CCL21 mediates CD4⁺ T-cell costimulation via a DOCK2/Rac-dependent pathway

Kathrin Gollmer, François Asperti-Boursin, Yoshinori Tanaka, Klaus Okkenhaug, Bart Vanhaesebroeck, Jeffrey R. Peterson, Yoshihiko Fukui, Emmanuel Donnadieu, and Jens V. Stein

Theodor Kocher Institute, University of Bern, Bern, Switzerland; Institut Cochin, Université Paris Descartes, Centre National de la Recherche Scientifique (UMR 8104), Paris, France; Inserm (U567), Paris, France; Division of Immunogenetics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; Japan Science and Technology, CREST, Tokyo, Japan; Laboratory of Lymphocyte Signaling and Development, Babraham Institute, Babraham Research Campus, Cambridge, United Kingdom; Center for Cell Signaling, Institute of Cancer, Queen Mary University of London, London, United Kingdom; and Department of Basic Sciences, Fox Chase Cancer Center, Philadelphia, PA

CD4⁺ T cells use the chemokine receptor CCR7 to home to and migrate within lymphoid tissue, where T-cell activation takes place. Using primary T-cell receptor (TCR)-transgenic (tg) CD4⁺ T cells, we explored the effect of CCR7 ligands, in particular CCL21, on T-cell activation. We found that the presence of CCL21 during early time points strongly increased in vitro T-cell proliferation after TCR stimulation, correlating with increased expression of early activation markers. CCL21 costimulation resulted in increased Ras-GTP formation and enhanced phosphorylation of Akt, MEK, and ERK but not p38 or JNK. Kinase-dead PI3KΔδ910A/D916A or PI3Kγ-deficient TCR-tg CD4⁺ T cells showed similar responsiveness to CCL21 costimulation as control CD4⁺ T cells. Conversely, deficiency in the Rac guanine exchange factor DOCK2 significantly impaired CCL21-mediated costimulation in TCR-tg CD4⁺ T cells, comonal with impaired Rac-GTP formation. Using lymph node slices for live monitoring of T-cell behavior and activation, we found that G protein-coupled receptor signaling was required for early CD69 expression but not for Ca²⁺ signaling. Our data suggest that the presence of CCL21 during early TCR signaling lowers the activation threshold through Ras- and Rac-dependent pathways leading to increased ERK phosphorylation. (Blood. 2009;114:580-588)

Introduction

Naïve T cells continuously traffic to secondary lymphoid organs, including peripheral lymph nodes (PLNs), where they screen antigen-presenting cells (APCs), in particular dendritic cells (DCs), for the presence of specific peptide Ag presented on MHC (pMHC) complexes. In proinflammatory conditions, DCs expressing cognate pMHC and costimulatory signals, such as B7 molecules, induce efficient T-cell activation through the T-cell receptor (TCR) and CD28. This leads to an early signaling response characterized by activation of multiple signaling pathways, including tyrosine kinase cascades, sustained increase in intracellular Ca²⁺, and activation of phosphoinositide-3-kinase (PI3K), small GTPases of the Ras and Rho family, mitogen-activated protein kinase, nuclear factor of activated T-cell, and nuclear factor-κB. Activated T cells subsequently increase surface expression of early activation markers, such as CD69 and CD25, produce interleukin-2 (IL-2), expand clonally, and differentiate into effector cells.

Direct observations of lymphocytes and DCs presenting cognate pMHC complexes in explanted PLNs or live mice using 2-photon microscopy have uncovered a dynamic range of cellular interactions within lymphoid tissue. In some settings, T cells almost immediately arrest on encountering DCs. Alternatively, T cells were observed to continue to migrate during the first several hours after entry into lymphoid tissue along the stromal network formed by fibroblastic reticular cells (FRCs), where they underwent brief serial contacts with DCs. This first phase of high motility is reminiscent of naïve T-cell migration in the absence of pMHC-loaded DCs. Of note, even during continuous motility during initial DC encounters, T cells integrate TCR-derived signals, as they gradually increase CD44 and CD69 surface levels. Differences in T-cell deceleration in distinct models are probably the result of variations in total Ag load and TCR-MHC affinity, which influence the time T cells require to reach a threshold for efficient arrest and formation of long-lasting contacts with DCs.

