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# CCL20 and $\beta$ -Defensin-2 Induce Arrest of Human Th17 Cells on Inflamed Endothelium In Vitro under Flow Conditions

Soufiane Ghannam,<sup>\*,1</sup> Cécile Dejou,<sup>\*,1</sup> Nathalie Pedretti,<sup>†,‡</sup> Jean-Philippe Giot,<sup>\*</sup> Karim Dorgham,<sup>§</sup> Hassan Boukhaddaoui,<sup>\*</sup> Virginie Deleuze,<sup>¶</sup> François-Xavier Bernard,<sup>†,‡</sup> Christian Jorgensen,<sup>\*</sup> Hans Yssel,<sup>\*</sup> and Jérôme Pène<sup>\*</sup>

CCR6 is a chemokine receptor that is expressed at the cell surface of Th17 cells, an IL-17- and IL-22-secreting population of CD4<sup>+</sup> T cells with antipathogenic, as well as inflammatory, properties. In the current study, we have determined the involvement of CCR6 in human Th17 lymphocyte migration toward inflamed tissue by analyzing the capacity of its ligands to induce arrest of these cells onto inflamed endothelium in vitro under flow conditions. We show that polarized, in situ-differentiated, skin-derived Th17 clones activated via the TCR-CD3 complex produce CCL20 in addition to IL-17 and IL-22. The latter cytokines induce, in a synergic fashion, the production of human  $\beta$ -defensin (hBD)-2, but neither hBD-1 nor hBD-3, by epidermal keratinocytes. Both CCL20 and hBD-2 are capable of inducing the arrest of Th17 cells, but not Th1 or Th2 cells, on HUVEC in an CD54-dependent manner that is CCR6 specific and independent from the expression of CXCR4, reported to be an alternative receptor for hBD-2. In addition, Ag-specific activation induces a transient loss of CCR6 expression, both at the transcriptional and protein level, which occurs with slow kinetics and is not due to endogenous CCL20-mediated internalization of CCR6. Together, these results indicate that Ag-specific activation will initially contribute to CCR6-mediated Th17 cell trafficking toward and sequestration in inflamed tissue, but that it eventually results in a transitory state of nonresponsiveness to further stimulation of these cells with CCR6 ligands, thus permitting their subsequent migration out of the inflamed site. *The Journal of Immunology*, 2011, 186: 1411–1420.

The interactions between lymphocytes in the circulation and endothelial cells lining the blood vessels are essential for in vivo lymphocyte trafficking into inflamed tissue. They are the consequence of a multistep process that involves lymphocyte rolling on endothelial cells, which is mediated by selectins and their receptors, rapid activation of integrins as a result of the interaction between lymphocyte-expressed chemokine receptors and their ligands, followed by firm adhesion of lymphocytes to endothelium through activated integrins, and, finally, diapedesis, resulting in lymphocyte infiltration into tissue (reviewed in Ref. 1). Chemokines and their receptors are important in this process because of their capacity to rapidly induce a conformational change in the integrin structure, in particular the  $\beta_2$  integrin LFA-1 (CD11a/CD18), leading to the expression of a high-affinity receptor that, following binding to its ligand ICAM-1 (CD54), will trigger immediate arrest of lymphocytes onto inflamed endothelial

cells (2–4). The rapidity of this event is important because the adhesion trigger must be delivered before the lymphocytes leave the area of inflammation. Lymphocyte arrest to endothelium near the site of diapedesis is precisely coordinated to allow lymphocyte locomotion over the endothelial cells while resisting detachment from the vessel wall by disruptive shear forces. In an in vitro model of leukocyte arrest under physiological conditions of shear flow, several chemokines, such as CXCL12, CCL19, and CCL21, that are ligands for the CXCR4 and the CCR7 chemokine receptors, respectively, have been shown capable of triggering adhesion of human peripheral blood T lymphocytes to HUVEC or to a surface coated with purified CD54 (5). Moreover, because of their selective expression pattern, chemokine receptors confer specificity to lymphocyte migration. It has been reported that CXCR3 and CCR5 on the one hand and CCR3, CCR8, and CRTH2 on the other hand are selectively expressed by human Th1 and Th2 lymphocytes, respectively, and are therefore likely to favor the trafficking of these T cell populations. However, few experimental data about the capacity of the ligands of these receptors to direct chemokine-mediated arrest in vitro have been reported. Finally, chemokine receptors might also play a role in tissue-specific lymphocyte homing, thereby defining and segregating regional immune responses, as has been demonstrated for CCR4 that reportedly mediates arrest of cutaneous, but not intestinal, mouse memory T cells to vascular endothelium (6).

CCR6 (NM A000629) is a CCR (7, 8) that is involved in host defense and inflammation, especially at epithelial surfaces (9–14). CCR6 is expressed on immature dendritic cells, whereas its expression is lost during their maturation (15). It is furthermore present on naive and memory B cells, but not on germinal center B cells (16), and is also expressed on a small fraction of memory T cells (17). In particular, the expression of CCR6 has been tightly associated with IL-17- and/or IL-22-producing T cells, referred to as Th17 and Th22 cells, respectively (18–22), both of which

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Abbreviations used in this article: [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; CHO, Chinese hamster ovary; hBD, human  $\beta$ -defensin; MFI, mean fluorescence intensity; NHEK, normal human epidermal keratinocyte; PPD, purified protein derivative; RHE, reconstituted human epidermis.

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represent Th populations with a highly inflammatory potential, notably in the skin, gut, and brain. CCR6 is highly selective for MIP-3 $\alpha$ /CCL20 (23, 24), a chemokine that is secreted by a broad spectrum of cells and tissues, including lymph nodes, appendix, liver, lungs, and endothelial cells and the production of which is induced by IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  as well as by LPS (reviewed in Ref. 25). Moreover, human  $\beta$ -defensins (hBD)-1 and -2, members of a family of small cationic antimicrobial peptides that are released by epithelial cells at mucosal surfaces and in the skin following microbial invasion or proinflammatory stimuli, also are able to bind to CCR6 and induce chemotaxis in immature dendritic cells and memory T cells (26). Furthermore, hBD-3, the antimicrobial ortholog of mouse  $\beta$ -defensin-14 (27, 28), has been reported to bind CCR6 as well. It has been shown that, despite the lack of sequence similarity between human hBD-2 and CCL20, both molecules share a part of their tertiary structure, as well as the position of several positively charged residues, thereby offering a possible explanation for their high selectivity for CCR6 and their overlap in biological functions (26, 29). Although hBD-2 has a lower affinity for CCR6, as compared with CCL20, both ligands compete for binding to their receptor (26).

Results from experimental animal models have shown that CCR6 and CCL20 play an important role in the migration of IL-17-producing T cells to inflamed sites *in vivo*. CCR6-expressing Th17 cells were found to be recruited to inflamed joints in a model of spontaneous arthritis (30), as well as to migrate through the blood-cerebrospinal fluid barrier into the CNS in a model of experimental autoimmune encephalomyelitis (31), which was associated with the expression of CCL20 in inflamed synovium (30) and the constitutive expression of this chemokine by epithelial cells of the choroid plexus (31), respectively.

In the current study, we have investigated the capacity of human tissue-infiltrating Th17 cells to directly or indirectly modulate the production of chemokine and nonchemokine ligands for CCR6 in the inflammatory environment. Furthermore, we have determined whether CCR6 ligands are able to mediate arrest of these cells onto inflamed endothelial cells *in vitro* under conditions of shear stress. Finally, as the migratory process is dependent on the activation state of the migrating T cells, we have analyzed whether CCR6 expression is stable or is subject to modulation during Ag-specific activation.

