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*J Immunol* 2008; 181:7681-7688; doi: 10.4049/jimmunol.181.11.7681
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Dynamic Modulation of CCR7 Expression and Function on Naive T Lymphocytes In Vivo

Mirjam R. Britschgi, Alexander Link, Tonje Katrine A. Lissandrin, and Sanjiv A. Luther

The chemokine receptor CCR7 is critical for the recirculation of naive T cells. It is required for T cell entry into secondary lymphoid organs (SLO) and for T cell motility and retention within these organs. How CCR7 activity is regulated during these processes in vivo is poorly understood. Here we show strong modulation of CCR7 surface expression and occupancy by the two CCR7 ligands, both in vitro and in vivo. In contrast to blood, T cells in SLO had most surface CCR7 occupied with CCL19, presumably leading to continuous signaling and cell motility. Both ligands triggered CCR7 internalization in vivo as shown in Ccl19−/− and plt/plt mice. Importantly, CCR7 occupancy and down-regulation led to strongly impaired chemotactic responses, an effect reversible by CCR7 resensitization. Therefore, during their recirculation, T cells cycle between states of free CCR7 with high ligand sensitivity in blood and occupied CCR7 associated with continual signaling and reduced ligand sensitivity within SLO. We propose that these two states of CCR7 are important to allow the various functions CCR7 plays in T cell recirculation. The Journal of Immunology, 2008, 181: 7681–7688.

Naive T lymphocytes continually patrol the body in search of Ags. They use blood and lymph to travel between secondary lymphoid organs (SLO), such as lymph nodes (LN) and spleen. Within SLO, T cells spend several hours scanning the Ags presented to them by dendritic cells (DCs). The recirculation of T lymphocytes is guided by various receptors recognizing chemokines, adhesion molecules, or sphingolipids (1, 2). In this study, we focus on the chemokine receptor CCR7 that plays a key role in the entry of T lymphocytes into SLO, in their migration within, as well as in their exit from these organs (2–4).

CCR7 is highly expressed on naive T cells and mature DCs while its two ligands, CCL19 and CCL21, are constitutively expressed by T zone reticular cells (TRC) within SLO. In addition, CCL21 is expressed by high endothelial venules (HEV) and lymphatic vessels (2, 3, 5). The importance of CCR7 and its ligands in vivo has been demonstrated in Ccr7−/− mice and in “paucity of lymph node T cell” (plt/plt) mice that lack the Ccl19 and Ccl21 genes expressed in lymphoid organs (6–10). Both mice have severe defects in T cell and DC migration and positioning as well as immune response and tolerance induction (2, 3, 6, 7, 11–13). This is thought to enhance their chances of encountering DCs attached to TRC and to lead to more efficient T cell priming (22, 23).

Several differences in expression and function have been described for CCL19 and CCL21. TRC in LN and spleen produce ~10 times more transcripts and 100 times more protein of CCL21 compared with CCL19 (5, 24). CCL19 binds with slightly higher affinity than CCL21 to human CCR7 (25–27). Although both ligands induce Ca²⁺ mobilization, chemotaxis, and integrin-mediated adhesion, CCL19 appears to be more potent at low concentrations (25, 28–35). Sequential stimulation with the two ligands led to cross-desensitization of CCR7, with CCL19 being more effective than CCL21 (25, 32, 33, 35, 36). Incubation of activated human peripheral blood lymphocytes or CCR7-transfected cells with CCL19 induced CCR7 internalization whereas CCL21 had a much weaker effect (35, 37, 38). After internalization, CCL19 was degraded and CCR7 recycled back to the plasma membrane (38).

We propose that these two states of CCR7 are important to allow the various functions CCR7 plays in T cell recirculation. The Journal of Immunology, 2008, 181: 7681–7688.

In this study we investigated CCR7 expression and function on naive murine T cells both in vitro and in vivo. We show that both CCR7 ligands regulate receptor occupancy, internalization and reexpression at the cell surface. As a consequence, T cells within LN are much less responsive to CCR7 ligands than T cells in blood where little ligand is present.