FRCs in the T-cell area of PLN express the homeostatic chemokines CCL19 and CCL21. Their G-protein coupled receptor (GPCR) CCR7 is highly expressed on naïve T cells and contributes to random motility during DC scanning in vitro and in vivo. TCR signaling events are thus spatiotemporally tightly connected to chemokine receptor signaling, suggesting a potential crosstalk between both pathways during early T-cell activation. Indeed, chemokines have been shown to contribute to T-cell activation in at least 2 ways. First, in vitro assays showed that CCL19 and CCL21 indirectly contribute to lymphocyte activation by allowing efficient screening of rare Ag-bearing DCs. CCL21 was also found to sensitize CD4⁺ T cells to pMHC complexes on neighboring DCs, correlating with efficient DC scanning of the leading edge of polarized T cells. These observations support a role for CCR7 ligands in promoting efficient encounters with other cell types present in lymph nodes, such as DCs or B cells, through increased motility and scanning.

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Independent of their chemotactic and guiding activities, chemokines also act directly as costimulatory factors and consequently modulate the outcome of an immune response.\textsuperscript{18,20} CCL5 costimulates Jurkat T cells by recruiting its receptor CCR5 and G\(\alpha_{i1}\) to the immunologic synapse without inducing migration.\textsuperscript{21} Similarly, CXCL12 and CCL21 increase anti-CD3-induced T-cell activation, suggesting that migration-independent chemokine receptor- and TCR-triggered signals are combined for optimal T-cell activation.\textsuperscript{19,22} Thus far, the underlying molecular mechanisms of chemokine-mediated costimulation and intracellular integrators acting both downstream TCR and chemokine receptors remain incompletely described. In addition, it is not known whether chemokines costimulate T cells in a lymphoid environment.

Chemokine-induced cell migration, scanning of Ag-bearing DCs, and subsequent formation of a stable immunologic synapse require cytoskeletal reorganizations in T cells.\textsuperscript{23,24} The small GT-DCs, and subsequent formation of a stable immunologic synapse is incompletely described. In addition, it is not known whether chemokine-mediated costimulation and intracellular integrators acting both downstream TCR and chemokine receptors remain incompletely described. In addition, it is not known whether chemokines costimulate T cells in a lymphoid environment.

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Methods

Mice

Four- to 12-week-old control or p110\textsuperscript{y}-deficient and p110\textsuperscript{y}\textsuperscript{D910A/D910A} knock-in mice backcrossed to the OT-II TCR tg C57BL/6 background and 2B4 TCR tg DOCK2-deficient mice on a B10.RR background were described before.\textsuperscript{29,37} Female Marylin (anti–H-Y peptide called DBY) TCR-transgenic Rag\textsuperscript{2−/−}/CD45.1\textsuperscript{1+/-} mice were obtained from the CDTA. C57BL/6, BALB/c, and DO11.10 mice were bred at the Theodor Kocher Institute or purchased from Harlan. All experiments were performed in accordance with approval from the Swiss Kanton of Bern Veterinary Office and French veterinary animal experimentation regulations.

Reagents

All antibodies and other reagents are listed in supplemental data (available on the Blood website; see the Supplemental Materials link at the top of the online article).

Proliferation assays

Primary T cells were purified from spleen and PLN single-cell suspensions via negative selection using antibody-coated magnetic beads (\textgt;90% purity; Dynal). Isolated T cells (5 \times 10\textsuperscript{5} cells/mL) were either plated on 96-well plates coated with anti-CD3e monoclonal antibodies (mAbs) or stimulated with soluble anti-CD3e mAb and cultured in RPMI 1640/10% fetal calf serum/standard supplements, in the presence or absence of anti-CD28 mAb or chemokines (100 nM final concentration). \textsuperscript{[3H]Thymidine was added for the last 16 hours of a 48- or 72-hour culture, followed by quantification in a scintillation counter. Where indicated, T cells were pretreated with 400 ng/mL pertussis toxin (PTX; 30 minutes at 37°C, 7% CO\textsubscript{2}); washed twice, and cultured as described. For activation of TCR-transgenic CD4\textsuperscript{+} T cells, isolated T cells (4 \times 10\textsuperscript{5} cells/mL) were cultured for 48 or 72 hours with irradiated congenic spleen cells (5 \times 10\textsuperscript{5} cells/mL) in the presence or absence of 100 nM chemokine and indicated concentrations of agonist peptide. \textsuperscript{[3H]Thymidine incorporation was determined as detailed earlier in this paragraph.

