Imprinting of CCR9 on CD4 T Cells Requires IL-4 Signaling on Mesenteric Lymph Node Dendritic Cells[1]

Raul Elgueta,[2*†] Fernando E. Sepulveda,[2*†] Felipe Vilches,[*] Leonardo Vargas,[*†] J. Rodrigo Mora,[‡§] Maria Rosa Bono,[*†] and Mario Rosemblatt[3*†‡¶]

It has recently been shown that IL-4 can educate dendritic cells (DC) to differentially affect T cell effector activity. In this study, we show that IL-4 can also act upon DC to instruct naive T cells to express the gut-associated homing receptor CCR9. Thus, effector T cells generated after coculture with mesenteric lymph node (MLN)-DC show a higher expression of CCR9 when activated in the presence of IL-4. In contrast, IL-4 had no effect on CCR9 expression when naive T cells were polyclonally activated in the absence of MLN-DC, suggesting that the effect of IL-4 on CCR9 expression passed through DC. Indeed, T cells activated by MLN-DC from IL-4R$\alpha^{-/-}$ mice showed a much lower CCR9 expression and a greatly reduced migration to the small intestine than T cells activated by wild-type MLN-DC even in the presence of IL-4. Consistent with the finding that the vitamin A metabolite retinoic acid (RA) induces gut-homing molecules on T cells, we further demonstrate that IL-4 up-regulated retinaldehyde dehydrogenase 2 mRNA on MLN-DC, a critical enzyme involved in the synthesis of RA. Moreover, LE135, a RA receptor antagonist, blocked the increased expression of CCR9 driven by IL-4-treated MLN-DC. Thus, besides the direct effect of RA on T cell gut tropism, our results show that the induction of a gut-homing phenotype on CD4$^+$ T cells is also influenced by the effect of IL-4 on gut-associated DC.

Homing of T cells depends on their activation status. Whereas naive T cells are continuously migrating through secondary lymphoid organs (SLO)[4] (lymph nodes, spleen, and Peyer's patches (PP)), effector T cells migrate preferentially to extralymphoid tissues (1, 2).

Lymphocyte homing is determined by the differential expression of adhesion molecules on different subtypes of T cells (3–5). Naive T cells recirculate through SLO due in part to the expression of L-selectin (CD62L) and the chemokine receptor CCR7 that recognize peripheral lymph node (PLN) addressin and chemokines CCL19 and CCL21, respectively (6). In contrast, effector/memory T cells have been separated into at least two groups, depending on their gut-homing vs skin-homing receptor expression. Lymphocytes with a gut-homing potential express the chemokine receptor CCR9 and integrin $\alpha_4\beta_7$ (that bind CCL25 and mucosal addressin cell adhesion molecule-1, respectively) and preferentially home to the small bowel lamina propria and intraepithelial compartments (7–9). In contrast, lymphocytes with skin-homing potential express ligands for E- and P-selectin and chemokine receptors CCR4 and/or CCR10 (that bind CCL17/CCL22 and CCL27, respectively) and home to inflamed skin (10–12).

It has been described that the acquisition of homing potential by T cells occurs after activation in the SLO, and that this depends on the microenvironment in which Ag presentation occurs (13, 14). In fact, it has been shown that the site of entry of the Ag determines the combinatorial expression of adhesion molecules and chemokine receptors that T lymphocytes will acquire (13). We (15) and others (16–20) have shown that dendritic cells (DC) from GALT (GALT-DC) induce on CD8 T cells and CD4 T cells a higher expression of the gut-homing receptors CCR9 and $\alpha_4\beta_7$ and the capacity to home into the small intestinal mucosa (15, 19, 20). Conversely, PLN-DC induce skin-homing receptors such as E-selectin ligand and CCR4 on naive CD8 T cells (17). All together, these results indicate an essential role for DC in the establishment of the homing potential of T lymphocytes.

Previous evidence demonstrated that DC from PP (but not from spleen) induce IL-4 synthesis by T cells (21, 22). More recently, Matzinger and coworkers (23) reported that effector T cells specific for an orally administered Ag could instruct DC through signals that include IL-4. These DC may be the targets of early IL-4 secretion, an event that could lead to further IL-4 production after activation of naive T cells. Recently, Mora et al. (24) reported a reciprocal (skin vs gut) control of CD8 T cell homing and that IL-4 generated an increase in CCR9 expression on CD8 T cells when activated by GALT-DC, although IL-4 did not seem to be necessary for the induction of gut homing by these cells. These results, plus a report showing that IL-4 caused a down-regulation and in some cases a total loss in the expression of the T cell skin-homing

*Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile; †Millennium Institute for Fundamental and Applied Biology, Santiago, Chile; ‡Fundacion Ciencia para la Vida, Santiago, Chile; §Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; and ¶Universidad Andres Bello, Santiago, Chile

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from Fondo Nacional de Investigación Científica y Tecnológica 1060834 and DI 03-02 from Universidad Andrés Bello (to M.R.) and Fondo Nacional de Investigación Científica y Tecnológica 1060253 (to M.R.B.). R.E. was supported by doctoral fellowships from Programa de Mejoramiento de la Calidad y Equidad en la Educacion Superior and Consejo Nacional de Ciencia y Tecnologia and a grant from the Departmento de Postgrado and Postitulo from Universidad de Chile. J.R.M. was supported in part by a Pew Fellowship and a Career Development Award from The Crohn's & Colitis Foundation of America.

[2] R.E. and F.E.S. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Mario Rosemblatt, Fundación Ciencia para la Vida, Avenida Zanartu 1482, Santiago, Chile. E-mail address: mrosembl@bionova.cl

[4] Abbreviations used in this paper: SLO, secondary lymphoid organ; DC, dendritic cell; HI, homing index; KO, knockout; LP, lamina propria; MFI, mean fluorescence intensity; MLN, mesenteric lymph node; PLN, peripheral lymph node; PP, Peyer's patches; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; TRITC, tetramethylrhodamine isothiocyanate.

