Chapter 24

T-Cell Homing to the Gut Mucosa: General Concepts and Methodological Considerations

Jaime De Calisto, Eduardo J. Villablanca, Sen Wang, Maria R. Bono, Mario Rosemblatt, and J. Rodrigo Mora

Abstract

Effector/memory T cells can migrate to most extra-lymphoid tissues in the body. However, migration to the intestinal mucosa requires the expression of very specific homing receptors on T cells, integrin α4β7 and chemokine receptor CCR9. These receptors are induced by all-trans retinoic acid (RA), a vitamin A metabolite that is specifically synthesized by gut-associated dendritic cells (DC), but not by extra-intestinal DC. Here we summarize some general concepts on T cell homing with an emphasis on the gut mucosa. We also discuss experimental strategies to generate gut-homing T cells in vivo and in vitro and the techniques to track gut-homing T cells.

Key words: Gut homing, α4β7, CCR9, CCL25, Retinoic acid, Small intestine, RALDH, GALT, DC, Homing, Chemotaxis

1. Introduction

Leukocyte adhesion to the endothelium and subsequent migration into different tissues is at the heart of both protective and pathological immune responses. On one hand, leukocyte adhesion is essential for protecting the body against pathogenic organisms, as illustrated by genetic defects in leukocyte adhesion, such as leukocyte adhesion deficiency (LAD)-I (lack of β2 integrins), LAD-II (defective generation of selectin ligands) or LAD-III (defects in integrin activation), which are characterized by variable degrees of immunodeficiency (1). On the other hand, the key role of lymphocyte adhesion in immune-driven pathology is clearly demonstrated by the clinical effectiveness of therapies targeting specific adhesion receptors, as seen in multiple sclerosis,
psoriasis, and inflammatory bowel disease (1). Thus, understanding how lymphocytes are targeted to different organs is important not only from a basic immunological standpoint, but also has clinical implications. Lymphocyte adhesion is a multistep process (2–4), involving the “capture” of lymphocytes by the endothelial cells (tethering), followed by loose adhesion (rolling) and then by an activation step (activation) that finally leads to firm arrest (sticking) and transmigration of lymphocytes into different tissues. Each of these steps is specifically controlled by distinct sets of adhesion and chemokine receptors expressed on lymphocytes and by their respective ligands, which are displayed on endothelial cells. Upon initial interaction with an endothelial cell a lymphocyte must follow a sequential algorithm with multiple yes/no decision points, all of which must be successful for a lymphocyte to finally adhere and transmigrate to the extravascular space (5). It is important to realize that the multistep nature of lymphocyte adhesion allows for a higher versatility and specificity in lymphocyte migration and that a particular migratory code for a given tissue (molecular “zip code”) is determined by the combination of homing receptors involved in each step and not by a single adhesion-receptor pair. This versatility and specificity also makes lymphocyte adhesion readily amenable to manipulation for experimental or therapeutic purposes.

First we will summarize our understanding of lymphocyte migration with a special emphasis in homing to the gastrointestinal mucosa and the skin, which are the two major surfaces exposed to pathogens in the body and are also the tissues with the best characterized homing requirements. Next, we will focus on describing some useful methods and techniques aimed at exploring lymphocyte adhesion and homing to the gut mucosa.

1.1. Lymphocyte Migration to Lymphoid and Nonlymphoid Compartments

Naïve T and B cells constantly transit between the blood and secondary lymphoid organs (SLO) with very high efficiency. Classic experiments using thoracic duct cannulation determined that most lymphocytes recirculate between the blood and the lymph around ten times per day (6, 7). More recent experiments using the immunosuppressant drug FTY720, a sphingosine 1-phosphate analogue that blocks the exit of lymphocytes from secondary lymphoid organs, showed that a high proportion of lymphocytes disappeared from the blood in only a few hours upon administration of the drug due to sequestering of lymphocytes in the lymphoid compartments. These observations illustrate the steady and massive migration of lymphocytes into SLO.

Migration of Naïve T and B cells to SLO, in particular lymph nodes (LN) and Peyer’s patches (PP) depends on the expression of L-Selectin (CD62L), integrin LFA-1 (αLβ2) and chemokine receptor CCR7 on lymphocytes (5). Naïve B cells are less dependent on CCR7 and they can also use CXCR4 and CXCR5 to migrate to LN and PP (1). The integrin α4β7, which is key for
lymphocyte homing to the gut mucosa, also plays a relevant role in naïve lymphocyte migration to PP, where it can partially compensate for the lack of L-selectin \((1)\). Lymphocyte adhesion in LN and PP occurs in the high endothelial venules (HEV), specialized postcapillary venules that express the necessary adhesion ligands and chemokines (collectively termed \textit{addressins}) (Table 1) \((1)\). If T cells are activated by their cognate antigen presented by dendritic cells (DC) they proliferate and become effector and/or memory T cells and then leave the lymphoid compartments in order to reach different peripheral/effector sites in the body. Some activated T cells maintain their expression of L-selectin and CCR7 and can migrate to LN. These T cells are referred to as central memory T cells \((T_{CM})\) \((8)\). On the other hand, a large proportion of T cells loses their expression of L-selectin and/or CCR7 and therefore cannot migrate back to the LN. Those T cells are termed effector \((T_{EFF})\) or effector memory \((T_{EM})\) T cells. While initially defined as cells with no effector capacity, it has become clear that \(T_{CM}\) can exhibit potent effector activity and can also confer protection against infectious challenges \((9–12)\). Moreover, in addition to L-selectin and CCR7, \(T_{CM}\) might also express homing receptors involved in extra-lymphoid migration, similar to \(T_{EFF}/T_{EM}\) \((12)\). Therefore, the main functional difference between \(T_{CM}\) and \(T_{EFF}/T_{EM}\) is that \(T_{CM}\) can migrate to LN,

<table>
<thead>
<tr>
<th><strong>Table 1</strong></th>
<th><strong>T cell homing receptors: inductive and effector sites</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target tissue</strong></td>
<td><strong>Inductive sites</strong></td>
</tr>
<tr>
<td><strong>T cell subtype</strong></td>
<td><strong>Lymph nodes</strong></td>
</tr>
<tr>
<td><strong>Tethering/rolling</strong></td>
<td>L-selectin/PNAd (\alpha4\beta7/\text{MAdCAM-1}^a) (\text{only MLN})</td>
</tr>
<tr>
<td><strong>Integrin activation</strong></td>
<td>CCR7/CCL19, CCL21</td>
</tr>
<tr>
<td><strong>Firm Adhesion</strong></td>
<td>LFA-1/ICAM-1</td>
</tr>
</tbody>
</table>

\(^a\)Pairs of homing receptors expressed on T cells/ligand on endothelial cells
\(^b\)T_{CM}: central memory T cells
\(^c\)T_{EFF}/T_{EM}: effector/memory T cells
\(^\ast\)Expressed constitutively and increased during inflammation
\(^\ast\ast\)Expressed only during inflammation
whereas $T_{\text{EFF}}/T_{\text{EM}}$ cannot home to this lymphoid compartment (12).

