but not in those of the SPL-DC culture or those without DC (Figure 6E and data not shown). MLN-DC and T cells without antigen or MLN-DC alone also induced RA production but somewhat less significantly (data not shown). These results suggest that MLN-DC and PP-DC can convert retinol to RA and that the interaction with T cells via antigen may affect the conversion.

To examine if the ability of MLN-DC and PP-DC to imprint T cells with the gut tropism is dependent on RA production, we used the RALDH inhibitor citral (Connor and Smit, 1987), the RARβ (and α) antagonist LE135 (Umemiya et al., 1997; Li et al., 1999), and the RXR pan-antagonist PA452 (Takahashi et al., 2002). The expression of $\alpha4\beta7$ integrin on naive $CD4^+$ T cells was enhanced upon antigenic stimulation with MLN-DC and PP-DC and was partly but significantly suppressed by citral and LE135, but not by the RXR pan-antagonist PA452 (Figures 7A and 7B). Citral and LE135 at the concentrations used here did not significantly affect the recovery of viable T cells (Figure 7C). These results indicate that DC-derived RA is at least partly responsible for the imprinting of T cells with the gut tropism by these DCs and that RARβ and/or RARα, but not RXR, are involved in the effect.

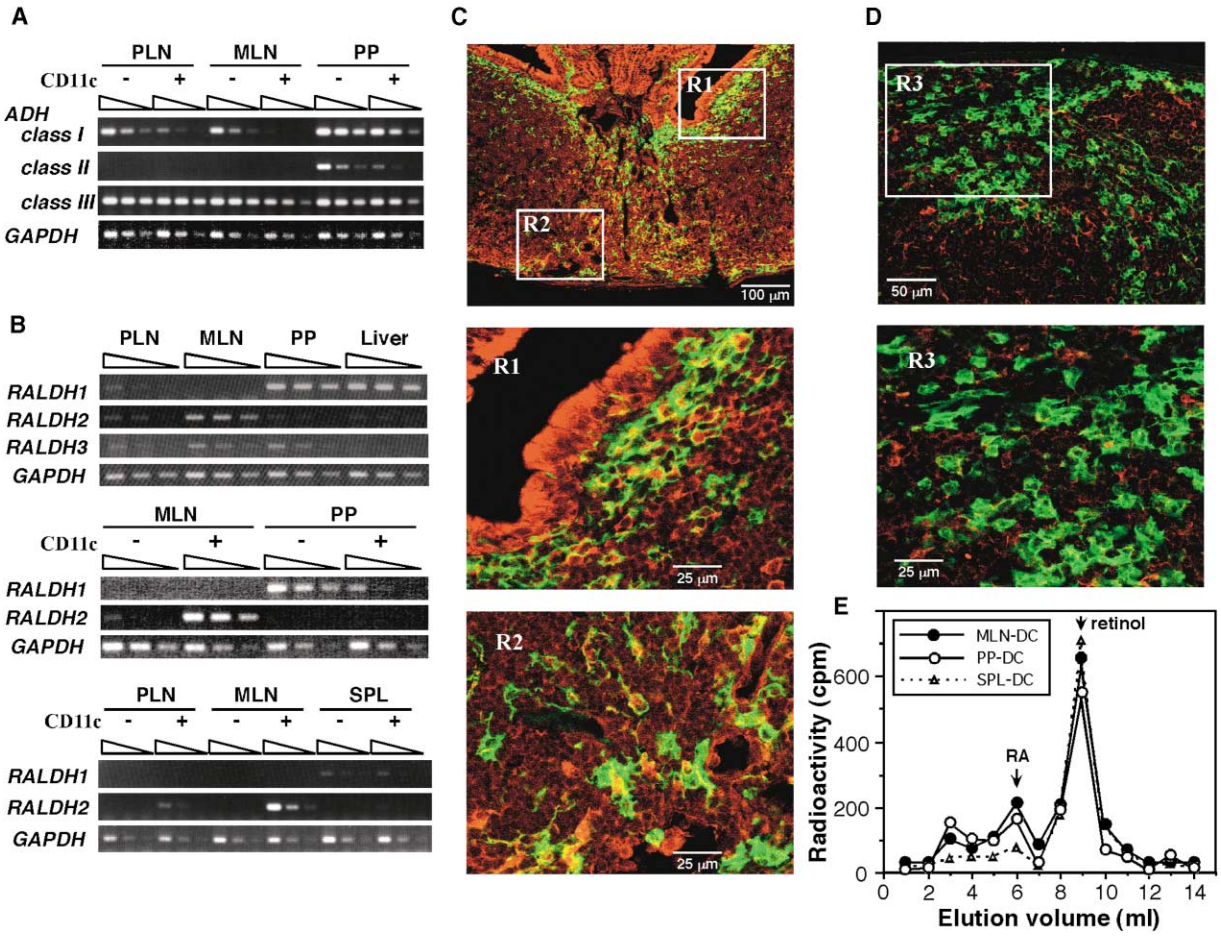

Figure 6. MLN-DC and PP-DC Express RA-Producing Enzymes and Produce RA from Retinol

Single cell suspensions from PLN (inguinal, brachial, and axillar LN), MLN, PP, liver, or SPL of normal BALB/c (A) or B10.D2 (B) mice were fractionated into $CD11c^-$ and $CD11c^+$ cells. RT-PCR of ADH (A) and RALDH (B) isozyme mRNAs from the fractionated and unfractionated cells. (C and D) Immunohistochemical analyses were performed for $RALDH1^+$ cells and $CD11c^+$ DCs in PP and its vicinity in the small intestine (C) and SPL (D) of normal B10.D2 mice with anti-RALDH1 (red) and anti-CD11c (green) antibodies. (E) MLN-DCs, PP-DCs, or SPL-DCs were cultured with naive $CD4^+$ T cells from DO11.10 transgenic mice, antigen, and [^{3}H]-all-*trans*-retinol. After 18 hr of culture, cell extracts were fractionated with an HPLC system, and radioactivity of each fraction was determined. The internal retinoid standards, all-*trans*-RA, all-*trans*-retinol, and all-*trans*-retinal, were eluted at 6.0, 8.8, and 17.4 ml, respectively. A representative result of at least three independent experiments is shown.

