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Lymph Node Stromal Cells Support Dendritic Cell-Induced Gut-Homing of T Cells

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T cells are imprinted to express tissue-specific homing receptors upon activation in tissue-draining lymph nodes, resulting in their migration to the site of Ag entry. Expression of gut-homing molecules \( \alpha_\beta \) and CCR9 is induced by retinoic acid, a vitamin A metabolite produced by retinal dehydrogenases, which are specifically expressed in dendritic cells as well as stromal cells in mucosa-draining lymph nodes. In this study, we demonstrate that mesenteric lymph node stromal cell-derived retinoic acid can directly induce the expression of gut-homing molecules on proliferating T cells, a process strongly enhanced by bone marrow-derived dendritic cells in vitro. Therefore, cooperation of sessile lymph node stromal cells with mobile dendritic cells warrants the imprinting of tissue specific homing receptors on activated T cells. *The Journal of Immunology, 2009, 183: 6395–6402.

Upon activation by dendritic cells (DCs), coming from either peripheral or mucosal sites, T cells are induced to express adhesion molecules and chemokine receptors to ensure that activated T cells will preferentially migrate to the site where DCs initially encountered the Ag (1–5). For migration to the intestines, T cells require expression of integrin \( \alpha_\beta \), and chemokine receptor CCR9, although the requirement for CCR9 may not be absolute (6–11). The ligand for \( \alpha_\beta \), mucosal addressin cell adhesion molecule-1, is expressed in the lamina propria of the colon and small intestine (12–14), while CCL25, the ligand for CCR9, is mainly produced by epithelial cells of the small intestine (15–20). In recent years more insight into the mechanisms by which homing receptors are induced has been obtained and an important role for the vitamin A metabolite retinoic acid (RA) in the up-regulation of \( \alpha_\beta \) and CCR9 on activated T and B cells has been described (6, 21). Vitamin A (retinol) is first reversibly oxidized by alcohol dehydrogenases to form retinal, which in turn is irreversibly metabolized to RA by three members of the aldehyde dehydrogenase gene family, the retinal dehydrogenases 1–3 (RALDH1–3) (22, 23). In mice, RALDH1 through 3 are differentially expressed in DCs from gut-draining lymphoid tissues, forming the basis for the RA-induced gut tropism (6). This differential expression suggests a role for the mucosal environment in the induction of RALDH enzymes. This regulation could occur at the site where Ag is initially captured by DCs, for example the intestinal epithelium and lamina propria, as suggested by several reports (4, 24–26). In addition, it can be envisaged that the microenvironment of the draining mucosal lymph nodes (LNs), where the interaction and activation of lymphocytes takes place, also provides the appropriate signals for induction of T cell tropism, as shown in a recent paper by Hammerschmidt et al. (27). Such a role can further be inferred from our earlier experiments in which we showed with transplantation studies that mucosa-draining cervical LNs are unique in their capability to induce mucosa-associated immune tolerance, as peripheral, nonmucosal, LNs (PLNs) transplanted to the site of cervical LNs were not able to induce immune tolerance (28).

To elucidate the role of the LN microenvironment in providing signals for T cell tropism, either gut or skin draining LNs were transplanted into a peripheral site, the popliteal fossa, so that DCs entering these LNs are bringing in Ag from the periphery. These transplantations show that the microenvironment of mucosal mesenteric LNs (MLNs) and not PLNs, supports the induction of \( \alpha_\beta \), but not CCR9, on T cells upon their activation. Furthermore, MLN stromal cells showed expression of vitamin A converting enzymes allowing production of RA by these cells. Although in vitro cultured MLN stromal cells were able to induce gut-homing tropism on activated T cells directly, addition of RALDH-low unpulsed bone marrow-derived DCs (BM-DCs) strongly enhanced the expression of gut-homing molecules \( \alpha_\beta \) and CCR9. Altogether, our data demonstrates a crucial role for MLN stromal cells in creating an instructive mucosal microenvironment in which three obligatory parties, the stromal cells, DCs, and lymphocytes cooperate for efficient differential imprinting of tissue tropism.
Materials and Methods

Mice

BALB/c and C57BL/6 mice aged 8 to 12 wk were purchased from Charles River Laboratories and DO11.10, β-actin-GFP/C57BL/6, MHC-II–/–, OT-I, and OT-II transgenic mice and C57BL/6-CD45.1 and C57BL/6-CD45.2 congenic mice aged 6 to 8 wk were bred at our own facilities. The Animal Experiments Committee of the VU (Vrije Universiteit) University Medical Center approved all of the experiments described in this study.

