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Inhibition of CCR6 Function Reduces the Severity of Experimental Autoimmune Encephalomyelitis via Effects on the Priming Phase of the Immune Response[1]

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Chemokines are essential for homeostasis and activation of the immune system. The chemokine ligand/receptor pairing CCL20/CCR6 is interesting because these molecules display characteristics of both homeostatic and activation functions. These dual characteristics suggest a role for CCR6 in the priming and effector phases of the immune response. However, while CCR6 has been implicated in the effector phase in several models, a role in the priming phase is less clear. Herein we analyze the role of CCR6 in these two important arms of the immune response during experimental autoimmune encephalomyelitis (EAE). Both CCR6 and its chemokine ligand CCL20 were up-regulated in the draining lymph nodes and spinal cord during EAE, and CCR6 was up-regulated on $CD4^+$ T cells that had divided following induction of EAE. The functional role of this expression was demonstrated by impaired development of EAE in gene-targeted CCR6-deficient mice and in mice treated either with a neutralizing anti-CCR6 Ab or with a novel receptor antagonist. Inhibition of EAE was due to reduced priming of autoreactive $CD4^+$ T cells probably as a result of impaired late-stage influx of dendritic cells into draining lymph nodes. This was accompanied by reduced egress of activated lymphocytes from the lymph nodes. These results demonstrate a novel role for CCR6 in the mechanism of autoreactive lymphocyte priming and emigration to the efferent lymphatics. *The Journal of Immunology,* **2009, 182: 3121–3130.**

The immune response, which protects us throughout life from a number of challenges, is reliant on cell migration. Chemokines, a structurally related group of small chemotactic cytokines, are primary mediators of migration during the immune response. The principal immunological function of chemokines is two-fold: homeostatic (these are involved in basal leukocyte trafficking and/or the formation of the architecture of secondary lymphoid organs) and inflammatory (these are responsible for the recruitment of leukocytes into peripheral tissues in response to infection) (1). CCL20, also known as macrophage inflammatory protein (MIP)-3α, Exodus-1, and LARC (2), induces migration through its cognate receptor, CCR6 (3). However, CCL20-CCR6 does not constitute a monogamous binding pair, as recent studies have demonstrated that the β-defensin group of antimicrobial peptides are able to bind and activate CCR6 (4).

CCL20 shows an unusual expression pattern for a chemokine, with both constitutive and inducible expression (5). Primary regions of constitutive CCL20 expression include most host-environment interfaces, such as the skin and mucosal surfaces, and the basal expression rate is increased through exposure to inflammatory agents including IL-1β, PMA, ionomycin, LPS, and dsRNA, as well as to microbial pathogens (5, 6). Cellular distribution of CCR6 is limited to leukocytes, with expression in mature lymphocytes, especially memory cells (3) and immature dendritic cells (DCs)[5] of particular lineages (7). In most cases it is absent from granulocytes (except activated neutrophils), monocytic cells, immature lymphocytes, and mature DC (8–10).

Expression profiles of the ligand-receptor system have led to speculation regarding the function of CCR6 during homeostasis and inflammation. Homeostatic production of CCL20 and β-defensins occurs under normal, healthy conditions (which may be aided by normal microfloral production of LPS (11)). The basal expression level is thought to regulate migration of CCR6-expressing immature DC and memory lymphocytes from the blood for homeostatic surveillance. Up-regulation of CCL20 due to danger signals during inflammation may enhance migration of both of these cell types into the tissue, increasing the speed of initiation and the extent of both primary and secondary adaptive immune responses. This process is thought to occur in both the mucosa (12, 13) and skin (13–15).

This dual role of the CCR6 axis in migration of immature DC and memory lymphocytes suggests a potentially important role for

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[5] Abbreviations used in this paper: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; NRIgG, normal rabbit IgG; PLP, proteolipid protein; S1P, sphingosine-1-phosphate.