## Materials and Methods

### *mAbs, flow cytometry analysis, and FACS sorting*

The following mAbs were used for immunostaining: FITC- or PE-conjugated anti-CD4 mAbs (BD Biosciences, Le Pont-De-Claix, France), a FITC- or PE-conjugated anti-CCR6 mAb (R&D Systems, Lille, France), and FITC-conjugated anti-CD54, CD62E, and CD106 mAbs (BD Biosciences). Flow cytometry analysis and FACS sorting was carried out using the procedure described by Scheffold et al. (32), using an FACSCalibur or FACSARIA flow cytometer, respectively (BD Biosciences). Data were collected for 10,000 events/sample using CellQuest Pro software (BD Biosciences).

### *T cells*

PBMC were isolated from freshly collected, heparinized peripheral blood of healthy donors (Etablissement Français du Sang, Montpellier, France) or patients with Crohn's disease (Department of Digestive Surgery, Centre Hospitalier de l'Université St. Eloi, Montpellier, France) by centrifugation over Ficoll-Hypaque. Patients' samples were collected after informed consent according to the protocol validated by the Ethics Committee of the University Hospitals of Montpellier (Declaration DC-2008-417). CCR6<sup>+</sup> and CCR6<sup>-</sup> T cell lines were generated following staining of PBMC with an FITC-conjugated anti-CD4 mAb and a PE-conjugated anti-CCR6 mAb and electronic flow cytometry sorting using an FACSARIA cell sorter (BD Biosciences). CD4<sup>+</sup>CCR6<sup>+</sup> and CD4<sup>+</sup>CCR6<sup>-</sup> T cells, each obtained from the

same donor, were stimulated with magnetic beads, coated with anti-CD3 and anti-CD28 mAbs (Expander beads; Invitrogen, Cergy Pontoise, France) at a T cell/bead ratio of 4:1, and propagated with 2 ng/ml rIL-2 (a kind gift of Dr. John Wijdenes, Genprobe, Besançon, France). Cloned CCR6-expressing T cells specific for purified protein derivative (PPD) were generated by stimulation of 10<sup>6</sup> PBMC with 1  $\mu$ g/ml PPD (Serum Statens Institute, Copenhagen, Denmark) in a 24-well tissue culture plate (BD Biosciences). After 14 d of culture, the cells were stained with the PE-conjugated anti-CCR6 mAb, and CCR6<sup>+</sup> T cells were electronically cloned by an FACSARIA cell sorter, cultured in a feeder cell mixture consisting of irradiated (45 Gy) allogeneic PBMC, irradiated (50 Gy) JY cells, and 2  $\mu$ g/ml PHA (Remel Europe; Oxoïd, Dardilly, France) in 96-well culture plates, and propagated with rIL-2, as described (33). After 14 d of culture, T cell clones were analyzed based on the expression of CCR6, measured by flow cytometry as well as on their capacity to proliferate in response to stimulation with PPD in the presence of autologous PBMC, measured by Cell Titer-Glo luminescent cell viability assay (Promega, Charbonnières-les-Bains, France), and selected T cell clones were propagated or used in subsequent experiments. Fully *in vivo*-differentiated human Th1, Th2, or Th17 cell clones were generated from tissue-infiltrating T cells as described previously and selected on the basis of their specific cytokine production profile and the presence or absence of the lineage-specific transcription factors GATA-3 and retinoic acid-related orphan receptor C, respectively (34). T cell lines and clones were cultured in the above-mentioned feeder cell mixture, propagated with rIL-2, and used in experiments between 10 and 14 d after the onset of each propagation. All cultures and experimental procedures were carried out in Yssel's medium (35) supplemented with 1% human AB<sup>+</sup> serum (Etablissement Français du Sang).

### *Generation of T cell culture supernatants and ELISA*

Th17 clones were cultured in keratinocyte serum-free medium (Invitrogen), without supplements, at a concentration of 2.10<sup>6</sup> cells/ml in the presence of plate-bound anti-CD3 mAb (SPV-T3b; Beckman-Coulter, Roissy, France); coated for 4 h at 37°C at a concentration of 10  $\mu$ g/ml in PBS) and 1  $\mu$ g/ml anti-CD28 mAb (L293; BD Biosciences). The negative control consisted of serum-free medium incubated only with the latter mAbs. Culture supernatants were collected after 48 h of culture, and IL-17 and/or IL-22 were removed from as follows: 250  $\mu$ l goat anti-mouse IgG Ab-coated magnetic beads (Invitrogen) were incubated with 100  $\mu$ l either the neutralizing anti-human IL-17 mAb (1 mg/ml; clone 41809; R&D Systems), anti-IL-22 mAb (20E5.4; a generous gift of Dr. Wenjun Ouyang, Genentech, South San Francisco, CA), or both for 45 min at 4°C under orbital shaking. After washing with PBS supplemented with 2% FCS, the mAb-coated beads were incubated with 1 ml culture supernatant for 45 min at 4°C under orbital shaking. After removal of the beads using a magnetic device (Miltenyi Biotec, Paris, France), the culture supernatants were collected and stored at -80°C prior to their use in the experiments. The production of IL-17, IL-22, and CCL20 in the culture supernatants was measured by ELISA using DuoSet kits (R&D Systems).

### *Keratinocyte cultures and in vitro reconstituted human epidermis*

Normal human epidermal keratinocytes (NHEK) and *in vitro* reconstituted human epidermis (RHE) were generated as described previously (36) and cultured in keratinocyte serum-free medium, supplemented with bovine pituitary extract (25  $\mu$ g/ml) and recombinant epidermal growth factor (0.25 ng/ml; all purchased from Invitrogen Life Technologies) or in BIOalternatives differentiation medium, respectively. NHEK and RHE were starved for 24 or 48 h, respectively, in culture medium without growth factor supplements, prior to incubation with rIL-17 and rIL-22 (R&D Systems) or culture supernatants and the secretion of hBD-2 production determined by immunohistochemistry after 48 h of culture.

### *Chinese hamster ovary-CCR6, HUVEC, and mouse L cell transfectants*

The Chinese hamster ovary (CHO)-K1 cell line was transfected with the pcDNA3.1-CCR6 vector obtained from the Missouri S&T cDNA Resource Center (No. CCR0600000; <http://www.cdna.org>), using JetPEI 9 transfection reagent (Polyplus-Transfection, Illkirch, France) according to the manufacturer's instructions and selected with G418 sulfate (Invitrogen) at 1 mg/ml. G418-resistant cells were subcloned by limiting dilution in 96-well plates and analyzed for CCR6 expression by flow cytometry. Primary HUVEC, obtained from Clonetics (Lonza, Levallois Perret, France) were cultured in complete EGM-2MV (Lonza) supplemented with 3% FCS (HyClone; Perbio Science, Brebières, France). For experiments measuring cell arrest under flow conditions, HUVEC were seeded onto gelatin-coated

glass coverslips and cultured until a confluent monolayer was obtained. Forty-eight hours prior to the experiment, cells were stimulated with IL-1 $\beta$  and TNF- $\alpha$  (10 ng/ml each) to induce the expression of CD54. Mouse L cells, stably transfected with human CD54 (a kind gift of Dr. René de Waal Malefyt, Merck Biopharma, Palo Alto, CA) were cultured in IMDM supplemented with 10% FCS (Biowest, France), 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

#### Quantitative RT-PCR

Total RNA was extracted from cell lysates obtained with TRIzol reagent (Invitrogen). For mRNA analysis, 500 ng total RNA was reverse transcribed using Multiscribe reverse transcriptase (Applied Biosystems, Carlsbad, CA). The expression of CCL20 and hBDs was measured using the following primers, designed using Primer3 software (<http://frodo.wi.mit.edu/>): CCR6: sense 5'-TGAGCGGGGAATCAATGAATT-3' and antisense 5'-TCCTGC-AAGGAGCACAGTAACA-3'; CCL20: sense 5'-GCAACTTTGACTGCTGTCTTGA-3' and antisense 5'-TTTGGATTTGCGCACACAGA-3'; hBD-1: sense 5'-CTGGAAGCCTCTGCAGCTC-3' and antisense 5'-CAGGTGCCTTGAATTTTGGT-3'; hBD-2: sense 5'-GCC ATC AGC CAT GAG GGT CTTG-3' and antisense 5'-AAT CCG CAT CAG CCA CAG CAG-3'; hBD-3: sense 5'-AGCTGTGGCTGGAACCTTTA-3' and antisense 5'-CTTCTTCG-CAGCATTTC-3'; and ribosomal protein S9: sense 5'-AAGCCGGCC-GGGAAGTCTGAC-3' and antisense 5'-ACCACCTGCTGCGGACCT-GATA-3'.

