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Pan–CC Chemokine Neutralization Restricts Splenocyte Egress and Reduces Inflammation in a Model of Arthritis

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Chemokines are key regulators of leukocyte trafficking and play a crucial role under homeostatic and inflammatory conditions. Because chemokines are involved in multiple pathologies, they represent an attractive class of therapeutic targets. However, because of the redundancy of this system, neutralizing a single chemokine may be insufficient to achieve therapeutic benefit. Our strategy was to use a Fc-fusion recombinant protein form of the poxvirus-derived viral CC chemokine inhibitor protein (vCCI-Fc) that has the ability to specifically bind to multiple CC chemokines and neutralize their activity. In this study, we demonstrate first that, in vivo, vCCI-Fc prevents CC chemokine-dependent migration of macrophages into inflamed tissue of carageenan-challenged mice. We next studied this effect of inhibiting CC chemokine activity in a model more relevant to human disease, collagen-induced arthritis. Mice receiving vCCI-Fc revealed a striking retention of splenocytes, including activated and IFN-γ-secreting CD4+ and CD8+ T cells, that was associated with a concomitant decrease of cells in the draining lymph nodes. These phenomena resulted in a significant decrease in the incidence of disease and a reduction in clinical score, joint inflammation, and cartilage destruction as compared with mice receiving isotype control. Taken together, these results define a role for CC chemokines in the control of disease, as interfering with their function leads to a previously unappreciated role of controlling inflammatory cell trafficking in and out of secondary lymphoid organs. The Journal of Immunology, 2010, 185: 2544–2554.

The trafficking of cells has been shown to be regulated by chemokines, a protein superfamiliy that is divided into four subfamilies based on the arrangement of their amino-terminal cysteine residues (C, CC, CXC, and CX3C) (1). Their biological effects are mediated through seven transmembrane G protein-coupled receptors (2). In general, CC chemokines are chemoattractant for monocytes and T cells, CXC for neutrophils, whereas lymphotactin (the only C chemokine) attracts T and NK cells (3, 4). Soluble fractalkine (the only CXC chemokine) attracts monocytes, T cells, and NK cells (3, 4). Although the expression of most chemokines is induced during infection and inflammation, some chemokines, including CCL19 and CCL21, are constitutively expressed and control cell movement during homeostasis (5). Inflammatory chemokines control cell migration to the inflamed tissue due to their potential to attract and activate leukocytes (6). Chemokines have been shown to play an important role in multiple inflammatory and autoimmune diseases (1). Therefore, inhibiting cell traffic toward sites of inflammation is an actively pursued therapeutic strategy (7, 8). However, antagonizing chemokine signaling by neutralizing a single ligand or receptor might be limited by the redundancy of this biological system.

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Poxviruses, a family of complex DNA viruses, have evolved unique strategies to evade the host immune response by producing immunomodulatory factors (9–11). Viral CC chemokine inhibitor (vCCI) is a soluble 35-kDa protein encoded by the vaccinia virus. This viral protein displays a multiple chemokine binding capacity for both human and murine CC chemokines, albeit with different affinities (12), and it consequently neutralizes function by preventing interaction with cognate receptors on target cells (13, 14). Thus, because of its capacity to bind to a broad range of CC chemokines with high affinity, vCCI represents a powerful tool for dissecting the role of CC chemokines in cell migration in homeostatic conditions and in diseases driven by inflammation. Indeed, vCCI has been shown to alter both allergic murine airway hyperreactivity (15) as well as arteriole occlusion in a model of atherosclerosis (16).

To better understand the role of CC chemokines in the trafficking of cells under inflammatory conditions, we chose to study collagen-induced arthritis (CIA), a well-established murine model that shares clinical and pathological features of the human disease rheumatoid arthritis (RA) (17, 18). This autoimmune disorder is characterized by synovial membrane hyperplasia and erosion of bone and cartilage leading to destruction of joints, which is associated with the recruitment of inflammatory cells, including T cells, macrophages, and dendritic cells (19). In this disease, proinflammatory chemokines are thought to play a pivotal role in the attraction of leukocytes to sites of inflammation leading to the initiation, maintenance, and progression of the inflammatory process (20, 21). Indeed, a number of CC chemokines, including CCL5, CCL2, CCL3 and CCL4, are present at elevated levels in the joints of arthritic patients (22–25) as well as in animal models of this disease (26, 27). These levels coincide with the recruitment of, for example, CCR5+ monocytes and T cells into human synovial tissues (28). The functional importance of individual CC chemokines in eliciting inflammation has been assessed using murine models of RA in conjunction with neutralizing agents, such as...
blocking Abs (26, 29), low–molecular mass compounds (30, 31), or a fusion protein (32). However, the heterogeneity of the results and marginal potency of the effect suggest that redundancy in chemokine biology may compensate for neutralizing a specific ligand or receptor.