**Materials and Methods**

**Mice**

C57BL/6 mice were obtained from Janvier. Ccl19−/− (5) and plt/plt (6) mice were backcrossed 12 and Ccr7−/− mice (7) were backcrossed 9 generations, onto C57BL/6 background. All mice were maintained in pathogen-free conditions and were age- and sex-matched for experiments. All mouse experiments were authorized by the Swiss Federal Veterinary Office.
MFI of stainings. The anti-CCR7 or CCL19-Fc. Depicted are histograms and bar plots showing were incubated for 1 h at 37°C, cooled on ice for 15 min, and then incubated change much anti-CCR7 or CCL19-Fc staining, as tested in a control experiment where LN, spleen, and blood were RBC lysed using the same conditions. Cells were kept in complete DMEM containing 5% FBS.

**FIGURE 1.** Flow cytometric analysis of naive (CD62Lhigh) CD4⁺ T lymphocytes from spleen. The anti-CCR7 Ab recognizes all surface CCR7 and CCL19-Fc recognizes CCR7 free of bound CCL19. A. Splenocytes from wild-type (WT) and Ccr7⁻/⁻ mice were stained on ice with anti-CCR7, CCL19-Fc or isotype controls. Stainings with isotype controls on wild-type and Ccr7⁻/⁻ splenocytes were identical (data not shown). Depicted are histograms and bar plots showing MFI of stainings. Data are representative of over 10 wild-type and two Ccr7⁻/⁻ mice. B. Splenocytes were incubated for 1 h at 37°C, cooled on ice for 15 min, and then incubated with 30 min on ice with different chemokines before staining with anti-CCR7 or CCL19-Fc. Depicted are histograms and bar plots showing MFI of stainings. The 1 µg/ml of the irrelevant chemokine CXCL12 did not influence the anti-CCR7 staining or the CCL19-Fc binding (data not shown). Data are representative of three independent experiments.

**Flow cytometry**

Stainings were performed as described previously (5) for 25 min per incubation step, with the exception that cells were blocked with 2% normal mouse serum. Cells were stained with anti-CCR7 (4B12; a gift from J. Zwirner, Georg August University, Goettingen, Germany; on the online datasheet, eBioscience reports that the anti-CCR7 staining quality is improved by staining at 37°C; in this study stainings were done at 4°C to avoid CCR7 modulation by experimental procedures) or the isotype control anti-keyhole limpet hemocyanin (BioLegend) followed by PE-coupled donkey anti-rat IgG (Jackson ImmunoResearch Laboratories). Before other Abs were added, a blocking step with 4% normal rat serum was performed.

Murine CCL19-Fc (40) and the control human Fn14-Fc (a gift from P. Schneider, University of Lausanne, Epalinges, Switzerland) were both fusion proteins with a human IgG1 Fc portion. The fusion proteins (8 µg/ml) were added to the cells and detected using a biotinylated goat anti-human IgG (Jackson ImmunoResearch Laboratories) that had been pretreated for 30 min with 4% normal mouse serum and normal rat serum. Finally, streptavidin-PE (eBioscience) was added, along with other surface markers. Other Abs used were anti-CD62L-FITC (eBioscience), anti-CD4-Alexa647 (BioLegend), anti-CD8α-Alexa647 (53–67), and anti-CD8α-PE (BioLegend). Data were acquired on a FACSDuo flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**Chemotaxis assay**

Murine CCL19, CCL21, and CXCL12 were obtained from PeproTech. Migration of splenic lymphocytes was assessed in Transwell plates (96 wells, 5-µm pores, ChemoTx 101-5; NeuroProbe) according to the manufacturer’s protocol and using DMEM + Glutamax medium containing 0.5% fatty acid-free BSA (CultiBiochem) and 10 mM HEPES. Chemokine dilutions were added to the plate and incubated at 37°C for at least 15 min before loading 250,000 cells per well. Migration assays were run at 37°C with 5% CO₂. To measure the number of migrated cells, two wells were
pooled, stained for flow cytometry, and cell numbers counted with a FACS-Canto at high flow (for 30 s; normalized using a titration of cells).

Adaptive cell transfers

Lymphocytes (25 × 10^6) from spleen and LN of wild-type mice were labeled with 20 μM CFSE (Molecular Probes) and transferred into recipient mice by i.v. injection. Two hours or 2 days later, cells isolated from LN were stained for flow cytometry, and the levels of CCR7 and CCL19-Fc were analyzed on transferred CFSE+ and endogenous CFSE+ cells.

Statistical analysis

Results of experimental points are reported as means ± SD. Statistical significance was determined using an unpaired two-tailed Student’s t test for unequal variance.