Recently activated T cells can migrate to multiple extra-lymphoid tissues upon activation, including liver and lungs, apparently without specificity (13, 14). It should be noted, however, that liver, lungs, and the spleen do not have tight postcapillary venules and exhibit a more open circulation due to the presence of sinusoids, which probably explains why T-cell migration to these organs does not seem to be selective (15, 16). However, whether lymphocytes require tissue-specific adhesion or chemokine receptors for their interstitial migration in these organs (e.g., to the alveolar space in the lungs) remains to be determined.

T-cell migration to other tissues in the body, in particular the intestinal mucosa, the skin, and the central nervous system, requires the expression of specific adhesion and chemokine receptors on lymphocytes and their respective ligands to be expressed in the postcapillary venules of these tissues. Homing to the central nervous system requires P-selectin ligands, $\alpha 4\beta 1$ integrin and probably CCR6 on T cells (1, 17). However, the best-characterized tissues in terms of specific homing are the gut mucosa and the skin, which are also the largest surfaces exposed to the environment in the body (the total surface area of the intestinal mucosa in an adult is similar in size to a tennis court). T cell homing to the skin requires P- and E-selectin ligands (P- and E-Lig, respectively) (12, 18), whose synthesis relies on the expression of fucosyltransferase-VII (FucT-VII) (19), among other enzymes (12). In addition, T-cell migration to the skin requires the integrins LFA-1 (and probably $\alpha 4\beta 1$) and the chemokine receptors CCR4 (20) and/or CCR10 (12, 21, 22). T-cell migration to the small intestine requires the integrin $\alpha 4\beta 7$ (23, 24) and also LFA-1 (25) and CCR9 (12, 26–28). Interestingly, although recently activated CD8 T cells clearly need CCR9 for migrating to the small bowel, homing of CD4 T cells to this compartment is, at least in part, CCR9-independent (29). Homing to the colon has some very distinctive features, as it requires either $\alpha 4\beta 7$ or $\alpha 4\beta 1$, but not CCR9 (1, 12). Even though IgA-ASC rely on CCR10 for migrating to most mucosal sites, including the colon (1), it is currently unknown which is/are the chemokine receptors involved in T-cell homing to this tissue and why migration to the small bowel and colon is differentially regulated.

**1.2. Induction of Gut-Tropic T Cells**

How do naïve T and B cells acquire specificity for migrating to the gut mucosa? Whereas systemic immunization induces T cells with multiple homing capacities (13, 14, 17), the site of antigen entry determines, at least in part, the adhesion receptors that T cells acquire (30). Vaccination via the oral route induces preferential expression of $\alpha 4\beta 7$, whereas parenteral immunization does not significantly induce the expression of this gut-homing integrin (1, 12).
Moreover, T cells activated in mesenteric lymph nodes (MLN) upregulate α4β7 and CCR9, whereas those activated in skin-draining peripheral lymph nodes (PLN) acquire the expression of E- and P-Lig, but not gut-homing receptors (30, 31). In fact, in the lymphoid microenvironment DC and stromal cells are sufficient to imprint tissue-specific tropism on T cells upon activation ex vivo (15, 28, 30, 32–35). DC from MLN, PP, and small intestine lamina propria (gut-associated DC), but not DC from extra-intestinal tissues, induce the expression of α4β7 and CCR9 on T and B cells. This gut-specific imprinting property depends on the selective capacity to metabolize vitamin A (retinol) into all-trans retinoic acid (RA) (36) and RA is necessary and sufficient to induce gut-tropism in vitro and in vivo in murine (36, 37) and human T and B cells (37, 38) (Fig. 1). RA not only induces gut-tropic T cells, but also prevents the generation of skin-homing
receptors by blocking the induction of FucT-VII (39) and P- and E-Lig on T cells (36). In addition, RA decreases the expression of CCR4 mRNA (36, 39). Thus, RA is sufficient to induce gut-tropic T cells, while it reciprocally inhibits the induction of skin-homing T cells.

RA exerts its gut-homing imprinting effect on lymphocytes by acting via RA receptors of the RAR family (36). RAR need to heterodimerize to nuclear receptors of the RXR family, which can also homodimerize and heterodimerize with other nuclear receptors, such as peroxisome proliferator-activated receptor-γ (PPARγ), Vitamin D receptor (VDR) and liver X receptor (LXR) (40). Given this promiscuity in RXR dimerization, it is possible that ligands for other RXR-dependent nuclear receptors might modulate the effect of RA on lymphocytes. In fact, ligation of VDR-RXR by 1,25(OH)\(_2\)D\(_3\) (the physiologically most active form of vitamin D) can inhibit RA-mediated induction of gut-homing T cells (39, 41). Of note, although 1,25(OH)\(_2\)D\(_3\) induces CCR10 (on human T cells) (41), it blocks the expression of FucT-VII and P-/E-Lig on T cells and it also decreases delayed-type hypersensitivity responses in the skin (39). Therefore, in spite of inducing CCR10 on T cells and being synthesized by skin-derived DC (41), 1,25(OH)\(_2\)D\(_3\) might not be sufficient to promote skin-tropic T cells.

