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Cross-Talk Between TCR and CCR7 Signaling Sets a Temporal Threshold for Enhanced T Lymphocyte Migration

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Lymphocyte homing to, and motility within, lymph nodes is regulated by the chemokine receptor CCR7 and its two ligands CCL19 and CCL21. There, lymphocytes are exposed to a number of extracellular stimuli that influence cellular functions and determine the cell fate. In this study, we assessed the effect of TCR engagement on CCR7-mediated cell migration. We found that long-term TCR triggering of freshly isolated human T cells through CD3/CD28 attenuated CCR7-driven chemotaxis, whereas short-term activation significantly enhanced CCR7-mediated, but not CXCR4-mediated, migration efficiency. Short-term activation most prominently enhanced the migratory response of naive T cells of both CD4 and CD8 subsets. We identified distinct roles for Src family kinases in modulating CCR7-mediated T cell migration. We provide evidence that Fyn, together with Ca^{2+}-independent protein kinase C isoforms, kept the migratory response of naive T cells toward CCL21 at a low level. In nonactivated T cells, CCR7 triggering induced a Fyn-dependent phosphorylation of the inhibitory Tyr505 of Lck. Inhibiting Fyn in these nonactivated T cells prevented the negative regulation of Lck and facilitated high CCR7-driven T cell chemotaxis. Moreover, we found that the enhanced migration of short-term activated T cells was accompanied by a synergistic, Src-dependent activation of the adaptor molecule linker for activation of T cells. Collectively, we characterize a cross-talk between the TCR and CCR7 and provide mechanistic evidence that the activation status of T cells controls lymphocyte motility and sets a threshold for their migratory response. ***The Journal of Immunology*****, 2011, 187: 5645–5652.**

Directed migration of lymphocytes is a prerequisite to maintain the immune surveillance and to acquire an efficient immune response. Chemotaxis depends on local expression of diverse chemokines, which guide lymphocytes through the lymphatic system by activating the corresponding chemokine receptors (1). Under homeostatic conditions, naive T cells circulate continuously between the blood and lymphoid tissues, where they scan dendritic cells for their cognate Ag. T cell homing to lymph nodes is mainly enabled by the chemokine receptor CCR7 and its two ligands CCL19 and CCL21 (2). CCR7 belongs to the family of G-protein coupled, seven-transmembrane domain receptors and is expressed on thymocytes, naive lymphocytes, central memory T cells, regulatory T cells, NK cells, and mature dendritic cells (2). Triggering of CCR7 with one of its ligands induces the onset of various signaling cascades, leading to cell polarization and directional migration toward the ligand source. Both CCR7 ligands are constitutively expressed mainly by fibroblastic reticular cells distributed throughout the T cell zone of secondary lymphoid organs (3, 4). Although CCR7 ligands are also produced by human DCs, murine CCL21 is additionally produced in high endothelial venules (3, 5, 6). CCR7-deficient mice show strongly impaired lymphocyte homing accompanied by severe alterations in the architecture of lymphoid organs, manifested by a delayed induction of an adaptive immune response (7). In addition to its ability to recruit lymphocytes into lymphoid organs, CCR7 enables their intranodal motility (8, 9). Because the number of naive T cells specific for a given Ag is extremely low, naive T cells must scan large areas for the appropriate APC to permit the initiation of an effective immune response (10–12). Upon TCR engagement by its cognate peptide/MHC complex expressed on APCs, T cells become activated and start to proliferate, resulting in clonal expansion of Ag-specific T cells.

Before a T cell starts to proliferate, it enters a complex priming phase, in which the T cell consistently interacts for short intervals with several APCs and begins to upregulate early activation markers, such as CD69 (13). Within this initial priming phase, T cells stay highly motile. In a second step, the interaction of the APC with the cognate T cell becomes prolonged, leading to the production of IL-2 and IFN-γ (13). During this priming phase, naive T cells are exposed simultaneously to a number of different stimuli, including signals arising from chemokine receptors and the TCR, which together finally build up and tune a specific cellular response. Hitherto, only a few studies have investigated the interplay between chemokine receptor and TCR signaling. For instance, TCR triggering was demonstrated to downregulate the

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Abbreviations used in this article: BimI, bisindolylmaleimide I; LAT, linker for activation of T cells; PKC, protein kinase C isoform; SFK, Src family kinase; siRNA, small interfering RNA.

surface expression of CXCR4, leading to a reduced migratory response toward CXCL12 (14), and to attenuate CXCR3-induced phosphorylation of ZAP70 and linker for activation of T cells (LAT), resulting in reduced chemotaxis (15). These phenomena might contribute to the stop-and-go model in which TCR engagements during synapse formation deliver stop signals to chemokine receptors, facilitating prolonged T cell–APC interaction and efficient T cell activation (16). However, there is recent evidence that TCR stimulation acts via Rac1 to reduce the phosphorylation of ERM proteins required for cell polarization and chemokine-independent random motility (17). Moreover, the TCR-mediated stop signal can be reverted by CTLA-4 to enhance random motility (18). These two studies suggested that the proposed TCR stop signal does not directly interfere with chemokine receptor-mediated migration. In contrast, CXCR4 was also shown to directly interact with the TCR upon chemokine stimulation (19). The physical interaction between TCR and CXCR4 resulted in prolonged ERK-1/2 phosphorylation, strong transcriptional activity of AP-1, and enhanced cytokine secretion. Furthermore, CXCR4 was shown to use ZAP70 to enhance the migratory response of T cells (19–21). These findings are in line with in vivo observations demonstrating that tonic TCR signaling is required for normal naive CD4 T cell motility (22), as well as for a sustained active migration of tumor-infiltrating effector T cells through the tumor microenvironment (23).