In this study, we first established that vCCI is able to block cell migration into local inflamed tissue using an in vivo air pouch model. From those results, we were able to administer vCCI-Fc such that it also interfered with establishing arthritis in the CIA model. The striking effect of vCCI-Fc on disease was surprisingly via alteration of leukocyte trafficking through the architecture of secondary lymphoid tissues. The data demonstrated the retention of cells, including IFN-γ-producing T cells and activated T cells in the splenic compartment, suggesting that CC chemokines play a major role in cell trafficking out of the spleen. Adopting a strategy to interfere with multiple chemokines designated as part of the CC subset may thus prove highly beneficial when designing new therapeutic agents to treat inflammatory diseases, such as RA.

Materials and Methods

Recombinant chemokines

The genes encoding mouse (m) CCL5, mCCL2, mCCL22, mCCL3, and mCCL4 were cloned from human cDNA in the pEAK8 expression vector (Edge Biosystems, Gaithersburg, MD), and an AviTag biotinylation site was introduced at the C terminus of the protein for single-site in vivo biotinylation (Avidity, Aurora, CO). Biotinylated mCCL2 and mCCL10 were produced as transcription elongation protein nusA fusion proteins as previously described (33). Recombinant chemokines used for BIACore analysis and chemotaxis assays were purchased from PeproTech (London, U.K.).

vCCI-Fc cloning, expression, and purification

The entire coding sequence of the vCCI gene from vaccinia virus (Lister strain) was fused to the hinge region of the human IgG1 (hIgG1) Fc fragment and cloned into the pFastBacDual baculovirus plasmid (Invitrogen, Basel, Switzerland). Protein expression was performed using the Bac-to-Bac baculovirus expression system (Invitrogen) in Sf9 cells. Supernatants containing vCCI-Fc were centrifuged and filtered before being concentrated on a 30-KDa membrane (Sartorius, Goettingen, Germany). The concentrate was loaded on a HiTrap protein G HP column (GE Healthcare, Rockford, IL) using an ÄKTA prime chromatography system (Amer sham Biosciences, Buckinghamshire, U.K.).

Cells and culture media

Murine pre-B lymphoma L1.2 cells were stably transfected with either human (h) CCR5, hCCR4, hCCR7, hCCR2, or hCXCR3 cultured as previously described (33). PEAK cells were cultured in DMEM (D5796; Sigma-Aldrich), supplemented with 10% FCS (F7524; Sigma-Aldrich), 2 mM l-glutamine (G7513; Sigma-Aldrich), and 25 μg/ml gentamycin (G1397; Sigma-Aldrich). Spodoptera frugiperda Sf9 cells were cultured in suspension in SF-900 II serum-free medium (Invitrogen) supplemented with 2 mM l-glutamine and incubated at 27°C with shaking. Splenocytes and cells obtained from draining lymph nodes (dLN)s) were cultured in complete RPMI (eRPMI) consisting of RPMI 1640 medium (D5796; Sigma-Aldrich) supplemented with 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate (S8636; Sigma-Aldrich), 0.5 μM β-mercaptoethanol (31350-010; Invitrogen), and 25 μg/ml gentamicin.

Chemokine ELISA

Eight-well StreptaWell strips (Roche, Rotkreuz, Switzerland) were coated with 2 μg/ml transcription elongation protein nusA fusion chemokine or biotinylated chemokine-containing desalted PEAK supernatant. The strips were then washed with PBS/0.05% Tween 20. vCCI-Fc, diluted in 1% BSA-PBS, was added at a concentration of 2 μg/ml and incubated for 1 h at room temperature. Strips were washed again and incubated with an anti-hIgG (Fcγ)-specific mAb conjugated to HRP (1:4000; Jackson ImmunoResearch, West Grove, PA) in 1% BSA-PBS for 30 min at room temperature. After washing, the interaction was revealed with 3,3′,5,5′-tetramethylbenzidine substrate (TMB, T5569; Sigma-Aldrich) and the reaction was stopped with H2SO4. The absorbance at 450 nm was recorded using a Synergy HT multidetection microplate reader (BioTek Instruments, Winooski, VT).

Surface plasmon resonance analysis

Nine hundred fifty response units of anti-hIgG (Fcγ-specific mAb) (Jackson ImmunoResearch Europe) was immobilized onto a C1 sensor chip using a BIACore 2000 system (Biacore, Oettingen, Switzerland). vCCI-Fc was injected at 20 μl/min and captured at a level of ~190 response units. Chemokines were diluted in running buffer HBS-EP (10 mM HEPES [pH 7.4]/150 mM NaCl/3 mM EDTA/0.005% surfactant polysorbate 20 [v/v]) to a final concentration of 20 μg/ml. For association phases, 15 μl of chemokine was injected and 300 s were allowed for dissociation in HBS-EP at the same flow rate of 20 μl/min. The surface was regenerated using 10 mM glycine, HCl (pH 1.5).