Gut-associated DC, but not extra-intestinal DC, express high levels of retinal dehydrogenases (RALDH1 and RALDH2 isoforms), which are critical enzymes for synthesizing RA (36, 42). Moreover, among MLN-DC, only CD103\(^+\) DC, which arrive from the small intestine lamina propria, express RALDH2 and imprint gut-tropic lymphocytes, whereas CD103\(^-\) DC do not exhibit gut-imprinting properties (32, 43). Interestingly, radioresistant stromal cells from MLN, but not from PLN, also express RALDH and can imprint gut-tropic T cells (33–35). In addition, intestinal epithelial cells (IEC) express RALDH and can also synthesize RA (36, 44). It will be important to determine the interplay and relative contribution of DC, stromal cells, and IEC in the generation of gut-homing lymphocytes in vivo and how gut-associated DC acquire the capacity to synthesize RA and hence imprint gut-tropic lymphocytes.

Finally, a deeper understanding of the mechanisms by which lymphocytes acquire gut-tropism and how DC are educated in the gut could offer simple and straightforward methods for boosting intestinal immune responses for vaccination purposes, such as in infections by Salmonella, rotavirus, and HIV, where the small intestine has been identified as a major reservoir for the virus (45) and where recent major vaccine trials have been mostly unsuccessful (46). Below we describe some methods to induce and study gut-tropic T cells in vitro and in vivo.
2. Materials

2.1. Animals

1. C57BL/6 mice: commonly used for DC isolation.
2. OT-1/RAG1−/−, OT-2/RAG1−/− and P14/TCRα−/− (Taconic Farms, Germantown, NY): used for or T-cell isolation. In addition, CD45.1 and Thy1.1 congenic strains are available through Jackson Laboratories (Bar Harbor, ME).
3. CCR9−/− mice: There are three published mouse strains with deficiencies in gut-homing receptors, but they are not yet commercially available (47, 48). Although these mice have only a mild phenotype in the steady state, recently activated T cells migrate around 10 times less to the small bowel as compared to wild-type T cells. Similarly, homing of IgA-ASC to the small bowel is impaired in these mice (49).
4. β7 integrin−/− mice (Jackson labs): These mice have a dramatic decrease in the number of lymphocytes even in the steady state. In addition, Peyer’s patches are very small in this strain (50).

2.2. Culture Media

1. IMDM (Iscove’s Modified Dulbecco’s Medium + l-Glutamine + HEPES) plus 10% heat-inactivated FBS (Fetal Bovine Serum) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 0.5 mg/ml fungizone/amphotericin B, and 50 μM β-mercaptoethanol.
2. FBS-free (retinol-free) media (X-vivo 15, Lonza, Basel, Switzerland): regular FBS contains variable amounts of retinol. This culture media support mouse and human T-cell activation without needing the addition of FBS.
3. Red Blood Cell Lysis buffer (RBC, 10 mM KHCO₃, 150 mM NH₄Cl, 0.1 mM EDTA, pH 8.0), adjust to pH 7.2–7.4 and store at room temperature.
4. PBS (Phosphate-Buffered Saline).
5. Flow cytometry (FACS) media (PBS or IMDM +2% FBS +5 mM EDTA): When staining using Selectin-Fc chimeras, media with 2 mM Ca²⁺ should be used in all steps (including FACS acquisition). IMDM is recommended in this case.

2.3. T-Cell Labeling and Adoptive Transfer

1. CFSE (carboxyfluorescein diacetate, succinimidyl ester).
2. CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine) (Molecular Probes®, Invitrogen, Carlsbad, CA). 1,000× stocks should be made in DMSO (5 mM CFSE, 20 mM CMTMR) and stored at −20°C.
2.4. Flow Cytometry (FACS) Staining

1. Polyclonal activation: CD3 (145-2C11), CD28 (37.51).
2. Lineage mAb: CD4 (GK1.5), CD8a (53-6.7), CD11c (N418), CD90.1 (Thy1.1/HIS51), CD90.2 (Thy.12/53-2.1), CD45.1 (A20), CD45.2 (104).
3. Antibodies to gut-homing receptors: purified CCR9 (CD199/eBioCW-1.2, eBioscience, San Diego, CA) plus secondary reagent F(ab’)2 fragment of goat anti-mouse (H+L), α4β7 (LPAM-1/DATK3), isotype control (IgG2a, k), α4 (R1-2), β7 (M293), and αE/CD103 (M290) integrin chains.
4. Skin-homing receptors: P-selectin-Fc (Purified Mouse P-Selectin – IgG Fusion Protein, BD Pharmingen™, San Jose, CA), E-selectin-Fc (Recombinant Mouse E-Selectin/Fc Chimera, R&D Systems, Minneapolis, MN) plus corresponding secondary reagent goat F(ab’)2 anti-human IgG R-PE.

2.5. Retinoids and RALDH Inhibitors

1. All-trans retinoic acid, retinol (both from Sigma, St. Louis, MO): resuspended in absolute ethanol or DMSO, use a yellow bulb or an indirect source of light during the preparation. Store aliquots in glass vials at −80°C and protect from light at all times.
2. Synthetic RAR-agonists: Am80 (Wako Chemicals, Richmond, VA).
4. RALDH inhibitors: Citral (Sigma, St. Louis, MO), diethylaminobenzaldehyde (DEAB) and bisdiamine (both from Acros Organics, Geel, Belgium).

2.6. Protein and Peptide for Transgenic Activation

1. OT-1 peptide: chicken ovalbumin257–264 (SIINFEKL).
2. OT-2 peptide: chicken ovalbumin323–339 (ISQAVHAAHAEINEAGR).

2.7. Primers Real-Time PCR Using SYBRGreen

1. FucT-VII (Fuct7): forward, ACTGATGTTGAAACCAAAAGG, and reverse, GCCCAGTCTCTCCTTATATCC
2. CCR4: forward, GGTACCTAGACTACGCCATCC, and reverse, ATGTACTTGCGGAATTTCTCC
3. α4 integrin chain: forward, AAACACTGGGATTAGCATGG, and reverse, ATTGCCCTGTAGTTGTCTGG
4. CCR9: forward, AGTATAGTCAGGCCAATGTACGCC, and reverse, ATCCTTTTCTAGTTGCTCTGC
5. RALDH1 (Aldh1a1): forward, ACAAGGTGGCCCTTCACTGGA, and reverse, GCACACACATGCAAGGGCT
6. RALDH2 (Aldh1a2): forward, TGGGTGAGTTTGGCTTACGG and reverse, AGAAACGTGGCAGTCTTGGC
7. RALDH3 (Aldh1a3): forward, TGTAGAAAGGGACCGAGCGAT, and reverse, CCCGGCAAAATATCTGAAGGT
8. GAPDH: forward, CAACTTTGTCAAGCTCATTTCC and reverse, GGTCCAGGGTTTCTTACTCC. mRNA levels can be expressed relative to GAPDH mRNA for each sample.