Results

CCL19-Fc binding measures CCR7 occupancy by CCL19 but not CCL21

To investigate CCR7 surface expression on naive CD4+ T cells, a monoclonal anti-CCR7 Ab and a CCL19-Fc fusion protein were used. Because our goal was to get accurate measures of in vivo CCR7 expression, cells were not resensitized by incubation at 37°C, a process known to increase the staining intensity. Both reagents labeled CCR7 on wild-type CD4+ T cells but did not stain Ccr7−/− cells, confirming that both reagents were CCR7 specific (Fig. 1A). Because CCL19-Fc but not anti-CCR7 binds to CCR7 via the ligand-binding site, we investigated the influence of bound ligand on the staining intensity. To this end, naive CD4+ T cells were incubated with either CCL19 or CCL21 on ice (to minimize CCR7 internalization), washed, and then labeled with anti-CCR7 or CCL19-Fc. Doses of CCL19 (0.01 μg/ml) and CCL21 (1 μg/ml) thought to be physiological (24) did not influence the anti-CCR7 staining (Fig. 1B); however, a superphysiological CCL19 dose (1 μg/ml) led to a slight reduction in CCR7 staining. As expected, CCL19 bound to CCR7 diminished CCL19-Fc staining in a dose-dependent manner. Surprisingly, the presence of a high dose of CCL21 did not reduce CCL19-Fc binding. In conclusion, CCR7 Abs can be used to get an estimate of total CCR7 surface levels, while CCL19-Fc labels CCR7 that has no CCL19 bond and is therefore an indirect readout of CCR7 occupancy by CCL19.

CCR7 occupancy is high on naive CD4+ T cells from SLO

During recirculation, lymphocytes are thought to encounter CCR7 ligands in SLO but not in blood or efferent lymph. To see how

FIGURE 3. Free CCR7 correlates with increased migratory capacity of T lymphocytes. A and B. MFI values of CCR7 or CCL19-Fc stainings on naive (CD62L+CD4+) T lymphocytes isolated from the indicated organs and incubated at 37°C for various lengths of time before staining (n = 3). CCR7 data for blood cells after 180 min incubation were not obtained. Data are representative of three independent experiments. As a control, IL7Rα levels were determined which remained constant over the entire incubation period (data not shown). C, Transwell migration of naive (CD62L+CD4+) T cells toward the indicated concentrations (μg/ml) of CCL19 and CCL21. The cells were either freshly isolated from spleen (and warmed up to 37°C for 5 min) or resensitized for 1 h at 37°C (n = 2). The time for the transwell migration assay was only 45 min to counter the rapid adjustment of cells to the environment. Due to the low efficiency in migration, background migration levels toward medium were subtracted. Data are representative of two independent experiments with two to three data points per condition.

FIGURE 4. CCL19 and CCL21 rapidly occupy CCR7 and lead to CCR7 internalization and desensitization. A and B, Flow cytometric analysis of naive (CD62L+CD4+) T lymphocytes isolated from spleen, incubated at 37°C for 1 h and then incubated for different lengths of time with the indicated amounts of CCL19 or CCL21 before staining with anti-CCR7 (A) or CCL19-Fc (B). MFI values were normalized to the staining intensity observed on cells not incubated with CCR7-ligands (0 min = 100%) (n = 1). Data are representative of 2 independent experiments. C and D, Transwell migration assay of naive CD4+ T lymphocytes from spleen. Cells were incubated for 1 h at 37°C, then for 10 min with 1 μg/ml CCL19 or CCL21 at 37°C before washing and performing a 45 min transwell migration assay toward CCL19 or CCL21 at a high (C) or low (D) concentration (n = 3–4). The number of migrated cells was normalized to the migration of control cells that had not been pretreated with chemokines. Pretreatment with 1 μg/ml CXCL12 did not influence migration (data not shown). Data are representative of two to three (C) or one (D) independent experiments with three to four data points per condition. *, p < 0.05; **, p < 0.01.
CCR7 reacts to these different conditions, we analyzed CCR7 on lymphocytes isolated from LN, spleen, and blood. Both the levels of total (anti-CCR7) and “free” CCR7 (CCL19-Fc; CCL19-free) were lowest on LN cells, intermediate on spleen cells, and highest on blood cells (Fig. 2, A and B). The staining levels in each tissue were homogeneous except in the spleen, where 10–15% of CD4+ T cells bound higher amounts of CCL19-Fc (Fig. 2B). Strikingly, the anti-CCR7 stain in blood was only 30% higher than in LN, although CCL19-Fc staining was 400% higher (Fig. 2, A and B). The CCR7 dependence of both stainings was confirmed using Ccr7−/− cells and argues against the possibility that CCL19-Fc bound to other molecules present on the T cell surface (Fig. 2C). These findings suggest that a part of surface CCR7 is internalized in CD4+ T cells in LN and becomes re-expressed in cells found in blood. An even larger part of CCR7 on LN T cells is occupied with CCL19 and probably becomes liberated on cells found in blood.