In vitro chemotaxis assay

L1.2 cells expressing hCCR5, hCCR4, hCCR7, hCCR2, or hCXCR3 were cultured in cRPMI. The day before the experiment, cells were incubated with 0.6 mg/ml butyric acid. Different concentrations of purified vCCI-Fc were incubated with 0–40 μM mouse chemokine and placed in the bottom chamber of chemotaxis 96-well plates (NeuroProbe, Gaithersburg, MD). The filter plate was placed on top of the chemotaxis plate, and each well was overlaid with 20 μl of a 105 cells/ml suspension. The plate was incubated for 3 h at 37°C. Cells that migrated through the filter were stained with a 10 μM final concentration of DRAQ5 (Alexis, Lausen, Switzerland) and counted on an FMAT 8200 reader (Applied Biosystems, Foster City, CA).

Mice

All in vivo experimentation was performed using 8-wk-old male DBA/1J mice (Janvier Laboratories, Le Genest-St-Isle, France) and female C57BL/6 mice (Charles River Laboratories, L’Arbresle, France). Experiments were performed in compliance with Swiss experimental animal regulations.

Air pouch model

This model of local inflammation was performed using female C57BL/6 mice as described by Romano et al. (34). Briefly, anesthetized mice were injected with 6 ml filtered air under the skin on a dorsal site. After 4 d, 4 ml air was reinjected into the air pouches. On day 7, mice received i.v. either 200 μg vCCI-Fc or 200 μg control hIgG1 mAb (Novimmune, Geneva, Switzerland). Six hours later, 1 ml of 1% α-carrageenan (Sigma-Aldrich) was injected into the pouch. After 72 h, mice were euthanized and pouches were flushed with 2 ml PBS and this fluid was collected. The lavage fluid was immediately put on ice and the volume recorded. The samples were centrifuged and cells of the pellets were analyzed by flow cytometry.

CIA induction and treatments

Lyophilized bovine collagen type II (CII) (MD Biosciences, Zurich, Switzerland) was dissolved in 0.1 N acetic acid at 2 mg/ml and emulsified in an equal volume of CFA (Difco, San Jose, CA). Male DBA/1J mice were immunized intradermally with 100 μl (i.e., 100 μg bovine CII) of the emulsion at the base of the tail. After 21 d, mice received a secondary intradermal immunization with 100 μg bovine CII emulsified in IFA. On days 20, 21, 22, 25, 27, 29, 32, 34, 36, and 39 after the first bovine CII immunization, 200 μg vCCI-Fc or control hIgG1 mAb was given i.v.

Clinical assessment of the disease

Clinical scores were assessed immediately after the secondary immunization three times per week. CIA severity was graded by overall assessment of inflammation on individual paws, applying a scale ranging from 0 to 4. Each paw was graded according to the following system: 0, no inflammation; 1, swelling of at least one digit; 2, swelling of all digits and inflammation of tissue surrounding digits; 3, severe inflammation of whole paw and digits or ankylosis; and 4, necrosis. The sum of these four individual scores is represented as the total clinical score, that is, with the maximal clinical score of 16 for an individual mouse.

Histology

Specimens were collected and fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 10 d, rinsed in PBS, and transferred to an EDTA-based decalcifying solution (Osteosoft, Merck, Germany) for 3 wk. Samples were then rinsed in PBS, transferred through a succession of alcohol concentrations, and processed into paraffin blocks using an automated tissue processes.
sor (STP 120 from Microm Microtech, Francheville, France). Sagittal sections (7 μm) of the whole knee joint were stained with either H&E or safranin O, with counterstaining using fast green. Histological sections were graded by an assessor who was blinded to the corresponding treatment group, using an established scoring system for synovial hyperplasia and cell infiltration (from 0, indicating no hyperplasia, no cell infiltration, to 3, indicating most severe hyperplasia and cell infiltration) and cartilage damage as determined by loss of safranin O (red) staining (from 0, indicating no hyperplasia, no cell infiltration, to 3, indicating total loss of safranin O staining).