Transplantation of LNs to the popliteal site

Transplantation of donor MLNs (mesenteric) or PLNs (from axial, brachial or inguinal sites) to the popliteal fossa was performed as described before (29).

T cell enrichment, CFSE labeling, transfer and antigenic stimulation

Spleens and LNs from DO11.10, OT-I, OT-II, or C57BL/6 mice were minced through a 100-μm gauze to obtain single cell suspensions. To deplete erythrocytes from spleen cell suspension, cells were incubated for 2 min on ice in lysis buffer (150 mM NH4Cl, 1 mM NaHCO3 (pH 7.4)). CD4+ or CD8+ T cells were enriched to at least 60% and 85%, respectively, using the CD4 or CD8 negative selection kit (Dynal). Cells were labeled with 5 μM of 5,6-CFSE (Molecular Probes, Invitrogen) at 3 × 10^7 cells/ml for 10 min at 37°C. Cells were used for in vitro cultures with stromal cells and/or DCs or used for in vivo i.v. injections. Transplanted and control BALB/c or C57BL/6 mice were injected with −10^7 OVA-specific T cells and were subsequently stimulated 24 h later by i.m. or intragastric administration of 200 μg OVA in 10 μl saline or 50 μg OVA in 200 μl saline respectively. After antigenic stimulation, transplanted LNs were isolated and used as single cell suspensions for FACS analysis.

Immunofluorescence and flow cytometry

Single cell suspensions were made by cutting LNs with scissors, followed by digestion at 37°C for 20 min, using Blendzyme 2 (Roche) and 100 U/ml DNase I (Roche). Abs were used anti-ERTR7 (affinity purified from hybridoma cell culture supernatant), anti-CD4 (clone GK1.5, BD Pharmingen), biotinylated anti-mouse DO11.10 TCR (clone KT2–26, Caltag Laboratories), PE-Cy7 conjugated CD8 (eBiosciences), PE-Cy7 conjugated CD4 (eBiosciences), PE conjugated anti-mouse OT-I/OT-II TCR (Vα2, eBiosciences), anti-αβ integrin (clone DATK32, provided by Dr. Alf Hamann, Charité Universitymedizin Berlin, Germany), rat anti-mouse CCR9 (clone 7E7), rat anti-mouse peripheral lymph node addressin (PNAd) (clone Mec-19, provided by Dr. E.C. Butcher, Stanford University, Stanford, CA), anti-B220 (clone 6B2), PE-conjugated anti-CD11c (clone N418, eBioscience), anti-CD3 (clone KT3), Alexa Fluor 647 conjugated CD45 (clone MP33, biotin-conjugated anti-MHC-II (clone M5/114), and 7-amino-actinomycin D (Molecular Probes, Invitrogen) or Sytox Blue (Invitrogen) to discriminate live vs dead cells. Secondary Abs were Alex-a-conjugated goat-anti-rat-IgG and Alex-a-conjugated streptavidin (Molecular Probes, Invitrogen). Cells were analyzed with a FACS Calibur (BD Biosciences) or with a Cyan Advanced Digital Processing High-Performance Research flow cytometer (Beckman Coulter). Cells were sorted using a MoFlo sorter (DakoCytometry). Tissue stainings were analyzed on a Leica TCS-SP2-AOBS confocal laser-scanning microscope (Leica Microsystems) and images were obtained with Leica confocal software.