In some experiments, T cells were loaded with carboxyfluorescein succinimidyl ester (CFSE) (1 \muM, 30 minutes at 37°C, 7% CO\textsubscript{2}) before activation and analyzed for dye dilution by flow cytometry (FACS caliber, BD Biosciences). From obtained histograms, a proliferation index was calculated as follows. The percentage of divided cells in the presence of anti-CD3e mAb alone (without chemokines), or, in case of APC-triggered proliferation, at a peptide concentration of 0.1 \muM without chemokines, was arbitrarily normalized to “1.” Other percentages were adjusted accordingly, with their ratio to “1” being the proliferation index. For Figure 2A, the cpm value of 0.1 \muM without chemokines was normalized to “1” and the other cpm values expressed as proliferation index accordingly.

Flow cytometry

TCR Tg DO11.10 T cells were activated by chicken or turkey OVA\textsuperscript{323-339}-pulsed irradiated splenocytes in the presence or absence of CCL21 (100 nM) for indicated times and stained for CD25 and CD69. For measurement of intracellular IL-2, DO11.10 T cells were restimulated with ionomycin/phorbol myristate acetate in the presence of brefeldin A for 3 hours and labeled with mAbs against Thy1.2, KJ-26, and IL-2 following the manufacturer’s instructions (BD Biosciences PharMingen).

Immunoblotting

Isolated T cells (5 \times 10\textsuperscript{5} cells/mL) were incubated with anti-CD3e mAb (5 \mug/mL; 4°C, 20 minutes) after overnight incubation in RPMI 1640/0.5% fatty acid free bovine serum albumin. Cells were stimulated by crosslinking of primary antibody with goat anti–hamster-IgG Ab (G94-56, 20 \muM/mL) at 37°C for indicated times, in the presence or absence of CCL21 (100 nM). For quantification, all blots were normalized to the loading control. Fold increase over background was calculated using ImageJ software or according to the manufacturer’s instructions (LI-COR Biosciences). Additional information is available in the supplemental data.

Time-lapse imaging

Splenocytes were washed in Hanks Balanced Salt Solution and 4 \times 10\textsuperscript{5} cells were left to settle to glass coverslips for 15 minutes at 37°C. In parallel, 5 \times 10\textsuperscript{5} T cells prepared from peripheral and mesenteric lymph nodes were incubated for 5 minutes at 37°C with 0.5 \muM 5-chloromethyl-fluorescein diacetate (CMFDA). When indicated, T cells were treated with 1 \muM CCL21 for 5 minutes at 37°C before adding to the splenocytes layer. Imaging was performed as described\textsuperscript{16} on an inverted microscope in a heating chamber (37°C). Images were acquired every 10 seconds during 10 to 15 minutes using MetaFlour software (Molecular Devices). Cell displacements were analyzed with Imaris software (Bitplane).
PLN slice preparation and video imaging

PLN slice preparation was performed as previously described with some modifications (supplemental data). The preparation was perfused at a rate of 1 mL/minute with RPMI without phenol red medium bubbled with a mixture of 95% O₂ and 5% CO₂. A single section located 20 to 30 μm from the surface of slice was acquired every 30 seconds. Five minutes after beginning of image acquisition, slices were perfused with oxygenated RPMI medium containing DBY peptide at indicated concentrations. Fura-2-loaded T cells were alternatively excited at 350 and 380 nm and emission at 510 nm was used for analysis of Ca²⁺ responses using MetaFluor software. Ca²⁺ values were represented as the fluorescence intensity ratio at 340/380 nm. T cells were considered responsive when the amplitude of their responses reached at least twice that of the background. When several Ca²⁺ traces were averaged, the rising phases of the traces were synchronized. T-cell motility was analyzed with Imaris software.

Measurement of CD69 expression on T cells activated within PLN slices

PLN slices overlaid with CMFDA-loaded T cells were treated with indicated concentrations of DBY peptide at 37°C, 6% CO₂. After 2 hours, slices were washed and mechanically dissociated using 30-G needles to obtain single-cell suspensions. Cells were stained with phycoerythrin (PE)–labeled anti-CD69 and PE-Cy5-labeled anti-CD45.1 for analysis on a FACScan (BD Biosciences).

Statistical analysis

Data were analyzed using Prism software (GraphPad Software). Student t test was used for statistical analysis, unless indicated otherwise. Significance was set at P less than .05.