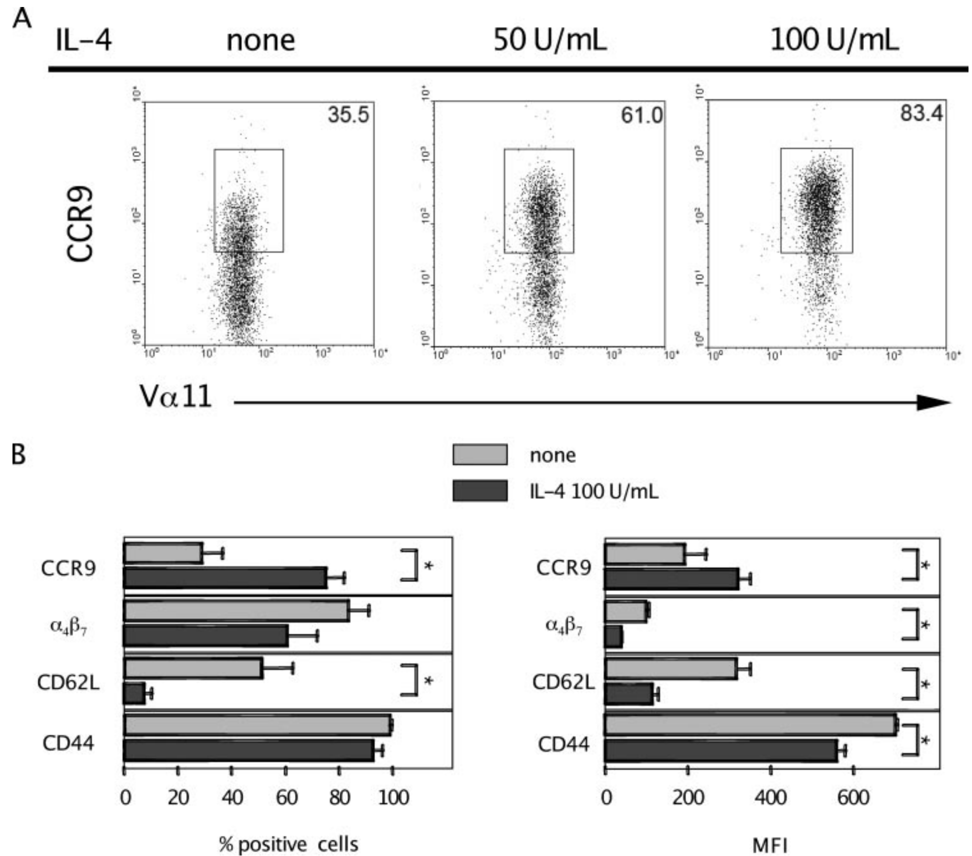

FIGURE 1. IL-4 increases CCR9 expression on CD4 T cells activated by MLN-DC. Naive transgenic TCR AND CD4 T cells were activated with MLN-DC in the presence or absence of IL-4. After 5 days of coculture, the expression of homing receptors and activation markers on the resulting effector T cells was determined by flow cytometry. *A*, Representative results for CCR9 expression on T cells activated with MLN-DC in the presence of 0, 50, and 100 U/ml IL-4. *B*, Quantification of the percentages of CD4 T cells (mean ± SD) expressing homing receptors (CCR9, $\alpha_4\beta_7$) or activation markers (CD62L, CD44) on Vα11-positive cells (*left panel*). MFI (*right panel*) of double-positive cells (n = 3–4). *, $p < 0.01$ comparing T cells activated with MLN-DC in the presence or absence of 100 U/ml IL-4.

receptor cutaneous lymphocyte Ag (25), lead us to hypothesize that, in a reciprocal fashion, IL-4 may affect CD4 T cell homing to gut-associated tissues.

In agreement with our hypothesis, in the present study we demonstrate that IL-4 induces a higher CCR9-imprinting potential on mesenteric lymph node (MLN)-DC. Moreover, IL-4 seems to play a necessary in vivo role in the education of DC to imprint gut-tropic T cells, because MLN-DC from IL-4R$\alpha^{-/-}$ mice were impaired for inducing CCR9 expression on T cells. Because we also found that IL-4 induces an increase in the mRNA expression for retinaldehyde dehydrogenase (RALDH) 2 on MLN-DC, a critical enzyme involved in the synthesis of RA, we propose a relationship between the effect of IL-4 reported in this study and that of retinoic acid (RA) reported by Iwata el al (26). Thus, our results correlate IL-4, a cytokine present in the gut microenvironment (21), with the production of RA by GALT-DC and the acquisition of gut-homing potential by CD4 T lymphocytes.

Materials and Methods

Mice

The B10.BR, transgenic AND, transgenic DO11.10, IL-4R$\alpha^{-/-}$, and BALB/c mice strains were from The Jackson Laboratory. AND × B10.BR F_1 and other mice were bred and maintained at Millennium Institute for Fundamental and Applied Biology. All animal handling was done according to institutional guidelines.

Antibodies

The following mAbs from BD Biosciences were used: FITC-conjugated anti-Vα11 (RR8-1); PE-conjugated anti-$\alpha_4\beta_7$ (DATK32); PE-conjugated anti-CD11c (HL3); PE-conjugated anti-CD25 (3C7); PE-conjugated anti-CD62L (MEL-14); PE-conjugated anti-rat IgM (G53-238); PE-conjugated rIgGa,k (R35-95) isotype control; PE-Cy5-conjugated anti-CD4 (RM4-5); CyChrome-conjugated anti-CD44 (IM7); purified anti-I-E^k (14-4-4S); and purified anti-I-A^d (34-5-3). FITC-conjugated anti-mouse OVA-specific TCR (KJ1-26) was obtained from Invitrogen. Anti-mouse CCR9 (5F2) and rIgM (MAC158) isotype control were provided by M. Briskin (Millennium Pharmaceuticals, Cambridge, MA). Anti-mouse CD3 (2C11) and anti-CD28 (PV-1) were donated by R. Noelle (Dartmouth Medical School, Lebanon, NH).

Cell isolation, cocultures, and polyclonal activation

DC were obtained, as previously reported (15). Naive CD4 T cells were obtained from thymus of AND × B10.BR F_1 or transgenic DO11.10 4- to 6-wk-old mice depleted of APCs by negative selection using I-E^k or I-A^d mAb plus MACS conjugated with anti-mouse IgG Abs (Miltenyi Biotec) (27). Transgenic thymocytes contained >75% of mature CD4 T cells, of which >90% expressed the transgenic TCR. After removal of RBC, splenic naive CD4 transgenic T cells AND × B10.BR were purified to >95%, by MACS conjugated with anti-mouse CD4 Ab.

DC and T cells (1:5 ratio) were cocultured in the presence of 5 μM pigeon cytochrome *c* peptide 87–104 (27) or $OVA_{323-339}$ (17) (New England Peptide) for 5 days and then analyzed by flow cytometry (27). In some experiments, cocultures were supplemented with rIL-4 (BD Pharmingen) in varying doses (10, 50, and 100 U/ml) with or without 1 μM LE135 (Tocris Bioscience). In some experiments, MLN-DC were treated with 100 U/ml IL-4 for 2 h, washed twice with medium, and then cocultured, as above.