**CCR7 occupancy determines the potency of the chemotactic response**

To test the hypothesis that entering into a ligand-free environment liberates CCR7 and allows its re-expression on the cell surface, CD4+ T lymphocytes from SLO and blood were isolated and incubated at 37°C for different lengths of time before staining with anti-CCR7 or CCL19-Fc. Incubation of LN CD4+ T cells led to the up-regulation of both total CCR7 (Fig. 3A) and free CCR7 (Fig. 3B). Spleen levels of both markers were reached after 15 min and blood levels after 1 h incubation. Over the entire incubation period, the mean fluorescent index (MFI) of CCR7 doubled and CCL19-Fc binding increased 10-fold. All anti-CCR7 and CCL19-Fc staining on resensitized CD4+ T cells was CCR7-specific (data not shown). These data corroborate with our in vivo results, suggesting that the absence of CCR7 ligands leads to more total and ligand-free surface CCR7. This appears to occur by re-expression of internalized receptor and to an even greater extent by dissociation of receptor-ligand complexes at the cell surface.

To address the question whether different amounts of free CCR7 lead to differences in migratory responses, the chemotaxis of freshly isolated CD4+ T cells was compared with resensitized CD4+ T cells. Resensitization of splenocytes was performed during 1 h to reach a CCR7 phenotype comparable to blood lymphocytes with ∼5 times more free CCR7 on the cell surface than freshly isolated splenocytes (Fig. 3B). Resensitized CD4+ T cells migrated much better than freshly isolated cells to both CCR7 ligand complexes at the cell surface.

**Both CCL19 and CCL21 can modulate CCR7 function**

Given the rapid liberation and re-expression of CCR7 when cells enter into a chemokine-free environment, we predict the opposite scenario of rapid CCR7 occupation and internalization when T cells transit from blood into the T zone of SLO. To try to mimic this situation in vitro and test the capacity of the two CCR7 ligands to induce these processes, resensitized splenic CD4+ T cells were
incubated with either ligand at 37°C for various lengths of time. Then CCR7 surface expression and occupancy were determined. Within minutes, both CCL19 and CCL21 led to a strong decrease in CCR7 surface expression on CD4+ T cells (Fig. 4A). CCL19 was more potent than CCL21 in inducing CCR7 internalization consistent with earlier descriptions of human T cells (35, 38). As expected, the amount of free CCR7 decreased even more rapidly and potently upon addition of CCL19. The CCL19-Fc staining on CD4+ T cells decreased 80–90% within 3 min of CCL19 addition (Fig. 4B) and is a combined effect of receptor occupancy and internalization. Addition of CCL21 also induced a reduction in CCL19-Fc binding on T cells. As CCL19-Fc can still bind to CCR7 in the presence of CCL21, this reduction is largely due to CCR7 internalization. Also in this setting, the changes in anti-CCR7 and CCL19-Fc staining were CCR7 specific (data not shown).

The increased capacity of CCL19 to cause CCR7 internalization might lead to more pronounced CCR7 desensitization than with CCL21. Evidence for such differences have previously been reported, mainly using human CCR7 ligands (25, 32, 33, 35, 36, 39). To test this hypothesis using murine ligands, resensitized CD4+ T cells were incubated for 10 min at 37°C with either CCL19 or CCL21 and then tested for their migration potential toward another source of CCR7 ligands. Pretreated T cells migrated far less efficiently toward high doses of CCL19 or CCL21 than untreated cells (Fig. 4C). Typically, CCL19 induced a slightly stronger receptor desensitization than CCL21. When pretreated cells were tested in a migration assay toward a low dose of CCL19, migration was even more impaired, especially in the case of CCL19 pretreatment (Fig. 4D). In summary, pretreatment leads to a loss of sensitivity toward CCR7 ligands. However, under all conditions tested, desensitized CD4+ T cells kept some responsiveness to high concentrations of CCL7 ligands.