Discussion

Dietary vitamin A appears to be critical for the gut immunity. We found here that the vitamin A metabolite RA enhanced the expression of the gut-homing receptors $\alpha4\beta7$ integrin and CCR9 on T cells and suppressed that of E-lig, P-lig, and FucT-VII in vitro. The $CD4^+$ T cells that were activated in the presence of RA showed an enhanced chemotactic activity toward TECK and a preferential homing to the gut-associated secondary lymphoid organs MLN and PP after the adoptive transfer to normal mice. There was some homing to MLN and PP of control cells activated under the unskewed condition (Figure 3). It may reflect the fact that these cells showed some expression of $\alpha4\beta7$ and CD62L. The RA-treated T cells but few control T cells migrated into the intestinal LP. Thus, RA is capable of imprinting T cells with the gut-homing specificity. To examine whether the RA effect is physiological, we produced vitamin A-deficient mice, since RA is derived from dietary vitamin A. Most of vitamin A is stored in the liver as retinyl esters. Prior to entering the circulation from the liver, hepatic retinyl esters are hydrolyzed. The plasma retinol level is maintained within a certain range, owing to the hepatic reserves. To decrease the plasma retinol level, a vitamin A-free diet needs to last until the hepatic reserves are depleted (Carman et al., 1989; Molrine et al., 1995). In vitamin A-deficient mice, $\alpha4\beta7^+CD4^+$ memory/activated T cells were markedly reduced in secondary lymphoid organs. More strikingly, T cells were depleted from the intestinal LP in these mice, whereas T cells were not depleted from the liver or lung (Figures 4E and 5). The results indicate that vitamin A is physiologically essential for the migration of T cells into the gut tissues. Since retinol and retinal showed only weak activities at physiological concentrations, RA is most likely to take charge of this role of vitamin A.