In vitro experiments

To obtain stromal cells for in vitro cultures, MLNs and PLNs from C57BL/6 mice were dissociated with Blendzyme 2 as described above. One × 10^8 or 4 × 10^6 LN cells were grown well per 96-wells or 24-wells flat-bottom plates, respectively (Greiner Bio One) in IMDM (Life Technologies) containing 10% FCS (HyClone), 50 μM 2-ME (Merck), 1% t-glutamine, and 1% penicillin-streptomycin (Biowhittaker) for 24 h. Subsequently, nonadherent cells were removed, fresh medium was added, and the culture was continued for 1 wk.

Bone marrow was isolated from the femur and tibia using a mortar and pestle, and used as single cell suspensions for FACS analysis. To investigate this, we transplanted MLNs from β-actin-GFP/C57BL/6 mice into the popliteal fossa of C57BL/6 mice and addressed which cells were donor-derived as reported for LN transplantation (27). The majority of the high endothelial venules expressing PNA-d (detected with MECA79) were of donor origin as well (Fig. 1D and data not shown). To further prove that indeed hematopoietic cells were derived from the host, we transplanted LNs from CD45.1 mice into CD45.2 congenic hosts and observed that 5 wk after transplantation very few donor-derived hematopoietic CD45.1+ cells, predominantly B cells, could be detected in the transplanted LNs (Fig. 1E). In conclusion, we established that after transplantation of lymph nodes virtually all hematopoietic cells were derived from the host, while the stromal cells were donor-derived.

μM LE135 (RA receptor β inhibitor, Tocris Bioscience), and 1 μM LE540 (RA receptor β inhibitor, Wako Chemicals) as indicated and activated with 5 × 10^5 CD3/CD28 T cell expander Dynabeads (Dynal, Invitrogen) in presence or absence of 1 × 10^5 unpulsed BM-DCs. In all T cell activation assays, responding T cells were analyzed with flow cytometry after 96 h.

RNA isolation and real time PCR

Sorted MHC-II+ CD11c+ DCs, CD45- MHC-II+ CD11c- LN stromal cells, and stromal cells after 7 days of culture were lysed in TRIzol (Life Technologies) or RLT buffer (Qiagen Benelux). RNA was isolated by precipitation with isopropanol or by using the RNeasy kit (Qiagen Benelux) according to the manufacturer’s protocol and cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences) according to the manufacturer’s protocol. RALDH1 (Adh1b1), RALDH2 (Adh1b2), and RALDH3 (Adh1b3) specific primers and probes for housekeeping genes β-actin, Ubiquitin C, HPRT, and GAPDH were designed across exon-intron boundaries using Primer Express software (PE Applied Biosystems). Real time PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Total volume of the reaction mixture was 10 μl, containing cDNA, 300 nM of each primer and SYBR Green Mastermix (PE Applied Biosystems). To correct for primer efficiency, a standard curve was generated for each primer set with cDNA from a pool of nonactivated LNs. Expression levels of transcripts obtained with real time PCR were analyzed and normalized with geNORM v.3.4 software (30).

Statistics

Statistical analysis was conducted using 2-tailed Student’s t test for differences in mean fluorescence intensity, ratio of αβ TCR, or CCR9 expression on activated T cells in LNs or cocultures, or differences in relative RALDH1, RALDH2, or RALDH3 mRNA expression levels in sorted DCs and stromal cells. Differences were considered significant when p < 0.05 or p < 0.02 as indicated.