CCR7 surface expression and occupancy are fine-tuned by the amount of CCL19 and CCL21 present in the in vivo environment

It is unclear whether both CCL19 and CCL21 contribute to the CCR7 internalization observed in CD4+ T cells in SLO. To address this question, CCR7 levels and CCL19-Fc binding were measured on naive CD4+ T cells isolated from SLO of wild-type, Ccl19−/−, and plt/plt mice. CCR7 surface levels on cells from Ccl19−/− and from plt/plt mice were 40 and 200% increased, respectively, compared with cells from wild-type mice (Fig. 5A and B), suggesting that both ligands participate in CCR7 internalization in vivo. A 4-fold increase in CCL19-Fc staining was observed for CD4+ T cells from Ccl19−/− relative to wild-type LN, and cells from plt/plt LN showed a 15-fold increase (Fig. 5, A and B). This correlates with higher total CCR7 levels on T cells in these mice and the increased availability of CCR7 for CCL19-Fc binding. CCL19-Fc staining was intermediate on CD4+ T cells from Ccl19−/− relative to wild-type and Ccl19−/− LN (data not shown), suggesting that CCR7 surface and occupancy levels reflect fairly precisely the amount of chemokine present in the environment.

To rule out a developmental defect leading to the high CCR7 expression on T cells from plt/plt mice, wild-type splenocytes were transferred into plt/plt mice. Two days after transfer, CCR7 expression and occupancy on transferred CD4+ T cells within LN had completely adapted to the high level of endogenous cells (Fig. 5C). Transfer of wild-type cells into wild-type or Ccl19−/− mice led to a lowered staining level, which was indistinguishable between transferred and endogenous cells. This indicates that environmental rather than developmental signals determine CCR7 expression levels. Already 2 h after transfer, CCR7 levels had adjusted to the chemokine-deficient environment (Fig. 5D). In contrast, CCL19-Fc binding studies suggested that wild-type T cells transferred into plt/plt mice retained some CCL19 bound to their CCR7 receptors (Fig. 5E).

**FIGURE 6.** Reduced migration of CD8+ T cells toward CCR7 ligands. A–C, Flow cytometric analysis of anti-CCR7, CCL19-Fc, or isotype control staining of naive (CD62Lhigh) CD8+ T lymphocytes isolated on ice from LN, spleen, and blood of wild-type mice (A); LN and blood of wild-type and Ccr7−/− mice (B); LN of wild-type, Ccl19−/−, and plt/plt mice (C). D, Three hours transwell migration assay of naive T cells toward various concentrations of CCL19 and CCL21. Cells isolated from wild-type spleen were incubated 1 h at 37°C before migration. Data are representative of two independent experiments with three data points per condition. *, p < 0.05; ***, p < 0.001.

**Increased CCR7 level on plt/plt T cells conveys higher migratory capacity**

To test whether the increased level of surface and free CCR7 on CD4+ T cells isolated from chemokine-deficient mice had a functional consequence, their chemotactic capacity was assessed. Indeed, at all chemokine concentrations tested, splenic CD4+ T cells
from plt/plt mice migrated far better than those from Ccl19−/− mice, which in turn migrated better than those from wild-type mice (Fig. 5E). These results further support the notion that CCR7 function is critically regulated by the amount of CCR7 expressed at the cell surface and its ligand accessibility.

**Reduced CCR7 levels and migratory response of CD8+ compared with CD4+ T cells**

The results reported so far have focused on CD4+ T cells. However, CCR7 expression and function was also assessed for naive CD8+ T cells. Total CCR7 levels were lower on CD8+ than CD4+ T cells, while still being well above background levels of CCR7-deficient cells (Fig. 6, A and B). CCR7 surface levels were indistinguishable between CD8+ T cells from SLO and blood of wild-type mice (Fig. 6A). Even CD8+ T cells from wild-type and plt/plt LN showed only small differences (Fig. 6C). The low capacity of CCR7 to be modulated on CD8+ T cells was reproduced in re-sensitization or desensitization assays in vitro (data not shown). In contrast to the total CCR7 levels, the amount of free CCR7 on CD8+ T cells was modulated between SLO and blood of wild-type mice (Fig. 6A), indicating that most CCR7 on CD8+ T cells in SLO are occupied with CCL19, similar to CD4+ T cells. This notion is supported by the finding of increased CCL19-Fc staining on CD8+ T cells from Ccl19−/− or plt/plt compared with wild-type LN (Fig. 6C).