Quantitative PCR was performed using the FastStar DNA Master SYBER Green 1 kit and a Light Cycler 480 Detection system (both from Roche, Mannheim, Germany), as specified by the manufacturer. For quantification, values express the relative mRNA level of specific gene expression as obtained using the 2<sup>- $\Delta$ CT</sup> method and *Rsp9* housekeeping gene for normalization.

#### Immunohistochemical analysis

Formalin-fixed RHE samples were ethanol-dehydrated and then embedded in Paraplast X-tra paraffin (NEYCO, Paris, France). The 5- $\mu$ m transversal sections were deparaffinized, washed, and incubated with hydrogen peroxide. The sections were incubated (1 h, room temperature) with the SC-20798 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) that is specific for hBD-2 and does not cross react with hBD-3. Specific binding was revealed using the peroxidase detection kit (Vectastain Universal Quick Kit, PK7800; Clinisciences, Montrouge, France) and an AEC substrate (K3461; Dako-Cytomation, Trappes, France). Nuclei were counterstained with a solution of hematoxylin.

#### Measurement of cell adherence under flow conditions

Arrest of T cells to HUVEC under flow conditions was measured using the technique described by Alon et al. (37). HUVEC were cultured on gelatin-coated glass coverslips until a confluent monolayer was obtained, which was then placed in a flow chamber (Flow Chamber System; Provitro, Berlin, Germany). HUVEC were either cultured in medium alone or preincubated with IL-1 $\beta$  and TNF- $\alpha$  (each at 10 ng/ml) for 48 h prior to their use in the assay. T cells were labeled with CFSE (10  $\mu$ M/ml for 10<sup>6</sup> T cells; Molecular Probes, Invitrogen), resuspended at a concentration of 1  $\times$  10<sup>6</sup> cells/ml in Yssel's medium, supplemented with 1% of human serum, and injected into the flow chamber, under conditions of shear stress, using a precision peristaltic pump IPC4N (Ismatec, Glattbrugg, Switzerland). To induce arrest of the rolling T cells, various concentrations of CCL20 and hBD-2 were added during the 3 min prior to the moment that the cells reached the flow chamber. All flow experiments were performed at 37°C using a heated microscope chamber and prewarmed medium. At first, the cells were drawn through the chamber as a bolus at 1 dyne/cm<sup>2</sup> until the front of cells entering the chamber was observed under the microscope. The shear flow was then reduced to 0.2 dyne/cm<sup>2</sup> for 1 min to allow T cell accumulation and was subsequently increased to 0.75 dyne/cm<sup>2</sup> for a 5-min period. Finally, after washing the flow chamber with medium, lymphocyte arrest was recorded by analyzing 20 fields adjacent to the center of the chamber, using an Axiovert 25 B/W epifluorescence inverted microscope, coupled to an AxioCam high-resolution numeric camera (both from Zeiss, Le Pecq, France), and all cell counts were integrated for each measurement.

#### Calcium mobilization assay

CHO-CCR6 were plated 24 h before the assay (3  $\times$  10<sup>5</sup>/well in 100  $\mu$ l) into 96-blackwell clear-bottom culture plates (Costar) in IMDM supplemented with 10% FCS. Culture medium was removed, and cells were loaded for intracellular Ca<sup>2+</sup> concentration [Ca<sup>2+</sup>]<sub>i</sub> measurement with 100  $\mu$ l HBSS, 20 mM HEPES (pH 7.4), and Calcium No Wash Plus Dye (DiscoverRx

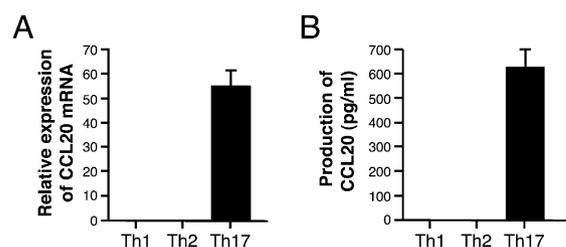
Calcium No Wash Assay Kit, Fremont, CA), supplemented with 2.5 mM probenecid (Sigma-Aldrich) to avoid calcium dye leakage from the cells. Cells were incubated at 37°C for 1 h in 5% CO<sub>2</sub> atmosphere, then 30 min at room temperature in the dark. A volume of 25  $\mu$ l/well hBD-2 and CCL20 diluted in assay buffer was added after 30 s of baseline acquisition to achieve the final indicated concentrations. The reference agonist CCL20 was added 60 s after the first compound in a volume of 25  $\mu$ l/well to reach the EC80 (350  $\mu$ g/ml) determined beforehand. Increase of [Ca<sup>2+</sup>]<sub>i</sub> was monitored for 180 s using the Flexstation 3 fluorometer system (Molecular Devices, Invitrogen) with excitation, emission, and cutoff filter set to 485, 525, and 515 nm, respectively. Maximum change in fluorescence over baseline (peak signal) was used to determine agonist response, as quantified using SoftMax Pro software.

## Results

### *Ag-activated Th17 cells produce CCL20 and induce the production of hBD-2, but neither hBD-1 nor hBD-3, by NHEK via the synergistic action of IL-17 and IL-22*

Th17 cells selectively express CCR6, a chemokine receptor that interacts with its ligand CCL20. The latter chemokine is produced by a variety of cell types in the skin, such as epidermal keratinocytes, dermal fibroblasts, and microvascular endothelial cells and is strongly enhanced under inflammatory conditions. In addition, differentiated Th17, but neither Th1 nor Th2 clones, isolated from inflamed lesional skin of patients with psoriasis were found to express CCL20 mRNA and produce CCL20 following activation via CD3 and CD28 (Fig. 1). The polarized nature of these cells was confirmed by their specific cytokine production profile, as determined in the culture supernatants of anti-CD3/CD28 mAb-activated cells (Table I). CCL20 was also produced by a PPD-specific, CCR6-expressing T cell clone following stimulation with PPD in the presence of autologous PBMC (500  $\pm$  65 pg/10<sup>6</sup> cells), confirming that the induction of CCL20 production is the result of a TCR-CD3 complex-mediated, Ag-specific activation event.

In addition to binding its chemokine ligand CCL20, CCR6 also interacts with all three members of the hBD family: hBD-1, hBD-2, and hBD-3. Human DBs are secreted by epithelial and non-epithelial cells under inflammatory conditions. We therefore investigated whether Th17 cells via their production of inflammatory cytokines could induce the production of hBDs by epithelial keratinocytes. Culture supernatants of differentiated Th17 cell clones, activated with anti-CD3 and anti-CD28 mAb, induced transcripts for hBD-2, but neither hBD-1 nor hBD-3, transcripts in NHEK (Table II). Depletion of IL-22 from the culture supernatants (Table I) only marginally (20%) inhibited hBD-2 mRNA expression, in contrast to depletion of IL-17 that completely (>98%) abolished the induction of hBD-2 transcripts. These results were mirrored by those obtained with rIL-22 and rIL-17; whereas IL-22 by itself had little effect, it strongly enhanced, in a synergistic fashion, the capacity of IL-17 to induce the expression of hBD-2 transcripts in NHEK, as well as the secretion of



**FIGURE 1.** Activated Th17 cells produce CCL20. A total of 10 Th1, 5 Th2, and 5 Th17 T cell clones were activated with anti-CD3/CD28 mAbs and the expression of CCL20 mRNA in cell lysates (A) as well as the secretion of CCL20 in culture supernatants (B) was analyzed after 6 and 24 h of culture, respectively. Values represent mean  $\pm$  SD.