In this study, we investigated the effect of TCR engagement through CD3/CD28 activation on CCR7-mediated cellular response of freshly isolated primary human T cells. We demonstrated that short-term activation of human naive T cells modified CCR7 signaling manifested by an enhanced chemotactic response toward low concentrations of the CCR7 ligands CCL19 and CCL21. We found that this enhanced migratory response depends mainly on Src family kinases (SFKs). Altogether, our data propose a mechanism that permits short-term activated T cells to move in a CCR7-dependent manner within the lymph nodes to scan APCs.

Materials and Methods

Isolation and activation of primary human T cells

Human PBMCs were isolated from blood samples of healthy donors by Ficoll density-gradient centrifugation. PBLs were obtained by privation of monocytes with $CD14^+$ magnetic sorting (Miltenyi Biotec). $CD3^+$ T cells were isolated from PBLs by negative selection using the Pan T Cell Isolation Kit II (Miltenyi Biotec), yielding a purity of 90–97%. T cells were permitted to recover from the isolation procedure by maintaining the cells in RPMI 1640 medium supplemented with 0.5% human AB serum for 1 h. For longer cultivation and migration assays, T cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 1% 2-ME, and 1% nonessential amino acids. A total of 200 U/ml human IL-2 was added at day 3 of cultivation. For short-term activation, T cells were plated on dishes precoated with 5 μg/ml anti-CD3 (clone OKT3; Janssen-Cilag) and 2 μg/ml anti-CD28 Abs (clone CD28.2; eBioscience), for indicated time points. A fraction of cells was kept under the same condition but on uncoated dishes.

Cell surface expression of proteins was determined by flow cytometry (LSRII; BD Biosciences) using the following Abs: PE- or fluorescein-labeled anti-CCR7 mAb (Clone 150503) and PE-labeled anti-CXCR4 mAb (clone 12G5) from R&D Systems and PE-labeled anti-CD62L (clone FMC46) and PE-labeled anti-CD69 (clone FN50) from AbD Serotec.

In several experiments, cells were treated with the inhibitory compounds MG132 (Calbiochem), LY294002 (Cell Signaling), Y-27632 (Sigma-Aldrich), SU6656 (Biaffin), PP2 (Calbiochem), bisindolylmaleimide I (Sigma-Aldrich), or Gö6976 (Calbiochem).

Chemotaxis

Human $CD3^+$ T cells (1×10^5) were placed in Transwell filters (Corning Costar), with 5 μm pore size, and allowed to migrate to the lower compartment containing the indicated concentrations of recombinant human CCL19, CCL21, or CXCL12 (PeproTech) for 3 h. The numbers of input and migrated cells were determined by flow cytometry (LSRII; BD Biosciences) by gating on live, TOPRO-3^- (Molecular Probes) cells and acquiring events for 90 s. Specific cell migration was calculated by subtracting the number of cells migrating in the absence of chemokines. Results are shown as percentage of specifically migrated cells relative to the number of input cells.

T cell stimulation by chemokines and Western blot analysis

PBLs (1×10^6 cells/assay point) were stimulated with 1–2 μg/ml chemokine for the indicated times at 37°C and immediately lysed on ice in 1% Nonidet P-40, 0.25% sodium desoxycholate, 0.4% *N*-dodecyl maltoside, 50 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, and 1 mM NaF (pH 7.4), supplemented with protease- and phosphatase-inhibitor mix (Roche). Total-cell lysates were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes by wet blotting. Western blot analysis was performed using the following Abs: anti–phospho-p44/42 MAPK (pERK-1/2, Thr202/Tyr204), anti-p42 MAPK (ERK-2), anti-pLck (Y505), anti-Fyn, anti-LAT, and anti-pLAT (Y191) (all from Cell Signaling Technology). Anti-Lck (clone 3A5) was purchased from Millipore, and anti-pFyn (pY528/c-Src pY530) was purchased from BD Bioscience. To investigate the role of SFKs, cells were either preincubated for 30 min with 10 μM PP2 before TCR engagement or for 1 h with 5 μM SU6656 before chemokine triggering, respectively.

Statistical evaluation

Statistical analysis was performed using GraphPad InStat (GraphPad Software, La Jolla, CA), applying one of the following algorithms as specified in the figure legends: paired *t* test, repeated-measures ANOVA with Bonferroni multiple comparison posttest, or one-way ANOVA with Bonferroni multiple comparison posttest. Data displaying statistical analyses were collected at least five times independently using lymphocytes from different donors.

Results

Modulation of CCR7-driven migration of primary human T cells upon TCR triggering

The chemokines CCL19 and CCL21 recruit $CCR7^+$ naive and central memory T cells to lymph nodes. Within this chemokine-rich area of lymph nodes, naive T cells scan dendritic cells searching for cognate Ags. It is poorly understood how T cells integrate diverse signals derived from CCR7 and the TCR. To gain new insights about the influence of T cell activation on the chemotactic response toward lymphoid chemokines, we first examined CCR7-mediated migration of T cells at different activation stages. Therefore, we isolated $CD3^+$ human T cells from healthy donors, cultured them for increasing periods of time on anti-CD3/CD28 Ab-coated plates in the presence of human IL-2, and assessed the migratory capacity in Transwell-migration assays. Freshly isolated $CD3^+$ T cells migrated readily toward CCL19, and CD3/CD28 triggering steadily attenuated the migration efficiency between days 2 and 6 (Fig. 1*A*), similar to a reduced CXCL10/CXCL12-dependent (14, 15) and chemokine-independent (17) T cell motility observed after TCR engagement. In contrast, CCL21-mediated chemotactic responses were rather poor in freshly isolated $CD3^+$ T cells, but they transiently increased upon CD3/CD28 triggering, reaching the greatest responses at day 2 before progressively declining down to baseline levels (Fig. 1*A*). Similar results were observed in PBLs cultured in the presence of IL-2 alone (5). Next, we determined surface expression levels of CCR7 by flow cytometry. CCR7 expression increased slightly at day 2 after TCR stimulation, but it declined at day 3 and, more profound, at day 6 (Fig. 1*B*). This result is in line with previous data showing that TCR ligation led to an enhanced CCR7 surface expression that was accompanied by increased responsiveness (24). Supposing that small differences in receptor surface expression influence the migratory re-