Polyclonal T cell restimulation

LNs located in the popliteal fossa, which represented the dLNs, were harvested and pooled per group. Spleens were processed and analyzed individually. Both organs were digested with collagenase (2.4 mg/ml; Invitrogen) and DNAse (1 mg/ml; Sigma-Aldrich) for 30 min, resuspended in RPMI, and the cell numbers were obtained using trypan blue. Spleen or dLN cells (1 × 10^6) were resuspended in RPMI, placed in 96-well plates precoated with 2 μg/ml purified anti-CD3 mAb (clone 145-2C11; BD Biosciences, San Jose, CA), and 2 μg/ml soluble anti-CD28 mAb (clone 37.51; BD Biosciences) was then added for a 6-h culture at 37°C. During the last 2 h, GolgiPlug (BD Biosciences), containing brefeldin A, was added. Cells were then processed for flow cytometry.

Flow cytometry analysis

For cell surface or intracellular labeling, the following mAbs were used (all from BD Biosciences, unless indicated otherwise): FITC-conjugated anti-F4/80 (clone A3-1; AbD Serotec, Düsseldorf, Germany), -CD3 (clone 145 2C11), and -CD4 (clone H129.19); PE-conjugated anti-CD8 (clone 53-6.7), -CD4 (clone IM7), -Gr1 (clone RB6-8C5); PerCP-conjugated anti-CD4 (clone RM4-5) and -CD8 (clone 53-6.7); PerCP-Cy5.5-conjugated anti-CD19 (clone 1D3); or allophycocyanin-conjugated anti-CD62L (clone ME14) or -CD45 (clone 30-F11). For intracellular staining, cells were then permeabilized and fixed following the instructions of the BD Cytofix/ Cytoperm Plus kit and labeled with allophycocyanin-conjugated anti–IFN-γ (clone XMG1.2). Absolute cell number in blood was quantitated using BD TruCount tubes (BD Biosciences). Analysis was performed using the FACSCalibur flow cytometer (BD Biosciences).

Detection of Abs to vCCI-Fc

Immunogenicity to vCCI-Fc was assessed by ELISA. Briefly, serially diluted serum samples were added to each well of vCCI-Fc– (2 μg/ml; R&D Systems, Minneapolis, MN) or hIgG1 (2 μg/ml)-precoated immunoplates (MaxiSorp; Nunc, Roskilde, Denmark). After incubation for 2 h at 37°C, plates were washed with PBS/0.05% Tween 20, and anti–vCCI-Fc or anti-hIgG1 Abs were measured using an anti-mouse IgG (Fcγ-specific) conjugated to HRP (Jackson ImmunoResearch Europe). After washing, the interaction was revealed with TMB and the reaction was stopped with H2SO4. The absorbance at 450 nm was recorded using a Synergy HT multidetection micro plate reader (BioTek Instruments).

Measurement of serum vCCI-Fc concentration

The concentration of vCCI-Fc in sera was assessed by ELISA. Briefly, diluted serum samples (1/1000), or known concentrations of vCCI-Fc (R&D Systems), were added to vCCI-Fc-specific capture mAb (2 μg/ml; R&D Systems, Abingdon, U.K.)-precoated immunoplates (MaxiSorp; Nunc, Roskilde, Denmark). After incubation for 2 h at 37°C, plates were washed with PBS/0.05% Tween 20, and bound vCCI-Fc was measured using anti-human IgG (Fcγ-specific) conjugated to HRP (Jackson ImmunoResearch Europe). After washing, the interaction was revealed with TMB and the reaction was stopped with H2SO4. The absorbance at 450 nm was recorded using a Synergy HT multidetection micro plate reader (BioTek Instruments).

Statistical analysis

For the clinical score, each time point was assessed using a two-tailed Mann-Whitney U test. For others analyses an unpaired t test was applied. All data were expressed as means ± SEM or means ± SD.

Results

vCCI-Fc selectively binds and neutralizes multiple CC chemokines in vitro

Certain characteristics of the vaccinia virus–encoded protein, vCCI, have previously been described (12–14, 35, 36). vCCI binds to a large panel of CC chemokines with varying affinities and is able to neutralize their activity in vitro (12–14, 35, 37). We generated a vCCI-Fc fusion protein and tested its specificity and neutralization potential in vitro. Using an ELISA-based format, vCCI-Fc was found to bind to members of the CC family, including mouse (m) CCL2, mCCL22, mCCL3, mCCL4, mCCL21 (Fig. 1A), and human (h) CCL5 (data not shown), but not the CXC chemokines mCXCL10 (Fig. 1A) or hCXCL10 (data not shown).

In contrast to published results (12), binding was also detected for mCCL5 (Fig. 1A). To further confirm this observation, these interactions were investigated by surface plasmon resonance using BLIcore technology. As expected, hCCL5 bound to immobilized vCCI-Fc, and this interaction was characterized by clear association and dissociation phases (Fig. 1B). Similarly, an interaction between mCCL5 and vCCI-Fc was also observed, thus supporting the ELISA data (Fig. 1B). In contrast, the interaction of mCXCL10 with vCCI-Fc was characterized by an extremely rapid dissociation phase indicative of a very low binding affinity of mCXCL10 for vCCI-Fc (Fig. 1B).