To test whether the difference in CCR7 levels and modulation between CD8+ and CD4+ T cells had a functional consequence, we compared the two T cell subsets in a chemotaxis assay. Both were equally potent in their migratory response to high CCL19 concentrations (Fig. 6D). However, at low CCL19 and all CCL21 concentrations tested, CD8+ T cells migrated significantly less than CD4+ T cells, indicating that the lower CCR7 expression level and/or the reduced modulation was associated with a decreased capacity to sense chemokine gradients.

**Discussion**

In this study, we showed that CCR7 occupancy and internalization are processes occurring continuously in naive T cells in vivo. They are observed in LN and spleen, where both CCL19 and CCL21 are abundantly present and directly affect the capacity of T cells to respond to new sources of these ligands.

To measure CCR7 expression, we relied on two widely used reagents, which so far have been used interchangeably. We confirm that anti-CCR7 Ab binding to CCR7 is not influenced by CCL21 (41) or physiological doses of CCL19. The anti-CCR7 Ab can therefore be used to assess total CCR7 surface expression.

The recognition of CCR7 by CCL19-Fc is inhibited by bound CCL19. Although previous studies indicate overlapping binding sites for the human CCR7 ligands (25, 36), murine CCL21 did not interfere with the binding of the murine CCL19-Fc-fusion. This may be because the higher affinity of CCL19 compared with CCL21 for CCR7 (25–27), allows CCL19-Fc to displace bound CCL21. CCL19-Fc is therefore a readout of CCR7 occupancy by CCL19 and provides information different from the anti-CCR7 staining.

Total CCR7 levels and levels of free CCR7 were higher on CD4+ T cells from blood compared with SLO, both in wild-type and Ccl19−/− tissues. This higher CCL19-Fc staining level was due to a combination of higher surface CCR7 expression and decreased CCR7 occupancy. Importantly, it correlated with a higher capacity of “blood-phenotype” cells to sense chemotactic gradients. These results suggest that the level of free CCR7 and therefore responsiveness is regulated both at the level of receptor internalization/re-expression and occupancy. The higher dynamic range of CCL19-Fc vs total CCR7 staining suggests a more important role for receptor occupancy than internalization in modulating CCR7 responses.

CCL19-Fc staining allowed us to establish a hierarchy of CCR7 occupancy in vivo. In blood, most CCR7 is ligand-free, but in SLO most is occupied. Interestingly, CCR7 occupancy is even higher in LN than in spleen, in accordance with our previous observation that CCL19 expression is 2-fold higher in LN than in spleen. Relative to CCL21, CCL19 protein levels are more than 100-fold lower in both tissues and hard to detect (24). In this light, the high CCR7 occupancy by CCL19 comes as a surprise, as it suggests that T cells migrating within T zones of SLO have continual access to CCL19.

CCR7 occupancy by CCL19 was strikingly homogeneous for the whole T cell pool within LN. In contrast, splenic T cells could be divided into two populations with distinct CCR7 occupancy. Although most splenic T cells displayed an intermediate CCR7 occupancy by CCL19, 10–15% of cells had as much free CCR7 as cells isolated from blood, indicating that they reside in an environment with little or no CCL19. Recirculating T cells enter the spleen via open arteries, flushing them into the marginal zone of the white pulp cords. From there they migrate into the T zone of the white pulp. To exit the spleen, T cells need to access venous blood vessels found in the red pulp (2). Based on CCL19 mRNA analysis, both the marginal zone and the red pulp are regions lacking detectable CCL19 expression (31). Therefore, the splenic T cells with lots of free CCR7 are likely to comprise arriving T cells localizing to the marginal zone and exiting T cells that have left the white pulp at least 20 to 30 min ago.