2.8. TaqMan qPCR for Aldh1a2 (Codifying for RALDH2)
1. RNeasy (Qiagen).
2. iScript cDNA synthesis kit (Bio-Rad).
3. TaqMan PCR master mix and the following TaqMan kits (Applied Biosystems): Aldh1a2 (Mm00501306_m1) and β-actin (4352933) for normalization.

2.9. Aldefluor Assay for RALDH Activity
1. ALDEFLUOR FACS-based assay kit (StemCell Technologies, Vancouver, British Columbia, Canada): used for measuring RALDH activity (42).

3. Methods

3.1. Ex Vivo Induction of Gut-Homing Receptors
Gut-homing receptors can be induced on T and B cells either by coculturing them with gut-associated DC or by activating them in the presence of RA or a synthetic RA receptor (RAR) agonist, such as Am80 (40). Gut-associated DC allow us to study defined DC subsets involved in imprinting gut-homing lymphocytes. However, obtaining high numbers of gut-associated DC is technically challenging and logistically impractical for studies involving human T cells. On the other hand, RA or RAR-agonists allow the generation of large number of gut-tropic T cells expressing high levels of both α4β7 and CCR9.

3.1.1. Ex Vivo Induction of Gut-Tropic T Cells Using Gut-Associated DC
Dendritic Cell Isolation
Gut-associated DC can be isolated from either unmanipulated mice (in which DC have not been expanded) or from mice that have been pre-treated with the cytokine Flt3L (see Note 1).
1. C57BL/6 mice are injected subcutaneously with B16 melanoma cells secreting Flt3-L to expand DC in vivo as described (15, 37).
2. After 12–17 days, the mice are sacrificed and single-cell suspensions are generated by digesting lymph nodes (PP, MLN, spleen) using 250 μg/ml Liberase CI (Roche, Indianapolis, IN) and 100 μg/ml DNase I (Roche, Indianapolis, IN) 40 min at 37°C with mild agitation.
3. DC are immunomagnetically isolated using MACS CD11c microbeads (Miltenyi Biotec, Auburn, CA) according to the
manufacturer’s instructions. DC purity will be around 90%, as determined by CD11c staining. FACS-based sorting can follow magnetic separation in order to improve DC purity or for isolating specific DC subsets, such as CD103+ DC. If unmanipulated mice are used, one should start from at least 3–5 mice. In Flt3L-treated mice (recombinant cytokine or injected with B16-Flt3L melanoma), 1 mouse might be enough to obtain around $1 \times 10^6$ CD11c+ cells from PP and $4 \times 10^6$ from MLN.

4. Collect SLO (MLN, PP and PLN as controls) and isolate DC by negative or positive immunomagnetic selection. Check purity by FACS (see Note 2).

5. Load DC with peptide or protein (see Subheading 2 for working concentrations) in complete IMDM for 2 h at 37°C. Wash twice and resuspend in complete IMDM (see Note 3).

Labeling with CFSE

Naive CD4+ and/or CD8+ T cells can be obtained from splenocytes after red blood cell lysis using ACK buffer (see Subheading 2), followed by either negative or positive immunomagnetic selection. The proportion of T cells will be much higher when using splenocytes from TCR transgenic mice in a RAG−/− background due to the lack of B cells (see Note 4).

1. Splenocytes from C57BL/6 mice or TCR transgenic mice are used for T-cell isolation. Cells are centrifuged at 300 × g for 5 min and resuspended in 2 mL of RBC lysis buffer for 3 min at room temperature (longer incubation times might cause significant decrease in T-cell viability).

2. Cells are washed with IMDM, centrifuged as before and finally resuspended in 10 ml of complete IMDM. T cells can be isolated by negative selection, as described (15) or by using CD4/CD8 microbeads for positive selection or the Pan T-cell isolation kit for negative selection (both from Miltenyi Biotec). Negative selection might be preferred, as it does not “touch” T cells.

3. For CFSE labeling, lymphocytes are washed twice with warm PBS to remove the serum. The cell concentration is adjusted to $10^7$/ml in PBS (see Note 5).

4. Add CFSE to a final concentration of 1–5 µM, vortex and incubate for 10–15 min in a water bath or an incubator at 37°C. The optimal working concentration should be determined depending on the final application. For in vitro studies it is better to use <5 µM, since it makes it easier to adjust the compensations for FACS analysis. On the other hand, 5–10 µM CFSE gives more consistent results for in vivo adoptive transfer experiments.
5. After the incubation period, add 1 volume of FBS to quench the CFSE and incubate for 1–5 min at room temperature. Then, dilute ten times with warm PBS and centrifuge for 5 min at 300 × g. After that, perform an additional washing step with PBS and resuspend the cells in complete IMDM. It should be kept in mind that around 20–30% of the cells can be lost during the labeling, so incubation times should be limited to a minimum.

Polyclonal T-Cell Activation

1. 24-well or 96-well plates are incubated for 2 h at 37°C with 300 μL or 50 μL PBS, respectively, containing 10 μg/ml anti-CD3 plus anti-CD28 antibodies. After that, the culture plates are washed two times with PBS and used immediately for T-cell culture. Plates can be stored at 4°C for 24 h, provided that the wells are covered with PBS to avoid drying.

2. Dynabeads coated with anti-CD3/anti-CD28 (“artificial APC,” Dynal, Invitrogen, Carlsbad, CA) can also be used for T-cell activation instead of plate-bound antibodies. However, it should be kept in mind that the expression levels of homing receptors might vary depending on the method of T-cell activation. Therefore, the method of choice should be adapted depending on the final aim of the experiment.

DC/T Cell Co-cultures

1. Flat-bottom 96-well or 24-well plates can be used. For polyclonal activation use anti-CD3 plus anti-CD28 pre-treated plates (see Note 6).

2. Mix 1–2 × 10^6 naïve T cells at 1:1 T:DC ratio (antigen pulsed-DC). Coculture in complete IMDM in a final volume of 1.5–2.0 mL for 24-well plates or 150–200 μL for 96-well plates. Culture at 37°C and 5% CO₂ for 5–7 days. At day 3 replace half of the media if necessary. After that, analyze the cells by FACS (see Note 7).