Polyclonal T cell activation was done in plates coated with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28. In some experiments, T cells were supplemented with 100 U/ml rIL-4.

Homing assays

Competitive homing assays were performed with DO11.10 CD4 T cells activated for 5 days with MLN-DC from BALB/c or IL-4R$\alpha^{-/-}$ mice and differentially labeled with calcein or tetramethylrhodamine isothiocyanate (TRITC; Molecular Probes), as previously described (15). In some experiments, we transferred the cells into IL-4R$\alpha^{-/-}$ mice. Mice were sacrificed 2 days later, and single-cell suspensions from PLN, spleen, MLN, PP, and lamina propria (LP) were generated. Cell samples were labeled and gated with anti-CD4 PE-Cy5 for flow cytometry analysis. The homing index (HI) was calculated, as previously described: HI = $(\text{ratio calcein/TRITC})_{\text{tissue}}$:$(\text{ratio calcein/TRITC})_{\text{input}}$ (15).

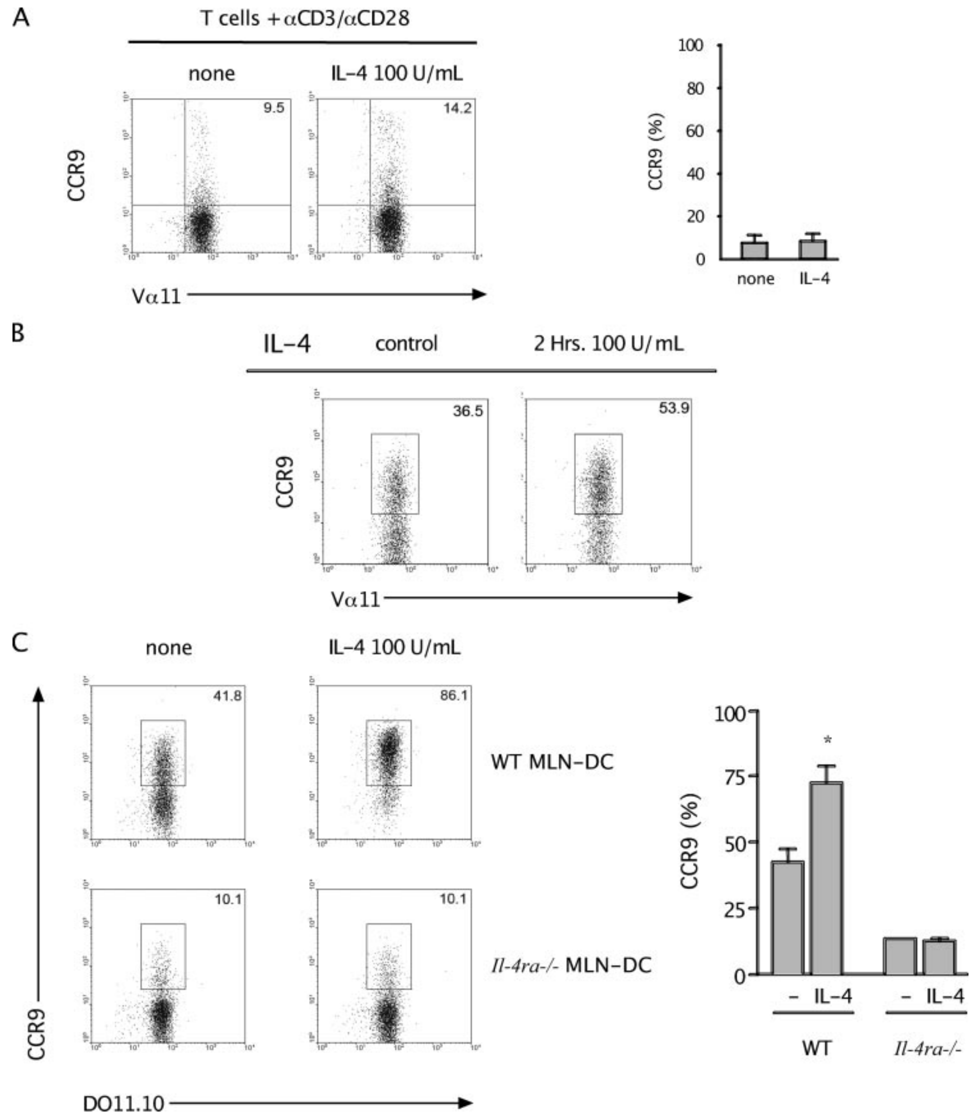

FIGURE 2. The increase of CCR9 expression on CD4 T cells induced by IL-4 requires the presence of MLN-DC. *A*, T cells polyclonally activated with anti-CD3 plus anti-CD28 with (*right panel*) or without (*left panel*) 100 U/ml IL-4. Bar graph at *right* shows the results of three independent experiments. *B*, Representative results for CCR9 expression on T cells activated by MLN-DC pretreated (*right panel*) or not (*left panel*) for 2 h with 100 U/ml IL-4. (One representative experiment of two with similar results is shown.) *C*, Representative results for CCR9 expression on transgenic DO11.10 CD4 T cells activated with MLN-DC from wild-type (WT) BALB/c mice (*upper row*) or IL-4R$\alpha^{-/-}$ mice (*lower row*) in the presence (*right panel*) or absence (*left panel*) of IL-4. Bar graph at *right* shows a statistical analysis of three to four independent experiments. *, $p < 0.01$.

Quantitation of gene expression using real-time PCR

Total RNA was purified from control or IL-4-treated DC-MLN from wild-type or IL-4R$\alpha^{-/-}$ mice using RNAeasy kits (Qiagen). cDNA synthesis was synthesized in 25 μl reaction containing 3 μg of total RNA, 500 ng of oligo(dT) (Invitrogen), 2 U of Moloney murine leukemia virus-reverse transcriptase (Promega), 15 U of RNAsin (Promega), 0.5 mM dNTP, and Moloney murine leukemia virus-reverse transcriptase buffer (Promega) for 1 h at 42°C (27). Primers used for murine RALDH2 and murine GAPDH were from Genearray (Affymetrix). Quantitative PCR were performed using Quantitect Primer Assays (Qiagen) with SYBR Green PCR Master Mix (Invitrogen). cDNA samples were assayed in triplicate using a Chromo4 detection system (MJ Research), and gene expression levels for each individual sample were normalized to GAPDH. Mean relative gene expression was determined, and the differences were calculated using the $2C_t$ method (28).