The potential of vCCI-Fc to neutralize chemokine-mediated cell migration was first assessed in an in vitro chemotaxis assay. Inhibition of cell migration induced by different murine CC chemokines, such as mCCL5, mCCL22, mCCL3, and mCCL4, was observed in a dose-dependent manner with vCCI-Fc (Fig. 1C and Supplemental Fig. 1) and, to a lesser extent, to mCCL22, mCCL19, and mCCL21, whereas the protein had no effect on mCXCL10-induced cell migration (Fig. 1C). These data further confirmed the ability of vCCI-Fc to bind mCCL5 and to block the biological function of several mCC chemokines.

Reduced macrophage migration in vivo by vCCI-Fc

The first experiment evaluating the ability of vCCI-Fc to inhibit CC chemokine–dependent migration in vivo employed the air pouch model of acute inflammation in which local migration of macrophages and neutrophils, induced by carrageenan, has been described (34). Indeed, macrophage migration is primarily dependent on CC chemokines, whereas neutrophil traffic is more reliant on CXC chemokines (38–40). vCCI-Fc or a control hIgG1 were injected i.v. 6 h prior to the injection of carrageenan into the air pouch. Three days later, exudates from the dorsal pouch were analyzed. vCCI-Fc decreased the total cell infiltration by significantly altering the number of macrophages, but not neutrophils, recruited to the inflammatory site (Fig. 1D). Taken together, these results further validated the use of vCCI-Fc as an antagonist for CC chemokine–induced migration in vivo.

Splenocyte egress is altered in vCCI-Fc–treated CIA mice

The inflammatory cascade driving recruitment of a plethora of cells including macrophages has been associated with the pathogenesis observed in the CIA model (41, 42). We thus wanted to test whether vCCI-Fc blocking capacity of CC chemokine activity was sufficient to stem cellular trafficking in CIA. To this end, vCCI-Fc or the control hlgG1 was administered on day 20, 1 d prior to the secondary injection with CIA in adjuvant. On day 34 (after primary immunization) and after seven consecutive injections, spleens from vCCI-Fc–treated mice exhibited increased global cellularity in the spleen as compared with control animals that received hlgG1 (Fig. 2A, upper panel), which was associated with a significant increase in the absolute number of CD4+ T cells, B cells, and macrophages and, to a lesser extent, of CD8+ T cells (Fig. 2A, lower panel). In contrast, dLNs from vCCI-Fc–treated mice exhibited a decrease in the total cell
number for these populations, although this reduction did not reach significance, except for CD19+ B cells (Fig. 2B).

Due to the observed alteration of cell numbers in the spleens of vCCI-Fc–treated mice, an effect of a generalized lymphopenia was evaluated. As shown in Fig. 2C, neither the total CD45+ cell number (Fig. 2C, upper panels) nor the CD4+, CD8+ T cells or CD19+ B cell numbers (Fig. 2C, lower panels) in whole blood were affected by vCCI-Fc treatment as compared with the isotype control (hIgG1). Increased number of splenic IFN-γ-secreting T cells in vCCI-Fc–treated mice

The next objective was to evaluate whether the observed accumulation of cells in spleens from vCCI-Fc–treated mice also resulted in the retention of IFN-γ-secreting Th1 cells, which are associated with the pathogenesis of CIA (43). Thus, IFN-γ–producing T cells in the spleen and dLN(s) were enumerated ex vivo.
creased proportion of activated splenic CD4+ T cells (58.4 and low level for CD62L. vCCI-Fc treatment resulted in an inactivation include a concomitant high level of expression for CD44 lymphoid organs was analyzed. Established markers of T cell

FIGURE 2. Blockade of cell egress from the spleen in CIA mice treated with vCCI-Fc. Mice received either 200 µg of vCCI-Fc (n = 5) or 200 µg of hlgG1 (n = 5) on days 20, 21, 22, 25, 27, 29, and 32 after primary immunization. At day 34, spleen (A), popliteal dLNs (B), and blood (C) were harvested. dLNs were pooled per group of treatment, whereas spleens were analyzed individually. These compartments were analyzed for CD4+ T cells, CD8+ T cells, CD19+ B cells, and F4/80+ macrophages by flow cytometry. Graphs represent the total cell number (upper panels) and the CD4+ T, CD8+ T, CD19+ B, and F4/80+ macrophage cell numbers (lower panels) in each compartment. In whole blood, cells are gated on CD45+ cells. Data are the means ± SEM. Results are representative of three independent experiments. *p < 0.05; **p < 0.01.