3.1.2. Ex Vivo Induction of Gut-Tropic T Cells with RAR-Agonists

1. Resuspend T cells at 1 × 10^6/ml in complete IMDM and add all-trans RA (or RAR-agonists, such as Am80 or Am580) to a final concentration of 10–200 nM. The induction of gut-homing receptors is dose-dependent and reaches a plateau around 100–200 nM RA (see Note 8).

2. Add 1.5–2.0 mL of the cell suspension to each well of a 24-well plate pre-treated with anti-CD3 plus anti-CD28 and culture at 37°C and 5% CO₂.

3. After 72 h, transfer the cells (in the same media) into a new untreated 24-well plate and culture for an additional 2–3 days. Depending on cell density and proliferation the media might become yellow (acidic), in which case it might be necessary to replace half of the media with fresh complete IMDM.

4. Evaluate the expression of homing markers (see Note 9).
The induction/expression of homing markers can be analyzed at the mRNA level using quantitative RT-PCR, and/or at the protein level by FACS. The mRNA levels are usually expressed relative to a housekeeping gene such as GAPDH or β-actin (see Note 10). Among the receptors analyzed are the gut-homing receptors α4β7 and CCR9 and skin-homing receptors including E- and P-selectin ligands and some of the enzymes involved in their synthesis (Core-2/C2GlcNAcT-I, FucT-VII, FucT-IV), and chemokine receptors CCR4 and CCR10. Of note, gut- and skin-homing receptors are reciprocally regulated by RA. Whereas RA induces α4β7 and CCR9, it markedly suppresses the expression of P- and E-selectin ligands, CCR4 and FucT-VII (40) (see Note 11).

Different time points can be used for analyzing the expression of homing receptors on T cells, starting as early as day 2 of culture. Gut-homing receptors (α4β7 and CCR9) are clearly upregulated starting at day 3 of culture with gut-associated DC or RAR agonists.

### 3.1.4. FACS Analysis

1. For each staining collect between 0.2–0.5 × 10^6 cells and centrifuge for 5 min at 300 × g at 4°C.

2. Incubate with the primary antibodies anti-α4β7 or anti-CCR9 for 20 min at 4°C in the dark. E-selectin-Fc and P-Selectin-Fc chimeric proteins can be used to detect the skin-homing receptors P- and E-selectin ligands (see Note 12).

3. After the incubation, centrifuge for 5 min at 300 × g 4°C. Remove the media and incubate with the appropriate fluorochrome-conjugated secondary antibody. In the case of P- and E-selectin-Fc chimeras, an anti-human Fc antibody can be used as secondary reagent. Incubate for no more than 30 min at 4°C.

4. Centrifuge for 5 min at 300 × g 4°C, resuspend in staining buffer, and analyze by FACS.

### 3.2. Functional Assessment of Gut-Homing T Cells

#### 3.2.1. Chemokine Receptor Functionality

Chemotaxis experiments can be performed after 4–5 days of culture. Each assay should be performed at least in duplicate.

1. Make 10 μM chemokine stocks in PBS + 0.1% BSA and store them at −80°C. At the moment of using the chemokines dilute them in complete IMDM to reach a final working concentration of 0.1–500 nM, depending on the chemokine (see Note 13).

2. Add 600 μL complete IMDM (control condition) or complete IMDM + chemokine in the lower chamber of the 24-well transwell (Corning, 5 μm pore size for T cells).

3. Carefully place the transwells into the wells (see Note 14).

4. Add 100 μl of the cell suspension (2–5 × 10^6/ml) in the upper chambers (i.e., 2–5 × 10^5 cells/well). See Note 15.
5. Cover the plate and incubate 1 h at 37°C and 5% CO₂ (see Note 16).

6. After the incubation period, carefully remove the transwell inserts from each well and resuspend the media in the lower chamber. Take an aliquot and read by FACS (see Note 17).

7. The *chemotactic index* is calculated as: the number of cells migrating to the media plus chemokine divided by the number of cells migrating to the media alone. The *percentage of migration* is calculated as the number of cells migrating to the media alone, or the media plus chemokine divided by the total number of cells (obtained from the well in to which the total number of cells were added). See Note 18.

In vivo induction of gut-homing T cells is a fast and relatively simple strategy to assess the impact of genetic and/or pharmacological manipulations in the generation of gut-tropic T cells. TCR transgenic T cells are commonly used to evaluate the expression of gut-homing molecules upon in vivo immunization. T cells can be pre-labeled with CFSE in order to discriminate transferred T cells from the endogenous population and to track T-cell activation and proliferation. In addition, CFSE labeling can also be combined with congenic markers (CD45.1/2 or Thy1.1/2), which makes it easier to identify the transferred T cells by FACS.

1. Inject 3–5 × 10⁶ CFSE⁺-labeled TCR transgenic T cells i.v. (via tail vein) into C57BL/6 recipient. OT-1 or OT-2 T cells are commonly used for this purpose and they can be in a RAG⁻/⁻ background to exclude the possibility of having preformed effector/memory T cells. In order to easily discriminate the transferred from the endogenous T cells, congenic Thy1.1⁺ recipient mice can be used. Given that only T cells will be activated upon immunization, total splenocytes (without T-cell isolation) can also be used for CFSE labeling and adoptive transfer (see Note 19).

2. Next day, immunize with specific protein or peptide plus adjuvant i.p. or via oral gavage (see Subheading 2). The adjuvant is usually LPS, but other adjuvants, such as alum, can also be used (28) (see Note 20). If OT-1 CD8 or OT-2 CD4 T cells are used, most protocols immunize using 5 mg ovalbumin plus 100 µg LPS i.p in 300 µl PSB (total volume).

3. Mice can be euthanized at different time points, starting on day 2 post-immunization. However, clear induction of gut-homing receptors in MLN (and skin-homing receptors in PLN) is only seen starting at day 3. At that time, T cells can also be found in the small bowel lamina propria and IEL compartment (see Note 21).

4. The expression of α4β7 and CCR9 should be significantly higher when analyzing T cells activated in MLN and PP as
compared to those activated in PLN. Conversely, P- and E-selectin ligands (detected by the corresponding P- and E-selectin-Fc chimeras) are readily induced in PLN, but at a much lower degree in MLN and PP (31).