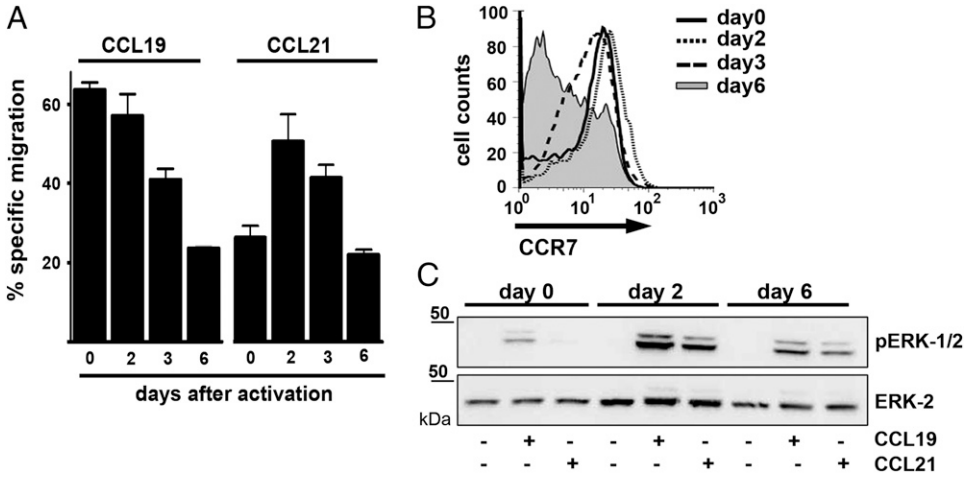

FIGURE 1. Temporarily modified CCR7 signaling and migration upon T cell activation. *A*, Freshly isolated human $CD3^+$ T cells were activated or not on anti-CD3/CD28 Ab-coated dishes and cultured for up to 6 d. T cell migration toward 1 μg/ml CCL19 and CCL21 was determined by Transwell-migration assay. The percentage of cells migrated to the lower compartment within 3 h was determined by flow cytometry. Cells migrated in the absence of chemokines were subtracted. Mean values ± SEM derived from four independent experiments are shown. *B*, Simultaneously, cell surface expression of CCR7 was assessed by flow cytometry using an FITC-labeled CCR7-specific Ab. *C*, Freshly isolated $CD3^+$ T cells and CD3/CD28-stimulated cells were stimulated for 2 min with 2 μg/ml CCL19 or CCL21 at 37°C. Chemokine-induced ERK-1/2 activation was determined by Western blotting using a phospho-specific anti–ERK-1/2 Ab. Reprobing the same blot with a total ERK-2 Ab served as protein loading control. A representative of three independent experiments is shown.

sponse, observed changes in CCR7 expression could explain the chemotaxis results for one of the ligands but not the other. To investigate the consequence of T cell activation on CCR7 functions other than migration, we examined the activation of the MAPKs ERK-1/2 in nonactivated and CD3/CD28-activated human T cells. Strikingly, CCR7-mediated ERK-1/2 activation was profoundly enhanced by both ligands at day 2 after T cell activation compared with nonactivated T cells (Fig. 1*C*). At day 6, the ability of CCR7 to trigger ERK-1/2 phosphorylation was much lower compared with day 2, but it was still greater than in freshly isolated $CD3^+$ T cells. Given that receptor expression at day 6 was significantly reduced, the enhanced chemokine-mediated ERK-1/2 phosphorylation did not correlate with the expression levels of CCR7. These data suggested that T cell activation via CD3/CD28 modifies CCR7 signaling and cellular responses.

Short-term TCR triggering is sufficient for enhanced CCR7-mediated migration

To assess whether early TCR signals account for the enhanced CCR7 signaling, we performed Transwell-migration assays with short-term activated and nonactivated T cells. To this end, we isolated primary human $CD3^+$ T cells from blood samples and activated them on anti-CD3/CD28–coated plates. After 2 h of incubation, short-term CD3/CD28-activated and nonactivated $CD3^+$ T cells were allowed to migrate toward CCL19, CCL21, and CXCL12. Interestingly, short-term CD3/CD28-activated $CD3^+$ T cells migrated significantly better toward CCL19 and CCL21 at concentrations near the K_d values (~0.01–0.1 μg/ml) (5, 25, 26) of the two chemokines (Fig. 2*A*). Maximal migration was observed at 0.5 μg/ml of CCR7 ligands, and it decreased again at greater chemokine concentrations, independently of whether T cells were activated. Remarkably, enhanced CCL21-induced migration upon