Results

Homeostatic chemokines act as costimulatory factors during mAb- or APC-induced T-cell activation

The lymphoid tissue–expressed chemokines CCL21 and CXCL12 have been shown to act as costimulatory factors during T-cell activation. In CFSE dilution experiments, we confirmed that CCL21 enhanced proliferation of T cells activated with plate-bound anti-CD3 mAb alone or in combination with anti-CD28 mAb (Figure 1A-B). CXCL12 and CCL19 were not effective, although CCL19 showed a nonsignificant tendency to increase proliferation on plates coated with anti-CD3 mAb alone (data not shown). Similar results were obtained when stimulating with soluble anti-CD3 mAb (data not shown). No increased proliferation was observed in the presence of human CCL2 or heat-inactivated CCL21 (data not shown). Thus, CCR7 ligands, especially CCL21, possess higher costimulatory potency than the CXCR4 ligand CXCL12, reflecting receptor expression levels on murine naive T cells. In the conditions used here, chemokines alone did not induce T-cell proliferation (Figure 1B), whereas both CCL19 and CCL21 slightly increased the percentage of live (7-amino-actinomycin D-negative) T cells after 3 days of culture from 18.4% plus or minus 1.1% to 27% plus or minus 1% and 27.4% plus or minus 0.7%, respectively. For subsequent experiments, we focused on CCL21, which is expressed by FRCs at 100-fold higher levels than CCL19 and therefore probably physiologically more relevant.

To evaluate the costimulatory potential of CCL21 during physiologic TCR activation, we activated DO11.10 TCR tg CD4⁺ T cells with either chicken or turkey OVA323-339 peptide-pulsed irradiated splenocytes as APCs. Proliferation induced by both peptides was increased in the presence of CCL21 (Figure 1C). The relative increase in the presence of CCL21 was particularly noticeable at low peptide concentrations and with the low affinity turkey OVA323-339 peptide, representing suboptimal T-cell activation conditions (Figure 1C). In line with previous observations, inhibition of Goi signaling by preincubation of T cells with PTX completely blocked the costimulatory effect of CCL21, indicating that CCL21 acted directly on T cells (Figure 1D).

CCL19 and CCL21 have been reported to induce cell motility under certain experimental conditions, thus increasing the likelihood of T-cell–DC encounters. We examined whether motility, rather than direct signaling, was involved in CCL21-mediated costimulation. Using time-lapse videomicroscopy, we followed individual CD4⁺ T cells coincubated with splenocytes on glass coverslips during 10-minute observation periods in the presence or absence of CCL21.
absence of CCL21. In the absence of CCL21, very few T cells displayed a motile behavior. CCL21 did not increase the percentage of motile T cells under these conditions, although cells were more elongated (supplemental Figure 1; supplemental Videos 1-2). Taken together, our data suggest that, in the conditions used here, CCL21 acts mainly as a costimulatory factor independent of motility.

**CCL21-mediated costimulation is required during an early time window**

In lymphoid tissue containing few pMHC complexes, T-cell activation starts with an early phase of continuous motility most probably codependent on CCL21, followed by stable T-cell–DC interactions.4,5,8,39 We thus examined the effect of CCL21 on T-cell activation triggered by low and high pMHC levels during early or late time points in vitro. Addition of CCL21 to DO11.10 TCR tg CD4+ T cells at 8 or 24 hours after activation led to a gradual decrease in pMHC-stimulated proliferation, indicating that for efficient costimulation, presence of CCL21 is required within an early time frame of T-cell activation (Figure 2A). Similarly, addition of neutralizing anti-CCL21 Abs to the T-cell culture only blocked chemokine-induced costimulation when added from the start but not when added 8 hours after activation (data not shown).

In line with a priming effect of CCL21, the percentage of T cells expressing the early activation marker CD69 was doubled as soon as 8 hours after activation in the presence of CCL21, with cells expressing higher mean CD69 levels (Figure 2B). We also observed an increase in the percentage of CD25+ DO11.10 CD4+ T cells 8 hours after activation (Figure 2B), paralleled by enhanced IL-2 production 24 hours after antigen-specific stimulation (Figure 2C). Taken together, T-cell costimulation through CCL21 is effective during an early time window and directly increases the percentage and expression levels of early activation markers.

To examine whether CCR7-mediated costimulation was a general phenomenon, we stimulated peripheral blood human T cells with anti-CD3 mAbs in the presence or absence of CCL19 or CCL21. Addition of CCL19 accelerated the initiation of TCR-triggered Ca2+ increase but did not affect the amplitude of the response (supplemental Figure 2A). Furthermore, simultaneous activation of TCR and CCR7 resulted in a marked increase in cells expressing CD69, with CCL19 being more potent than CCL21, in contrast to mouse lymphocytes (supplemental Figure 2B). Altogether, these results support a conserved role for CCR7 during T-cell activation.