The homogeneously high CCR7 occupancy on LN T cells suggests that upon entry into the chemokine-rich environment of the T zone, most CCR7 on T cells is rapidly saturated by CCL19, and possibly CCL21. Therefore, CCR7 occupancy appears to be independent of the amount of time a T cell has spent in the T zone or its precise localization. If a T cell would move up a steep CCL19 gradient within the T zone this should have been detected at the level of CCR7 occupancy. Consistent with the absence of striking chemokine gradients within T zones is the uniform distribution previously reported for CCL19 and CCL21 transcripts and CCL21 protein (5, 9, 17, 29–31, 42). Further support for this concept comes from intravital imaging experiments showing that naive T cells display continuous and non-directional migration along the TRC network all over the T zone (22, 23, 43). Interestingly, part of this motility is dependent on CCR7, implying that T cells receive continuous CCR7 signals (14–21). Here we provide direct evidence that, in SLO, most CCR7 on a given T cell is continually occupied by CCL19, and presumably by CCL21, thereby supporting T cell motility.

The notion of continuous CCR7 signaling raises the question of whether T cells within SLO maintain responsiveness toward CCR7 ligands by continuously internalizing and recycling receptor. Receptor internalization upon ligand binding has been demonstrated in vitro for human CCR7 as well as for several other chemokine receptors (35, 37, 38). We demonstrate that CCR7 internalization indeed also occurs in vivo. CCR7 on CD4+ T cells from LN and spleen was down-regulated 35% and 10%, respectively, when compared with blood. The moderate level of internalized CCR7 observed for T cells ex vivo may be a reflection of the fast kinetics of receptor internalization and re-expression preventing complete CCR7 desensitization.

Down-regulation and desensitization of human CCR7 was much more pronounced upon CCL19 than CCL21 binding (35, 38). Both ligands showed a similar capacity to induce the internalization of murine CCR7 when taking into account the 100-fold difference in
expression of CCL19 and CCL21 protein within SLO (24). The in vivo role of CCL19 in CCR7 down-regulation was indicated by the 40% increase in CCR7 expression on T cells from CCL19-deficient tissues. The precise role of the much more abundant CCL21 in CCR7 modulation could not be addressed as Ccl21<sup>−/−</sup> mice have not been reported. However, we obtained some evidence that CCL21 may contribute to this process in vivo as CD4<sup>+</sup> T cells transferred into plt/plt mice increased their CCR7 expression much more strongly than cells transferred into <em>Ccl19</em><sup>−/−</sup> mice. This CCR7 expression level is considerably higher than on T cells from wild-type blood or LN T cells resensitized in vitro for 3 h, suggesting that de novo production of CCR7 might contribute. Importantly, the 3-fold higher CCR7 level on CD4<sup>+</sup> T cells from plt/plt relative to wild-type correlated with a much higher chemotactic response. A role for CCL21 in modulating CCR7 expression and function is also supported by data from transgenic studies where superphysiological CCL21 concentrations led to reduced CCR7 levels on T cells and consequently reduced responsiveness (44, 45).

CD4<sup>+</sup> T cells isolated from SLO showed a striking reduction in their capacity to migrate toward CCR7 ligands, similar to T cells preincubated with CCR7 ligands. Incubation at 37°C for 1 h was sufficient to restore responsiveness. In fact, resensitization by incubation at 37°C is a common practice in laboratories working on tissue lymphocytes and empirically known to improve the efficiency of CCR7 staining and chemotaxis (29, 46). Here we provide mechanistic insight into this resensitization process. It appears to involve both re-expression of internalized and liberation of occupied CCR7, as CD4<sup>+</sup> T cells isolated from SLO and incubated in medium reach blood levels of free and total surface CCR7 within 30 min to 1 h. The kinetic is comparable to studies of CCR7 re-expression on human cells (35, 38). We provide evidence suggesting similar kinetics of CCR7 re-expression in vivo: 1) Upon entry into the blood circulation, CD4<sup>+</sup> T cells swiftly increased their CCR7 expression relative to cells in SLO and displayed homogeneously high CCR7 levels; and 2) CCR7 on adoptively transferred CD4<sup>+</sup> T cells rapidly adjusted to the environment, most drastically shown in the highly increased CCR7 expression on wild-type cells that had been transferred into plt/plt mice. Importantly, the re-expression of internalized CCR7 and liberation of occupied receptor were associated in both settings with a strongly increased migratory response. Therefore, we think that these processes are critical for the maintenance of CCL19/21 sensitivity and continuous cell motility within T zones.