d了 vivo. Cells obtained from the spleen or dLNs were restimulated with anti-CD3 and anti-CD28, and the IFN-γ intracellular levels were analyzed by flow cytometry. The hlgG1-treated group demonstrated a higher proportion of IFN-γ-secreting CD8+ T cells than IFN-γ-secreting CD4+ T cells (Fig. 3A, 3B, left panels). vCCI-Fc did not affect the proportion of IFN-γ-producing T cells in the spleen but significantly increased the absolute number of IFN-γ-secreting CD8+ and CD4+ T cells in this compartment as compared with hlgG1 control (Fig. 3A). A similar analysis on cells from pooled dLNs demonstrated that vCCI-Fc decreased >3-fold the proportion and the absolute number of IFN-γ-secreting CD8+ T cells, whereas IFN-γ-secreting CD4+ T cells were undetectable (Fig. 3B).

Next, the activation state of the T cells in these secondary lymphoid organs was analyzed. Established markers of T cell activation include a concomitant high level of expression for CD44 and low level for CD62L. vCCI-Fc treatment resulted in an increased proportion of activated splenic CD4+ T cells (58.4 ± 4.4 vs 43.7 ± 5.2 in vCCI-Fc vs hlgG1 treatments) and CD8+ T cells (34.3 ± 2.2 vs 26.6 ± 6.0 in vCCI-Fc vs hlgG1 treatments). In the dLNs, similar to the decrease of IFN-γ-secreting cells, a decrease of activated CD8+ T cells (12.4 ± 0.6 vs 22.4 ± 5.8 in vCCI-Fc vs hlgG1 treatments), and to a lesser extent of activated CD4+ T cells (24.8 ± 1.4 vs 28.0 ± 5.0 in vCCI-Fc vs hlgG1 treatments), was observed in vCCI-Fc–treated mice as compared with the hlgG1 control group (Fig. 3C). Furthermore, in the dLNs, as the activated population decreased, the proportion of naive CD4+ T cells (18.8 ± 3.5 vs 9.2 ± 2.0 in vCCI-Fc vs hlgG1 treatments) and naive CD8+ T cells (43.5 ± 1.2 vs 24.6 ± 10.2 in vCCI-Fc vs hlgG1 treatments) compartments increased.

vCCI-Fc treatment reduces the incidence and severity of CIA

The development of arthritis was followed in the treated and control animals to determine whether the vCCI-Fc–mediated retention of cells in the spleen could have an impact on the development of the disease. The hlgG1-treated mice presented a disease incidence of 82% whereas the vCCI-Fc–treated mice demonstrated a maximum incidence of 55% (Fig. 4A) as well as a delayed onset of disease. Furthermore, a significant decrease in the severity of disease was observed in vCCI-Fc–treated mice (Fig. 4B). This robust inhibition of disease was sustained for 14 d after the first injection of vCCI-Fc (until day 34).

Histological preparations were also processed to determine the level of local inflammation and cartilage degradation. For this purpose, knee joints, harvested at day 34, were stained with H&E and safranin O/fast green. As expected, the CIA observed within the hlgG1 control group resulted in infiltration of inflammatory cells, hyperplasia, and degradation of the cartilage (Fig. 4C, upper panels) within the knee joint. In contrast, vCCI-Fc–treated mice histologically exhibited lower cell infiltration and hyperplasia as well as reduced degradation of cartilage (Fig. 4C, lower panels). A summary of these results, plotted in Fig. 4D, demonstrated the significant effect of CC chemokine antagonism via vCCI-Fc in reducing the inflammation as well as cartilage degradation in the knee joints.

As the efficacy of vCCI-Fc treatment diminishes, cells exit from spleen and mice succumb to disease

We observed that the vCCI-Fc treatment began to lose efficacy around day 34. Serum analysis of vCCI-Fc–treated animals indicated that vCCI-Fc induced a strong immunogenic response that
could explain this loss in potency (Supplemental Fig. 2). Alternatively, the development of the disease could be due to a CC chemokine–independent mechanism that cannot be blocked by vCCI-Fc.

In additional experiments, the vCCI-Fc treatment was stopped at day 39 and cell trafficking exiting the spleen was assessed. Ten days after the cessation of treatment, the distribution of T cells, B cells, and macrophages in the secondary lymphoid organs was analyzed. The number of these cells was found to be equivalent between the vCCI-Fc and hIgG1 control groups (Fig. 5A, 5B). The numbers of CD4⁺ T, CD8⁺ T, and B cells were also not altered in the blood compartment (Fig. 5C), and the number of IFN-γ-secreting CD4⁺ T and CD8⁺ T cells, both within the spleen or dLNs, was equivalent in all groups following end of treatment.