Results

Donor-derived cells in transplanted LNs are stromal cells

In earlier studies, we showed by transplantation of LNs that differences exist between LNs with respect to their ability to allow the induction of mucosal tolerance (28). Because DCs as well as lymphocytes are mobile cells that are likely to be replated rapidly after transplantation, these results suggested that the specific features that make up the differences between MLNs and PLNs reside within the stromal elements of the LN. To investigate this, we transplanted MLNs from β-actin-GFP/C57BL/6 mice into the popliteal fossa of C57BL/6 mice and addressed which cells were donor-derived at 12 wk after transplantation. By staining for podoplanin (gp38) expressed on fibroblastic reticular cells (FRCs) and pericytes and for ERTR7+, an extracellular matrix glycoprotein produced by these cells, we could demonstrate that most podoplanin+ cells which colocalized with ERTR7+ extracellular matrix structures were GFP expressing donor-derived cells (Fig. 1, A and B), thus confirming that FRCs were donor-derived as reported for LN transplantations into gut mesenteries (27). The majority of the high endothelial venules expressing PNA-d (detected with MECA79) were of donor origin as well (Fig. 1C). Staining for hematopoietic cells revealed that CD4+ and CD8+ T cells, B cells, as well as DCs were lacking GFP expression and thus were host derived (Fig. 1D and data not shown). To further prove that indeed hematopoietic cells were derived from the host, we transplanted LNs from CD45.1 mice into CD45.2 congenic hosts and observed that 5 wk after transplantation very few donor-derived hematopoietic CD45.1+ cells, predominantly B cells, could be detected in the transplanted LNs (Fig. 1E).
MHC-II deficient mice were transplanted to the popliteal fossa with T cell activation in the transplanted LNs, LNs from DCs might still be present after transplantation and interfere shown). To exclude the possibility that donor-derived mucosal orthotopic MLNs upon intragastric administration of OVA (Fig. 2A–D). Nonhematopoietic cells remain in transplanted LNs, while all donor-derived hematopoietic cells disappear. A–D, MLNs or PLNs from β-actin-GFP/C57BL/6 mice were transplanted into the popliteal fossa after removal of the popliteal LNs. Twelve weeks later, LNs were collected and stromal cells and hematopoietic cells in the transplanted LNs were analyzed with immunofluorescence for expression of podoplanin (gp38, red) (A), ERTR7 (extracellular matrix marker, red) (B), PNAd (high endothelial venule marker, red) (C), and CD11c (DCs, red) (D), while GFP+ donor-derived cells appear in green in all pictures. Results shown are representative for six LNs transplanted for each group. E, MLNs from C57BL/6-CD45.1 mice were transplanted into the popliteal fossa of C57BL/6-CD45.2 recipients and collected after 5 wk for FACS analysis of hematopoietic cells present in the transplanted MLN (tLN) or orthotopic PLN (coLN). The expression of B220, CD3, and CD11c on CD45.1+ cells and all (total) hematopoietic cells is shown for transplanted MLN (tLN). Results were comparable to transplanted PLN. Results shown are representative for five LNs transplanted for each group.

**FIGURE 1.** Nonhematopoietic cells remain in transplanted LNs, while all donor-derived hematopoietic cells disappear. A–D, MLNs or PLNs from β-actin-GFP/C57BL/6 mice were transplanted into the popliteal fossa after removal of the popliteal LNs. Twelve weeks later, LNs were collected and stromal cells and hematopoietic cells in the transplanted LNs were analyzed with immunofluorescence for expression of podoplanin (gp38, red) (A), ERTR7 (extracellular matrix marker, red) (B), PNAd (high endothelial venule marker, red) (C), and CD11c (DCs, red) (D), while GFP+ donor-derived cells appear in green in all pictures. Results shown are representative for six LNs transplanted for each group. E, MLNs from C57BL/6-CD45.1 mice were transplanted into the popliteal fossa of C57BL/6-CD45.2 recipients and collected after 5 wk for FACS analysis of hematopoietic cells present in the transplanted MLN (tLN) or orthotopic PLN (coLN). The expression of B220, CD3, and CD11c on CD45.1+ cells and all (total) hematopoietic cells is shown for transplanted MLN (tLN). Results were comparable to transplanted PLN. Results shown are representative for five LNs transplanted for each group.