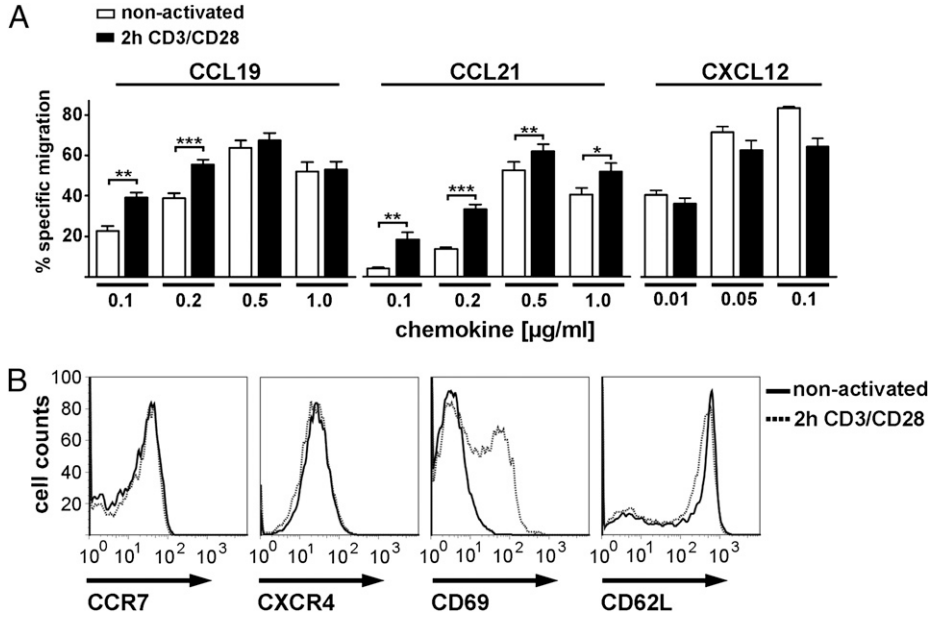

FIGURE 2. Enhanced migratory response of short-term activated human $CD3^+$ T cells is specific for CCR7. *A*, Migratory responses of freshly isolated, nonactivated or 2-h short-term CD3/CD28-activated human $CD3^+$ T cells toward graded concentrations of CCL19, CCL21, and CXCL12 were determined by Transwell-migration assays. Cells were allowed to migrate toward the ligand source for 3 h at 37°C, and the number of migrated cells was measured by flow cytometry. Mean values ± SEM derived from six independent experiments are presented. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (paired *t* test). *B*, Surface expression of the chemokine receptors CCR7 and CXCR4, the T cell activation marker CD69, as well as the lymphocyte homing marker CD62L, was determined by flow cytometry. A representative result from the above-mentioned experiment is depicted.

short-term T cell activation was observed at any chemokine concentration tested, whereas more efficient CCL19-mediated chemotaxis was only noted at concentrations close to the K_d value (Fig. 2*A*). In contrast, CXCL12-mediated migration was slightly attenuated in activated T cells (Fig. 2*A*), confirming previous observations for CXCR4 (14). As depicted in Figure 2*B*, short-term activation of T cells strongly induced CD69 expression, a marker for early T cell activation, but it did not significantly alter the expression levels of the homing receptors CCR7, CXCR4, and CD62L. These data clearly indicated that the enhanced T cell migration mediated by TCR triggering is restricted to CCR7, is most prominently observed at chemokine concentrations near the K_d values, and cannot be attributed to changes in chemokine receptor surface expression. These observations also suggested that the TCR-signaling pathway differentially cooperates with distinct modules of chemokine receptor signaling. All subsequent chemotaxis assays were performed at 0.2 μg/ml of CCR7 ligands, for which differences in cell-migration efficiency between nonactivated and TCR-triggered T cells are most prominent.

Next, we tested whether even shorter TCR triggering was sufficient for inducing an enhanced T cell-migratory phenotype. Therefore, we activated primary human $CD3^+$ T cells for 45 min on anti-CD3/CD28–coated plates, transferred the cells to uncoated dishes, and cultured them for an additional 75 min before the migratory response was analyzed. Even this shortened period of time was sufficient to provoke a significantly enhanced CCR7-dependent T cell-migration phenotype (Fig. 3*A*). TCR triggering for 45 min seemed to be the minimal activation time, because a further reduction in the duration of TCR stimulation no longer induced the enhanced migratory phenotype (data not shown). In addition, this result also demonstrated that the promigratory effect remained after TCR ligation for ≥1 h and did not require simultaneous stimulation of the TCR and the chemokine receptor.

Enhanced migration by short-term TCR triggering is most effective for naive T cells

Next, we aimed to determine whether different T cell subpopulations are more prone to develop an enhanced migratory phenotype. To this end, we stained $CD3^+$ T cells for CD45RO, CD45RA, CD4, and CD8 before and after migration toward CCR7 ligands and determined the percentage of specifically migrated T cell subsets relative to the input of cells. As depicted in Figure 3*B*, all T cell subpopulations showed an enhanced migratory phenotype induced by short-term CD3/CD28 activation, but the most prominent effect was observed for $CD45RA^+$ naive T cells, irrespective of whether they were $CD4^+$ Th cells or $CD8^+$ cytotoxic T cells.

SFKs are important for the enhanced CCR7-mediated migration of short-term activated T cells

Because we obtained no evidence for the regulation of CCR7 expression by short-term activation of T cells that could account for the enhanced migratory capacity, we next focused on potential signaling cross-talks between the TCR and CCR7. First, we used pharmacological inhibitors and assessed the migratory capacity of drug-treated T cells. Importantly, we first stimulated $CD3^+$ T cells with anti-CD3/CD28 for 1 h (which was sufficient for inducing the enhanced migratory phenotype, as shown in Fig. 3*A*). Cells were then further incubated in the presence of inhibitors or solvent controls for another hour prior to assessing their migratory capacities. Inhibiting PI3K by LY294002 or the Rho pathway using the ROCK inhibitor Y-27632 partially diminished the overall migratory response but did not abrogate the enhanced migration of short-term activated T cells toward CCL19 and CCL21 (data not shown). T cells treated with the proteasome inhibitor MG132 prior to T cell activation to prevent degradation of potential signaling proteins hampering CCR7-mediated migration still displayed enhanced migration upon TCR triggering (data not shown). Inhibiting SFKs using PP2 led to an overall attenuation of CCR7-mediated migration, but it additionally significantly reduced the enhanced migratory capacity of short-term activated $CD3^+$ T cells (Fig. 4*A*). The SFKs Fyn and Lck are well-known tyrosine kinases downstream of the TCR that play crucial roles in T cell activation (27). To investigate whether one of these SFKs accounts for the enhanced T cell migration, we performed small interfering RNA (siRNA) experiments in primary human T cells to specifically knockdown individual kinases. Unfortunately, siRNA transfection itself, whether specific or nonspecific, resulted in T cell activation manifested by enhanced CD69 and reduced CD62L expression. Despite multiple approaches, we were unable to establish conditions to introduce siRNA in primary T cells while keeping the enhanced CCR7-driven migration phenotype upon CD3/CD28 triggering (data not shown). As an alternative strategy, we exploited the pharmacological compound SU6656, which selectively inhibits the SFK members Fyn and Src with only minor or no effect on Lck activity (28). Primary human $CD3^+$ T cells were pretreated for 2 h with SU6656 before being subjected to migration assays. Surprisingly, SU6656 treatment significantly enhanced migration of nonactivated T cells toward CCL19 and CCL21, but it did not influence the migratory re-