Table I. Cytokine production by Th17 T cells

Treatment <sup>b</sup>	Cytokine Production (pg/ml) <sup>a</sup>		
	IL-17	IL-22	TNF- $\alpha$
Nondepleted	74,624 $\pm$ 27,328	134,159 $\pm$ 44,389	24,153 $\pm$ 3,070
-IL-17	278 $\pm$ 14	127,779 $\pm$ 38,242	25,308 $\pm$ 3,842
-IL-22	77,079 $\pm$ 28,524	4,687 $\pm$ 2,464	22,864 $\pm$ 3,471
-IL-17-IL-22	721 $\pm$ 209	5,340 $\pm$ 2,327	22,166 $\pm$ 3,365

<sup>a</sup>Th17 T cell clones were activated with anti-CD3 and CD28 mAb, and cytokine production was measured in the culture supernatant by ELISA after 48 h.

<sup>b</sup>The Th17 cell culture supernatants were depleted of contents in either IL-17, IL-22, or both cytokines, as described in *Materials and Methods*.

hBD-2 by these cells (Table II). IL-17 and IL-22, neither alone nor in combination, were able to induce the expression hBD-1 or hBD-3 transcripts. The synergistic action of IL-17 and IL-22 on the induction of hBD-2 production was confirmed by immunohistochemistry in RHE stimulated with recombinant cytokines or with Th17 cell-derived supernatants that were untreated or depleted for either or both cytokines (Fig. 2).

Taken together, these results show that, in addition to producing the chemokine ligand for CCR6, Th17 cells are also able to induce the release of the nonchemokine ligand hBD-2 by epithelial cells via the production of IL-17 and IL-22, characteristic for this lymphocyte population.

#### *CCL20 and hBD-2 mediate CD54-dependent arrest of Th17 cells to endothelial cells in vitro under conditions of shear stress*

To determine whether interaction of CCR6 with its ligands results in the arrest of Th17 cells that roll over inflamed endothelium, we used an in vitro adhesion assay with cytokine-stimulated HUVEC permitting to analyze this process under flow conditions. No rolling of T cells was observed on untreated HUVEC (results not shown). Consistent with previous reports, preincubation of HUVEC with a combination of the proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  induced, in a synergistic manner, the expression of the selectin CD54, the ligand of CD11a/CD18 (Fig. 3A, 3B), as well as that of CD62E (E selectin) and the sialoglycoprotein CD106 (VCAM-1) (Fig. 3B), thus providing the proper experimental conditions for

lymphocytes to roll and adhere. Although Th17 cells were indeed found to roll over proinflammatory, cytokine-pretreated HUVEC, no cell arrest was observed in the absence of CCR6 triggering (Fig. 4A). However, the addition of either CCL20 or hBD-2 induced a significant and consistent arrest of Th17 cells to TNF- $\alpha$ - and IL-1 $\beta$ -treated HUVEC in a dose-dependent manner (Fig. 4A, 4C). In contrast, neither CCR6 ligand was able to induce the arrest of Th2 cells that were used as a negative control (Fig. 4B, 4C) nor the arrest of Th17 cells on nonactivated HUVEC (Fig. 4C). Human BD-2 and hBD-3 reportedly can bind to another chemokine receptor, CXCR4 (38), which could potentially result in Th17 lymphocyte arrest. Therefore, to confirm the specificity of CCR6-mediated arrest of Th17 lymphocytes on TNF- $\alpha$ - and IL-1 $\beta$ -treated HUVEC, experiments were repeated with CCR6<sup>+</sup> and CCR6<sup>-</sup> cells obtained from the same donor and each expressing

Table II. Induction of hBD-2 by rIL-17 and rIL-22 or culture supernatants of differentiated Th17 cell clones

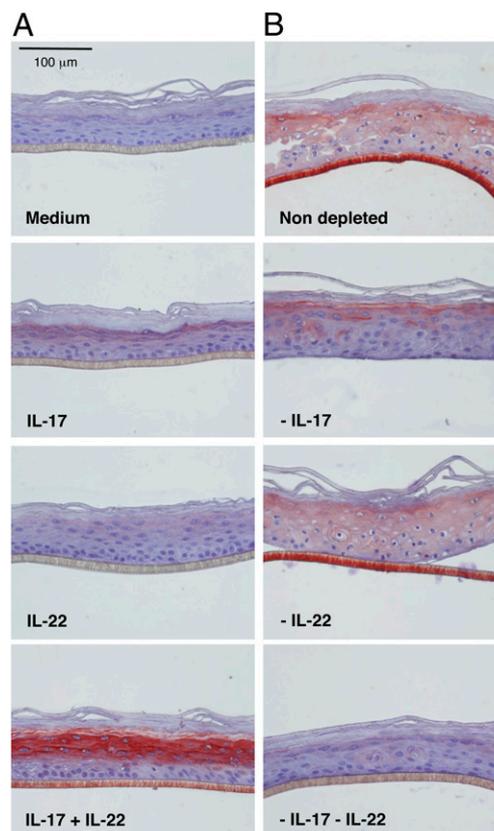
Culture Condition <sup>a</sup>	Transcripts (Relative Units)			Secretion (pg/ml)
	hBD-1	hBD-2	hBD-3	hBD-2
<b>Recombinant cytokines<sup>b</sup></b>				
Medium	1	1	1	129 $\pm$ 4
IL-17	1	363	ND	430 $\pm$ 18
IL-22	1	2	ND	535 $\pm$ 52
IL-17 + IL-22	1	442	ND	1673 $\pm$ 73
<b>CCR6<sup>+</sup> Th17 cell clone supernatant<sup>c</sup></b>				
Nondepleted	3	8304	ND	1701 $\pm$ 88
-IL-17	4	169	ND	430 $\pm$ 13
-IL-22	4	6812	ND	1377 $\pm$ 10
-IL-17-IL-22	4	258	ND	494 $\pm$ 58

<sup>a</sup>Primary epidermal keratinocytes were cultured in the presence of recombinant cytokines or culture supernatant from anti-CD3/CD28 mAb-activated Th17 cell clones.

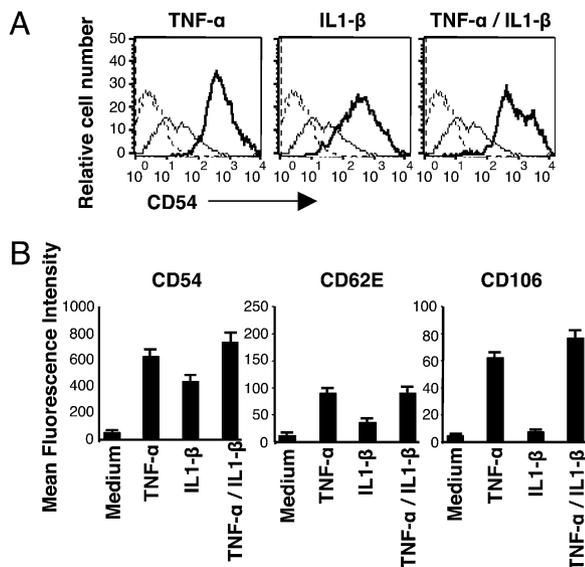
<sup>b</sup>IL-17, IL-22, or both cytokines were added at a concentration of 10 ng/ml.

<sup>c</sup>T cell culture supernatant was depleted of its contents in IL-17, IL-22, or both cytokines as described under Table I.

ND, not detectable.



**FIGURE 2.** Activated Th17 cells induce the production of hBD-2 by RHE via the synergistic action of IL-17 and IL-22. RHE were cultured in the absence or in the presence of rIL-17, rIL-22, or the combination of both cytokines (10 ng/ml) (A) as well as culture supernatants of Th17 clones ( $n = 2$ ) (B), either untreated or depleted for the presence of IL-17, IL-22, or both, and the production of hBD-2 was analyzed by immunohistochemistry after 48 h of incubation.