**Activation of T cells in transplanted MLNs results in increased expression of α1β7 but not CCR9**

To study the mechanism of tissue tropism induction guided by stromal cells in vivo, independent of mucosal tissue drainage, MLNs and PLNs were transplanted to the popliteal fossa of C57BL/6 mice. At 12 wk after transplantation, C57BL/6 mice were injected with CFSE-labeled OVA specific transgenic CD8+Va2+ T cells (OT-I cells) and immunized with OVA in the sural muscle 24 h later. At 72 h after Ag administration, expression of mucosal homing molecules α1β7 and CCR9 was analyzed on proliferating OT-I cells by flow cytometry. We observed a significant difference between expression of α1β7 on OT-I cells activated in transplanted MLNs (tMLNs) vs transplanted PLNs (tPLNs) (Fig. 2A). Induced expression in tMLNs was comparable to levels on activated OT-I cells in the orthotopic MLNs upon intragastric administration of OVA (Fig. 2A). No difference in α1β7 expression was visible on the CFSE-labeled nonproliferating T cell populations in tMLNs vs tPLNs, indicative of a uniform entry of injected cells in both types of transplanted LNs and the necessity of T cell activation for induction of this gut-homing molecule. Remarkably, the mucosal homing receptor CCR9 was not induced on proliferating OT-I cells in tMLNs, while its expression was readily induced in the orthotopic MLNs upon intragastric administration of OVA (Fig. 2B). Identical results were obtained when transplanted mice were injected with CFSE-labeled OVA specific CD4+Va2+ T cells (OT-II cells). To rule out strain-specific effects the transplantsations were also performed in the BALB/c mouse strain in similar results on the induction of α1β7 expression (data not shown). To exclude the possibility that donor-derived mucosal DCs might still be present after transplantation and interfere with T cell activation in the transplanted LNs, LNs from MHC-II deficient mice were transplanted to the popliteal fossa of C57BL/6 mice. These experiments showed similar induction of α1β7 integrin on activated OT-II cells as observed in wild-type transplanted LNs (Fig. 2A).

Together, these results indicate that the mucosal microenvironment in the transplanted MLNs is partially preserved and allows for the induction of the gut-homing molecule α1β7, but not CCR9, by host-derived DCs on Ag-specific T cells. For the induction of CCR9 on activated T cells additional factors might be required, presumably derived from the intestines and present within the intestinal lamina propria as well as in the lymph draining from the gut into the MLNs.

**RALDH enzymes are expressed in mucosal DCs and MLN stromal cells**

Because the induction of gut-homing molecules on T cells has been described to be dependent on RA (6), we analyzed mRNA expression levels of RA producing RALDH enzymes in sorted CD45+ MHC-II+ CD11c+ DCs vs CD45− stromal cells. As expected, sorted DCs from MLN (MLN-DCs) expressed high levels of RALDH2 as well as RALDH3, as shown before (6), while PLN-DCs expressed RALDH2 at much lower levels (Fig. 3A). Notably, RALDH2 and RALDH3 were found to be expressed by freshly sorted CD45− stromal cells from MLNs, but not by PLN stromal cells (Fig. 3C). Expression of RALDH1 was barely detectable in both PLN and MLN stroma (data not shown). Therefore, MLN stromal cells may contribute to the generation of gut-homing T cells by producing RA, either by directly influencing T cells as suggested before (27), through instruction of DCs or by affecting both.

**Gut-homing molecules are induced on activated T cells in the presence of MLN stromal cells and BM-DCs in vitro**

To see whether indeed stromal cells are instrumental to an instructive environment for induction of T cell tropism, we set up an in
vivo assay to address this question. Because the low number of freshly sorted stromal cells would limit such in vitro studies, we addressed whether MLN stromal cells maintained RALDH levels after expansion for 7 days in culture. Indeed, RALDH1, RALDH2, and RALDH3 expression were detected in cultured MLN stromal cells, and at very low levels in cultured PLN stromal cells (Fig. 3B). Flow cytometric analysis of these stromal cultures showed that the majority of the cells were CD45-negative. Only a small percentage of hematopoietic cells was present after 7 days of culture (Fig. 3D).