Statistical analysis

Data are presented as mean ± SD and were analyzed using one-way ANOVA or two-way ANOVA with Bonferroni correction where appropriate. HI were tested vs HI = 1 using a one-sample *t* test. Significance was set at $p < 0.05$.

Results

IL-4 acts upon MNL-DC to increase CCR9 expression on CD4 T cells

Recently, it has been reported that memory T cells generated in the GALT educate DC through an IL-4-dependent mechanism to generate a Th2 response on naive T cells (23). Given that IL-4 also blocks the acquisition of skin-homing receptors (25), we asked whether IL-4 may reciprocally help in the education of DC for the induction of gut-homing potential on CD4 T cells. To answer this question, we performed coculture experiments between enriched naive TCR transgenic CD4 T cells (TCR AND) from thymus and DC from MLN in the presence of the specific peptide pigeon cytochrome *c* peptide 87–104. After 5 days of coculture either without IL-4 or in the presence of different concentrations of the cytokine, we analyzed gut-homing receptors and activation markers on the resulting effector T cells. As shown in Fig. 1*A*, CCR9 expression on CD4 T cells activated by MLN-DC plus IL-4 increases in a dose-dependent manner. In contrast, when we analyzed the effect of IL-4 on $\alpha_4\beta_7$ expression, we did not observe any statistically significant change in the percentage of cells that express this receptor, except for a small reduction in the mean fluorescence intensity (MFI) (Fig. 1*B*). The IL-4 effect was associated with a significant down-regulation in the MFI for CD62L and a drastic fall in the percentage of T cells expressing this marker without any further increase on CD44 expression (Fig. 1*B*). We also analyzed the effect of IL-4 on thymus-derived CD4 T cells activated with DC isolated from PLN and spleen. The results show that IL-4 induces only a minor increase in the expression on CCR9 on CD4

T cells after activation with these cells, as compared with the significant increase seen on CD4 T cells activated with MLN-DC (data not shown).

Additionally, we evaluated the effect of IL-4 on CCR9 expression on CD4 T cells purified from spleen and activated with MLN-DC for 5 days. We observed that IL-4 also induced an increase in the expression of CCR9 on spleen-derived CD4 T cells to levels similar to those achieved with thymic CD4 T cells activated with MLN-DC (data not shown). These results demonstrate that the effect of IL-4 on CCR9 expression on CD4 T cells is independent of the origin of the T cells.

To ensure that the increase in CCR9 expression on CD4 T cells induced by IL-4 was not due to a more vigorous stimulation of T cells by healthier DC or due to a selective survival or expansion of a particular subset of DC by this cytokine, we analyzed the expression of several DC markers (CD103, CD8, B220, MHCII, CD86, and CD80) on MLN-DC and viability after 24 h of coculture either in the presence or absence of IL-4. No differences in marker expression and viability were observed between these two culture conditions (data not shown).

To determine whether IL-4 was acting directly on CD4 T cells or through DC in these cocultures, we performed polyclonal activation with anti-CD3 plus anti-CD28 Abs without DC and in the presence or absence of IL-4. The results show that IL-4 did not increase CCR9 expression when acting on CD4 T cells in the absence of DC (Fig. 2, *A* and bar graph), similar to what has been reported for CD8 T cells (24). Because CD4 T cells were not the target of IL-4 in these experiments, we sought to determine whether IL-4 was acting through DC. At this point, two different experimental strategies were used. First, MLN-DC were preincubated with 100 U/ml IL-4 for 2 h before coculture with naive TCR transgenic T cells and peptide. As seen in Fig. 2*B*, pretreatment of DC with the cytokine was sufficient to confer MLN-DC with the ability to induce an increase in CCR9 expression on naive CD4 T cells. In a second approach, MLN-DC from mice deficient for the α-chain of the IL-4R (IL-4R$\alpha^{-/-}$ MLN-DC) and from wild-type BALB/c (wild-type MLN-DC) mice were cocultured with enriched DO11.10 CD4 T cells from thymus plus 100 U/ml IL-4, in the presence of the specific peptide $OVA_{323-339}$. We observed that IL-4 had no effect on CCR9 expression on T cells activated by IL-4R$\alpha^{-/-}$ MLN-DC as compared with T cells activated by wild-type MLN-DC (Fig. 2*C*, *right panels* and bar graph), indicating that DC lacking the IL-4Ra are incapable of instructing CD4 T cells to increase CCR9 expression. Additionally, as Fig. 2*C* shows, MLN-DC purified from IL-4R$\alpha^{-/-}$ mice induce less CCR9 on CD4 T cells as compared with DC from wild-type mice (Fig. 2*C*, *left panels* and bar graph). Taken together, our results indicate that IL-4 acts on MLN-DC to increase their ability to induce CCR9 on CD4 T cells. One possible implication of this model is that gut lamina propria CD4 T cells from IL-4R-deficient mice should express less CCR9 than those from wild-type mice (see below).

Concerning $\alpha_4\beta_7$ expression, the BALB/c model (either wild-type or IL-4R$\alpha^{-/-}$ mice) behaves differently than B6 mice. BALB/c MLN-DC had only a modest effect on the expression of $\alpha_4\beta_7$, although similar to B6 MLN-DC, IL-4 had no effect on the capacity of BALB/c MLN-DC to induce $\alpha_4\beta_7$ on CD4 T cells (data not shown).

In view of the fact that CD4 T cells activated with MLN-DC from IL-4R$\alpha^{-/-}$ mice showed a much lower expression of CCR9 that those activated with wild-type DC, and a recent report indicating the existence of CCR9-dependent and -independent pathways for CD4 T cell entry to the gut (20), we aimed to determine whether the differential expression of CCR9 on CD4 T cells correlated with the capacity of these cells to home to gut-associated tissues in vivo. For this purpose, we conducted competitive homing experiments in an isogenic mouse model between CD4 T cells activated with MLN-DC from either IL-4R$\alpha^{-/-}$ or wild-type mice. We found that CD4 T cells activated with MLN-DC from wild-type mice home to the small intestinal lamina propria 3.8 $\pm$ 1.8 times better as compared with CD4 T cells activated with IL-4R$\alpha^{-/-}$ MLN-DC (Fig. 3). In contrast, we did not observe a privileged migration to MLN or PP (Fig. 3). These results suggest that the increase of CCR9 on CD4 T cells correlated with a higher capability of these cells to home to the small intestinal lamina propria.