The expression of $\alpha4\beta7$ was significantly enhanced

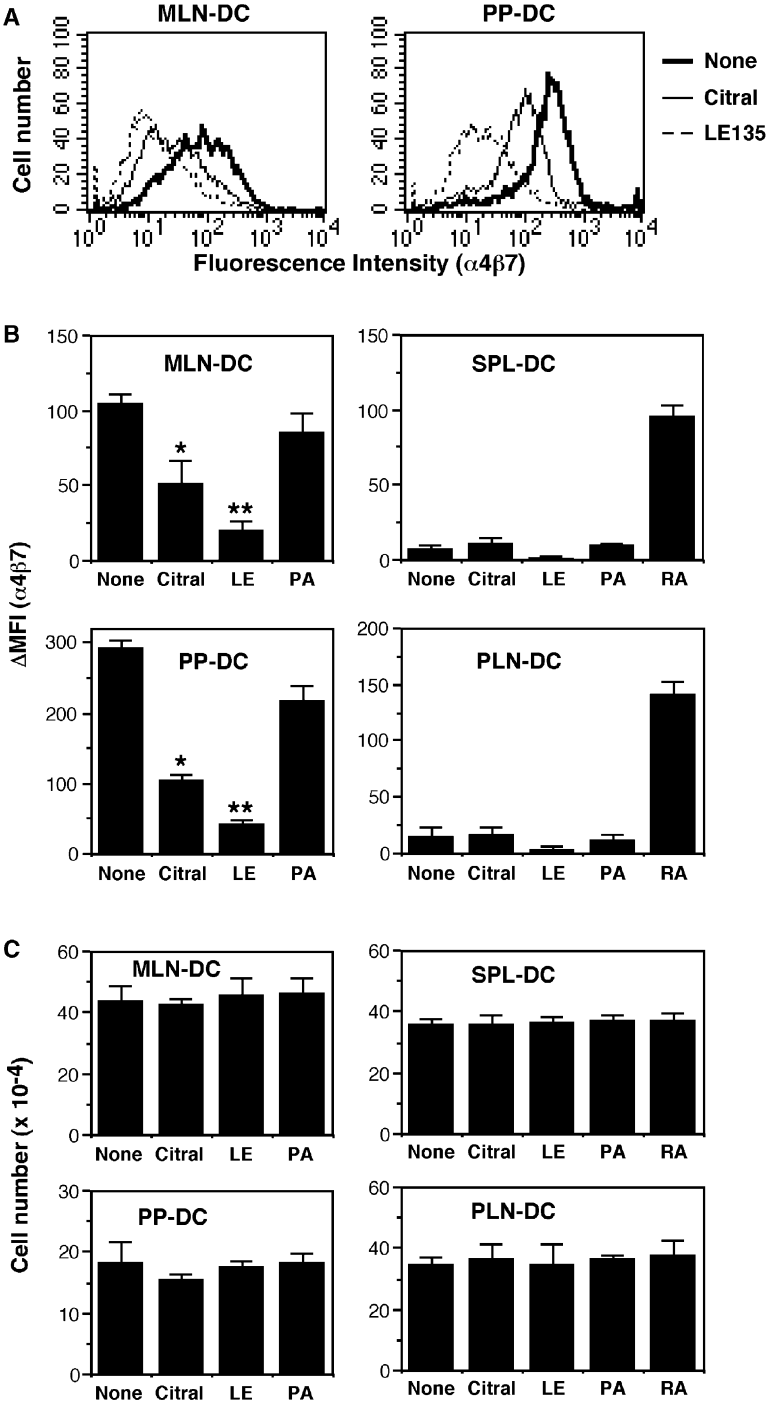

Figure 7. The Capacity of MLN-DC and PP-DC to Enhance the $\alpha4\beta7$ Expression on $CD4^+$ T Cells Is Suppressed by the RALDH Inhibitor Citral and the RAR Antagonist LE135

(A) Representative histograms for $\alpha4\beta7$ expression of $CD4^+$ T cells cultured with antigen-pulsed MLN-DC or PP-DC in the presence or absence of 10 μM citral or 1 μM LE135 for 4 days. (B and C) $\alpha4\beta7$ expression (B) or recovery of viable cells per culture (C) of $CD4^+$ T cells cultured with antigen-pulsed DCs from MLN, SPL, PP, or PLN in the presence or absence of 10 μM citral, 1 μM LE135, 1 μM PA452, or 1 nM all-*trans*-RA for 4 days. Vertical bars represent SD (n = 4 to 5 per group). Asterisks indicate that the difference with control is statistically significant with Kruskal-Wallis test with Dunn's posttest (*$p < 0.05$, **$p < 0.001$). A representative result of at least two independent experiments is shown.