To investigate the effect of LN stromal cells on the induction of T cell tropism, stromal cells from PLNs vs MLNs were cultured for 7 days, after which OVA peptide-loaded BM-DCs and CFSE-labeled OT-I cells were added. Although all our cultures were performed in the presence of 10% FCS, which should account for ~40 nM retinol, experiments were conducted in the presence or absence of additional retinol to circumvent potential loss of bioactivity of FCS-derived retinol. After 96 h, expression of mucosal homing molecules α4β7 and CCR9 on proliferating Ag-specific T cells was analyzed by flow cytometry. When OT-I cells were stimulated in the presence of MLN stromal cells, the ratio of α4β7+ to α4β7− T cells increased with each cell division upon addition of retinol, suggesting that RA production from retinol was involved in the induction of α4β7 expression (Fig. 4A). This effect was not seen with stromal cells from PLNs, where a stable ratio of α4β7+ to α4β7− T cells was found. Similar results were found for the induction of CCR9. Only in the presence of MLN stromal cells and retinol, a robust expression of CCR9 on dividing T cells was seen (Fig. 4B). Addition of retinol to the PLN stromal cell cultures allowed a slight induction of CCR9 on activated T cells, however not to the levels seen on T cells activated on MLN stromal cells and only during the first cell divisions. These results suggest that only in the presence of MLN stromal cells, but not PLN stromal cells, peptide-pulsed BM-DCs can strongly induce α4β7 and CCR9 expression on activated T cells and that this process requires the addition of retinol, which is converted to RA by RALDH enzymes.

**BM-DCs are not induced to express RALDH enzymes in vitro**

Because we have observed that BM-DCs, which were cultured together with small intestinal epithelial cells, were induced to express RALDH enzymes (25), we reasoned that a similar instruction could be mediated by MLN stromal cells. Therefore BM-DCs were cocultured with MLN stromal cells or PLN stromal cells for 48 h, after which stromal cells and DCs were sorted and analyzed for RALDH2 mRNA expression levels. BM-DCs alone or cultured with PLN stromal cells express low levels of RALDH2 mRNA, in agreement with an earlier report (31), either in the presence or absence of added retinol. Moreover, MLN stromal cells were unable to further induce RALDH2 mRNA levels in BM-DCs, while MLN stromal cells expressed high levels of RALDH2 (Fig. 4C). Therefore, in the in vitro cultures that allow the induction of gut-homing molecules on activated T cells, MLN stromal cells are the main source of RALDH enzymes, which are needed to convert retinol into RA.
RALDH-low BM-DCs greatly enhance induction of gut-homing molecules by MLN stromal cells

To address whether indeed stromal cells alone are sufficient for the induction of gut-homing molecules on T cells, CD8\(^+\) T cells were activated with anti-CD3 and anti-CD28 coated beads on LN stromal cells in the absence or presence of BM-DCs. Retinol was either added as a substrate for RA production by stromal cells or omitted from the cultures. After 96 h, \(\alpha_{\delta}\beta_7\) and CCR9 expression on activated CD8\(^+\) T cells was determined. When CD8\(^+\) T cells were stimulated on MLN stromal cells in the absence of BM-DCs and retinol, the ratio of \(\alpha_{\delta}\beta_7\) to \(\alpha_{\delta}\beta_7\) T cells was significantly higher when compared with T cells activated on PLN stromal cells. Addition of retinol slightly increased the ratio of \(\alpha_{\delta}\beta_7\) to \(\alpha_{\delta}\beta_7\) T cells when activated on MLN stromal cells (Fig. 4D). Notably, addition of unpulsed BM-DCs strongly increased the expression of \(\alpha_{\delta}\beta_7\) on bead-activated CD8\(^+\) T cells in MLN stromal cocultures. This increased expression was already observed when no retinol was added, suggesting that BM-DCs have a synergizing effect on the induction of gut-homing molecules on T cells (Fig. 4D). Increased expression levels were not seen in PLN stromal cocultures, where a stable ratio of \(\alpha_{\delta}\beta_7\) to \(\alpha_{\delta}\beta_7\) T cells was observed and thus did the low levels of RALDH in BM-DCs not contribute to this induction. Similarly, only in the presence of stromal cells from MLNs an increasing expression of CCR9 on activated T cells was seen upon addition of retinol, while this was not observed on PLN stromal cells. Also, addition of unpulsed BM-DCs increased the expression of CCR9 on activated T cells in presence of MLN stromal cells. Remarkably, the ratio of CCR9\(^+\) to CCR9\(^-\) activated T cells was greatly increased upon addition of retinol (Fig. 4E). These results show that MLN stromal cells can support the generation of gut-homing T cells directly, however the presence of BM-DCs makes this process much more efficient.