**FIGURE 3.** Cytokine-mediated induction of expression of adhesion molecules by HUVEC. HUVEC were preincubated for 48 h in medium alone or in the presence of either TNF- $\alpha$ , IL-1 $\beta$ , or the combination of both cytokines, each at 10 ng/ml, and the expression of CD54 (ICAM-1), CD62E (E-selectin), and CD106 (VCAM-1) was analyzed by flow cytometry. *A*, The x-axis represents fluorescence (four-decade log scale), and the y-axis represents the relative cell number. Histograms from cells stained with an isotype control mAb (dotted line, nearest the y-axis) are superimposed over the histograms of cells stained with an anti-CD54, anti-CD62E, or anti-CD106 mAb and either preincubated in medium alone (thin lines) or preincubated with cytokines (bold lines). *B*, Mean fluorescence intensity of CD54-, CD62E-, and CD106-expressing HUVEC cultured in the presence or absence of cytokines. Values of MFI are expressed as mean  $\pm$  SD;  $n = 3$ .

comparable levels of CXCR4 (data not shown; Fig. 4*B*). Whereas hBD-2 induced the arrest of CCR6<sup>+</sup> T cells, it did not affect CCR6<sup>-</sup> T cells, indicating that CXCR4 did not play a role in hBD-2-mediated arrest of Th17 cells. Finally, to confirm that the arrest of Th17 cells to HUVEC was dependent on the interaction between CD11a/CD18 and CD54, the capacity of CD54-expressing L cell transfectants (Fig. 4*D*) to mediate cellular adhesion of Th17 cells was analyzed. Th17 cells were found to adhere to CD54-transfected, but not to wild-type, L cells in the presence of CCL20 or hBD-2 (Fig. 4*E*).

The results of these experiments show that CCL20 and hBD-2 induce arrest of Th17 cells in an equipotent manner. An early signaling event following CCL20-mediated activation of CCR6 is the mobilization of [Ca<sup>2+</sup>]<sub>i</sub>. As both ligands have been reported to compete for binding to CCR6, we determined whether hBD-2 could affect the biological activity of CCL20 by measuring its capacity to block the CCL20-induced mobilization of [Ca<sup>2+</sup>]<sub>i</sub> by spectrophotometry. Whereas CCL20 induced robust [Ca<sup>2+</sup>]<sub>i</sub> responses, hBD-2 failed to do so, even at a concentration as high as 1  $\mu$ g/ml. Moreover, preincubation of Th17 cells or a CCR6-expressing transfectant with hBD-2 did not prevent subsequent induction of [Ca<sup>2+</sup>]<sub>i</sub> by CCL20 (Fig. 5).

#### Ag-specific activation results in downregulation of CCR6 mRNA and loss of CCR6 expression from the surface of Th17 cells

The expression of chemokine receptors at the cell surface is dynamic and associated with cellular activation, as well as with the cytokine environment (39). Because chemokine-induced, integrin-mediated arrest to inflamed endothelial cells is dependent on the

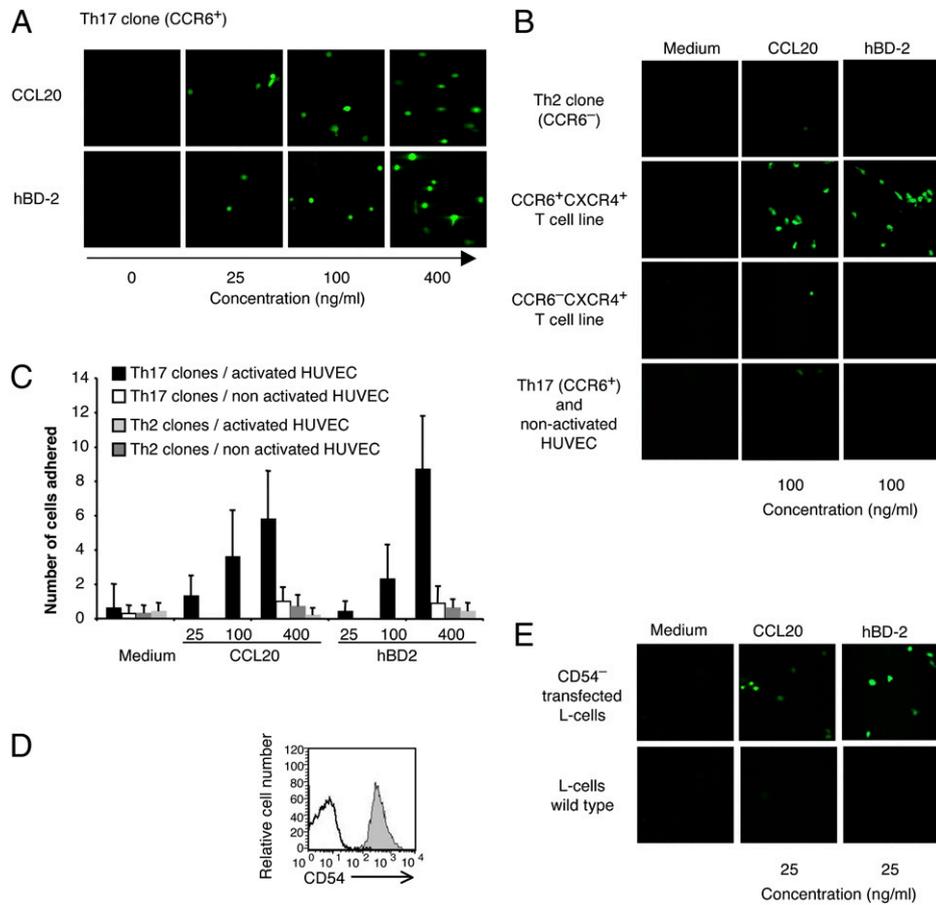
activation state of the migrating T cells, we investigated the stability of CCR6 expression on these cells following activation. Stimulation of Th17 cells with the combination of anti-CD3 and anti-CD28 mAbs resulted in a gradual decrease in CCR6 expression on the cell surface, with a complete loss after 72 h (Fig. 6*A*). Activation-induced downregulation of CCR6 expression was long lasting, and re-expression of CCR6 was generally not observed before 7 d of culture. Downregulation and loss of CCR6 expression followed similar kinetics on freshly isolated PBMC (results not shown). To confirm that activation-induced loss of CCR6 expression involved Ag-mediated TCR-mediated triggering, the experiments were repeated using a CCR6-expressing, PPD-specific T cell clone. As shown in Fig. 6*B*, stimulation of these cells with PPD and autologous monocytes also resulted in the loss of CCR6 expression with similar kinetics. Activation-induced loss of CCR6 expression was accompanied by a decrease in CCR6 mRNA expression, whereas, conversely, the restoration of CCR6 expression at the cell surface was preceded by the induction of CCR6 transcripts (Fig. 6*C*).

#### Downregulation of CCR6 expression is independent from endogenous CCL20 production

To rule out the possibility that downregulation of cell-surface CCR6 expression was due to activation-induced cell division and redistribution of this receptor on proliferating T cells, the experiments were repeated in the presence of the cell cycle inhibitor hydroxyurea. CD3-mediated activation under these experimental conditions also resulted in a decrease and subsequent loss of CCR6 expression with similar kinetics (results not shown), indicating that this process was independent of cell division. However, the capacity of Th17 cells to produce CCL20 following activation (Fig. 1) raises the possibility that the observed activation-induced decrease of CCR6 expression could be due to the interaction of this ligand with its receptor, which might result in the internalization of the CCR6–CCL20 complex. Indeed, stimulation of Th17 cells with CCL20 at concentrations as high as 100 ng/ml resulted in a rapid decrease, but no loss, of CCR6 expression in terms of the mean fluorescence intensity (Fig. 7*A*). Although maximal inhibition of CCR6 expression at the T cell surface was ~50%, measured after 30 min of incubation, the percentage of CCR6-expressing cells was not significantly affected. In addition, CCR6 expression levels were rapidly restored over time, even in the continuous presence of CCL20, which is in sharp contrast with those of TCR–CD3 complex-stimulated Th17 cells. Indeed, after 48–72 h of culture, when Ag-activated Th17 cells no longer expressed CCR6, the expression levels of CCR6 on Th17 cells incubated with CCL20 had recovered >80% of the initial levels (Fig. 7*B*). Taken together, these results show that activation-induced modulation of CCR6 expression is independent from the endogenous production of its ligand by Th17 cells and that it is, at least in part, transcriptionally regulated.