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this receptor in both the priming and effector phases of the immune response, an issue that has only been partially addressed using CCR6 knockout mice in which a lack of CCR6 expression impairs the effector phase (16). However, while a role in the effector phase of the immune response has been demonstrated, CCR6 has yet to be implicated in the priming phase, despite data indicating an important role in DC migration. Moreover, previously described minor developmental defects in the immune system of CCR6-deficient mice make them potentially unreliable for analysis of function during the immune response (13, 16, 17). Partial light has been shed through our previous analysis of CCL20 function in experimental autoimmune encephalomyelitis (EAE) through Ab-mediated neutralization (18), although the promiscuity of ligand-receptor binding in the chemokine network necessitates a direct analysis of the role of CCR6 in the immune response.

In the present study, we have therefore directly examined the role of CCR6 in the priming and effector phases of the immune response. Using a combination of gene knockout mice, receptor antagonism, and receptor neutralization during EAE, we show an important role for CCR6 in pathogenesis, as measured by the severity of clinical symptoms and histological damage to the spinal cord. Dissection of the mechanism of inhibition implicated CCR6 in the priming phase of the immune response and revealed novel actions of CCR6, with involvement in the late-stage influx of DC to the draining lymph node (LN) during priming and emigration of activated lymphocytes from the draining LN.

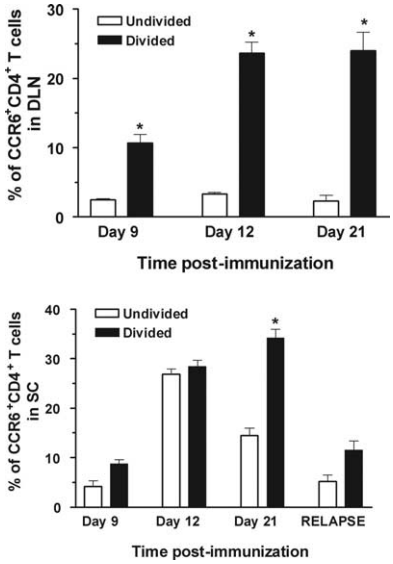

FIGURE 1. Changes in the expression of CCR6 on $CD4^+$ cells. Mice were immunized to induce EAE and fed BrdU starting on day 6 postimmunization. Spinal cord and draining LN were collected on days 9, 12, and 21 postimmunization. Within the $CD4^+$ T cell population, the percentage of $BrdU^+$ (divided) and $BrdU^-$ (undivided) cells expressing CCR6 was determined as a proportion of the total $BrdU^+$ and $BrdU^-$ populations. Data are presented as means ± SEM ($n = 5$).

Materials and Methods

Mice

Female SJL/J mice were purchased from the Australian National University (Canberra, Australian Capital Territory). Swiss, C57BL/6, and BALB/c mice were purchased from the Adelaide University Animal House (Adelaide, South Australia). CCR6 knockout mice were generated at the Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología, Universidad Autónoma, Madrid (16), and were backcrossed at least eight generations to the C57BL/6 background. Mice were kept under standard temperature and light conditions and afforded food and water ad libitum.

Experimental autoimmune encephalomyelitis

Six- to 8-wk-old female SJL/J mice were subcutaneously immunized in the hindflanks with 50 μg of proteolipid protein 139–151 ($PLP_{139-151}$; HSLGKWLGHPDKF) in CFA containing 0.5 mg/ml *Mycobacterium butyricum* (Difco Laboratories) and 8.33 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) (19). Two hours before immunization, and 2 days after, the mice were i.v. injected with 300 ng of pertussis toxin (List Biological Laboratories). Control mice were subjected to the same induction protocol, except that $PLP_{139-151}$ was absent. Spinal cord infiltrate was collected by maceration of spinal cord, filtration, and Percoll purification. The immunization of the CCR6-deficient mice was performed using 100 μg of myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}; MEVGWYRSPFSRVVHLYRNGK) peptide in CFA, as described (20). At days 0 and 2, 300 ng of pertussis toxin (List Biological Laboratories) was injected. In both models, clinical scores were measured by assessment of paralysis, as previously described (18). For passive transfer of EAE, donor mice were primed by s.c. immunization with 25 μg of $PLP_{139-151}$ in CFA. Ten days later, the draining LNs were collected and restimulated in vitro with 50 μg/ml $PLP_{139-151}$ for 96 h, before transfer of 5×10^7 cells to recipient mice via the i.v. route.