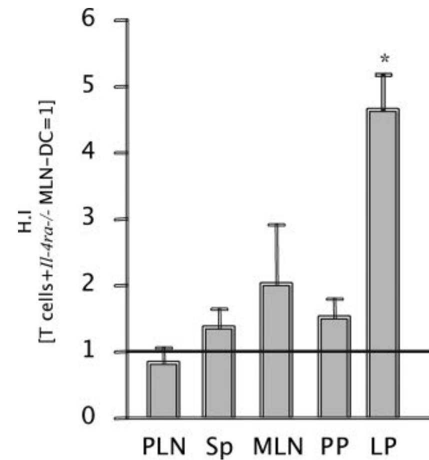

FIGURE 3. CD4 T cells activated with IL-4R$\alpha^{-/-}$ MLN-DC migrate significantly less to the gut. Naive TCR transgenic DO11.10 CD4 T cells were activated in the presence of the specific peptide ($OVA_{323-339}$) with MLN-DC from wild-type BALB/c or IL-4R$\alpha^{-/-}$ mice. After 5 days of coculture, the cells were labeled with calcein or TRITC and injected i.v. into an isogenic receptor. After 48 h, mice were killed and lymphocytes were isolated from different tissues of the recipient mice (n = 3–4). Cells were labeled with anti-CD4 PE-Cy5 and analyzed by flow cytometry. The HI was calculated as the ratio of (T cells + wild-type MLN-DC)$_{tissue}$/(T cells + IL-4R$\alpha^{-/-}$ MLN-DC)$_{tissue}$ to (T cells + wild-type MLN-DC)$_{input}$/(T cells + IL-4R$\alpha^{-/-}$ MLN-DC)$_{input}$. *, $p < 0.05$. Similar results were obtained when the labels were swapped between the two populations of cells (data not shown).

IL-4 regulates CCR9 expression on CD4 T cells in vivo

Next, we asked whether IL-4 has a role in CCR9 expression of T cells in vivo. For this, we first analyzed the expression of CCR9 on CD4 T cells from MLN, PP, and lamina propria of wild-type and IL-4R$\alpha^{-/-}$ animals. Our results showed almost identical percentages of CCR9$^+$ CD4 T cells in MLN and PP, whereas the lamina propria of wild-type mice presented a higher percentage of CCR9$^+$ CD4 T cells as compared with IL-4R$\alpha^{-/-}$ mice (Fig. 4*A*, 33.5 vs 9.8%). To rule out the possibility that the lower percentage of CCR9$^+$ CD4 T cells seen in the lamina propria of IL-4R$\alpha^{-/-}$ mice was due to a deficiency in the expression of specific endothelial addressins (i.e., CCL25 and/or $\alpha_4\beta_7$) rather than a decrease in CCR9 induction, we used IL-4R$\alpha^{-/-}$ mice as receptor animals to carry out competitive homing assays between CD4 T cells activated with wild-type or IL-4R$\alpha^{-/-}$ MLN-DC. We reasoned that if the differences observed in the percentages of CCR9$^+$ CD4 T cells are due to a decreased expression of addressins, then gut-tropic T cells imprinted by wild-type MLN-DC should be excluded from the small bowel LP of IL-4R$\alpha^{-/-}$ mice at a similar degree as T cells activated with MLN-DC from IL-4R$\alpha^{-/-}$ mice (regardless of the differences in CCR9 expression), therefore approaching the HI