**FIGURE 3.** Retention of IFN-γ⁺–secreting T cells in spleen following vCCI-Fc treatment. Mice received either 200 μg of vCCI-Fc (n = 5) or 200 μg of hIgG1 (n = 5) on days 20, 21, 22, 25, 27, 29, and 32 after primary immunization. At day 34, popliteal dLNs and spleens were harvested. A and B, A total of 1 × 10⁶ cells per spleen or pooled dLNs were restimulated with immobilized anti-mouse CD3 and soluble anti-mouse CD28 for 6 h, and GolgiPlug was added during the last 2 h. IFN-γ was detected after intracellular staining.

Data represent in spleen (A) and in dLNs (B) the percentage of IFN-γ-secreting cells gated on CD8⁺ CD3⁺ T cells and CD4⁺CD3⁺ T cells (A, B, *left panel*) and the absolute cell number of CD8⁺ T-IFN-γ–secreting and CD4⁺ T-IFN-γ–secreting cells (A, B, *right panel*). Data are the means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. C, The expression of CD44 and CD62L was analyzed on CD4⁺CD3⁺ T cells and CD8⁺CD3⁺ T cells in spleen (*upper panels*) and dLNs (*lower panels*). Quadrant numbers represent the proportion of each subpopulation based on differential expression of CD44 and CD62L. Results are representative of two independent experiments.
Furthermore, disease flared (Fig. 6A) and histological analyses (Fig. 6B) demonstrated that infiltration of inflammatory cells, hyperplasia, and degradation of the cartilage were similar to hIgG1 controls. Therefore, the development of disease correlated with an exit of cells from the spleen that could be either due to a loss of vCCI-Fc efficacy or to a CC chemokine–independent mechanism.

**Discussion**

The work presented in this study focused on using a pan–CC chemokine antagonist afforded by the viral protein vCCI to better understand the effects of blocking CC chemokine–induced cell migration in the course of an inflammatory process. Following the generation of a recombinant vCCI-Fc fusion protein, we confirmed its specificity for CC chemokines and blocking potential for mCCL2-, mCCL3-, mCCL4-, mCCL22-, mCCL19-, and mCCL21-mediated in vitro chemotaxis (Fig. 1A, 1C). In contradiction to previous studies (12), we found that vCCI-Fc can bind and neutralize mCCL5-induced cell chemotaxis. Following establishment of the potent neutralizing capacity of vCCI-Fc for several CC chemokines, its specificity was analyzed in vivo. We took advantage of the air pouch model of acute inflammation in which
different subfamily chemokine members can be assessed. Indeed, monocytes/macrophages are responsive to CC chemokines, such as CCL5 and CCL2 (38–40), whereas neutrophils are more dependent on the CXC family members, such as CXCL1, CXCL2, CXCL3, CXCL5, and CXCL8 (38–40). By using this model, we were able to effectively demonstrate vCCI-Fc specificity in vivo as the reagent selectively impaired CC- and not CXC-dependent trafficking (Fig. 1D).

In the context of a systemic inflammatory disease, such as a mouse model of RA, the major effect of vCCI-Fc on the distribution of different type of cells, such as T cells, B cells, and macrophages, through secondary lymphoid organs was not anticipated. The significant and reproducible increase of cells in the spleen, the concomitant reduction of these cells in dLNs, and the unaltered levels of circulating leukocytes suggest an organ-specific mechanism to control egress by CC chemokines from spleen. Nevertheless, we cannot exclude that the decrease of the number of cells in the LN draining the inflamed joints may also be due to impairment in the induction of inflammatory cells. Nevertheless, this hypothesis is unlikely, as we were able to detect IFN-γ-secreting T cells, following polyclonal activation, in the spleen and dLNs from all treated groups. Humoral responses, which involve activation of both T and B cells, were not affected, as titers of anti-murine or anti-bovine CII IgG were similar in vCCI-Fc– and control hIgG1-treated mice (data not shown). Furthermore, vCCI-Fc–treated mice developed antiviral protein Abs (Supplemental Fig. 2), which most likely contributed to the eventual decrease of vCCI-Fc. Indeed, the immunogenic response was deemed to be overwhelming despite the detectable levels of vCCI-Fc seen in the sera of injected mice (Supplemental Fig. 3). Immunogenicity to such
proteins is not uncommon, as similar antidrug reactions have been seen in RA patients treated with chimeric biological therapies (44). However, from a safety aspect, our observations demonstrate that a pan–CC chemokine–targeting strategy does not induce a general immunosuppression.