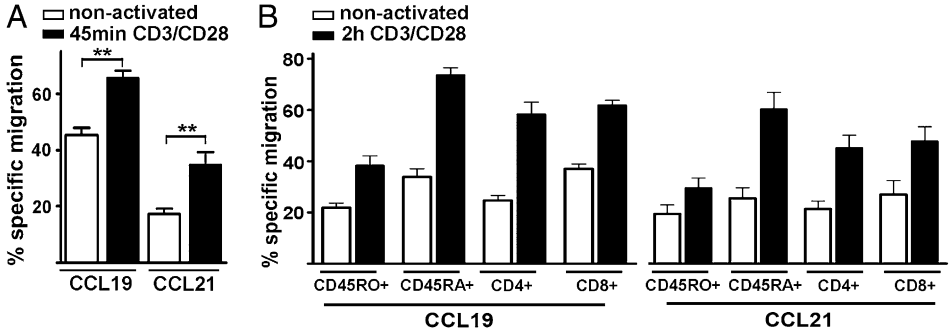

FIGURE 3. Enhanced migration by short-term TCR triggering is most effective for naive T cells. *A*, Human $CD3^+$ T cells were activated for 45 min on anti-CD3/CD28 Ab-coated dishes, followed by further incubation for 75 min on uncoated dishes. Cells were subsequently subjected to Transwell-migration assays toward 0.2 μg/ml CCL19 and CCL21. Mean values ± SEM derived from six independent experiments are shown. $^{**}p < 0.01$ (paired *t* test). *B*, Human $CD3^+$ T cells were activated or not for 2 h on anti-CD3/CD28–coated dishes and subjected to Transwell-chemotaxis assays. Input and migrated T cell populations were assessed for the expression of CD45RO, CD45RA, CD4, and CD8 by flow cytometry using specific Abs. The percentage of individual T cell subsets was calculated relative to the initial population. Mean values ± SEM derived from four independent experiments are presented.

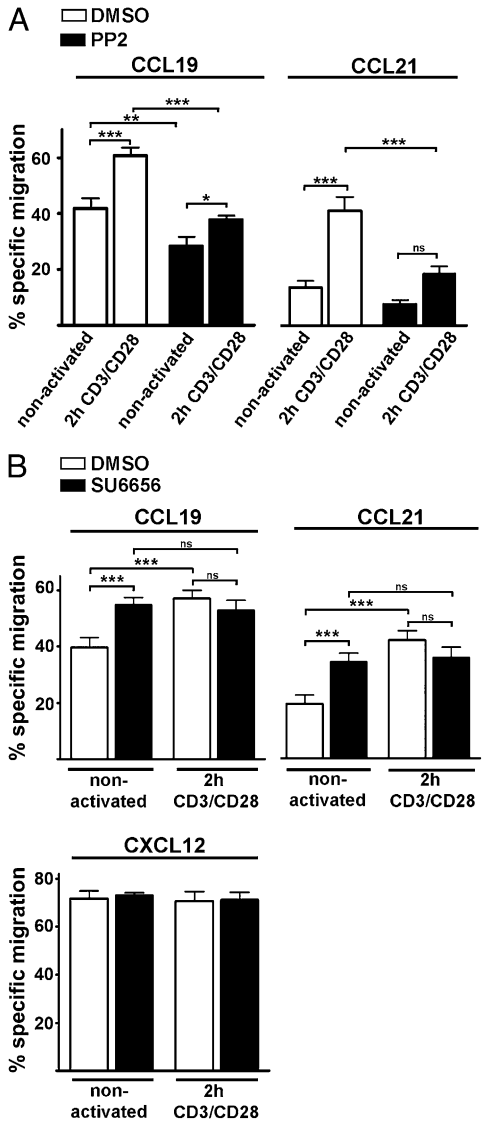

FIGURE 4. SFKs contribute to the enhanced CCR7-mediated migration of activated primary human T cells. Freshly isolated human $CD3^+$ T cells were either activated for 2 h by CD3/CD28 or left untreated. *A*, After the first hour of activation, 10 μM PP2 and equivalent amounts of its solvent control, DMSO, was added. *B*, Cells were treated for 2 h with the Fyn and Src inhibitor SU6656 (5 μM) prior to the chemotactic response being determined. Chemotaxis toward 0.2 μg/ml CCL19, 0.2 μg/ml CCL21, or 50 ng/ml CXCL12 was assessed by Transwell-migration assays. The numbers of migrated cells were determined by flow cytometry. Simultaneous TOPRO-3 staining was included to exclude that inhibitor treatment affected cell viability. Mean values ± SEM derived from seven (*A*) or eight (*B*) independent experiments are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (repeated-measures ANOVA, with Bonferroni multiple comparison posttest).

sponse of CD3/CD28-activated T cells (Fig. 4*B*). Interestingly, cell-migration efficiency was similar in SU6656-treated nonactivated cells and in short-term activated T cells without inhibitor treatment. SU6656 treatment did not affect CXCR4-mediated migration of activated or nonactivated T cells (Fig. 4*B*), demonstrating again a specificity for CCR7. These data indicated that Fyn plays a role in the TCR–CCR7 cross-talk leading to enhanced migration of short-termed TCR-triggered T cells.