Discussion

We have demonstrated in this study that stromal cells within MLNs mediate the induction of gut tropism by influencing proliferating T cells directly, a process strongly enhanced by the addition of BM-DCs in vitro. We were able to show that, despite the fact that a MLN was transplanted to a site where it drains the skin rather than the intestine, MLN stromal cells can still provide the necessary microenvironment for the induction of gut-homing molecule \(\alpha_{\delta}\beta_7\) expression on T cells, regardless of Ag transport by DCs from the intestine. Our in vitro studies revealed that MLN stromal cells can directly induce expression of gut-homing molecules on T cells, supporting the recent publication by Hammerschmidt et al. (27). However, our data extend their observation because we showed that in the presence of BM-DCs the induction of these molecules was markedly increased, while these DCs failed to further up-regulate the expression of RALDH enzymes. Because an intimate interaction between LN stromal cells and DCs has been observed by immunofluorescence as well as by intravital microscopy (32–34), we conclude that activation of T cells by DCs within

**FIGURE 3.** RALDH enzymes are expressed in MLN stromal cells and MLN DCs. RNA was isolated from indicated cell types. Relative expression levels of RALDH1, RALDH2, and RALDH3 enzymes were measured by real time PCR. Expression of transcripts was normalized to endogenous references genes using geNORM v.3.4 software. Relative expression levels in PLN-DCs (A) and PLN stroma (B and C) was set at 1. Significant differences \((p < 0.02)\) are indicated by *. A, CD11c\(^+\) MHC-II\(^+\) DCs were FACS-sorted from PLNs and MLNs from six C57BL/6 mice of age 5–9 wk, analyzed for RALDH1, RALDH2, and RALDH3 mRNA expression levels and normalized to GAPDH and HPRT. RALDH1 and RALDH3 expression were not detectable in these cells. The experiment was performed three times. B, Seven-day cultured MLN stromal cells and PLN stromal cells were analyzed for relative expression levels of RALDH1, RALDH2, and RALDH3 enzymes and normalized to GAPDH and HPRT. The experiment was performed seven times. C, CD45\(^+\) MLN stromal cells and PLN stromal cells, FACS sorted from freshly isolated MLN and PLN, were analyzed for relative expression levels of RALDH1, RALDH2, and RALDH3 enzymes and normalized to GAPDH and Ubiquitin C. RALDH1 expression was not detectable in these cells. RALDH2 was not detectable in PLN stroma. The experiment was performed three times. D, After 7 days of culture, LN stromal cells were trypsinized and analyzed with flow cytometry for expression of CD45 (hematopoietic cell marker), CD11c, CD11b, CD4, CD8, and 6B2. Shown are percentages of the total cell suspension. Three wells were pooled for analysis and experiment was performed four times.
FIGURE 4. MLN stromal cells support induction of gut-homing molecules on activated CD8+ T cells, which is greatly enhanced by the presence of BM-DCs. A and B, 1 x 10^5 CFSE-labeled OT-I cells were cultured in vitro with 5 x 10^5 OVA peptide-pulsed BM-DCs on a layer of MLN or PLN stromal cells in absence or presence of 50 nM retinol. After 96 h, activated OT-I cells were analyzed by FACS for the expression of gut-homing molecules αβ7 (A) and CCR9 (B). Representative FACS plots are shown. Boxes indicate percentage of αβ7 or CCR9 expressing dividing T cells of total cell suspension. Experiment was performed two times. Data are calculated as the ratio of αβ7+ to αβ7− (A, right column) and CCR9+ to CCR9− (B, right column) activated Ag-specific T cells upon cell division on MLN or PLN stroma in absence (gray dots) or presence (black dots) of 50 nM retinol. C. One x 10^5 BM-DCs per well were cultured on a layer of MLN stromal cells (M-DC) or PLN stromal cells (P-DC) in presence of 50 nM retinol for 48 h. Per condition, four wells were pooled for FACS sorting. CD45+CD11c+ MHC-II+ DCs and CD45− stromal cells were sorted from the cocultures. Relative mRNA expression levels of RALDH2 were measured by real time PCR. Expression of transcripts was normalized to HPRT and Ubiquitin C using geNORM v.3.4 software. Relative expression in PLN stroma was set at 1. The experiment was performed five times. D and E, 5 x 10^5 CD8+ T cells were cultured in vitro with 5 x 10^5 T cell expander Dynabeads on a layer of MLN stromal cells (●) or PLN stromal cells (○) in the absence or presence of unloaded BM-DCs as indicated. 50 nM retinol, 10 μM citral plus 1 μM of RA receptor β inhibitors LE540 and LE135 was added to the cultures as indicated. After 96 h, activated CD8+ T cells were analyzed by flow cytometry for the expression of gut-homing molecules αβ7 and CCR9. Data represent the ratio of αβ7− to αβ7+ (D) and CCR9− to CCR9+ (E) of activated CD8+ T cells. Per condition, two wells were pooled for analysis. The experiments have been performed three times. *, p < 0.05; **, p < 0.02.