**Signal transduction analysis in primary T cells after TCR and CCR7 costimulation**

To explore the biochemical basis of CCL21-induced costimulation, we investigated signaling events activated downstream of both TCR and chemokine receptors. The TCR on primary mouse T cells was cross-linked for various times in the presence or absence of CCL21 and lysates analyzed by quantitative Western blotting. As shown in Figure 3A (left panel), simultaneous activation of TCR and CCR7 resulted in additive Rac-GTP formation up to 2 minutes (supplemental Table 1). Unexpectedly, early Ras-GTP formation was mainly mediated by CCR7 at early time points, whereas at 5 minutes, Ras-GTP was only observed in CCR7 and TCR-costimulated cells (2.2 ± 0.7-fold increase over added values of single stimulation; mean ± SEM). This was paralleled by increased phosphorylation of MEK1/2 when both anti-CD3 and CCL21 were combined and by synergistic phosphorylation of its downstream target ERK1/2 (2.4 ± 1.3-fold and 2.3 ± 0.1-fold increase over added values of single stimulation at 5 and 10 minutes, respectively). Flow cytometric analysis uncovered that both percentage of pERK+ cells as well as cellular pERK levels were strongly increased when CCL21 and TCR signaling coincided (Figure 3B).

Conversely, the phosphorylation of JNK (Figure 3A), p38, and Raf (supplemental Table 1) did not increase during chemokine-mediated costimulation. We also analyzed PI3K activity by quantifying phosphorylation of its downstream target Akt. CCL21 triggered robust Akt phosphorylation, which was less pronounced in CD3-crosslinked T cells at the early time points measured here. Simultaneous TCR and CCR7 activation by CCL21 had an additive effect on Akt phosphorylation (Figure 3A). In summary, our data suggest a rapid and pronounced activation of Rac, Ras, and MEK1/2 on simultaneous TCR and CCR7 triggering, resulting in enhanced ERK1/2 phosphorylation.
PI3K activity is not required for CCL21-mediated costimulation

We wanted to examine whether PI3K activity had an effect on baseline and CCL21-enhanced T-cell proliferation, focusing on the lymphocyte-enriched PI3Kγ and PI3Kδ isoforms. To this end, we compared the proliferation of control, PI3KγD910A/D910A, or PI3Kγ−/− OT-II TCR-tg CD4+ T cells in response to increasing concentrations of chicken OVA323-339. Similar to our previous results with DO11.10 T cells, OT-II TCR-tg T cells proliferated more in the presence of CCL21, in particular at low peptide concentrations (Figure 4). Similarly, PI3KδD910A/D910A and PI3Kδ-deficient OT-II TCR-tg T cells OT-II T cells showed comparable antigen-induced and CCL21-costimulated proliferation (Figure 4). Furthermore, pretreatment of human lymphocytes with the pan-PI3K inhibitor Wortmannin had no effect on chemokine-induced enhancement of T-cell activation as measured by Ca2+ and CD69 responses (supplemental Figure 2A; and data not shown). Similar results were obtained with murine T lymphocytes (data not shown). In summary, these data suggest that PI3K activity downstream TCR or CKR is not required for CCL21-mediated costimulation (Figure 4). Similarly, pharmacologic inhibition of the Rac effector Pak1 using a novel, highly specific compound40 strongly reduced the costimulatory effect of CCL21 in the absence of DOCK2. This was particularly noticeable comparing the costimulatory effect of CCL21 at similar baseline proliferation (eg, 0.03 μg/mL MCC peptide in control vs 0.1 μg/mL MCC peptide in DOCK2−/− 2B4 TCR-tg CD4+ T cells). In addition, CCL21-stimulated CD69 and CD25 expression was reduced in DOCK2-deficient cells (supplemental Figure 3A). Similarly, pharmacologic inhibition of the Rac effector Pak1 using a novel, highly specific compound40 strongly reduced CCL21-induced proliferation and CD69 expression (supplemental Figure 4; and data not shown).

Next, we determined whether the decreased costimulatory effect in DOCK2−/− T cells correlated with impaired intracellular signaling. We performed anti-CD3ε crosslinking experiments in the presence and absence of CCL21. As reported,29 DOCK2−/− 2B4 TCR-tg T cells did not show any detectable Rac-GTP even when TCR and CCR7 were simultaneously activated (Figure 5B). In contrast, both wild-type and DOCK2−/− T cells showed normal Rac-GTP formation after activation of TCR and CCR7, or CCR7 alone (Figure 5C). Simultaneous activation of TCR and CCR7 in both control and DOCK2−/− 2B4 TCR-tg T cells synergistically increased ERK phosphorylation (Figure 5C; supplemental Table 1). However, absolute ERK phosphorylation levels at 2 minutes were

![Figure 3. Biochemical analysis of CCL21 costimulation.](image)

![Figure 4. CCL21-mediated costimulation in the absence of PI3Kγ- or PI3Kδ-activity.](image)
reduced in DOCK2−/− T cells by 72% plus or minus 5% (mean ± SD), with a recovery 5 minutes after stimulation (Figure 5C). CCL21 elicited similar Akt phosphorylation in both control and DOCK2−/− T cells, indicating normal PI3K function (supplemental Figure 4B). Taken together, these data suggest that DOCK2/Rac contributes to efficient ERK activation downstream both TCR and CCR7 at early time points.

