CHAPTER 6

Lymph node stromal cells control dendritic cell-induced tissue-specific homing of T cells

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Summary

For effective immune responses, it is crucial that T cells are recruited to the site of antigen entry. To bring about this tissue-specific homing, T cells meet with dendritic cells (DCs) that have taken up the antigen and have migrated to the tissue draining lymph nodes (LNs). Here T cells are imprinted to express tissue-specific homing receptors during their activation by DCs. This ensures the ability of T cells to migrate to tissues where the antigen was originally picked up by the DC and transported to the LN. For homing to the lamina propria of the gut, expression of the $\alpha_4\beta_7$ integrin and CCR9 are of major importance. Their expression is induced by retinoic acid (RA), a vitamin A metabolite produced by retinal dehydrogenases (RALDHs), which are differentially expressed in DCs in mucosa draining LNs. In this study we transplanted gut-draining mesenteric lymph nodes (MLNs) into a non-mucosal site, creating a situation in which non-mucosal DCs enter a mucosal LN. Here we show that DCs entering transplanted MLNs but not peripheral LNs (PLNs) are induced to express RALDH2, enhancing the production of RA. Upon antigen administration, antigen-specific T cells in these transplanted MLNs expressed the mucosal homing receptor $\alpha_4\beta_7$, but not the mucosal chemokine receptor CCR9. These data show that stromal cells from MLNs, but not PLNs instruct DCs to express RALDH2, hereby inducing the production of RA and directing the tissue-specific homing of T cells.
**Introduction**

When antigen enters the body, it is taken up by dendritic cells (DCs), which present the antigen to T cells in tissue draining lymph nodes (LNs). Costimulatory molecules like CD80 and CD86 expressed by DCs are required for the induction of efficient immune responses. To ensure that activated T cells, upon LN exit, will return to the site where DCs initially encountered the antigen, DCs from either peripheral or mucosal sites are able to imprint the homing and chemokine receptors required for specific homing to these sites [1-5]. For migration to the gut, T cells require the expression of the integrin $\alpha_4\beta_7$ and the chemokine receptor CCR9, although CCR9 independent intestinal migration has been described [6-10]. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), expressed in the lamina propria of the colon and small intestine, is the ligand for $\alpha_4\beta_7$ [11-13], while CCL25, the ligand for CCR9, is mainly expressed in epithelial cells of the small intestine [14-17]. For efficient upregulation of $\alpha_4\beta_7$ and CCR9 on activated T and B cells, ligation of the retinoic acid (RA) receptor is required [7, 18]. RA is produced by DCs as a product of vitamin A (retinol) metabolism. Retinol is first reversibly oxidized by alcohol dehydrogenases (ADH) to form retinal. Retinal can be irreversible metabolized by retinal dehydrogenases (RALDH) to form RA. Four isotypes of RALDH have been identified and three of them are differentially expressed in DCs from gut-draining lymphoid tissues [19], allowing DCs to direct T and B cells for gut homing. For T cell migration to the skin it was recently shown that the active form of vitamin D3, $1,25(OH)_2D_3$, can induce expression of CCR10 on activated human T cells [20]. CCR10 responds to CCL27, which is produced by keratinocytes in the epidermis of the skin. Vitamin D3 is produced in the skin by sunlight, and also for the metabolic conversion to its active form $1,25(OH)_2D_3$, DCs were shown to express the proper enzyme. Similar to RA, $1,25(OH)_2D_3$ can bind to its nuclear hormone receptor, the vitamin D3 receptor, leading to the induction of CCR10 on activated T cells. However, induction of other skin tropic homing molecules such as E- and P-selectin and CCR4 are not induced by $1,25(OH)_2D_3$. The role of vitamin D for the induction of skin homing molecules in mice might be less important since the induction of CCR10 by $1,25(OH)_2D_3$ was less pronounced. Furthermore, mice are mostly nocturnal and murine skin is largely shielded from direct UV light by hair [20].

Within the mucosal environment DCs have to be instructed to express the RALDH enzymes. It has been suggested that the local tissue environment where DCs capture their antigen is critical in determining the homing phenotype of T cells, as was shown by transfer studies with DCs from different sites and by studying the induction of skin- or gut homing properties on T cells [21]. Here we wished to study to what extent the microenvironment of the LNs where the DC-T cell interactions takes place is crucial for the induction of...
tissue-specific homing molecules on T cells. That the outcome of an immune response can be different in anatomically distinct LNs can be inferred from our earlier experiments in which we showed by transplantation studies that mucosa-draining cervical lymph nodes (CLNs) are unique in their capability to induce mucosa-associated immune tolerance, as peripheral lymph nodes (PLNs) transplanted to the site of the CLNs were not able to induce immune tolerance [22].