Organized lymphoid tissues also involves LN stromal cells. We therefore propose that activation of lymphocytes within organized lymphoid tissues should no longer be viewed as an interaction between APCs and lymphocytes, but rather as a response that involves three obligatory parties, namely stromal cells, DCs, and lymphocytes.

Within the MLNs, CD103+ DCs are able to induce the expression of both αβ7 and CCR9, while CD103− DCs are only capable of inducing αβ7 (35, 36). Because also αβ7−CCR9− cells were shown to migrate to the intestines, both DC subsets can induce gut-homing tropism (9). It has been suggested that CD103+ DCs form the subset that constantly migrates from the intestinal lamina propria into the MLNs, while CD103− DCs enter the MLN via the blood (1, 35-38). In our experimental setting, the lamina propria-derived CD103+ DCs are absent from the transplanted MLNs, suggesting that the absence of CCR9 expression on OVA-specific T cells is due to absence of this DC subset. However, in our in vitro cultures, LN-derived stromal cells by themselves are able to induce both αβ7 and CCR9 expression on activated T cells, suggesting that stromal cells that have recently been removed from the intestinal environment are better equipped to induce gut-homing on T cells, possibly by producing higher amounts of RA. Although stromal cells within MLNs can directly induce gut-homing tropism on proliferating T cells, this process is markedly enhanced by the addition of BM-DCs. Whether the synergism between BM-DCs and MLN stromal cells that we observe in our in vitro experiments also occurs in vivo will require further study.

Because DCs initiate T cell activation by their presentation of Ag, we propose that stromal-derived RA might be transferred to
and presented by DCs to T cells, perhaps through the immunological synapse formed between T cells and DCs during T cell activation. Compatible with this are the reported observations that uptake and presentation of RA to T cells can occur by DCs upon preincubation with RA. This process did not involve newly formed RA, because RALDH blockade did not affect induction of αβ+, and CCR9 expression on T cells (39). Similarly, a cell-cell interaction between MLN stromal cells and mucosal DCs might be required for the transfer of RA to mucosal DCs. Alternatively, DCs may by interacting with MLN stromal cells, somehow increase RALDH activity within MLN stromal cells, resulting in the enhanced induction of gut tropism in T cells.

The stromal cells within LNs have been thought of as cells that simply provide a structure for immune cells to optimally interact with each other. However, recently it was shown that these stromal components also provide migratory guidance for T and B cells, while DCs have been reported to adhere to these stromal cells, somehow increase RALDH activity within MLN stromal cells, resulting in the enhanced induction of gut tropism in T cells. Furthermore, stromal cells can present endogenous Ag to T cells, hereby promoting peripheral tolerance induction (42, 43). Additionally, our studies have provided evidence that MLN stromal cells influence the final differentiation of T cells by differential expression of RALDH enzymes leading to the production of RA which creates an instructive mucosal microenvironment. Thus, our findings that unique stromal microenvironments exist in anatomically distinct LNs and that this may direct tissue-specific lymphocyte homing properties adds to the role stromal cells have in controlling immune responses.

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References