by RA even at 0.1 nM in vitro (Figure 1B). The concentration was approximately one-tenth of that required for another effect of RA on T cells, the modulation of Th1/Th2 balance (Iwata et al., 2003). The plasma RA level is usually in the order of 10 nM, but most of the plasma RAs are bound to albumin and other plasma proteins. RA may function as a signal of the body's vitamin A adequacy by regulating the expression of the RA-catabolizing enzymes lecithin:retinol acyltransferase and a cytochrome P450, CYP26, and others (Ross, 2003), while the expression level of RALDH1 is controlled by the vitamin A status (Bhat 1998; Zhai et al. 2001). RA is converted from retinol by ADHs and RALDHs. Normal retinol levels in mouse sera are 0.8–1.2 μM (Carman et al., 1989; Molrine et al., 1995). Although more than 95% of plasma retinol is normally bound to the retinol binding protein, the bound retinol is transferred into a cell, owing to a membrane bound protein and the cellular retinol binding protein. Thus, a cell with an active intracellular oxidative pathway to convert retinol to RA via retinal may be capable of providing enough RA to imprint T cells with the gut-homing specificity.

We found that MLN-DC expressed mRNAs of *ADH class III* and *RALDH2* and that PP-DC expressed those of *ADH class I, II, III*, and *RALDH1* and RALDH1 protein. Furthermore, MLN-DC and PP-DC could convert [^{3}H]-retinol into [^{3}H]-RA. Although the purity of CD11c$^+$ MLN-DC preparations was approximately 90%, the CD11c$^-$ fractions of MLN expressed only low levels of *RALDHs* (Figure 6B and data not shown), and thus it is unlikely that the contaminated CD11c$^-$ cells in the MLN-DC preparations were responsible for the conversion. On the other hand, the CD11c$^-$ fractions of PP expressed *RALDH1* even more strongly than the CD11c$^+$ fractions (Figure 6B). However, by immunohistochemical analyses, we detected many RALDH1$^+$CD11c$^+$ cells in the subepithelial dome areas and some in the interfollicular T cell regions of PP (Figure 6C), suggesting that a subpopulation of PP-DCs is capable of producing RA. It has been reported that there are at least three types of B220$^-$ PP-DCs: (1) myeloid DCs (CD11b^{high}) in the subepithelial dome, (2) lymphoid DCs (CD8$\alpha\alpha^+$) in the interfollicular region, and (3) double-negative DC (CD11b$^-$CD8α^-) at both sites (Iwasaki and Kelsall, 2000). Another subset CD11c$^+$B220$^+$ DC (plasmacytoid pre-DC) has been also found in the subepithelial dome and in interfollicular regions of PP (Castellaneta et al., 2004). It remains to be elucidated which DC populations produce RA and contribute to the imprinting of T cells and why they specifically exist in the gut-associated lymphoid organs. We detected RALDH1 protein (Figure 6) and mRNAs of *RALDH1* and *ADH class I, II*, and *III* in mucosal epithelial cells as well (data not shown). Thus, these cells adjacent to PP may also provide RA to T cells by simple diffusion. The intestinal mucosa is known to produce RA also from dietary provitamins such as β-carotene in certain species including human (Wang et al., 1992).

Since [^{3}H]-RA production was more significant in the presence of T cells and antigen with MLN-DC than in the absence of T cells or antigen, the interaction of DC and T cells may affect the conversion. Another possibility is that the close association of the two cell types via antigen simply made a larger reservoir for RA. We analyzed [^{3}H]-retinoids in the culture supernatant as well, but most of the labeled products other than retinol were more hydrophilic than RA and were likely to be more oxidized products, such as 3,4-didehydroretinoic acid, or degradation products of retinol. Retinoids are known to be highly labile to light, heat, oxygen, and chemical treatments. It was thus difficult to assess [^{3}H]-RA released into the culture supernatant, and the half-life of RA outside of the cell in vitro may be much shorter than that in vivo.