**Optimal CD4+ T-cell activation inside lymphoid tissue requires GPCR signaling**

Our in vitro results indicated that CCR7 ligands act as costimulatory factors during CD4+ T-cell activation. We next performed experiments to study the influence of the chemokine-rich lymphoid microenvironment on T-cell responses, using PLN slices. One of the advantages of this system is the possibility to interact acutely with the molecular composition of the tissue. PLN slices (320 μm thickness), containing previously overlaid Marylin TCR tg CD4+ T cells, were perfused with the cognate DBY peptide during the imaging experiment. This experimental setting enabled us to measure the initiation of Ca2+ responses of T cells after their antigen encounter. Large concentrations of the antigenic peptide (100-1000 nM) triggered a strong increase in the intracellular Ca2+ concentration, which was associated with a reduction in the T-cell velocity (supplemental Figure 5; supplemental Video 3). The perfused peptide probably binds rapidly to MHC molecules expressed by resident DCs that form a dense network within the T-cell zone.41 One to 2 hours after the first Ca2+ response, a significant proportion of Marylin CD4+ T cells showed increased CD69 levels (Figure 6, supplemental Figure 4).

Next, we investigated whether GPCR ligands are involved in T-cell activation leading to increased CD69 surface levels. Marylin TCR-tg CD4+ T cells pretreated or not with PTX for 15 minutes were overlaid on PLN slices. Our previous experiments revealed that the blocking effect of PTX is only complete after 2 hours, allowing efficient migration of both control and inhibitor-treated lymphocytes into the slice. After 2 hours of PTX treatment, interstitial T-cell motility was significantly impaired compared with that of cells treated with the B subunit (suB) of the toxin that does not possess a catalytic activity and was used as a control.16

Addition of 100 nM specific peptide to slices induced rapid induction of Ca2+ flux in both control and PTX-treated populations (supplemental Video 3). On average, the mean delay between the perfusion of the peptide and the initiation of the Ca2+ response was 12.3 plus or minus 4.1 minutes for suB-treated cells and 12.0 plus or minus 1.9 minutes (n = 4) for PTX-treated cells. Moreover, the amplitude of Ca2+-flux was not affected by PTX, indicating that early TCR signaling is intact in PTX-treated lymphocytes, although the percentage of responding cells was significantly decreased.
that CCR7 and TCR do not colocalize at the T-cell–APC interface.21 These data support the notion that GPCR ligands contribute to efficient CD4+ T-cell activation inside the lymphoid microenvironment containing chemokines and other promigratory factors.

**Discussion**

The aim of this study was to examine whether CCL21 and other homeostatic chemokines present in the lymph node paracortex have a direct effect on TCR-induced intracellular signaling leading to T-cell activation. Our data suggest that the presence of CCL21 results in higher T-cell proliferation, with the costimulatory action of CCL21 being more pronounced at suboptimal activation. We provide evidence that the presence of CCL21 during the early stages of CD4+ T-cell activation leads to a selective and synergistic increase in ERK but not JNK or p38 phosphorylation, concomitant with increased expression of the early activation markers CD69 and CD25. Furthermore, we provide evidence that CCL21-triggered costimulation correlates with increased and prolonged Ras- and Rac-GTP levels. The latter was mediated by the RacGEF DOCK2 activated downstream of CCR7 and TCR, whereas PI3K activity was not required for costimulation in our system. Finally, observation of T-cell activation in PLN slices supports a costimulatory function of GPCR ligands in the paracortex.

Chemokines have previously been shown to participate in T-cell activation. For example, CCL5 induces recruitment of its ligand CCR5 and coupling with Gq/11 proteins at the interface between APC and T cells, enhancing conjugate stability and proliferation.21 This mechanism probably does not underlie the CCL21-stimulated costimulation described here, as the increased proliferation we observed was pertussis toxin sensitive, and hence Gqi-dependent, and because CCR7 is not thought to accumulate at the immunologic synapse.21 Furthermore, CCL21 also increased Ab-elicited proliferation, in the absence of adhesive ligands. It is nonetheless conceivable that CCR7-triggered integrin avidity contributes to more stable T-cell–DC interactions and therefore influences the outcome of an immune response.22 In addition, T-cell adhesion leads to potentiation of TCR signaling through ERK activation.43 The participation of adhesion in chemokine-induced T-cell costimulation deserves further investigations.