To further elucidate the role of the microenvironment in providing signals for T cell tissue homing, either gut or skin draining LNs were transplanted into a peripheral site, the popliteal fossa, so that DCs entering these LNs are bringing in antigen from a peripheral site. We show that after transplantation, only non-hematopoietic, stromal cells remain in the transplanted LNs. When T cells are activated in the transplanted mucosal LNs, the gut homing receptor $\alpha_6\beta_7$ is induced, but this is not the case in transplanted PLNs. Induction of CCR9 expression is not observed. Our data shows that mucosal LN stromal cells provide a microenvironment that instruct DCs to express RALDH enzymes required for the induction of $\alpha_6\beta_7$ expression on T cells. For the induction of CCR9 on activated T cells additional factors are required, which are presumably derived from the intestines and abundantly present within the intestinal lamina propria as well as in the lymph draining from the gut into the mesenteric lymph nodes (MLNs).
Materials and methods

Mice
Female BALB/c and C57Bl/6 mice aged 8 to 12 weeks were purchased from Charles River (Sulzfeld, Germany) and kept under standard animal housing conditions. The DO11.10, β-actin-GFP/C57Bl/6, and OT-I transgenic mice and the C57Bl/6-CD45.1 and C57Bl/6-CD45.2 congenic mice were used at 8 to 12 weeks of age and were bred at our own facilities. The Animal Experiments Committee of the VU Medical Center approved all of the experiments described in this study.

Transplantation of MLNs to the popliteal site of peripheral LNs
Transplantation of LNs was performed as described before [22]. In short, donor MLNs or PLNs (from axial, brachial or inguinal sites) were aseptically collected from β-actin-GFP/C57Bl/6, C57Bl/6 or BALB/c mice and kept in sterile RPMI on ice. Recipients, either C57Bl/6 or BALB/c mice, were anaesthetized with a mixture of oxygen and isofluorane. The popliteal LNs of the recipients were removed and at this site, a MLN or PLN was placed. Transplanted LNs were left for 8-12 weeks after which they were used for further analysis.

Tissue isolation and stainings
Transplanted LNs were isolated and either frozen in TissueTek for immunofluorescence or used as single cell suspensions for FACS analysis or FACS sorting. Single cell suspensions were made by cutting LNs with scissors, followed by digestion at 37ºC for 30 min, using Blenzyme 2 (Roche, Penzberg, Germany) and 100 U/ml DNAse I (Roche, Penzberg, Germany). Cell clumps were removed by pipetting the cells over a nylon mesh. The LN cells were washed and resuspended in PBS with 2% New Born Calf Serum (PBS-NBCS). For sorting of DCs, cells were stained with biotin-conjugated anti-MHCII (clone M5/114), PE-conjugated anti-CD11c (clone N418, eBioscience) and 7AAD (Molecular Probes, Leiden, The Netherlands) to discriminate live versus dead cells. For immunofluorescence or FACS staining, anti-ERTR7 (affinity purified from hybridoma cell culture supernatant), anti-CD4 (clone GK1.5, BD Pharmingen, Woerden, The Netherlands), biotinylated anti-mouse DO11.10 TCR (KJ1-26, Caltag Laboratories, Burlingame, CA), PE-Cy7 labelled CD8 (eBiosciences), PE conjugated anti Vj2-PE (eBiosciences), anti-αdβ integrin (clone DATK32, kindly provided by Dr. Alf Hamann, University of Hamburg), rat anti-mouse CCR9 (clone 7E7, kindly provided by Dr. R. Förster), rat anti-mouse PNAd (clone Meca-79, kindly provided by Dr. E.C. Butcher, Stanford University) were used. Secondary antibodies were Alexa-conjugated goat-anti-rat-Fab and Alexa-conjugated streptavidin (Molecular Probes, Leiden, The Netherlands).
OVA-specific T cell enrichment, CFSE labelling, transfer and antigenic stimulation