The capacity of MLN-DC and PP-DC to enhance the $\alpha4\beta7$ expression on T cells was suppressed by the RALDH inhibitor citral and the RAR antagonist LE135, supporting that RA and RAR are involved. The RAR agonist Am80, but not the RXR pan-agonist HX600, exerted effects similar to those of RA on the expression of the homing receptors in T cells (Figure 1 and 2). LE135 is a potent antagonist of RARβ isotype and a less potent antagonist of RARα isotype, while Am80 is a potent RARα agonist and a less potent agonist of RARβ. Thus, RARα and/or RARβ, but not RARγ, may be involved in the effect. The RAR and RXR proteins interact with each other to form dimers RAR/RXR or RXR/RXR, mainly as RAR/RXR heterodimers under physiological conditions. The dimers are capable of binding to specific DNA sequences, RARE or RXRE, that are located within the 5′ regulatory region of retinoid-regulated genes or occasionally within introns. Around the first exons of CCR9 and $\beta7$ integrin genes, there are potential *cis*-elements including several RA boxes (T(G/C)AC(C/A)) (Lim et al., 1998) and half sites of RARE (A/G)G(G/T)(T/A)CA, which may contribute to these RA effects. Accordingly, mRNA expression of $\beta7$ and $\alpha4$, but not $\beta1$ integrin, was enhanced by RA and Am80 (data not shown).

Integrin $\alpha4\beta7^+$ T cells contribute to the development of insulitis in nonobese diabetic (NOD) mice (Faveeuw et al., 1995; Yang et al., 1997). The islet-infiltrating lymphocytes express $\alpha4\beta7$ integrin, while its ligand MAdCAM-1 is expressed on islet vessels. In insulin-dependent diabetes-prone BB/Wor rats, retinol deficiency reduces diabetes and insulitis, and retinoic acid can at least partly substitute for retinol in the development of insulitis (Driscoll et al., 1996). Thus, RA may contribute to endow diabetogenic T cells with the capacity to infiltrate into pancreatic islets. It has been suggested that initial priming of diabetogenic T cells take place in pancreatic lymph nodes or MLN in NOD mice (Gagnerault et al., 2002; Jaakkola et al., 2003). DC of pancreatic lymph nodes as well as MLN-DC in NOD mice may be able to produce RA.

We found that RA suppressed the expression of the skin-homing receptors E-lig, P-lig, and CCR4, on T cells upon activation, and their homing to PLN (Figures 1 and 2). Thus, RA may suppress the migration of T cells into the inflamed skin, where the ligands of these receptors are expressed. E-lig and P-lig are also expressed on endothelial cells at the atherosclerosis sites (Dong et al., 1998). This RA effect may be applied to regulate the migration of proinflammatory Th1 cells to the disease sites. It will be interesting to determine whether RA regulates the homing properties of other cell types including monocytes or macrophages.

Experimental Procedures

Mice and Cytokines

DO11.10 × Rag-2$^{-/-}$ mice (B10.D2 background), normal B10.D2, and BALB/c mice were obtained from Taconic (Germantown, NY), SLC (Hamamatsu, Japan), and Charles River Japan, respectively. Retinoids were obtained from Sigma (St. Louis, MO). Mouse IL-2, IL-4, IL-12, and IFN-γ were obtained from Genzyme-Techne (Cambridge, MA). To express IL-2 and IL-4 activities, U was used by calculating as 1 U/ml = 1 ED_{50} according to the manufacturer's definition of ED_{50}.

T Cell Isolation

Naive CD4$^+$CD62L^{high} DO11.10 × Rag-2$^{-/-}$ T cells were purified from splenocytes by using Dynabeads Mouse CD4, DetachaBead Mouse CD4 (Dynal, Oslo, Norway) and MACS CD62L Microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) as previously described (Iwata et al., 2003). In some experiments, CD4$^+$ T cells from secondary lymphoid organs were purified with Dynabeads Mouse CD4 and DetachaBead Mouse CD4, and the purity was at least 97%. T cells in the intestinal LP, lung, and liver were partially purified as described (Lefrançois and Lycke, 1999; Svensson et al., 2002), and analyzed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA).