Similar to the Ras-GTP formation downstream of CCR7 reported here, CXCL12- and PI3K-dependent signals trigger a physical interaction between CXCR4 and the TCR, which promotes Ras-GTP formation and ERK phosphorylation.44,46 The fact that CCR7 and TCR do not colocalize at the T-cell–APC interface21 and the refractiveness of CCL21 costimulation to PI3K inhibition argue against a physical interaction of CCR7 and TCR. Although the precise mechanisms by which CCR7- and TCR-derived signals are integrated for synergistic Ras activation remain unknown, we identified a role for the RacGEF DOCK2 in integrating TCR- and CCR7-triggered Rac activation. DOCK2-mediated Rac-GTP formation correlated with increased and prolonged ERK phosphorylation, possibly via the Rac-effector Pak, which can phosphorylate Raf and MEK.57 Further support for a downstream role for Pak1 is provided by pharmacologic inhibition, which largely phenocopies the DOCK2 deficiency. Although we did not detect increased Raf phosphorylation after costimulation with CCL21, levels of phosphorylated MEKI1/2 were increased in TCR- and CCR7-stimulated CD4+ T cells. Thus, both Ras- and Rac-GTP are efficiently formed after costimulation with homeostatic chemokines and, via activation of MEK1/2, mediate ERK phosphorylation (Figure 7). A digital all-or-nothing, highly amplified ERK phosphorylation is a central feature of T-cell activation in vitro48-49 and may help explain the narrow peptide concentration range between no T-cell proliferation and a full proliferative response observed in vivo.5

Although DOCK2 deficiency leads to delayed ERK phosphorylation downstream of TCR and CCR7, we still observed a significant chemokine-dependent costimulatory effect on ERK phosphorylation in these cells, potentially mediated by Ras-GTP. In contrast, the proliferation of DOCK2-deficient T cells was strongly impaired. These observations suggest additional roles for DOCK2 and Rac during TCR signal transduction, including TCR and lipid raft polarization.29,50 Lymphocytes also express the RacGEF Vav1, which acts downstream both TCR and chemokine receptors.51,52 Vav1-deficiency leads to decreased Rac and ERK activation,51,53,55 among other defects. As most Rac-GTP formation downstream the TCR is mediated by DOCK2,29 defective activation of Rac in Vav1-deficient T cells may be linked because of its adapter function, rather than its GEF activity. Alternatively, Vav-induced Rac activation may depend on preceding DOCK2-mediated F-actin formation.

Deficiency in Rac2 inhibits Th1 differentiation,56 and DOCK2−/− CD4+ T cells are skewed toward a Th2 phenotype because of inefficient down-regulation of the IL-4Rα chain.57 Conversely, exposure to CCL21 increases Th1 differentiation in vitro through increased IFN-γ production,22 and CCR7 ligands induce IL-12 production in DCs.58 Taken together, these observations indicate that the CCL21-rich paracortex favors Th1 differentiation through DOCK2-Rac activity. During infections, expression of homeostatic chemokines is strongly decreased,59,60 implying a change not only in streptolysin O architecture but also in lymphocyte differentiation pathways. Secondary challenges in mice with low lymphoid chemokine levels may thus show a preferential Th2 differentiation, although other cytokines are probably involved in decision-making.

We were unable to detect any defects in activation of PI3K-γ-deficient T cells or an impairment of CCL21-mediated costimulation in our experimental system. The discrepancy to other published reports32,33 is unclear at the moment but could be the result of different transgenic models. Although PI3Kδ activity is required for full ERK phosphorylation downstream TCR signaling,51,62 expression of inactive PI3Kδ did not reduce CCL21-mediated costimulation in the experimental model used here. PI3K activity thus appears dispensable for CCL21-mediated costimulation in vitro. It will be important to investigate the function of PI3K during T-cell activation under more physiologic conditions, in particular for the generation of cytokine-producing effector T cells. This is
especially relevant as directional migration of PI3Kγ-deficient T cells is affected in the paracortex,28 and PI3Kδ/δ010/A δ010A T cells show defective raft recruitment when stimulated with surrogate APCs in vitro.62