Spleens and lymph nodes from DO11.10 or OT-I 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 minutes on ice in lysis buffer (150 mM NH₄, 1 mM NaHCO₃, pH 7.4). Cells were washed and resuspended in PBS-NBCS and CD4⁺ or CD8⁺ T cells were enriched using the CD4 or CD8 negative selection kit (Dynal, Oslo, Norway). The enriched cell suspension consisted of at least 60% CD4⁺ T cells or 85% CD8⁺ cells, as determined by flow cytometry (FACS Calibur, Becton Dickinson). These cells were labeled with 5μM of CFSE at 10⁷/ml for 10 min and washed with ice-cold PBS. BALB/c or C57BL/6 mice were injected with approximately 10⁷ OVA-specific T cells and were subsequently stimulated by intramuscular (i.m.) or intragastral (i.g.) administration of 200-400 μg OVA in 10 μl saline or 50-70 mg OVA in 300 μl saline, respectively.

Real time PCR

MHCII⁺CD11c⁺ DCs were sorted using a MoFlo sorter (DakoCytomation, Glostrup, Denmark) and lysed in Trizol (Gibco BRL, Breda, The Netherlands). RNA was isolated by precipitation with isopropanol and cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer’s protocol. RALDH-1, -2, and -3 specific primers and primers for housekeeping genes β-actin and 18S RNA were designed across exon-intron boundaries using Primer Express software (PE Applied Biosystems, Foster City, CA). Real time PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Total volume of reaction mixture was 20 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 non-activated CLNs, PLNs and MLNs and expression of transcripts was related to β-actin and 18S RNA.

Statistics

Differences in mean fluorescence intensity (MFI) of αβ⁺ or CCR9 expression on OVA specific T cells at 72 hrs after antigen administration were compared by 2 tailed Student T test and considered significant when p<0.05.
Results

Figure 1: Non-hematopoietic cells remain in transplanted LNs, while all hematopoietic cells disappear. MLNs or PLNs from β-actin-GFP/C57BL/6 mice were transplanted into the popliteal fossa, after removal of the endogenous LNs. Transplanted LNs were collected 12 weeks later and results were comparable in tMLNs and tPLNs. Analysis by immunofluorescence revealed that (A) gp38⁻ fibroblastic reticular cells (FRCs) expressed GFP (green), and that these cells were directly adjacent to (B) ERTR7⁺ extracellular matrix molecules produced by these FRCs. (C) PNAd⁺ high endothelial venules (HEVs) were donor derived while (D) dendritic cells (DCs), stained by CD11c, were host derived. (E) LNs from C57BL/6-CD45.1 mice were transplanted to C57BL/6-CD45.2 recipients. FACS analysis of the transplanted LN (tLN) at 5 weeks after transplantation showed that virtually all hematopoietic cells within the graft were of host origin and that all donor cells had left the graft. Only few B⁺, T-lymphocytes and CD11c⁺ DCs could be detected. In acceptor LNs (aLN), no CD45.1 cells could be observed.

Transplantation results in depletion of hematopoietic cells from the donor LNs

In earlier studies we showed by transplantation of LNs that differences exists between LNs with respect to their ability to provide an environment allowing the induction of mucosal tolerance [22]. To address whether the LN microenvironment itself induces specific homing receptors on T cells, or that the LN environment influences DCs for imprinting of T cells, we decided to transplant MLNs to a peripheral location, away from the intestinal drainage area. To first address what remains of the microenvironment within
transplanted LNs, 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 weeks after transplantation. By staining for podoplanin (gp38) expressed on fibroblastic reticular cells (FRCs) and pericytes in combination with anti-ERTR7 which detects an extracellular matrix (ECM) glycoprotein produced by these cells, we could identify most GFP expressing cells to be FRCs and pericytes (fig. 1A, B). In addition, high endothelial venules (HEVs) expressing PNA were derived from the donor 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 (data not shown and fig. 1D). To further proof that indeed hematopoietic cells were derived from the host, we transplanted LNs from CD45.1 mice into CD45.2 congenic hosts and analyzed those at 5 weeks after transplantation. By immunofluorescence and FACS, we confirmed that indeed virtually all hematopoietic cells were derived from the host, since only very few CD45.1+ cells, predominantly B cells, could be detected in the transplanted LNs (fig. 1E).
Figure 3: MLN transplantation to the popliteal fossa provides an environment for the induction of α4β7 expression on activated CD8 T cells. CFSE-labeled OT-1 cells were transferred to transplanted or control C57BL/6 mice at 11 weeks after transplantation. Donor cells in transplanted MLNs (tMLN), transplanted PLNs (tPLN), control MLNs (co MLN) and control PLNs (co PLN) were analyzed by flow cytometry 3 days after immunization with OT-1 peptide. Data represent mean fluorescence intensity (MFI) ± SD of α4β7 (A) and CCR9 (B) expression on responding OT-1 cells (A). For each transplanted group 6 LNs were analyzed, while in each experiment 1 MLN and 2 popliteal LN served as controls. Significant differences (p<0.05) are indicated by *.