DC Isolation

Single-cell suspensions were generated by mechanical dissociation of the tissues of PLN, SPL, MLN, and PP as described (Lefrançois and Lycke, 1999). PPs were incubated in the medium containing 5 mM EDTA for 20 min prior to the mechanical dissociation and filtrated through Cell Strainers (BD Falcon, Franklin Lakes, NJ) after the mechanical dissociation. In some experiments, SPL-DC was enriched with Lymphosepar II (IBL, Gunma, Japan) before DC isolation. DCs were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD11c (BD Pharmingen, San Diego, CA) in the presence of a Fc receptor-blocking antibody (anti-FcR, 2.4G2) and were immunomagnetically isolated by positive selection by using anti-FITC microbeads and MS columns (Miltenyi). The purity of $CD11c^+$ cells was approximately 90%.

Culture Conditions

Naive $CD4^+$ T cells were added into the plates coated with 3 μg/ml of anti-CD3 (145-2C11) and 3 μg/ml of anti-CD28 as described previously (Iwata et al., 2003). After 2 days of culture with medium alone ("unskewed condition"), 5 ng/ml IL-12 and 2 μg/ml anti-IL-4 ("Th1 condition"), or 40 U/ml IL-4 and 2 μg/ml anti-IFN-γ and 2 μg/ml anti-IL-12 ("Th2 condition"), the cells were resuspended in three times as much volume of fresh medium containing IL-2 and the same concentrations of IL-12 or IL-4. The suspensions were transferred into new wells and cultured for 2–4 days. Retinoids were added at the start of cultures (first and secondary cultures) or indicated time. For T cell/DC cocultures, 5×10^4 DCs from B10.D2 mice were pulsed with 1 μM ovalbumin peptide P323-339 for 3 hr. The antigen-pulsed DCs were mixed with 5×10^4 $CD4^+$ T cells from DO11.10 mice in 0.2 ml of medium containing 10% FCS, which was not heat inactivated, and indicated reagents in 96-well round-bottom plates. After 4 days of culture, the recovered viable cells were mostly T cells and were enumerated by using trypan blue.

FACS Analysis of Surface Markers

The cells were stained with PE-conjugated monoclonal antibodies (MAb) to CD4 (RM4-5), α4β7 (DATK32), CD62L (MEL-14), or FITC-conjugated MAb to CD8α (53-6.7) (Pharmingen) in the presence of anti-FcR. Some cells were stained with mouse E-selectin/human IgG Fc chimera protein (Genzyme-Techne) or mouse P-selectin/human IgG Fc chimera protein (Pharmingen) and then PE-conjugated goat anti-human IgG Fc (Jackson ImmunoResearch, West Grove, PA). To analyze naive and memory/activated populations in $CD4^+$ T cells, the cells were stained with FITC-conjugated MAb to CD44 (1M7), and biotinylated MAb to CD45RB (16A) (Pharmingen) followed by streptavidin-Tri-Color (Caltag). In some experiments, surface protein expression levels were expressed as Δmean fluorescence intensity (ΔMFI) calculated as: (mean fluorescence intensity of the cells treated with fluorophor-conjugated antibody) – (mean fluorescence intensity of the cells without the fluorophor-conjugated antibody treatment).