3.2.3. Competitive Homing Assays

Competitive homing experiments are used to directly compare the migratory capacity of different T-cell populations in the same mouse. This allows us to minimize the effects of variables such as the number of injected T cells, number of isolated/analyzed T cells in each organ, or eventual difference in the animal size. In a competitive homing experiment, two different cell populations (e.g., T cells treated plus/minus RA or T cells from CCR9 knock-out vs. wild-type mice) are mixed in a 1:1 ratio and adoptively transferred into a recipient mouse (ideally a congenic Thy1.1+ or CD45.1+ host). The aim of the experiment is to determine the relative migration of one T-cell population with respect to the other (differentially labeled) T-cell population in a given tissue. Since the results are expressed as the ratio of two T-cell populations, the analyses do not rely on absolute cell counts. If absolute numbers are needed, special care needs to be taken in order to control the total number of T cells injected and recovered from each mouse. Moreover, total T cells need to be carefully counted in every tissue (see Note 22).

1. The different T-cell populations injected can be distinguished by using a congenic marker, e.g., CD45.1/CD45.2 or Thy1.1/Thy1.2. Alternatively, T cells can be differentially labeled, e.g., using CFSE (green) or CMTMR (orange) (see Note 23).

2. After labeling, both cell populations are mixed together (in equal proportions), resuspended in no more than 250 µl PBS (total volume), and adoptively transferred i.v. via tail vein injection (using a tuberculine syringe with a 30G needle). See Note 24. Some cells should be saved after injection for calculating the input ratio (which should be close to 1).

3. The mice can be euthanized at different time points, although significant T-cell migration to the gut is usually achieved only after 12 h post-injection, even when using bona fide gut-homing T cells (see Note 25).

3.2.4. Labeling T Cells with CFSE & CMTMR

1. Wash T cells twice with PBS to remove the serum. Adjust T-cell concentration to 1.0–1.5 × 10⁷/ml (maximum) in PBS. All media/buffers should be at 37°C.

2. Add CFSE or CMTMR to a final concentration of 5 and 10 µM, respectively, vortex, and incubate for 20 min at 37°C.
3. After the incubation, add 1 volume of FBS and incubate for 1–5 min at room temperature. Dilute ten times with warm PBS and centrifuge for 5 min at 300 \( \times g \). Wash twice with PBS and resuspend in complete IMDM. Count the cells after the labeling prior to injection.

4. Resuspend the cells in up to 250 \( \mu L \) of warm PBS. Inject i.v. via tail vein (see Note 26). Ideally, 10–20 \( \times 10^6 \) cells of each T population should be injected (1:1 ratio) (see Note 27).

5. To calculate the input ratio leave 5–10 \( \mu L \) of the injected cell suspension and dilute into 300 \( \mu L \) of FACS buffer and analyze by FACS (see Note 28).

The right time to analyze T-cell migration varies depending on the aim of the experiment, the T-cell populations injected, and the tissue analyzed. Analysis of naïve T-cell homing to PLN can be done as fast as 1 h post-injection. The advantage of shorter times is that any differences observed in T-cell homing can be more confidently ascribed to T-cell entry rather than to T-cell exit rate (or proliferation/survival). However, shorter times will not allow for observing clear T-cell migration to some other tissues, such as the gut or the inflamed skin, even when using bona fide gut- or skin-homing T cells. Typically, homing to the gut mucosa is analyzed 12–24 h after T-cell injection. Mice are euthanized and the different tissues are collected in order to make single cell suspensions for FACS analysis. Collect lymphoid tissues including the spleen, PLN, MLN, PP, bone marrow, and peripheral tissues including liver, lungs, small, and large bowel. Isolation of gut lamina propria and intraepithelial lymphocytes has been previously described (15) (see Note 29).

1. FACS staining: depending on the number of cells obtained, the samples will need to be diluted at a density no higher than 3.0 \( \times 10^6 \) cells/ml. Some tissues, including popliteal lymph nodes, gut lamina propria, and IEL, do not give high T-cell yields. In those cases the whole sample should be used for staining. If congenic CD45.1\(^+\) or Thy1.1\(^+\) mice were used as recipients, label the cells with the congenic marker (e.g., CD45.2 or Thy1.2) combined with some lineage marker (e.g., TCR\(\beta\) chain, CD4, CD8, CD45.1\(^+\)/CD45.2\(^+\)).

2. Analyze by FACS. During FACS analysis, transferred T cells are distinguished from the endogenous T cells by the congenic marker and because they are labeled with CFSE or CMTMR.

3. The data is usually expressed as the Homing Index (HI), which is calculated as the ratio CFSE/CMTMR (or CMTMR/CFSE) in each tissue divided by the input ratio. If the input
ratio is very close to 1, then the tissue ratios will be equivalent to the HI. In addition, when the HI is significantly different than 1 in the blood, tissue HI can be normalized by blood HI (see Notes 29 and 30).

\[ \text{HI} = \frac{[\text{CFSE}]_{\text{tissue}}}{[\text{CMTMR}]_{\text{tissue}}} : \frac{[\text{CFSE}]_{\text{input}}}{[\text{CMTMR}]_{\text{input}}} \]

### 4. Notes

1. Using unmanipulated mice has the advantage of eliminating any potential known or unknown artifacts derived from DC expansion. For instance, even though Flt3L dramatically expands all the classically described DC subsets in most tissues, including CD103+ DC, it might change the relative DC subset composition. Therefore, DC expansion should not be used when studying the physiological composition of DC subsets in different tissues. On the other hand, if the aim is to study gut-specific imprinting, including induction of gut-homing lymphocytes and/or IgA-ASC, we and others have demonstrated that gut-associated DC isolated from Flt3L-treated mice are equivalent to their counterparts isolated from unmanipulated mice (28, 36, 42) and to freshly isolated DC from human MLN (37, 38).

2. While the isolation protocols might affect DC’s maturation kinetics, they should not significantly affect the gut-homing imprinting capacity of gut-associated DC. Nonetheless, their viability tends to be lower than extra-intestinal DC, so they should be used for co-cultures immediately after isolation.