Activation of CD4+ T cells in transplanted MLNs results in upregulation of α4β7

The finding that donor hematopoietic cells, including DCs, are depleted from a LN after transplantation allowed us to specifically look at the properties of the LN microenvironment created by the stromal cells that remained in the graft and its influence on DC behaviour. In normal, orthotopic MLNs, T cells will upregulate the mucosal homing receptors α4β7 and CCR9 upon activation by gut-derived DCs through the involvement of vitamin A converting enzymes [3, 5, 7, 10, 23, 24]. To investigate the contribution of the LN stromal cells in this process, both MLNs and PLNs were transplanted to the popliteal fossa, creating a situation where two different LNs would receive antigen-carrying DCs from the same, peripheral site. At least twelve weeks after transplantation, mice were injected with CFSE-labeled, OVA-specific DO11.10 transgenic CD4+ T cells. Twenty-four hours later, OVA was injected into the sural muscle, and 72 hours after antigen administration, LNs were removed from the popliteal fossa and proliferating T cells within the LNs were analyzed by FACS (fig. 2). Proliferating OVA-specific T cells in transplanted MLNs showed increased expression of α4β7 with each cell division, while proliferating OVA-specific T cells from transplanted PLNs (tPLNs) showed a decline of α4β7 expression on dividing T cells. To rule out the possibility that this was due to selective homing of α4β7high cells into transplanted MLNs (tMLNs) but not into PLNs, KJ1-CFSE expressing cells, lacking the OVA TCR were also analyzed, showing that
expression of $\alpha_4\beta_7$ on non-antigen specific incoming T cells was similar in tPLNs and tMLNs. These data thus suggests that the LN microenvironment is instructive for DCs to acquire either the mucosal or peripheral features for differential imprinting of T cell homing molecules.

**Activation of CD8$^+$ T cells in transplanted MLNs results in upregulation of $\alpha_4\beta_7$, but not CCR9**

To see whether the observed instruction of DCs by the microenvironment could also result in induction of mucosal homing receptors on CD8$^+$ T cells, OVA specific CD8$^+$ OT-I transgenic T cells were analyzed. Hereto, MLNs and PLNs were transplanted to the popliteal fossa and at 11 weeks after transplantation, CFSE labelled OT-I cells were injected. Analysis of proliferating OVA-specific CD8$^+$ OT-1 transgenic T cells at 72 hours after antigen administration showed induced expression of $\alpha_4\beta_7$ on activated cells in tMLN but not in tPLN (fig. 3A). Induced expression was comparable to values of activated CD8$^+$ cells in the endogenous MLNs upon intragastric administration of OVA (fig. 3A). The difference in $\alpha_4\beta_7$ expression was not visible in the non-proliferating T cell populations, indicating that antigenic stimulation of T cells is necessary for inducing expression of this gut-homing molecule (data not shown). Furthermore, the levels of $\alpha_4\beta_7$ expression on these non-proliferating CFSE$^+$ T cells were similar in transplanted PLNs versus MLNs, showing again a uniform entry of the injected cells in the two types of transplanted LNs. Remarkably, the mucosal homing receptor CCR9 was not induced on proliferating OT-1 T cells in tMLNs, while its expression was readily induced in the endogenous MLNs upon intragastric administration of OVA (fig. 3B). Similar to control PLNs, proliferating OT-1 T cells in tPLNs showed low expression of CCR9 (fig. 3B). Identical results were obtained when transplanted LNs were analyzed at 8 weeks after transplantation (data not shown). These results indicate that
tMLNs still contain a microenvironment that allows for the induction of the gut-homing molecule $\alpha_4\beta_7$, but not CCR9, on antigen-specific T cells.