As CCL21 contributes to some extent to the exploratory behavior of T cells in lymphoid tissue,13-16 it is experimentally difficult to dissect a costimulatory effect of chemokines from their indirect facilitation of efficient scanning of rare DCs. Besides a direct signaling effect, the promigratory functions of CCR7 ligands and other Gqi-dependent factors probably influence T-cell responses by increasing the chances for T cells to encounter and interact with APCs. Using the PLN slice system, we were able to synchronize T-cell activation in a physiologic environment while following motility, Ca2+ responses, and CD69 up-regulation as functional readout. Although CCL21 is abundant inside lymphoid tissue, other GPCR ligands contribute to DOCK2-dependent interstitial T-cell migration13,15,28 and may thus add to T-cell functional readout. Although CCL21 is abundant inside lymphoid tissue, other GPCR ligands contribute to DOCK2-dependent interstitial T-cell migration13,15,28 and may thus add to T-cell activation in vivo. We therefore used the general Gqi inhibitor PTX, which reduced the percentage of T cells showing Ca2+ flux and CD69 up-regulation. This observation may reflect the decreased cellular motility and ability to engage in productive encounters with DCs, although the responding PTX-treated T cells increase their Ca2+ with a delay similar to that of control T cells, indicating that DCs are forming a tight network inside lymphoid tissue. Notably, PTX-treated T cells showed normal Ca2+-flux responses, indicating that Gqi signaling is not generally required for T-cell activation. Nonetheless, PTX exerted an inhibitory effect on the level of CD69 expressed by antigen-stimulated T cells at the early time points measured here, suggesting that GPCR ligands contribute to optimal T-cell activation within lymphoid tissue. Because CD69 levels continuously increase over the first 24 to 48 hours of an immune response, it is possible that PTX-treated cells eventually reach similar activation levels as untreated cells.

Our data support a scenario where, within lymphoid tissue, migrating CD4+ T cells measure and integrate TCR- and GPCR-derived signals, resulting in more efficient CD69 up-regulation and IL-2 production. The physiologic situation is probably more complex, as CCL21 and other promigratory factors may influence T-cell activation both in positive and negative manners. It has been suggested that promigratory and antimigratory signals mediated by CCR7 and TCR, respectively, compete with each other, only allowing efficient T-cell activation to take place when the TCR-triggered stop signal overrides promigratory signals from chemokine gradients.23 How may these seemingly contradictory propositions on the role of CCL21 be reconciled? One potential answer may lie in the ability of T cells to integrate activation signals over time. At low doses of antigen, T cells do not immediately form tight stable contacts with DCs but undergo brief serial interaction with APCs for several hours.5 On repeated encounters with low amounts of pMHC complexes, intracellular promigratory signaling molecules may contribute to reach a lower activation threshold, thus actively participating in T-cell activation. In case T cells do not repeatedly encounter peptide-MHC complexes with a threshold frequency, no signal integration takes place. Homeostatic chemokines may thus serve as tissue “chokeast” for recirculating lymphocytes, informing them on their presence inside lymphoid tissue by increasing their sensitivity to repetitive TCR-derived signals. This may also help prevent unwanted lymphocyte activation in the nonlymphoid tissue to which naive T cells occasionally migrate.63 Simultaneously, promigratory signals may act to “silence noise” by avoiding T-cell adhesion at individual DCs displaying low affinity antigenic peptide. At later time points, full effector T-cell differentiation requires prolonged interactions with DCs, in which cell displacement is suppressed by continuous TCR signaling.23

In conclusion, our data support a role for homeostatic chemokines, in particular CCL21, during CD4+ T-cell costimulation in vitro and a role for GPCRs for T-cell activation in situ. We hypothesize that CCL21 and other GPCR ligands lower the threshold for T-cell activation in the early promigratory phase of T-cell activation by sustaining increased ERK phosphorylation levels downstream Rac and Ras.

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Authorship

Contribution: K.G., F.A.-B., and Y.T. performed experiments and analyzed results; K.O., B.V., J.R.P., and Y.F. provided genetically modified mouse strains and synthesized compounds; and K.G., E.D., and J.V.S. designed the research and wrote the paper.

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Correspondence: Jens V. Stein, University of Bern, Theodor Kocher Institute, Freiestrasse 1, 3012 Bern, Switzerland; e-mail: jstein@tki.unibe.ch.

References


CCL21 mediates CD4+ T-cell costimulation via a DOCK2/Rac-dependent pathway

Kathrin Gollmer, François Aspeti-Boursin, Yoshihiko Tanaka, Klaus Okkenhaug, Bart Vanhaesebroeck, Jeffrey R. Peterson, Yoshinori Fukui, Emmanuel Donnadieu and Jens V. Stein