Although CCR7 on human CD4<sup>+</sup> and CD8<sup>+</sup> T cells is similarly modulated by its ligands in vitro (35), we found that CCR7 surface expression on murine CD4<sup>+</sup> T cells is higher and more dynamic than on CD8<sup>+</sup> T cells. In contrast, CCL19-Fc staining on CD8<sup>+</sup> T cells varied considerably among tissues, similar to CD4<sup>+</sup> T cells. Therefore, CCR7 function on CD8<sup>+</sup> T cells seems to be regulated almost entirely at the level of occupancy. The lower CCR7 surface expression of CD8<sup>+</sup> vs CD4<sup>+</sup> T cells correlated with a lower sensitivity of the former cells in chemotaxis assays. These findings may provide mechanistic insight into similar differences observed by others in chemotaxis assays (29, 31, 36) and suggest that CCR7 internalization is not a stringent requirement for efficient T cell migration toward CCL19 and CCL21 in vivo.

In summary, we propose the following model for CCR7 regulation on recirculating CD4<sup>+</sup> T cells (Fig. 7). During recirculation, T cells pass through two environments that are fundamentally different in their chemokine concentration – no or little CCL19/21 in blood and splenic red pulp vs high levels of CCL19/21 in T zones of SLO – and T cells rapidly adjust to them. Within T zones, CCR7 is engaged by ligands, leading to continuous CCR7 signaling and chemokinetic cell migration. While migrating, CD4<sup>+</sup> T cells internalize and degrade CCL19 (and possibly CCL21). The internalized receptor returns to the cell surface to prevent complete desensitization. The lower CCR7 level on T cells in SLO may reduce cell retention and prepare them for the sphingosine-1-phosphate receptor 1-mediated exit (4). Once T cells have emigrated from SLO into the circulation, CCR7 signaling stops and most internalized receptors are re-expressed. This process may be critical for efficient exit from blood into LN, when T cells need to be at their highest sensitivity toward CCR7 ligands displayed on HEVs. The internalized receptor returns to the cell surface to prevent complete desensitization. The lower CCR7 level on T cells in SLO may reduce cell retention and prepare them for the sphingosine-1-phosphate receptor 1-mediated exit (4). Once T cells have emigrated from SLO into the circulation, CCR7 signaling stops and most internalized receptors are re-expressed. This process may be critical for efficient exit from blood into LN, when T cells need to be at their highest sensitivity toward CCR7 ligands displayed on HEVs.

**References**


**Acknowledgments**

We thank J. Cyster, B. Marsland, P. Schneider, and J. Zwirner for providing reagents; S. Favre and T. Vogt for technical help; J. Cyster, P. Schneider, and M. Sixt for critical reading of the manuscript; and all members of the Luther laboratory for discussions.

**Disclosures**

The authors have no financial conflict of interest.

FIGURE 7. Model proposing how CCR7 activity is regulated on CD4<sup>+</sup> T cells recirculating through SLO and blood. Blood CD4<sup>+</sup> T lymphocytes have high CCR7 surface levels and most of it is ligand-free. Consequently, they are highly sensitive for the CCL21 displayed by HEV and efficiently home into the LN by CCR7-dependent transendothelial migration. Once inside the LN, T cells crawl along the 3D-network of TRC throughout the T zone while continually receiving CCR7 signals that increase their motility. This motility is thought to improve the efficiency of encounters between naive T cells and Ag-presenting DCs bound to TRC. The CCR7 ligands are produced in large amounts by TRC and are likely to be associated with proteoglycans at the TRC surface where migrating T cells can easily pick them up. Once the ligand has bound to the receptor, this complex is eventually endocytosed, the ligand degraded and the receptor recycled back to the cell surface. The cycling of CCR7 prevents complete desensitization of the cell, but the decreased surface expression of CCR7 diminishes ligand sensitivity and increases the propensity to exit the LN. Once returned to the efferent lymph or the blood circulation where little CCL19/21 is found, all CCR7 recycles back to the cell surface. At this stage the T cell has regained the maximal sensitivity toward CCR7 ligands, allowing it to respond potently to CCL19/21 presented on HEV. The duration needed for full CCR7 resensitization may influence how long T cells stay inside the blood. In that sense the cyclical modulation of CCR7 activity appears to be opposite to sphingosine-1-phosphate receptor 1, that is not expressed on the surface of blood T cells but becomes gradually expressed on T cells during their stay in SLO (47).
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