To substantiate this finding, we inhibited another protein kinase family, the protein kinase C isoforms (PKCs), which were shown to serine phosphorylate Fyn in platelets (29). To investigate the role of PKC and, indirectly, Fyn on CCR7-mediated T cell migration, we pretreated nonactivated and short-term activated $CD3^+$ T cells with the PKC inhibitor bisindolylmaleimide I (BimI). Strikingly, we observed an enhanced CCR7-mediated migration, especially toward CCL21, in BimI-treated nonactivated T cells, whereas in short-term activated T cells PKC inhibition diminished migratory responses (Fig. 5*A*). Similarly to SU6656 treatment, cell migration did not increase upon TCR ligation in BimI-treated cells. Because BimI inhibits several PKC isoforms, including PKCα, β1, β_2, γ, δ, and ε, we used a second, more specific, PKC inhibitor (Gö6976), which selectively inhibits the Ca^{2+}-dependent α and β1 isozymes. Pretreatment of nonactivated $CD3^+$ T cells with Gö6976, in contrast to BimI, attenuated CCL19- and CCL21-mediated cell migration. Based on these observations, we assumed that the SFK Fyn, together with Ca^{2+}-independent PKC isoforms, keeps the migratory response of naive T cells toward CCL21 at a low level.

The activation status of Lck and Fyn is reflected in their phosphorylation pattern. Thereby, phosphorylation of Tyr394 of Lck results in activation of the kinase, whereas Tyr505 is the regulatory tyrosine residue, and its phosphorylation leads to an inactive conformation of Lck. For Fyn, Tyr419 is the activating, whereas Tyr528 is the negative regulating, tyrosine phosphorylation site. To assess the involvement of these two kinases in the negative regulation of CCR7-mediated human T cell migration, we determined their phosphorylation pattern after chemokine triggering. To this end, we challenged freshly isolated human PBLs with CCL21 for different times and determined the phosphorylation status of the regulatory tyrosines by immunoblotting. CCL21 induced a strong Tyr505 phosphorylation of Lck in nonactivated PBLs, which was maximal after 30 min of stimulation (Fig. 6*A*). In contrast, the phosphorylation status of Tyr528

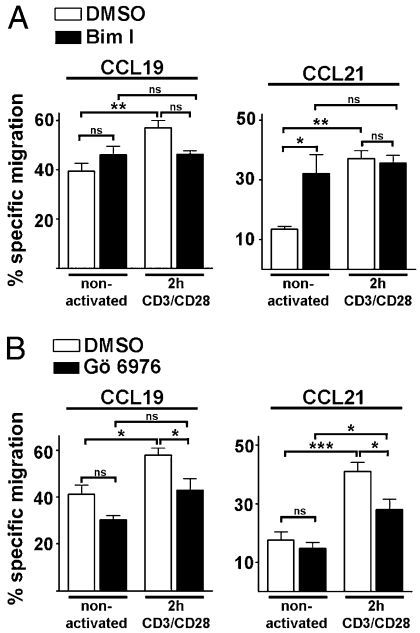

FIGURE 5. Contribution of PKCs to CCR7-mediated T cell migration. Nonactivated and short-term activated human $CD3^+$ T cells were treated with 10 μM of the PKC inhibitors BimI (*A*) or Gö6976 (*B*) 1 h before T cells were subjected to migration assays in response to 0.2 μg/ml CCL19 or CCL21. Cells were allowed to migrate for 3 h before the numbers of migrated cells were determined by flow cytometry. Cell viability after inhibitor treatment was assessed by TOPRO-3 staining. Mean values ± SEM derived from six (*A*) or seven (*B*) independent experiments are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (one-way ANOVA with Bonferroni multiple comparison posttest).

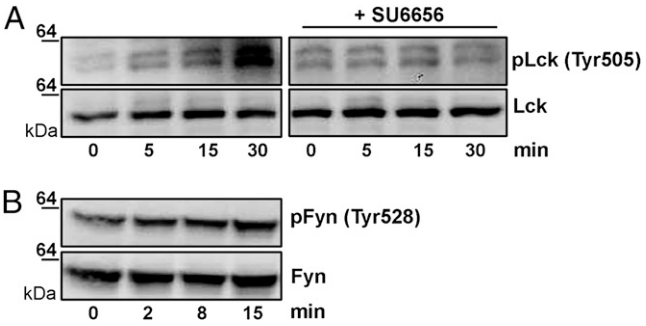

FIGURE 6. CCL21 induces inhibitory Tyr505 phosphorylation of Lck in a Fyn-dependent manner in nonactivated PBLs. Human PBLs were stimulated for the indicated times with 1 μg/ml CCL21. Cell lysates were separated on SDS-PAGE and immunoblotted for the inhibitory tyrosine of Lck, pTyr505 (*A*) or Fyn, pTyr528 (*B*), respectively. Blots were stripped and reblotted with Abs against total Lck and Fyn to assure equal protein loading. Where indicated, PBLs were pretreated with 5 μM of the Fyn inhibitor SU6656 for 2 h prior to chemokine stimulation.

of Fyn did not change upon CCR7 triggering (Fig. 6*B*). Because Fyn was postulated to act as a negative-feedback loop regulator of Lck (30), we investigated its role in CCR7-mediated phosphorylation of the regulatory Tyr505 of Lck. To this end, we treated human PBLs with SU6656 prior to CCR7 stimulation. Indeed, we found that SU6656 inhibited CCL21-mediated Tyr505 phosphorylation of Lck (Fig. 6*A*), suggesting a negative regulation of Lck by Fyn.