3. The concentration of antigenic peptide or protein used to pulse DC plays an important role in the final induction of gut-homing receptors (51). For instance, when activating OT-1 TCR transgenic T cells, pulsing DC with peptide concentrations above 200 nM dramatically decreases the induction levels of α4β7 and CCR9. The mechanism for this effect is unclear, but it also holds true when using exogenous RA instead of gut-associated DC to imprint gut-homing T cells.

4. Using T cells from TCR transgenic mice (like OT-1 or P14 mice), where T cells are activated by a specific peptide presented by antigen-presenting cells, is a valuable tool for studying both in vitro and in vivo induction and expression of gut-homing molecules. Nonetheless, polyclonal T cells can also be used to generate gut-tropic T cells by activating them with polyclonal activators (e.g., anti-CD3/CD28-coated plates or Dynabeads coated with anti-CD3/CD28) in the
presence of either RA or gut-associated DC in *trans* (without presenting antigenic peptide).

5. CFSE labeling is an optional step. It has the advantage of allowing the comparison in the expression of gut-homing receptors among cells that have proliferated equivalently.

6. Adding IL-2 improves T-cell viabilities and final yields. However, adding exogenous IL-2 also decreases the induction of gut-homing receptors in a concentration-dependent manner, so it should be avoided when generating gut-tropic T cells (15).

7. For CD8+ T cells the induction of gut-homing receptors can be analyzed as early as day 3 (52). However, the peak in the induction of gut-homing receptors is reached around day 5. Also, CD4 T cells tend to express lower levels of gut-homing receptors as compared to CD8 T cells.

8. RA is extremely sensitive to light, heat, and oxidation. Ideally, the stock solutions should be made at ~10 mM in ethanol (from an unopened bottle containing little or no water) and replaced every 3 months from a new vial. From those stocks, 1 mM working solutions should be prepared at least every month. All the stock solutions should be kept at −80°C in air-tight glass vials filled with N₂ gas and wrapped with aluminum foil. New RA vials should be opened in the dark (using only a small yellow light). Similarly, all the work involving RA (including co-cultures) should be done in the dark (Makoto Iwata, Tokushima Bunri University, Japan, personal communication).

9. If the protocol was successful, 90–100% of T cells should become CCR9+ and α4β7+. It is also important to consider the level of gut-homing receptor expression (MFI), as it is strongly correlated with the final T-cell gut-homing capacity (at least for α4β7) (15). The final MFI will depend on several factors, including the concentration of RAR agonist used, antigenic peptide/protein concentration used to load DC (lower concentrations will lead to higher MFI), addition of cytokines (e.g., IL-2 which has a negative effect), time of culture (>3 days for higher MFI), and cell viability.

10. Even though it is normally assumed that “housekeeping” genes do not change with different treatments and therefore can be used to normalize the expression of other genes, there might be some exceptions. In particular, GAPDH might not be a good choice when comparing naïve T cells vs. activated/effector T cells, as they markedly differ in their metabolic rate, which might cause significant variations in the GAPDH levels.

11. When looking at the expression of α4β7 integrin, it should be kept in mind that it is composed of two chains (as are all integrins)
and that therefore its final expression could be determined by the expression and interplay of at least four integrin chains. \( \alpha 4 \) can pair with either \( \beta 7 (\alpha 4\beta 7) \) or \( \beta 1 (\alpha 4\beta 1) \) integrin chains. On the other hand, the \( \beta 7 \) chain can pair with either \( \alpha 4 (\alpha 4\beta 7) \) or \( \alpha E/CD103 \) integrin (\( \alpha E\beta 7 \)) chains. Therefore, isolated analysis of individual \( \alpha 4 \) or \( \beta 7 \) integrin chains might not necessarily reflect the surface level of the \( \alpha 4\beta 7 \) heterodimer. The same consideration should be kept in mind when analyzing mRNA for individual integrin chains. Of note, the levels of \( \alpha 4 \) integrin chain (mRNA and surface protein) seem to correlate better with the levels of \( \alpha 4\beta 7 \) as compared to other integrin chains, at least for CD8 T cells (52). Since CD4 T cells do not normally express \( \alpha E\beta 7 \), the \( \beta 7 \) chain can be more reliably used in this case as a surrogate for \( \alpha 4\beta 7 \). Finally, a mAb is available to specifically label \( \alpha 4\beta 7 \) heterodimer in the mouse (DATK32 clone) (53). Another clone exists for labeling human \( \alpha 4\beta 7 \) (Act-1) (54), although it is currently not commercially available.

12. The binding of E- and P-selectin to E- and P-Lig on T cells is calcium dependent. Therefore, media with a physiological concentration of \( Ca^{++} \) should be used at all times (including FACS acquisition) when labeling with these selectin chimeras. DMEM is a good choice and can be supplemented with 1% FBS + 20 mM Hepes. Also, since the binding of selectins to their ligands has a much lower affinity as compared to antibodies, all pipetting and manipulations should be done gently. As negative controls, staining with E- and P-selectin chimeras can be done in a buffer with 5 mM EDTA (\( Ca^{++} \) chelator).

13. When using a chemokine for the first time in a given cell type it is recommended to perform a dose–response using 4–5 different chemokine concentrations (many chemokines exhibit a bell-shaped dose–response curve).

14. If media diffuses immediately to the upper chamber it could mean that the transwell is damaged and should be replaced.

15. Include additional wells with 500 \( \mu l \) media plus 100 \( \mu l \) of cells (directly added to the lower well, without transwells) in order to have a maximum total number of cells and then calculate percentages of migration with respect to total cells.

16. The optimal final incubation period will depend on the cell type, pore diameter, chemokine, etc. Nonetheless, try to use the same incubation times among different experiments in order to obtain more reproducible results.