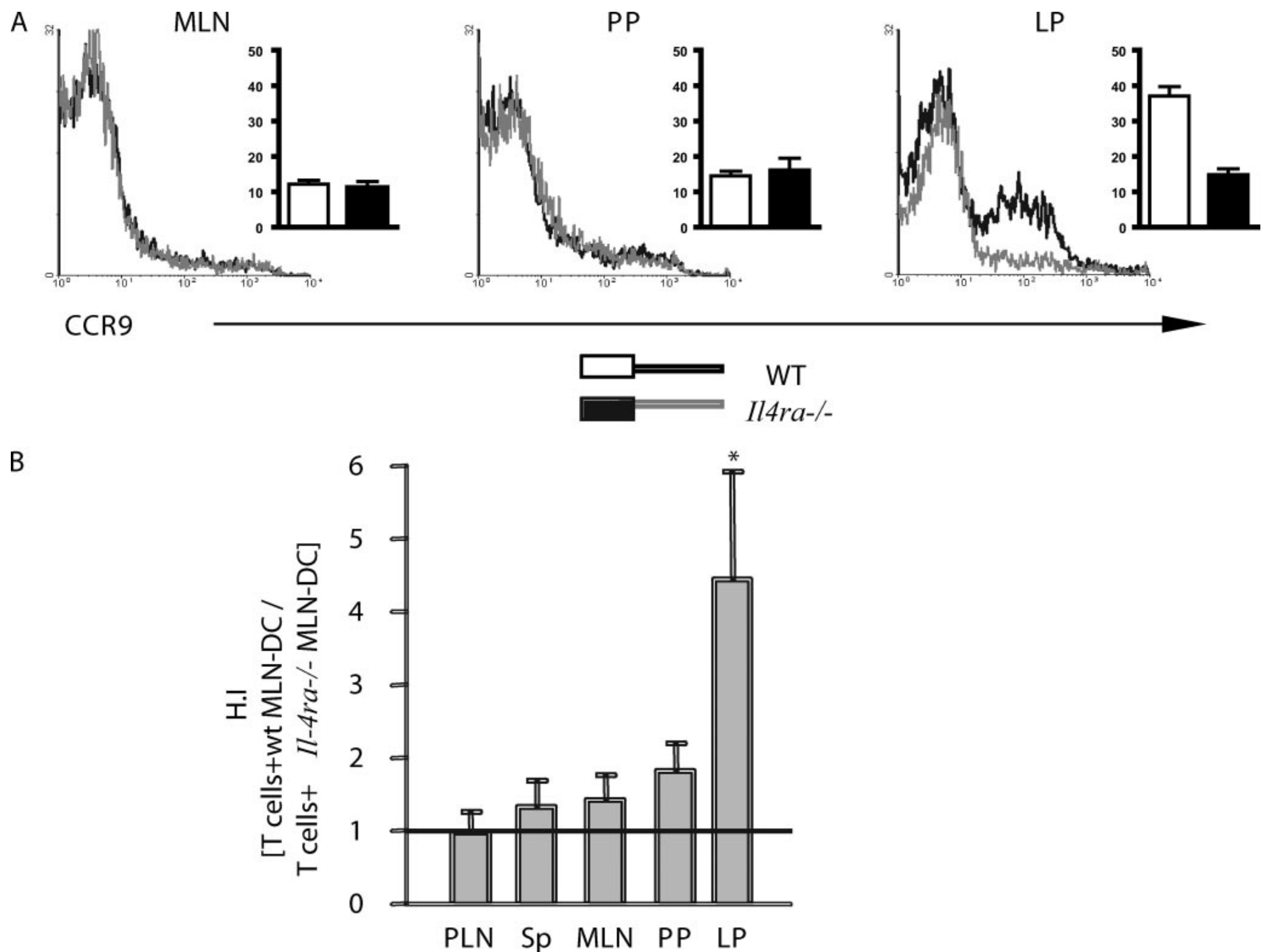

FIGURE 4. IL-4R-deficient mice have a reduced number of CCR9$^+$ CD4 T cells in the lamina propria despite a normal rate of entry of these cells to this tissue. *A*, CCR9 expression on CD4 T cells purified from MLN (*right panel*), PP (*central panel*), and lamina propria (*left panel*) of wild-type (WT) (black line) and IL-4R$\alpha^{-/-}$ (gray line) mice. *Inset*, Quantification of three independent experiments from wild-type (□) and IL-4R$\alpha^{-/-}$ (■) mice. *B*, A competitive homing experiment was performed with transgenic DO11.10 CD4 T cells activated with MLN-DC from wild-type and IL-4R$\alpha^{-/-}$ mice. After 5 days of coculture, the cells were labeled with calcein or TRITC and injected i.v. into an IL-4R$\alpha^{-/-}$ mice. After 48 h, mice were killed and lymphocytes were isolated from different tissues of the recipient mice ($n = 3$). Cells were labeled with anti-CD4 PE-Cy5 and analyzed by flow cytometry. The HI was calculated for different tissues as in Fig. 3 ($n = 3–4$). *, $p < 0.05$.

to 1 in a competitive homing experiment (i.e., no different migration). Indeed, although our results showed that IL-4Rα knockout (KO) mice have lower absolute numbers of T cells in LP as well as in other organs such as PLN (data not shown), the access of CD4 T cells activated with wild-type MLN-DC to the LP of IL-4R$\alpha^{-/-}$ mice is not affected, as reflected by the identical HI found in wild-type (Fig. 3) and KO mice (Fig. 4*B*). The latter data suggest that the decrease in the percentages of CCR9$^+$ cells found in the LP of IL-4R$\alpha^{-/-}$ mice is not due to a general restriction in the access of CCR9$^+$ T cells to the gut of these mice, but is rather consistent with a decrease in the imprinting of CCR9$^+$ CD4 T cells in IL-4R$\alpha^{-/-}$ mice.

IL-4 increases RALDH2 expression on MLN-DC

Because we showed that IL-4 increased the levels of CCR9 induced by GALT-DC on CD4 T cells (Fig. 1), we asked whether the effect of IL-4 on GALT-DC was mediated by an increase in RA production, a metabolite derived from vitamin A that has been shown to induce the gut-homing receptors $\alpha_4\beta_7$ and CCR9 expression on T cells (26). Because the RALDH enzymes are essential for the synthesis of RA, and RALDH2 is specifically expressed in MLN-DC (26), we postulated that IL-4 could increase RALDH2 expression in MLN-DC. As shown in Fig. 5*A*, there is a significant increase in RALDH2 mRNA in MLN-DC from wild-type mice treated with 100 U/ml IL-4 as compared with untreated cells. Moreover, in agreement with our previous results, IL-4 had no effect on RALDH2 expression in MLN-DC from IL-4R$\alpha^{-/-}$ mice (Fig. 5*A*). In addition, MLN-DC from these mice also tended to express lower basal levels of RALDH2 as compared with wild-type MLN-DC, suggesting that the lower induction of CCR9 by MLN-DC from IL-4R$\alpha^{-/-}$ mice can be explained, at least in part, by a decreased synthesis of RA. To determine whether the higher CCR9-imprinting potential induced by IL-4 on MLN-DC was mediated by an increase in RA production by MLN-DC, we activated CD4 T cells with MLN-DC in the presence of 100 U/ml IL-4 with or without 1 μM LE135, a RA receptor antagonist (26). As shown in Fig. 5*B*, LE135 treatment abolished the additional induction of CCR9 mediated by IL-4-treated MLN-DC and also decreased the basal induction of CCR9 mediated by untreated MLN-DC. Thus, our data suggest that IL-4 is important for inducing RA synthesis in MLN-DC, which is finally translated in a higher CCR9 imprinting on CD4 T cells.

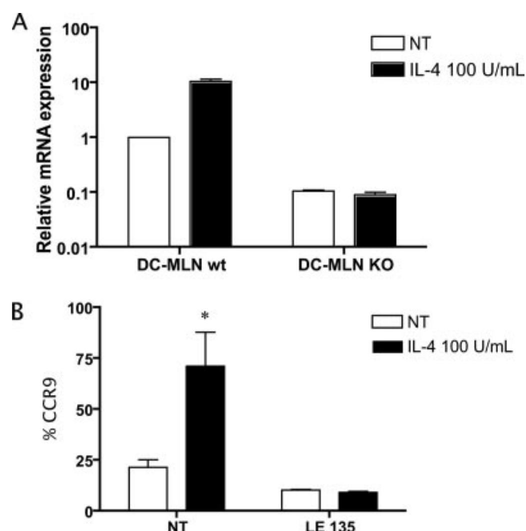

FIGURE 5. Increased expression of CCR9 by IL-4-treated MLN-DC dependents on RALDH2. *A*, MLN-DC from wild-type (wt) or IL-4R$\alpha^{-/-}$ mice were treated for 4 h with 100 U/ml IL-4. RALDH2 gene expression was assayed by quantitative PCR and normalized relative to expression of GAPDH. Data shown are representative of three independent experiments. *B*, Flow cytometry analysis of CCR9 expression on CD4 T cells activated by MLN-DC in the presence or absence of 100 U/ml IL-4 plus or minus 1 μM LE135 for 5 days. $*$, $p < 0.01$.

Discussion

The key role of IL-4 in the education of memory T cells by GALT-DC is well established, both in vitro and in vivo (23). Also, Mora et al. (24) recently demonstrated that IL-4 generated an increase on CCR9 expression on CD8 T cells activated by GALT-DC. Although it is known that IL-4 can block the up-regulation of skin-homing receptors (25), it remains unclear whether it also affects the regulation of gut-homing receptors on CD4 T cells. In this study, we demonstrate that IL-4 increases the percentage and MFI of CCR9 expression on CD4 T cells activated by MLN-DC (Figs. 1 and 2*C*, and data not shown). Furthermore, this effect was independent of the origin of the CD4 T cells (data not shown). We show that the effect of IL-4 is mediated via MLN-DC, because IL-4 had no effect on CCR9 expression when T cells were cocultured without DC or with MLN-DC from IL-4R$\alpha^{-/-}$ mice. In contrast, we demonstrated that IL-4 increased RALDH2 mRNA on MLN-DC, suggesting that the effect of IL-4 on MLN-DC-induced CCR9 expression on T cells depends on RA.