CCR7 triggering leads to LAT phosphorylation in activated T cells

TCR triggering is known to induce phosphorylation of LAT by ZAP70, which is itself activated by Lck (31). To gain further information about the activation state of Lck and Fyn, we tested whether CCR7 triggering induced LAT phosphorylation. In nonactivated PBLs, CCL19 and CCL21 barely induced LAT phosphorylation (Fig. 7*A*). In contrast, in short-term TCR-engaged PBLs, LAT phosphorylation was strongly enhanced upon CCR7 triggering. Of note, at day 6 of PBL activation, CCL19 and CCL21 no longer induced LAT phosphorylation (data not shown). Treating of CD3/CD28 short-term activated PBLs with PP2 completely blocked CCR7-triggered LAT phosphorylation (Fig. 7*B*), demonstrating that an Src kinase member, most likely Lck, is responsible for CCL19/CCL21-mediated LAT phosphorylation.

Taken together, we found that short-term activation of (naive) T cells resulted in a significantly enhanced migratory response to CCL19 and CCL21 but not toward CXCL12. We provided evidence that SFKs, presumably Lck, are responsible for these increased migratory responses. Moreover, CCR7 triggering induces LAT phosphorylation by SFKs in synergy with TCR engagement. This study demonstrated a profound signaling cross-talk between CCR7 and the TCR.

Discussion

Within lymph nodes, naive T cells are very motile and scan a large number of Ag-presenting dendritic cells in search of presented cognate Ags. Upon Ag recognition, T cells are activated and start to proliferate, resulting in clonal expansion and differentiation. However, TCR triggering alone is not sufficient for a full-blown T cell activation. It is now well established that the affinity of the Ag for the TCR, as well as T cell costimulation, is critical for the T cell-activation outcome (32, 33). Intravital microscopy revealed that naive T cells initially interact only temporarily with dendritic cells presenting cognate Ags (13). During this early priming phase, naive T cells upregulate the activation marker CD69 and stay highly motile. Of note, tonic TCR signaling in secondary lymphoid tissues was shown to be required for maintaining a basal Rac1 and Rap1 activation, which is critical for normal T cell motility and Ag receptivity of naive CD4 T cells (22). The high motility of T cells during the early priming phase is mediated by the chemokine receptor CCR7 (8, 9). It is poorly understood how the migratory behavior of T cells is regulated during early- and late-priming phases. In the current study, we investigated the influence of TCR ligation on the chemotactic response toward the lymph node chemokines CCL19 and CCL21 using human peripheral blood-derived T cells as a model system. We demonstrated that mainly $CD45RA^+$ naive T cells, but also $CD45RO^+$ memory T cells, migrate more readily toward CCL19 and CCL21 upon short-term triggering of the TCR. This promigratory effect was specific for the chemokine receptor CCR7 and did not occur for CXCR4. This is in line with the sole previous finding that CCL21, but not CXCL12, could override the TCR-mediated stop signal in a two-dimensional system where T cells were subjected to opposing gradients of coated Ags and chemokines (34). Moreover, our findings are also supported by intravital imaging studies describing a promigratory role for TCR signals in naive (22), as well as in effector T cells (23), without specifically investigating involved chemokines/chemokine receptors. Substantially more information is available about the interplay between CXCR4 and the TCR. In fact, CXCR4 was shown to physically interact with the TCR after chemokine binding (19). On

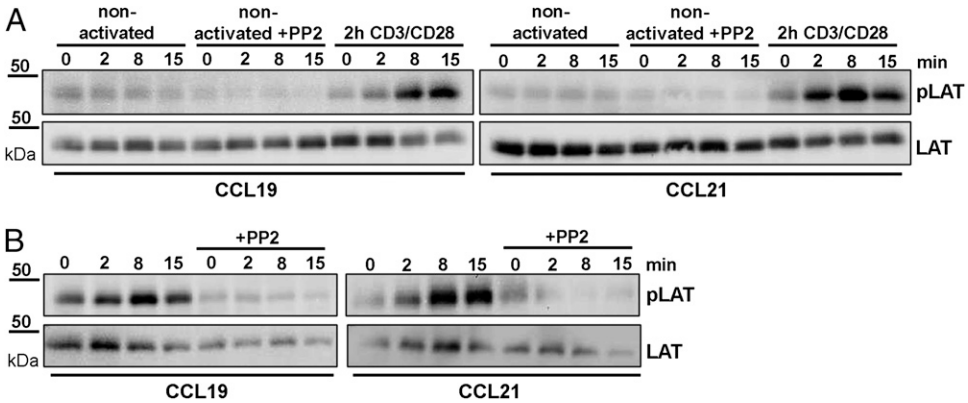

FIGURE 7. CCR7 stimulation synergistically induces LAT phosphorylation exclusively in TCR-triggered PBLs. Human PBLs, either untreated (*A*) or activated for 2 h with Abs against CD3 and CD28 (*B*), were stimulated for the indicated times with 1 μg/ml CCL19 or CCL21. Chemokine-induced LAT phosphorylation at Tyr191 was determined by Western blotting. Cells were lysed and immunoblotted for the detection of total and phosphorylated LAT. Where indicated, 10 μM PP2 was added 30 min prior to chemokine (*A*) or TCR (*B*) triggering. A representative Western blot of four derived from individual blood donors is depicted.

one hand, this heterodimer between CXCR4 and the TCR enabled the chemokine receptor to signal via the TCR ITAM motifs in a ZAP70-dependent manner, resulting in sustained ERK activation, altered transcriptional activity, and increased intracellular Ca^{2+} concentration mobilization (19, 21). On the other hand, CXCR4 triggering diminished TCR activation by lowering the phosphorylation of ZAP70, LAT, and SLP76 (14). The same investigators reported that TCR activation inhibited T cell migration toward CXCL12 and caused augmented CXCR4 internalization (14). Our observations confirmed the reduced CXCR4-mediated migratory response after TCR engagement in human T cells; however, this effect did not correlate with a decreased surface chemokine receptor expression (Fig. 2).