Our central mechanistic hypothesis is that vCCI-Fc blocks cell egress from the spleen toward other lymphoid organs and inflamed tissues. This hypothesis is consistent with the observation that during vCCI-Fc treatment cells are retained in the spleen and reduced in the dLNs, while upon loss of treatment efficacy, cells are able to equilibrate between secondary lymphoid organs. Several members of the CC chemokine family, such as CCL5, CCL3, CCL4, and CCL2, are implicated in the migration of T cells and macrophages out of the bloodstream and into tissues (21, 42, 45), and CCL19/CCL21–dependent entry of naive or memory T cells into lymph nodes has been established (46, 47). The ability of vCCI-Fc to bind and neutralize CCL19 and CCL21 (Fig. 1C), ligands of CCR7, demonstrated less potent effects than observed in CCR7-deficient mice where entry of T cells in the LN was significantly impaired (48, 49). In our study, albeit to a lesser degree, a decrease in lymphocyte migration to dLNs was observed (Fig. 2B). The described low affinity (12) and the less potent ability of vCCI-Fc to inhibit cell migration in a chemotaxis assay described in this study (Fig. 1A) for CCL19 and CCL21 chemokines, as compared with others ligands, could explain this discrepancy.

In contrast, nothing has been reported concerning the CC chemokine–dependent trafficking out of secondary lymphoid organs, a novel finding put forward by this study. Cellular egress has only recently been brought to light by animal experiments conducted with FTY720. This low–molecular mass compound is an antagonist of lipid G–coupled receptors (SIP receptor) that promotes lymphocyte egress after binding of its ligand SIP (46, 50). Injected into animals, this compound induces retention of lymphocytes in the LNs and Peyer’s patches, but not in the spleen (51), and, consequently, it influences the recirculation of naive T cells through secondary lymphoid organs (50, 52). Our results indicate that CC chemokines contribute to controlling the egress of cells from the spleen without affecting circulating populations in the blood. In contrast, FTY720 treatment induces lymphopenia, resulting in a general immunosuppression (52). In this respect, a pan–CC chemokine blockade strategy could be a more attractive therapeutic strategy compared with the use of FTY720 by reducing adverse effects during long-term therapies. However, a major limitation to using vCCI-Fc lies in the high rate of immunogenicity that this molecule will elicit in humans. To circumvent this issue, future studies should envisage the use of less immunogenic molecules, such as a combination of monoclonal Abs or pan-specific anti-chemokine monoclonal Abs, that have recently been described (53).

The specific retention of cells in the spleen, upon CC chemokine inhibition and in the context of CIA, is associated with a dramatic impairment of the incidence and severity of disease. A role for specific CC chemokines in processes instrumental to orchestrate and amplify autoimmune diseases, such as RA, has already been suggested. First, elevated levels of CC chemokines are associated with development of arthritis in mouse models (54) and patients (22–25). Second, the use of a mutated form of CCL5 as an antagonist of the natural chemokine decreased the severity of disease in the adjuvant-induced arthritis model (55), while an anti-CCL3 mAb decreased the incidence and arthritis index in CIA (20). Added to this, an antagonist to CCL2 ameliorated arthritis in MRL-lpr mice (56), as an anti-CCR2 neutralizing mAb (57) or an inhibitor of CCR2 (the fusokine GM-CSF-CCL2) (32) ameliorated arthritis in the CIA model. Nevertheless, other studies have shown no alteration in CIA disease evolution in, for example, CCR5-deficient animals (58). Similarly, experiments conducted in our laboratory using a neutralizing anti-mCCL5 mAb failed to ameliorate CIA (V. Buatois, N. Fischer, M. H. Kosco-Vilbois, and W. G. Ferlin, unpublished observations). Finally, an anti-CCL2 mAb also failed to induce disease amelioration in RA patients (59). Thus, targeting one CC chemokine or CC chemokine receptor may not be sufficient to inhibit complex inflammatory conditions. In our study, we clearly showed that targeting more than one CC chemokine has a drastic effect on the disease. Nevertheless, the timing of CC chemokine inhibition is critical, as treatment with vCCI-Fc, after the onset of the disease, fails to prevent disease (data not shown). As vCCI-Fc has numerous potential targets, including CCL5, CCL2, CCL3, CCL4, CCL22, and CCL19 (12), additional studies will be needed to identify the pivotal CC chemokines associated with control of splenic egress and disease.

An unexpected role for CC chemokines in the control of splenic egress in the context of an inflammatory disease was demonstrated. This phenomenon was associated in a model of RA with amelioration of disease. Thus, neutralizing combinations of CC chemokines warrants further investigation for the development of effective treatments while potentially limiting the toxicity associated with current therapy of autoimmune diseases, such as RA.
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Disclosures

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References