RT-PCR Assay

mRNA levels of genes were semiquantitatively assessed by RT-PCR assays as described previously (Adachi et al., 2000). The reverse-transcribed cDNA from each sample was serially diluted (three times for each step), and each diluted sample was subjected to PCR amplification. After the first denaturation step (94°C for 3 min), the amplification was performed for 25 or 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; (25 cycles for GAPDH, 30 cycles for CCR9, CCR7, CCR4, FucT-VII, ADHs, and RALDHs). The final cycle was followed by an extension step of 5 min at 72°C. Sequences of the sense and anti-sense primers were as follows: CCR9, 5′-TGCAGGCTGTTGACGCTTATG-3′ and 5′-CATCCCAGGTTCTTCAGGGTC-3′; CCR7, 5′-GAAACCCAGGAAAAACGTGCT-3′ and 5′-TACGGGGAGAAGGTTGTGGT-3′; CCR4, 5′-TGGAGAGGTACCTAGACTACG-3′ and 5′-GTCACAGTGTTTGCAGAGCAC-3′; FucT-VII, 5′-CGTCAGCATGAATGAGAGTCG-3′ and 5′-CAGGAGTTCAAGCCTGGAACCA-3′; ADH class I, 5′-TGGCTAAGAAGTTTCCGTTGGAC-3′ and 5′-GTGATGTGGCTGGTGCTTGA-3′; ADH class II, 5′-ACAGCTCAGACCTTGAAAGCAG-3′ and 5′-GCATGGGTCACCAGTAAGTCC-3′; ADH class III, 5′-GACGAATTTGTGACCGGCAATC-3′ and 5′-GTGCAGGATGGACAGTGTTCTC-3′; RALDH1, 5′-ATGGTTTAGCAGCAGGACTCTTC-3′ and 5′-CCAGACATCTTGAATCCACCGAA-3′; RALDH2, 5′-GACTTGTAGCAGCTGTCTTCACT-3′ and 5′-TCACCCATTTCTCTCCCATTTCC-3′; RALDH3, 5′-GGACAGTCTGGATCAACTGCTAC-3′ and 5′-TCAGGGGTTCTTCTCCTCGAGT-3′; GAPDH, 5′-CCAGGTTGTCTCCTGCGACTT-3′ and 5′-CCTGTTGCTGTAGCCGTATTCA-3′. PCR products were resolved by electrophoresis on a 1.3% agarose gel containing ethidium bromide. CCR9, CCR7, CCR4, FucT-VII, ADH class I, ADH class II, ADH class III, RALDH1, RALDH2, RALDH3, and GAPDH cDNA yielded PCR products of 184, 165, 167, 176, 193, 230, 129, 142, 160, 158, and 133 bp, respectively.

Chemotaxis Assay

Transwell chemotaxis assays were performed essentially as described (Campbell and Butcher, 2002). Briefly, 5×10^5 cells were added into the upper well of Transwell plates (Corning) with or without chemokines in the lower well. Experiments were performed with chemokine concentrations that induce maximal chemotaxis; 100 nM for TARC, 240 nM for TECK, 100 nM for CTACK (Genzyme-Techne), and 100 nM for IP-10 (Strathmann Biotec, Hamburg, Germany). The numbers of migrated cells into the lower wells were counted, and their percentages of the input cell number were calculated.

Homing Assay

The T cells cultured with or without RA were labeled for 15 min with 0.85 μM CFSE or 5 μg/ml TRITC (Molecular Probes, Eugene, OR) at 37°C. Thereafter, cells were centrifuged over fetal calf serum and extensively washed. Ten million cells from each preparation were mixed and injected intravenously into each recipient mouse. An aliquot was saved to assess the input cell population by flow cytometry. Recipients were sacrificed 16 hr after the injection. The homing index was calculated as the ratio of $CFSE^+$ cells (RA-treated) to $TRITC^+$ cells (control) in recipient tissues. For histological analyses, PLN, MLN, SPL, and blocks of small intestines with PP were immersion fixed in 4% paraformaldehyde. The fixed tissues were washed in 0.1 M phosphate buffer (pH 7.2), immersed in 20% sucrose in 0.1 M phosphate buffer for cryoprotection, and then embedded in O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and frozen on dry ice. Eight μm thick frozen sections of these tissues were cut out and thaw mounted onto silane-coated glass slides and coverslipped with glycerol. CFSE- and TRITC-labeled cells were observed by a confocal laser scanning microscopy (Bio-Rad). For statistical analysis of the labeled cells in LP, frozen, circular cross-sections of the small intestine were cut out at 10 μm. We surveyed the whole LP of each section and counted the labeled cells in 20 sections per mouse, omitting sections without a labeled cell.