## Discussion

The efficacy of the inflammatory and anti-inflammatory effector function of lymphocytes is strongly dependent on their capacity to traffic to sites of inflammation and transmigrate into inflamed tissue during the process of diapedesis. The recruitment of lymphocytes to inflamed and lymphoid target tissues, which is initiated by chemokine-induced arrest of the trafficking cells, is mediated by sequential adhesive interactions between cell-surface molecules and their endothelial counterligands (40, 41). Lymphocyte arrest must be delicately coordinated to allow leukocyte locomotion over the endothelial barrier toward sites of transendothelial migration while resisting detachment from the vessel wall by disruptive



**FIGURE 4.** Specific ligand-mediated triggering of CCR6 induces CD11a/CD18-CD54-dependent arrest of differentiated Th17 cells on HUVEC, stimulated with IL-1 $\beta$  and TNF- $\alpha$ , in vitro under flow conditions. Fully differentiated human Th17 and Th2 clones (A–C) or purified CCR6<sup>+</sup> and CCR6<sup>-</sup> cell lines (B), generated from the same donor, were loaded with CFSE and injected into a flow chamber containing a confluent monolayer of HUVEC (A–C), preincubated for 48 h in the presence or absence of TNF- $\alpha$  and IL-1 $\beta$  (each at 10 ng/ml) or CD54-expressing or wild-type mouse fibroblasts (L cells) (E). Arrest of the T cells under flow conditions was measured following the infection of different concentrations of rCCL20 or rhBD-2 or medium as a negative control. Images are representative of 20 adjacent fields, near the center of the chamber, that were recorded for each of the experimental conditions. C, Quantification of Th2 and Th17 T cell clones having adhered in the presence or absence of CCL20 or hBD-2 to HUVEC preincubated in medium alone or with TNF- $\alpha$  and IL-1 $\beta$ . Values express mean  $\pm$  SD of the 20 counted fields per experiment for a total of three experiments. D, Flow cytometric analysis of CD54 expression by L cells transfected with CD54 cDNA. Histograms from cells stained with an isotype control mAb (bold line, nearest the y-axis) are superimposed on those from cells stained with an anti-CD54 mAb (fine line). Original magnification of all images  $\times$ 12.5.

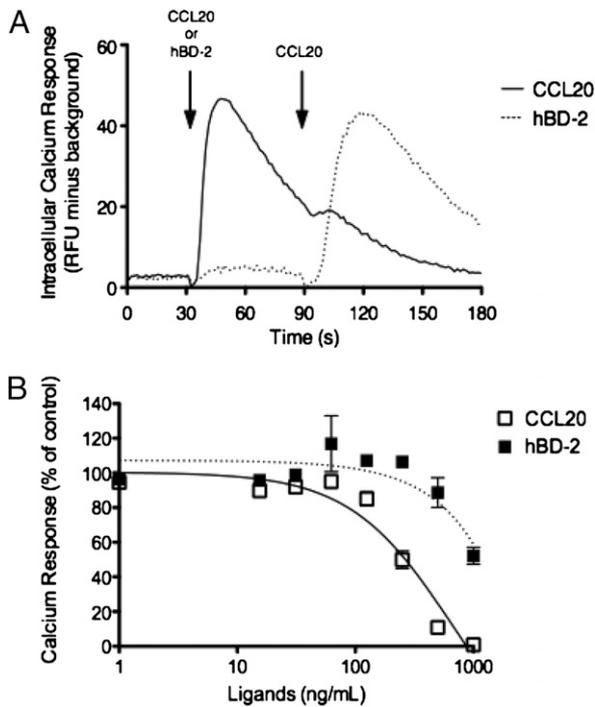
shear forces, and this process in vitro must therefore be studied under physiological conditions of shear flow (42).

Using an in vitro model of cell adhesion under shear flow conditions, we show in the current study that engagement of CCR6 with either one of its ligands, CCL20 or hBD-2, induces the arrest of IL-17- and/or IL-22-producing T cells on inflamed endothelium. Consistent with results from the literature, the presence of CD54, induced on endothelial cells by IL-1 $\beta$  and TNF- $\alpha$ , was necessary and sufficient for chemokine ligand-mediated T cell arrest, whereas the interaction of this ICAM with its counter-receptor, the integrin CD11a/CD18, was the result of the capacity of the CCR6-binding chemokines to bring about a rapid conformational modification of CD11a/CD18 (3). These results confirm and extend a previous report in the literature showing the importance of CCR6 in the arrest of subpopulations of human T cells on activated endothelium under physiologic flow conditions (40).

The involvement of CCR6 in Th17 cell trafficking to inflamed tissues has been underscored in animal models of inflammatory disease. It has been shown that CCR6 is also expressed by murine Th17 cells and that CCR6–CCL20 interaction is required for the migration of these cells to initiate self-destructive immune reactions in the joints, leading to the development of experimental

autoimmune arthritis (30). Moreover, although CCR6-deficient mice develop Th17 lymphocyte responses, they are resistant to the induction of experimental autoimmune encephalomyelitis, which was shown to be due to the requirement of CCR6 for the entry of T cells into the CNS in the early phase of the disease (31).

The migratory capacity of Th17 cells is determined by the availability of its ligands. In humans, CCL20 is constitutively expressed by epidermal keratinocytes in some dermal blood vessels in noninflamed skin (43, 44), as well as in the epithelial crypt in tonsils and epithelium of Peyer's patches (9, 45) and gut mucosa (15, 43, 46). Moreover, CCL20 was found to be constitutively produced by epithelial cells of the choroid plexus in mice and humans (31). Together, these observations indicate that Th17 cells may traffic to the skin, gut mucosal tissue, and the CNS for immune surveillance under noninflammatory conditions. However, under inflammatory conditions, the production of CCL20 is strongly upregulated in various tissues. In particular, IL-1 $\beta$  and TNF- $\alpha$  induce CCL20 production in epidermal keratinocytes (43), synovocytes (30), and endothelial cells (47). Moreover, the results of the current study show that Th17 cells, unlike differentiated Th1 and Th2 cells, express CCL20 mRNA and produce CCL20 following TCR–CD3 complex-mediated activation. Furthermore,