17. Usually 30 s of FACS acquisition per sample should be enough. Acquire only the cells that are in a gate of viable cells. An aliquot of fluorescent beads can be added to each well in order to normalize the cell counting (optative). In addition, the migrated T cells can be stained for different markers (optative).
18. Although chemotaxis is a convenient standard readout for chemokine functionality, it does not necessarily assess the actual physiological function of a given chemokine receptor. For instance, very high concentrations of CCL25/TECK (200–300 nM) are needed in order to detect CCR9 functionality with this assay (in comparison, peak responses to CCL21/SLC are obtained at 10 nM). However, the physiological concentrations of CCL25/TECK needed to signal via CCR9 and activate α4β7 in order to trigger T-cell arrest on endothelial cells might be much lower. In fact, clear calcium flux responses can be obtained at 1–10 nM CCL25/TECK (55), which is significantly lower than what is needed to trigger chemotaxis (200–300 nM). In order to test integrin activation by chemokines ex vivo, a better and more physiological assay is the flow chamber (for a detailed description on this technique, see Ref. (56)). Finally, the in vivo physiological roles of a chemokine–chemokine receptor pair (or any given adhesion receptor) should be assessed by performing homing assays and/or intravital microscopy.

19. Ideally T cells and recipient mice should be matched by sex. Nonetheless, cells can be adoptively transferred from male into male or from female into male, but not from male into female (male cells are rejected in female hosts).

20. Oral gastric gavage will mostly target gut-associated DC and will lead to a preferential activation of T cells in PP and draining MLN. By contrast, i.p. injection will induce T-cell activation in all SLO, including PP, MLN, PLN, and spleen.

21. Spleen should be analyzed first, as this is a tissue where T cells can be easily detected by FACS. Analysis of the spleen will also give a clear idea regarding the efficiency of T-cell activation (CFSE dilution) and additionally can be used to set the gating strategy and FACS compensations.

22. Homing experiments provide valuable information about the migration of total cell populations in any given tissue. The main advantages of homing experiments are that they are relatively fast to set up, they can be done competitively by co-injecting 2 differentially labeled T-cell populations, and they allow for the simultaneous examination of multiple tissues/organs and cell populations by FACS. However, it should be kept in mind that homing experiments do not directly assess endothelial adhesion and therefore do not discriminate in which step(s) of the multistep adhesion cascade (tethering/rolling, activation, or sticking) a given homing receptor is acting. The gold standard to define the specific role of a homing receptor in the adhesion cascade is intravital microscopy (IVM), in which individual fluorescently labeled T cells (or other cells, or even fluorescently labeled beads) are directly observed interacting
with endothelial cells in real time. IVM is a powerful technique that has greatly contributed to define the precise step where a homing receptor is acting in the adhesion cascade. Moreover, IVM can additionally provide information about other valuable parameters, such as lymphocyte velocity, vessel diameter, and the particular venules/vessels involved in lymphocyte adhesion in a given tissue. However, IVM is time consuming, involves mouse anesthesia and laborious surgical procedures and requires a large number of pure and homogeneous cell populations to label and inject. Moreover, some tissues, such as the central nervous system and the thymus, are difficult to access for IVM. Finally, IVM preparations require tissue immobilization that is difficult to attain in some organs/tissues, such as liver, lungs, and intestine. In fact, few studies have been done using IVM in the gut (57). For a detailed discussion on IVM techniques, see ref. (58). In summary, homing experiments and IVM provide unique and often complementary information about the role of specific homing receptors in tissue-specific cell migration in vivo.

23. In order to control for unwanted effects of the fluorescent labeling on lymphocyte migration (this is especially important for B cells (59), the labeling should be swapped among different experiments or among different injected mice (e.g., cells labeled with CFSE should be labeled with CMTMR in the next experiment and vice versa).

24. When labeling the two T-cell populations with CFSE and CMTMR, the differentially labeled cells should be mixed only immediately before i.v. injection (using 200 μl PBS). This will help to prevent label transfer among T cells and T-cell clumping.

25. It should be kept in mind that results obtained after longer time points might be influenced not only by T cell entry, but also by eventual differences in T-cell exit and, sometimes, by differential T-cell proliferation and/or death.

26. The mice can be warmed with an infrared lamp 5–10 min before the adoptive transfer to dilate the tail vein and make i.v. injection easier.

27. Mice should not be injected with more than $50 \times 10^6$ total cells (utilize a lower number if T cells are recently activated blasts), as it increases the likelihood of embolism, respiratory distress, and death of the animal.

28. Due to the high fluorescence intensity of CFSE and CMTMR sometimes it is hard to compensate the FL1-H and FL2-H channels properly. However, rather than perfect compensations, one should aim to clearly distinguish both T-cell populations in order to calculate their ratio.
29. Blood should also be obtained in order to determine whether both T-cell populations are present in the circulation or whether one T-cell population is decreased with respect to the other (e.g., preferential trapping of one T-cell population in lungs/liver or decreased viability might affect the homing index [HI] in blood). This might be a problem when comparing naïve or resting memory T cells vs. recently activated T cells, since the latter might be trapped to a greater extent in the lungs. If the HI in blood is significantly different from 1, one can normalize HI in tissues by the HI in blood. Blood can be obtained via cardiac puncture from mice anesthetized (with averitin or isoflurane prior to euthanasia) and should be lysed twice with ACK buffer before using for FACS staining.

30. The main advantage of expressing the data as HI is that it makes the results independent of the numbers of T cells injected/collected from every tissue or from the size of the animals. However, it does not provide information about the actual magnitude of T-cell migration into each tissue. In fact, in tissues with very poor T-cell migration and in which very few events can be detected by FACS (such as in the non-inflamed colon), the HI calculation can be very misleading. As a general rule, HI should be used only when a sufficient number of events can be detected by FACS, so that at least one of the labeled T-cell populations can be clearly identified in any given tissue. In fact, HI should be ideally combined with absolute counting and calculations of the numbers of T cells migrating to a given tissue with respect to the total number of transferred T cells (e.g., usually expressed as number of T cells in a tissue per $10^6$ transferred T cells). It should be kept in mind that determining absolute numbers of T cells in each tissue is time consuming, as it requires meticulous lymphocyte isolation and precise cell counting in each tissue analyzed. It also relies on careful i.v. injection technique to assure that most T cells are actually injected into the mice.

**Acknowledgments**

We thank Susan Davis and Allison McNulty for editorial assistance. JRM is grateful to Ingrid Ramos for constant support. EJV was supported by a grant from Crohn’s & Colitis Foundation of America (CCFA). MR was supported by Fondecyt grant 1100448. MRB was supported by Fondecyt grant 1100557. JRM was supported by grants from CCFA, Cancer Research Institute (CRI), Howard H. Goodman (MGH), Massachusetts Life Science Center (MLSC) and NIH Director’s New Innovator Award DP2 2009A054301.
References