A bulk of data suggests an important role for IL-4 in the induction of CCR9 expression by T cells primed in the GALT (23, 25, 29), and we do not rule out the possibility that, under physiological conditions, IL-4 effect may be also autocrine on MLN-DC.

In contrast, several studies have demonstrated that a large fraction of CD4$^+$ CCR9$^+$ T cells in the intestine and peripheral blood are actually IFN-γ-producing Th1 cells (30–32). Our data are in accordance with the mentioned studies, because we have found that treatment of MLN-DC with IL-4 increases IL-12 production by these cells, resulting in an increase in IFN-γ production by T cells (data not shown). This increase of IFN-γ is not observed on CD4 T cells activated with MLN-DC from IL-4R$\alpha^{-/-}$ mice.

Although the increase in CCR9 expression on CD4 T cells generated by the effect of IL-4 on MLN-DC translated in a higher localization of CD4 T cells to the intestinal lamina propria, CD4 T cells activated with MLN-DC from IL-4R$\alpha^{-/-}$ mice were clearly capable of entering this site (Fig. 3). These results lend further support to a previous report indicating the existence of CCR9-dependent and -independent mechanisms of gut homing (20). In contrast, the IL-4R$\alpha^{-/-}$ mice have lower absolute numbers of T cells in the LP as well as in other organs (i.e., PLN, data not shown). However, the access of CD4 T cells to the LP is not limiting in the KO mice, as reflected by the identical HI found in wild-type (Fig. 3) and KO mice (Fig. 4*B*).

Our results also suggest that IL-4 affects migration of CD4 T cells in vivo, because fewer CCR9$^+$ CD4 T cells were found on the intestinal lamina propria from IL-4R$\alpha^{-/-}$ mice (Fig. 4*A*). Furthermore, it should be noted that the lamina propria presents both CCR9$^+$ and CCR9$^-$ CD4 T cells, again consistent with previous data indicating the existence of CCR9-dependent and -independent pathways of T cell entry to the gut (20). However, CCR9 expression on CD8 T cells from LP from IL-4R$\alpha^{-/-}$ and wild-type mice was similar (data not shown), confirming previous reports indicating that IL-4 was not required for the induction of gut homing on CD8 T cells (24). Also, although CD4$^+$ and CD8$^+$ T cells from IL-4R$\alpha^{-/-}$ and wild-type mice show similar levels of CCR9 expression in the thymus (data not shown), only CD8 recent thymic emigrants can repopulate the small intestine, whereas CD4 recent thymic emigrants do not (33). Thus, CCR9$^+$ CD8 T cells in the small bowel of IL-4R$\alpha^{-/-}$ may represent (at least in part) recent thymic emigrants.

Interestingly, our results showed that IL-4 did not have a significant effect in the percentage of CD4 T cells that express $\alpha_4\beta_7$, both in the BALB/c (either wild-type or IL-4R$\alpha^{-/-}$ mice) and B6 murine models, although we observed a reduction in the MFI for $\alpha_4\beta_7$ without any noticeable effect on the capacity of these cells to migrate to the gut (data not shown). These results are in agreement with reports suggesting that gut-homing receptors CCR9 and $\alpha_4\beta_7$ may have distinct requirements for their expression (24, 34). In this regard, the role of IL-4 in the regulation of CCR9 expression on CD4 T cells described in this study may act as a complement to the role of OX40 on the regulation of $\alpha_4\beta_7$, as recently suggested by Agace (34).

In conclusion, our results show that IL-4 modulates the gut-homing potential induced by GALT-DC on naive CD4 T cells through the up-regulation of CCR9 expression. Of note, although IL-4 increases the induction of CCR9 by MLN-DC, it is clearly not sufficient to induce CCR9 expression in the absence of DC or when T cells are activated by nongut DC. However, it is possible that already differentiated peripheral DC are refractory to the IL-4-mediated effect on CCR9 expression because their precursors were not exposed to IL-4. In contrast, gut DC precursors may have received this education by IL-4 in a tissue-specific fashion. Because IL-4 increased the level of RALDH mRNA, it is possible that IL-4 also plays an important role in up-regulating RALDH during the differentiation of gut DC. The fact that RA also induces $\alpha_4\beta_7$ expression on CD4 T cells (26) and that IL-4 has no effect on the level of expression of this integrin suggests that IL-4 may raise the level of RA in the microenvironment in which the T cells are activated, resulting in an increased expression of CCR9 on CD4 T cells without affecting $\alpha_4\beta_7$ expression. The latter lends further support to the idea that these two gut-homing receptors are regulated in part by a common RA-dependent mechanism as well as by other distinct pathways (34).