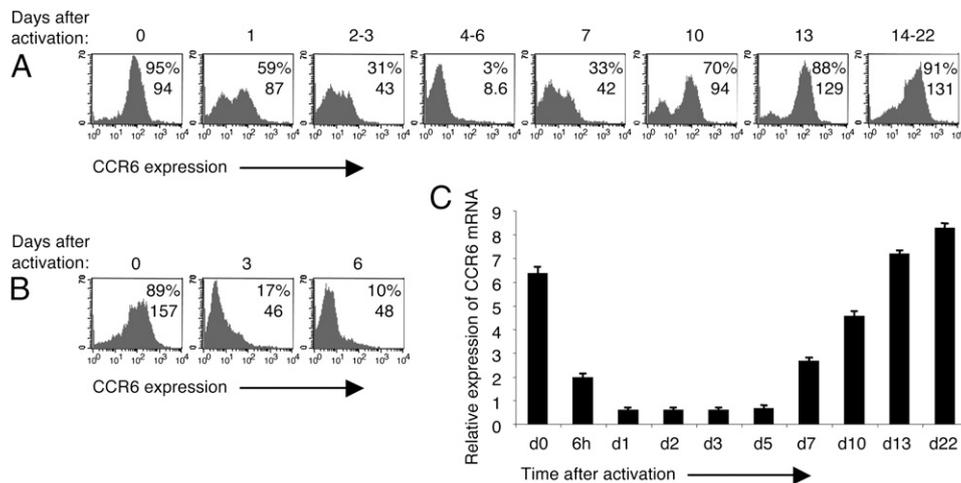


**FIGURE 5.** Calcium mobilization in CCR6-expressing CHO cells by CCL20 and hBD-2. CHO cells stably transfected with human CCR6 receptor were loaded with Ca No Wash Plus Dye and stimulated with CCL20 and hBD-2. To study CCR6 receptor desensitization, 350 ng/ml CCL20 was added 60 s after the first stimulation.  $[Ca^{2+}]_i$  concentration was monitored by measuring fluorescence for 180 s on a fluorescence plate reader. *A*, Kinetics study obtained with CCL20 and hBD-2 at 250 and 500 ng/ml final concentration, respectively. Arrowheads indicate time of application of each ligand.  $[Ca^{2+}]_i$  response was calculated as relative fluorescence units (RFU) minus background from each value. *B*, The inhibitory activity of hBD-2 (filled squares) and CCL20 (empty squares) was assayed at indicated concentrations on calcium response elicited in CHO-CCR6 by 350 ng/ml CCL20. Data are representative of three independent experiments.

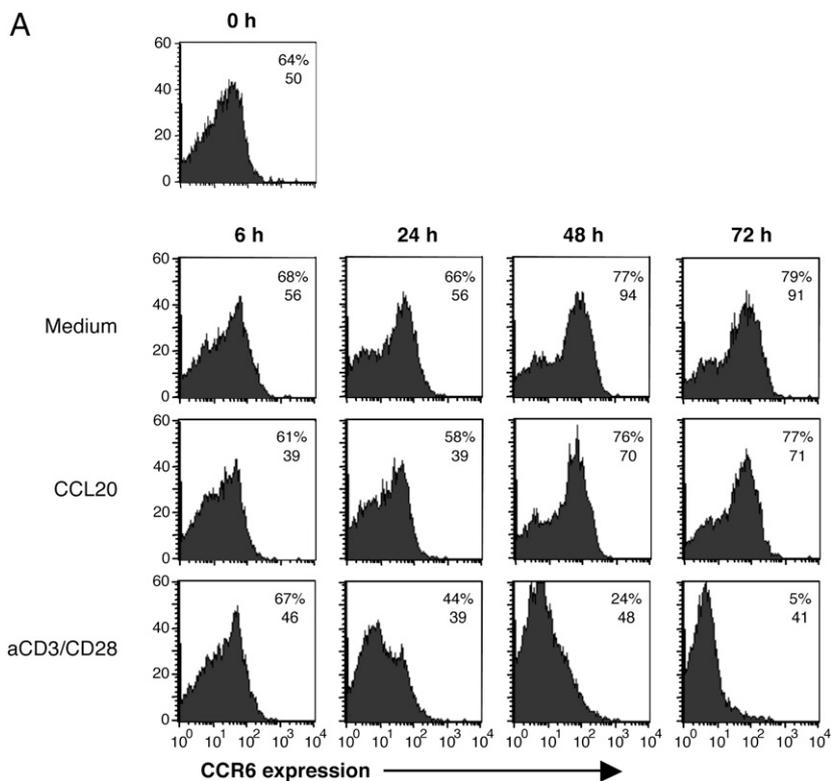
Th17 cells induce the production of hBD-2 by epidermal keratinocytes via the synergistic activity of IL-17 and IL-22 in mice (48) and human (this study). This second ligand for CCR6 (49) is a

member of a family of small, cationic antimicrobial peptides that are released by epithelial cells at mucosal surfaces or in the skin, either upon microbial invasion or in the presence of proinflammatory stimuli. In contrast, neither IL-17 nor IL-22 induced the expression of hBD-1 or hBD-3. It can, however, not be excluded that other cytokines secreted by proinflammatory cells induce the production of the latter hBDs. Indeed, culture of NHEK in the presence of rIFN- $\gamma$  was found to induce the expression of hBD-3 transcripts (results not shown), in agreement with an earlier report in the literature (27). It is of note that the differentiated Th17 clones used in the current study produce only very low levels of IFN- $\gamma$  ( $366 \pm 40$  pg/ml), which may explain their lack of hBD-3-inducing activity. Although the affinity of hBD-2 for CCR6 is reportedly lower than that of CCL20 (26), it was found to be as effective in inducing the arrest of CCR6-expressing cells in vitro, and this nonchemokine ligand is likely to play an important role in the recruitment of Th17 cells to and/or sequestration in inflamed tissue in distinct organs, in particular to the skin and the gut, where it is abundantly produced. Together, these observations indicate that Ag-activated Th17 cells not only favor their own arrest onto inflamed endothelium via the autocrine production of CCL20 and TNF- $\alpha$ , but also, once recruited to the site of inflammation, are able to induce the production of hBD-2 by epithelial cells that is likely to result in a prolonged sequestration and thus contributing to an intensified local inflammatory environment.

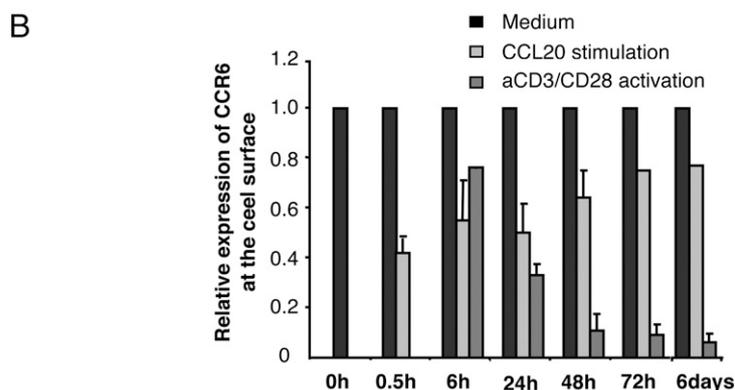
The results from multiple independent experiments carried out in the current study show that stimulation of CCR6-expressing Th17 cells with hBD-2 did not lead to  $Ca^{2+}$  mobilization in contrast to stimulation of these cells with CCL20. Moreover, preincubation of Th17 cells with hBD-2 did not inhibit the CCL20-induced  $Ca^{2+}$  mobilization. This finding is surprising because of the capacity of hBD-2 to induce cell arrest on endothelium and the generally accepted concept that CCR6-mediated signaling is associated with the mobilization of  $Ca^{2+}$  from intracellular stores. It has to be noted, however, that the scarce studies described in the literature on the possible role of  $Ca^{2+}$  in hBD2-mediated signaling via CCR6 have been carried out using transfected cell lines and that they have yielded contradictory results; whereas hBD2 was reported to induce both  $Ca^{2+}$  mobilization and chemotaxis in the human CCR6-expressing colonic epithelium cell line COLO2 (50), it had



**FIGURE 6.** Ag-specific activation results in downregulation of CCR6 mRNA and loss of CCR6 expression from the surface of Th17 cells. A Th17 cell clone was stimulated with the combination of anti-CD3 and anti-CD28 mAbs (*A*), or a PPD-specific CCR6-expressing T cell clone was stimulated with PPD and autologous monocytes (*B*), and the evolution of CCR6 expression at the cell surface was analyzed by flow cytometry. The x-axis represents fluorescence (four-decade log scale), and the y-axis represents the relative cell number. Values in histograms represent percentage of CCR6<sup>+</sup> T cells and mean fluorescence intensity. Isotype-matched control mAb as in Fig. 4*D*. *C*, Cells, stimulated as under *A*, were collected at the indicated time interval after their activation, lysed, and CCR6 mRNA expression was determined by real-time PCR. Values represent mean  $\pm$  SD of three experiments.