**DC-induced upregulation of $\alpha_4\beta_7$ on stimulated antigen-specific T cells in transplanted MLNs is directed by LN stromal cells**

To further dissect which RALDH enzyme is responsible for the generation of RA leading to the induction of $\alpha_4\beta_7$ in vivo, DCs were sorted from transplanted LNs at least 12 weeks after transplantation. Real time PCR analysis showed that, when compared to DCs from transplanted PLNs, DCs from transplanted MLNs had an enhanced expression of RALDH2. RALDH1 expression was not detectable in these LNs, while RALDH3 expression was too low to give any consistent difference (fig. 4 and data not shown). Thus, stromal cells within MLNs are able to specifically induce the expression of RALDH2 in DCs, leading to production of RA and subsequent induction of $\alpha_4\beta_7$ on activated T cells.
Discussion

After LN transplantation, hematopoietic cells are depleted from the LNs, while stromal cells remain within the graft. By placing a MLN into a location where it no longer drains the intestine but the skin, we could now study the effect of the microenvironment within the LN on the incoming hematopoietic cells. Here we show that stromal cells within LNs can instruct DCs to express RALDH2, allowing DCs to imprint $\alpha_4\beta_7$ expression on activated T cells, even when the antigen is not transported by DCs from the gut.

We showed that expression of RALDH2 by DCs in tMLNs was not sufficient for the induction of CCR9 on activated T cells. A similar divergence of CCR9 vs. $\alpha_4\beta_7$ expression on activated T cells has been described upon T cell activation by different DC subsets from MLNs [6, 8]. Within the MLNs, CD103$^+$ DCs are able to induce the expression of both $\alpha_4\beta_7$ and CCR9, while CD103$^-$ DCs are only capable of inducing $\alpha_4\beta_7$. Since also $\alpha_4\beta_7$-CCR9$^+$ cells were recently shown to migrate to the intestines, both DC subsets can induce gut homing tropism [10]. It has been suggested that CD103$^+$ DCs form the subset that has actually migrated from the intestinal lamina propria into the MLNs [1]. Furthermore, the level of RA available at the time of T cell activation might also control whether both $\alpha_4\beta_7$ and CCR9, or only $\alpha_4\beta_7$ is being upregulated. In this scenario $\alpha_4\beta_7$ would be induced at the lowest concentration of RA, while for additional CCR9 expression higher levels of RA are required [1]. Consequently, within the lamina propria higher levels of vitamin A converting enzymes or additional RALDH enzymes (RALDH1 or 3) could be induced, leading to production of more RA in intestinal DCs and in CD103$^+$ MLN-DCs than in DCs instructed by MLN stromal cells alone. We therefore propose that stromal cells within the MLNs are able to induce RALDH2 to levels that allow the generation of enough RA for the induction of $\alpha_4\beta_7$, but not CCR9. However, also soluble factors that drain from the intestine into the MLNs might, in addition to RA, be required for induction of CCR9 on activated T cells. Conversely, it cannot be excluded that in the situation we created with the ectopic transplantation, skin-derived factors may enter the transplanted LNs that affect the DCs in their enzymatic activity.

In contrast with our data are the observations by Calzascia et al. reporting that various imprinting programs can occur within one LN upon tumor implantation at anatomically different sites [25]. However, in these studies analysis of T cell homing molecules was analyzed at 4 days after tumor implantation. We have shown in earlier studies that by this time, T cells might already have migrated through multiple LNs [26]. At 4 days after antigen administration, but not at earlier time points, proliferating antigen-specific T cells can be found in other LNs than the LNs draining the antigen-containing tissues. Taken together, data
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from both studies in fact suggest that the induced T cell homing phenotype reflects the site of initial antigen encounter, even in distant LNs. The stromal cells within the LNs have been thought of as cells that simply provide a structure for the immune cells to optimally interact with each other. However, recently it was shown that for T and B cells, these stromal components also provide migratory guidance, while DCs have been reported to adhere to these cells [27, 28]. In addition, stromal cells within the T cell area of the LN, the fibroblastic reticular cells (FRCs), secrete extracellular matrix molecules as part of the conduit system, through which small size molecules can get rapid access to the LNs [29, 30]. Furthermore, LN stromal cells were recently shown to present endogenous antigen to T cells, hereby promoting peripheral tolerance induction [31]. Thus, our findings that unique stromal microenvironments exist in anatomically distinct LNs and that this may direct tissue-specific homing of activated T cells adds to the role stromal cells have in controlling immune responses.

In conclusion, our studies have provided evidence that LN stromal cells create a specific microenvironment that promotes tissue-specific lymphocyte homing properties. How in turn these stromal cells receive differential maturation stimuli and what molecules are involved in their instruction of DCs remains an intriguing question and will be subject of further studies.
References

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