Acknowledgments

We are grateful to Michael Briskin for the gift of anti-CCR9 mAb. We thank Drs. Randolph Noëlle and Sebastian Amigorena for helpful suggestions and critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Mackay, C. R., W. L. Marston, and L. Dudler. 1990. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* 171: 801–817.
2. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291: 2413–2417.
3. Bono, M. R., R. Elgueta, D. Sauma, K. Pino, F. Osorio, P. Michea, A. Fierro, and M. Rosemblatt. 2007. The essential role of chemokines in the selective regulation of lymphocyte homing. *Cytokine Growth Factor Rev.* 18: 33–43.
4. Butcher, E. C., and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science* 272: 60–66.
5. Kunkel, E. J., and E. C. Butcher. 2002. Chemokines and the tissue-specific migration of lymphocytes. *Immunity* 16: 1–4.
6. Von Andrian, U. H., and C. R. Mackay. 2000. T-cell function and migration: two sides of the same coin. *N. Engl. J. Med.* 343: 1020–1034.
7. Berlin, C., E. L. Berg, M. J. Briskin, D. P. Andrew, P. J. Kilshaw, B. Holzmann, I. L. Weissman, A. Hamann, and E. C. Butcher. 1993. $\alpha_4\beta_7$ integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 74: 185–195.
8. Svensson, M., J. Marsal, A. Ericsson, L. Carramolino, T. Broden, G. Marquez, and W. W. Agace. 2002. CCL25 mediates the localization of recently activated $CD8\alpha\beta^+$ lymphocytes to the small-intestinal mucosa. *J. Clin. Invest.* 110: 1113–1121.
9. Zabel, B. A., W. W. Agace, J. J. Campbell, H. M. Heath, D. Parent, A. I. Roberts, E. C. Ebert, N. Kassam, S. Qin, M. Zovko, et al. 1999. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J. Exp. Med.* 190: 1241–1256.
10. Campbell, J. J., G. Haraldsen, J. Pan, J. Rottman, S. Qin, P. Ponath, D. P. Andrew, R. Warnke, N. Ruffing, N. Kassam, et al. 1999. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 400: 776–780.
11. Homey, B., H. Alenius, A. Muller, H. Soto, E. P. Bowman, W. Yuan, L. McEvoy, A. I. Lauerma, T. Assmann, E. Bunemann, et al. 2002. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat. Med.* 8: 157–165.
12. Tietz, W., Y. Allemand, E. Borges, D. von Laer, R. Hallmann, D. Vestweber, and A. Hamann. 1998. $CD4^+$ T cells migrate into inflamed skin only if they express ligands for E- and P-selectin. *J. Immunol.* 161: 963–970.
13. Campbell, D. J., and E. C. Butcher. 2002. Rapid acquisition of tissue-specific homing phenotypes by $CD4^+$ T cells activated in cutaneous or mucosal lymphoid tissues. *J. Exp. Med.* 195: 135–141.
14. Kantele, A., J. M. Kantele, E. Savilahti, M. Westerholm, H. Arvilommi, A. Lazarovits, E. C. Butcher, and P. H. Makela. 1997. Homing potentials of circulating lymphocytes in humans depend on the site of activation: oral, but not parenteral, typhoid vaccination induces circulating antibody-secreting cells that all bear homing receptors directing them to the gut. *J. Immunol.* 158: 574–579.
15. Mora, J. R., M. R. Bono, N. Manjunath, W. Weninger, L. L. Cavanagh, M. Rosemblatt, and U. H. Von Andrian. 2003. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 424: 88–93.
16. Annacker, O., J. L. Coombes, V. Malmstrom, H. H. Uhlig, T. Bourne, B. Johansson-Lindbom, W. W. Agace, C. M. Parker, and F. Powrie. 2005. Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J. Exp. Med.* 202: 1051–1061.
17. Dudda, J. C., J. C. Simon, and S. Martin. 2004. Dendritic cell immunization route determines $CD8^+$ T cell trafficking to inflamed skin: role for tissue microenvironment and dendritic cells in establishment of T cell-homing subsets. *J. Immunol.* 172: 857–863.
18. Johansson-Lindbom, B., M. Svensson, O. Pabst, C. Palmqvist, G. Marquez, R. Forster, and W. W. Agace. 2005. Functional specialization of gut $CD103^+$ dendritic cells in the regulation of tissue-selective T cell homing. *J. Exp. Med.* 202: 1063–1073.
19. Johansson-Lindbom, B., M. Svensson, M. A. Wurbel, B. Malissen, G. Marquez, and W. Agace. 2003. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. *J. Exp. Med.* 198: 963–969.
20. Stenstad, H., A. Ericsson, B. Johansson-Lindbom, M. Svensson, J. Marsal, M. Mack, D. Picarella, D. Soler, G. Marquez, M. Briskin, and W. W. Agace. 2006. Gut-associated lymphoid tissue-primed $CD4^+$ T cells display CCR9-dependent and -independent homing to the small intestine. *Blood* 107: 3447–3454.
21. Everson, M. P., D. G. Lemak, D. S. McDuffie, W. J. Koopman, J. R. McGhee, and K. W. Beagley. 1998. Dendritic cells from Peyer's patch and spleen induce different T helper cell responses. *J. Interferon Cytokine Res.* 18: 103–115.
22. Iwasaki, A., and B. L. Kelsall. 1999. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J. Exp. Med.* 190: 229–239.
23. Alpan, O., E. Bachelder, E. Isil, H. Arnheiter, and P. Matzinger. 2004. 'Educated' dendritic cells act as messengers from memory to naive T helper cells. *Nat. Immunol.* 5: 615–622.
24. Mora, J. R., G. Cheng, D. Picarella, M. Briskin, N. Buchanan, and U. H. von Andrian. 2005. Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J. Exp. Med.* 201: 303–316.
25. Seneviratne, S. L., L. Jones, A. S. Bailey, R. V. Samuel, A. P. Black, and G. S. Ogg. 2005. Interleukin-4 induced down-regulation of skin-homing receptor expression by human viral-specific CD8 T cells may contribute to atopic risk of cutaneous infection. *Clin. Exp. Immunol.* 141: 107–115.
26. Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S. Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21: 527–538.
27. Sauma, D., P. Michea, A. M. Lennon-Dumenil, A. Fierro, J. Morales, M. Rosemblatt, and M. R. Bono. 2004. Interleukin-4 selectively inhibits interleukin-2 secretion by lipopolysaccharide-activated dendritic cells. *Scand. J. Immunol.* 59: 183–189.
28. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: e45.
29. Andrade, M. C., J. S. Menezes, G. D. Cassali, O. A. Martins-Filho, D. C. Cara, and A. M. Faria. 2006. Alcohol-induced gastritis prevents oral tolerance induction in mice. *Clin. Exp. Immunol.* 146: 312–322.
30. Kim, C. H., L. Rott, E. J. Kunkel, M. C. Genovese, D. P. Andrew, L. Wu, and E. C. Butcher. 2001. Rules of chemokine receptor association with T cell polarization in vivo. *J. Clin. Invest.* 108: 1331–1339.
31. Papadakis, K. A., C. Landers, J. Prehn, E. A. Kouroumalis, S. T. Moreno, J. C. Gutierrez-Ramos, M. R. Hodge, and S. R. Targan. 2003. CC chemokine receptor 9 expression defines a subset of peripheral blood lymphocytes with mucosal T cell phenotype and Th1 or T-regulatory 1 cytokine profile. *J. Immunol.* 171: 159–165.
32. Saruta, M., Q. T. Yu, A. Avanesyan, P. R. Fleshner, S. R. Targan, and K. A. Papadakis. 2007. Phenotype and effector function of CC chemokine receptor 9-expressing lymphocytes in small intestinal Crohn's disease. *J. Immunol.* 178: 3293–3300.
33. Staton, T. L., A. Habtezion, M. M. Winslow, T. Sato, P. E. Love, and E. C. Butcher. 2006. $CD8^+$ recent thymic emigrants home to and efficiently repopulate the small intestine epithelium. *Nat. Immunol.* 7: 482–488.
34. Agace, W. W. 2006. Tissue-tropic effector T cells: generation and targeting opportunities. *Nat. Rev. Immunol.* 6: 682–692.