**FIGURE 7.** Downregulation of CCR6 expression on Th17 cells is independent from endogenous CCL20 production. Th17 cells were incubated in medium only or in the presence of CCL20 (100 ng/ml) or were activated with anti-CD3 and anti-CD28 mAb. CCR6 expression was analyzed at the indicated time periods by flow cytometry. *A*, Flow cytometry analysis data from a representative experiment. The *x*-axis represents fluorescence (four-decade log scale), and the *y*-axis represents the relative cell number. Values indicated in the upper right quadrants represent the percentage  $\pm$  SD of CCR6-positive cells and their relative fluorescence intensity. *B*, Evolution of cell-surface CCR6 expression expressed as the relative fluorescence intensity  $\pm$  SD obtained in three independent experiments.



no effect on the human mast cell line RBL-2H3 cells that had been stably transfected with the CCR6 gene (51). It is most likely due to differences in the experimental tools and/or conditions, and further studies are needed to determine the molecular factors that underlie this discrepancy. Nevertheless, the results obtained with CCR6-expressing T cells in the current study are in agreement with a previous report in the literature showing that hBD-2-induced chemotaxis in human monocyte-derived macrophages is not accompanied by the mobilization of  $\text{Ca}^{2+}$  (51). A similar observation has been made in neutrophils showing that hBD2 was able to induce chemotaxis in a CCR6-dependent manner in the absence of mobilization of intracellular free  $\text{Ca}^{2+}$  (52). The results described in our study therefore strongly suggest that hBD-2-induced arrest of CCR6-expressing Th17 cells onto inflamed endothelium might occur in the absence of  $\text{Ca}^{2+}$  signals.

It was reported by Yang et al. (26) that hBD-2 displaces the binding of iodinated CCL20 to CCR6-expressing cells in a dose-dependent manner, which shows that both ligands compete for receptor occupancy. However, because either ligand induces Th17 cell arrest under flow conditions, the sequential incubation of the cells with CCL20 and hBD-2 will always lead to cell arrest, and

their competition for binding to CCR6, therefore, does not translate into a functional antagonistic effect.

Whereas CCL20 is truly specific for CCR6, hBD-2 has been reported to be a ligand for CXCR4 as well (38). To demonstrate the involvement of CCR6 in hBD-2-mediated arrest of Th17 cells *in vitro*, several CCR6<sup>+</sup> and CCR6<sup>-</sup> T cell lines, each pair obtained from the same donor, were used in the current study. Based on the selection procedure, using FACS sorting with a specific anti-CCR6 mAb, these cells differ only in their expression of CCR6, whereas their expression of CXCR4 is comparable. As the cell-surface expression of both CCR6 and CXCR4 is downregulated following activation (53), the T cell lines were used in a resting state expressing both chemokine receptors. The results from *in vitro* arrest experiments under shear stress with these CCR6<sup>+</sup> and CCR6<sup>-</sup> T cell lines are similar to those obtained with the Th17 and Th2 cell clones, respectively, showing that only the CCR6-expressing cells can be induced to adhere to inflamed endothelium under flow conditions following stimulation with hBD-2 or CCL20 and thus confirm that the involvement of hBD-2 is specific for CCR6 and that hBD-2-induced lymphocyte arrest is not mediated via interaction with CXCR4.

In addition to inducing the production of CCL20, TCR-CD3 complex-mediated activation of Th17 cells also caused a transient loss of CCR6 expression from their cell surface. Like many other chemokines and their specific receptors, interaction of CCL20 with CCR6 results in a rapid internalization of the receptor-ligand complex, as has been shown for dendritic cells (54), raising the possibility that activation-induced modulation of CCR6 expression could be mediated via the autocrine production of its ligand by the activated T cells. Several observations, however, point to an alternative mechanism involving a regulation at the transcriptional level. Activation-induced loss of the expression of this chemokine receptor was accompanied by an inhibition of CCR6 mRNA expression in the activated cells that preceded the production of CCL20 by activated Th17 cells. In addition, re-expression of CCR6 at the cell surface of the latter cells was also preceded by the expression of CCR6 transcripts. Furthermore, in contrast to ligand-induced internalization of CCR6 expression, TCR-CD3 complex-induced downregulation of CCR6 expression from the cell surface occurred with slow kinetics, as a complete loss of CCR6 expression was not observed before 72 h of activation. Although the duration of downregulation of CCR6 expression following interaction with its ligand *in vivo* is not known, the decreased expression at the cell surface is only transient, as prolonged internalization of chemokine receptors requires the constant presence of the specific ligand, whereas internalized chemokine receptors are quickly recycled and redistributed at the cell surface, as shown, for example, for CXCR4 (55). Although incubation of freshly isolated PBMC (results not shown) or CCR6<sup>+</sup> Th17 lymphocyte clones (Fig. 7) resulted in a decrease in CCR6 expression following incubation of the cells with a concentration of CCL20 at >100-fold excess as that detected in culture supernatants of activated Th17 cells, the decreased expression of the CCR6-CCL20 complex was incomplete and transient. Moreover, even in the continuous presence of CCL20, CCR6 expression levels were restored at the time that all of the TCR-CD3 complex-activated T cells had completely lost CCR6 expression.

The data described in this study confirm previously reported results that showed a decrease in CCR6 and CXCR3 expression on CD4<sup>+</sup> bronchial T cells following segmental allergen challenge of human atopic asthmatic patients (56). However, it was hypothesized from the latter results that these receptors were internalized due to their encounter with their respective ligands in the airways, not taking into account the direct effect of T cell activation on CCR6 expression, as underscored in the current study.

Although it is difficult to extrapolate *in vitro* production levels of CCL20 to the *in vivo* situation, in which local concentrations of this CCR6 ligand might be much more elevated, as a consequence to their binding to heparan sulfates, the results nevertheless suggest that the loss of CCR6 expression following Ag-mediated Th17 cell activation is independent from the autocrine production of its ligand by these cells. Another possible mechanism underlying the modulation of the expression of this chemokine receptor might involve lipid rafts, membrane-bound microdomains that are enriched with cholesterol, sphingolipids, and saturated fatty acids and that function as a scaffolding platform for a variety of cellular functions, including signal transduction events (57). It has recently been shown that cholesterol depletion of CCR6-expressing Jurkat T cells impairs CCL20-mediated CCR6 signaling, suggesting that lipid rafts are necessary for CCR6 activation (58). It can therefore be postulated that Ag-specific activation of CCR6-expressing T cells might result in a remodeling of the distribution of CCR6 at the cell surface, a subsequent exclusion of the receptor from the rafts and transport to clathrin-coated pits, thereby facilitating its internalization in the endosome and its subsequent degradation

by the proteasome. This hypothesis is currently under investigation in our laboratory.

In conclusion, the data described in this study support those obtained in experimental animal models showing that CCR6, through the action of both its ligands, plays an important role in the recruitment of Th17 cells to inflamed tissues, thereby contributing to the perpetuation of inflammation. Similar to dendritic cells that downregulate CCR6 expression following interaction with CCL20, the loss of this chemokine receptor in the inflammatory environment by Th17 cells, as a result of prior Ag-specific activation, will cause these cells to escape from the sequestration-inducing effects of CCR6/ligand interaction, permitting them to migrate out of the inflamed site to the draining lymph nodes.

Finally, because of the important role of CCR6 in the deleterious activity of IL-17- and/or IL-22-producing CCR6-expressing T cells in the pathogenesis of inflammatory disease, this chemokine receptor may represent a prime target for the development of therapeutics with antagonistic activity. However, CCR6 ligands perform poorly in the classic chemotactic assay, and the methodological approach to determine cell arrest under conditions of shear flow used in the current study is of greater relevance to determine the functional activity of such compounds that interfere with CCL20- and hBD2-induced cell arrest.

## Disclosures

The authors have no financial conflicts of interest.

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