Using a panel of pharmacological inhibitors, we determined that Src kinases substantially accounted for the enhanced CCR7-induced migration of T cells upon short-term triggering of the TCR. In our experimental set-up, inhibition of SFKs by PP2 led to an overall reduced lymphocyte migration, as well as abrogated the TCR-mediated promigratory phenotype. Surprisingly, we observed a significantly enhanced migration of nonactivated human T cells if the SFK Fyn was blocked by SU6656. This enhanced migration induced by Fyn inhibition could not be augmented by TCR triggering, strengthening the assumption that different members of SFK opposingly effect CCR7-driven T cell migration. Lck and Fyn are the major SFKs in T cells and are among the first downstream-signaling molecules of the TCR. Activation of these tyrosine kinases is, on the one hand, regulated by conformational changes that are due to the binding of target proteins to the SH3 and/or SH2 domains of the kinase and, on the other hand, by the phosphorylation status of two critical tyrosine residues (27). The activating tyrosine is located within the kinase domain, whereas the inhibitory/regulatory tyrosine is positioned in the C terminus (Tyr505 for Lck and Tyr528 for Fyn). After TCR engagement, SFKs, essentially Lck, phosphorylate the ITAM motifs within the γ-, δ-, ε-, and ζ-chains of CD3. Subsequently, ZAP70 is recruited to phosphorylated CD3 and is itself activated by Lck (31). Phosphorylation of the adaptor molecules LAT and SLP76 by activated ZAP70 is critical for an effective TCR triggering resulting in a fully activated T cell status (35). Although there is no doubt that TCR stimulation leads to activation of Lck and Fyn, differences in the phosphorylation status of these two kinases before and after TCR engagement were reported. It is well established that tyrosine residues within the kinase domain of Lck are phosphorylated after efficient T cell activation (36, 37). However, a recent study described that, in naive T cells, a significant proportion of Lck was constitutively active and, strikingly, partially also phosphorylated at the inhibitory Tyr505 (38). Moreover, in these cells, the amount of active Lck did not increase upon TCR/CD3 engagement but determined the extent of CD3ζ phosphorylation (38). Few studies have investigated the role of Lck and Fyn in chemokine receptor signaling and function: CXCR4 stimulation was reported to enhance Lck activity, which was important for the onset of different downstream pathways involving ZAP70, Dok-1 (39, 40), and Fyn (41). Lck was also reported to play a crucial role in CXCR4-mediated lymphocyte homing (42). Furthermore, a negative effect on TCR-mediated Lck activation was described for CXCL12-treated cells (43), suggesting an interplay between the chemokine receptor and the TCR signaling via the action of SFKs. In the current study, we identified a crucial role for SFKs in T cell migration toward the CCR7 ligands CCL19 and CCL21. Furthermore, we demonstrated that, in naive T cells, the regulatory Tyr505 of Lck was phosphorylated in a Fyn-dependent manner upon CCR7 triggering. Together with the finding that inhibition of Fyn led to an enhanced migratory response in nonactivated human T cells, our results provided evidence for a negative role for Fyn in CCR7-mediated migration, which presumably depended on the inhibition of Lck. Fyn was recently shown to control the time of TCR engagement, thereby regulating the threshold of T cell activation (30). Further, this study demonstrated a reduced phosphorylation of the regulatory Tyr505 of Lck in the absence of Fyn (30), assuming a negative-feedback loop, similar to our observation. The assumption that Lck is activated by CCR7 in activated T cells and, thereby, positively influences chemokine-mediated migration, is supported by the fact that we detected a profound SFK-dependent phosphorylation of LAT upon CCR7 triggering, which is in line with Lck-mediated ZAP70 activation upon TCR ligation (44).

In addition to SFK, we identified PKCs as a kinase regulating CCR7-mediated T cell migration. In fact, pharmacological inhibition of several PKC isoforms by BimI led to an increased migration of nonactivated human T cells toward CCL21. The CCR7-mediated migratory response of BimI-treated naive T cells resembles that of SU6656-treated cells. Again, TCR ligation could not augment the migratory response. No enhanced T cell migration was observed in cells in which Ca^{2+}-dependent PKC isoforms, like PKCα and PKCβ1, were inhibited, pointing to a major role for Ca^{2+}-independent PKC isoforms in naive T cell migration. Discrepant results have been reported for different PKC isoforms with regard to cell migration. For instance, T cell migration was reported to be resistant to PKC inhibitors (45). In contrast, PKCδ was required for CCR4-mediated chemotaxis (46, 47). Interestingly, it was noted that, upon culturing, T cells switch from a PI3K-dependent to a PKC-dependent chemotactic response to CXCL12, although the mechanism remains elusive (48). It was also speculated that different PKC isoforms differentially regulate actin reorganization and integrin activation and, hence, account for different mechanisms of migration-signaling paths (49, 50). From this perspective, it is worth noting that Fyn activity can be regulated by PKC (29), which could explain our migration data.

In summary, our data revealed that the migratory response of human primary T cells toward the lymph node chemokines CCL19 and CCL21 is linked to the activation status of the cells. We observed a significantly enhanced T cell migration upon short-term TCR activation that was independent of CCR7 surface expression. Moreover, we identified SFKs as integral signaling molecules between the TCR and CCR7. We provided evidence that, in naive human T cells, CCR7 activation led to Fyn-dependent Lck inhibition, whereas in short-term activated T cells, CCR7 triggering induced a strong chemokine-dependent LAT phosphorylation, presumably through Lck. Moreover, we determined a negative role for Ca^{2+}-independent PKC isoforms in CCR7-mediated migration, which faded away in activated T cells. These data clearly indicated that TCR engagement results in modified CCR7 signaling, leading to an altered migratory T cell phenotype. Our results provided mechanistic insights into how T cells acquire a highly motile phenotype during early-priming phases in lymph nodes, despite opposing stop signals provided by the TCR.

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Disclosures

The authors have no financial conflicts of interests.

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