Production of Vitamin A-Deficient Mice

B10.D2 mice were bred, and gravid females received either a chemically defined diet that lacked vitamin A (the modified AIN-93M feed, Oriental Yeast, Tokyo, Japan) or control diet containing retinyl acetate (25,000 IU/kg in the modified AIN-93M feed). These diets started at 7–10 days of gestation. The pups were weaned at 4 weeks of age and maintained on the same diet at least until 11 weeks of age before analysis was performed. The two populations showed no signs of inanition and had equivalent body weights. Serum retinol levels were analyzed by the method as described (Chatellard-Gruaz et al., 1998) with a modification. Briefly, 0.3 ml of serum was mixed with 0.3 ml of ethanol containing butylated hydroxytoluene and 6 μl of 1 M HCl. Retinoids were extracted from the mixture with 1 ml of hexane three times. The combined organic phases were evaporated under N_2 and the dry residue was dissolved in acetonitrile (30 μl). Aliquots of 25 μl were subsequently analyzed by HPLC with a Finepak SIL $C_{18}S$ column (4.6 × 150 mm; Nihon Bunko, Tokyo, Japan). Separation of retinol was performed with an isocratic elution by using a solvent system composed of 66% acetonitrile, 30% water (50 mM ammonium acetate, pH 7), and 4% tetrahydrofuran at a flow rate of 1.5 ml/min. The recovery of retinol after the extraction and HPLC processes was estimated as approximately 70% by using [11,12-^{3}H]-all-*trans*-retinol (Perkin Elmer, Norwalk, CT).

RA Production Assay

An aliquot of MLN-DC, PP-DC, or SPL-DC preparations (1×10^6) was mixed with 1×10^6 naive $CD4^+$ T cells from DO11.10 mice and

1 μM ovalbumin peptide P323-339 in 1 ml of the medium containing 10% FCS and 2.5 μCi of [^{3}H]-all-*trans*-retinol. The suspension was cultured for 18 hr, and the cells were washed three times with ice-cold PBS. The cells were then lysed in 0.5 ml of Tris-HCl buffer (50 mM Tris-HCl, 25 mM NaCl, 2 mM EDTA, 1 mM DTT [pH 7.5]) containing 100 μM citral and mixed with 0.5 ml of ethanol containing 1 nmole of each unlabeled retinoid (all-*trans*-retinol, all-*trans*-retinal, and all-*trans*-RA) and butylated hydroxytoluene (50 μg/ml). The mixture was adjusted to pH 3.0 by adding 10 μl of 1 M HCl. Retinoids were extracted, dried, and dissolved in 40 μl acetonitrile. 25 μl of the extract was fractionated with the HPLC system as described above. A half of each fraction (1 ml) was added into a piece of Ready Cap (Beckman, Fullerton, CA), dried, and its radioactivity was counted. Internal retinoid standards were detected by measuring ultraviolet absorption at 350 nm.

Immunohistochemical Analysis

Frozen tissue sections were prepared as described above and subjected to a series of blocking procedure with anti-FcR, 5% normal donkey serum (Jackson), and Avidin D/biotin solutions (Vector, Burlingame, CA). Sections were incubated with rat anti-CD4 (RM4-5, Pharmingen), anti-CD8α (53-6.7, Pharmingen), or goat anti-RALDH1 antibody (Abcam, Cambridge, UK) at 4°C overnight followed by the incubation with biotinylated anti-rat IgG (Jackson) or Cy3-conjugated anti-goat IgG (Jackson), respectively, for 30 min at room temperature. CD4$^+$ or CD8$^+$ cells were visualized with avidin-conjugated peroxidase and its substrate diaminobenzidine or with streptavidin-conjugated Cy3. The anti-RALDH1-treated sections were incubated with hamster anti-CD11c antibody at 4°C overnight followed by the incubation with biotinylated anti-Armenian hamster IgG (Jackson). CD11c$^+$ cells were visualized with streptavidin-conjugated Alexa Fluor 488 (Molecular Probes). The anti-RALDH1 antibody detected only a 54 kDa band in PP-DC but no significant bands in other DC preparations by Western blotting assay.

Statistical Analysis

Statistical comparisons were carried out by using the nonparametric two-tailed Mann-Whitney test and the Kruskal-Wallis test with Dunn's posttest.

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