Analysis of CCR6 and CCL20 mRNA levels

Spinal cord and inguinal LN samples were taken at days 0, 6, 9, 12, and 22 post-EAE induction, after perfusion. RNA was extracted using TRIzol total RNA isolation reagent (Invitrogen) and DNase-treated (Promega). cDNA was produced using SuperScript II reverse transcriptase (Invitrogen) and random hexamers (Amersham Life Sciences) as previously described (18). Semiquantitative PCR was performed using the following primers: GACT GACGTCTACCTGTTGA and GGCTCTGAGACAGACCTGTA (murine CCR6), TCTTGACTCTTAGGCTGAGG and CAGAAGCAGCAAG CAACTAC (murine CCL20), and TCCTTGGAGGCCATGTAGGCCAT and TGATGACATCAAGAAGGTGGTGAAG (GAPDH) (GeneWorks).

Treatment of mice with chemokine antagonists or neutralizing Abs

Chemokines, chemokine antagonists, and CCR6 peptides were synthesized as previously described (21). The agonistic activity of full-length chemokines was confirmed in calcium mobilization assays (22, 23). Mice were treated with 100 μg of $CCL20_{(6-70)}$ or the inert control peptide $CCL2_{(4Ala)}$ via i.p. injection on days 1, 3, 5, 7, 9, 11, 13, and 15 postinduction.

Polyclonal anti-CCR6 Abs were raised in New Zealand White rabbits by immunization with CCR6-TT (tetanus toxin 831–848 fused to $CCR6_{(18-39)}$). Affinity purification was performed with streptavidin Sepharose beads saturated with biotinylated $CCR6_{(18-39)}$ peptide. Mice were treated with neutralizing Abs 24 h before induction by i.p. injection of 100 μg of affinity-purified rabbit anti-murine CCR6 Abs or normal rabbit IgG (NRIgG).

Chemotaxis assays

In vitro chemotaxis assays were conducted using Transwell cell culture chambers (Costar) as previously described (18). Briefly, SJL/J splenocytes were cultured at 5×10^6 cells/ml in complete RPMI 1640 and 10 μg/ml LPS (Sigma-Aldrich) for 48 h. Cells were labeled with 2 μM calcein-acetoxymethyl ester (Molecular Probes), and resuspended at 1×10^7 cells/ml in chemotaxis buffer (0.5% BSA complete RPMI 1640). Agonists and antagonists were added in the lower chamber, and splenocytes and neutralizing Abs were added to the upper chamber. Migration after 3 h was measured using a Molecular Imager FX (Bio-Rad) with excitation at 488 nm and emission at 494 nm.

Dendritic cell migration studies were performed by injecting SJL/J mice with either 100 μg of $CCL20_{(6-70)}$, $CCL21_{(8-110)}$, or $CCL2_{(4Ala)}$ i.p. 1 h before immunization with 50 μg of CFA/PLP s.c. in each flank. Inguinal LNs were removed 48 or 96 h later, pooled for each group, and incubated with 1 mg/ml collagenase D (Sigma-Aldrich) for 30 min at 37°C. Single-cell suspensions were then prepared and incubated with MACS CD11c-conjugated MicroBeads (Miltenyi Biotec), and DC were positively selected for on MACS MS separation columns (Miltenyi Biotec), as per the manufacturer's recommendations, and enumerated by trypan exclusion.

Lymphocyte proliferation assays

In vitro proliferation assays were performed on draining inguinal LN cells from immunized mice at day 12 using a modification of previously published protocols (24, 25). Briefly, cells were labeled with CFSE (Molecular

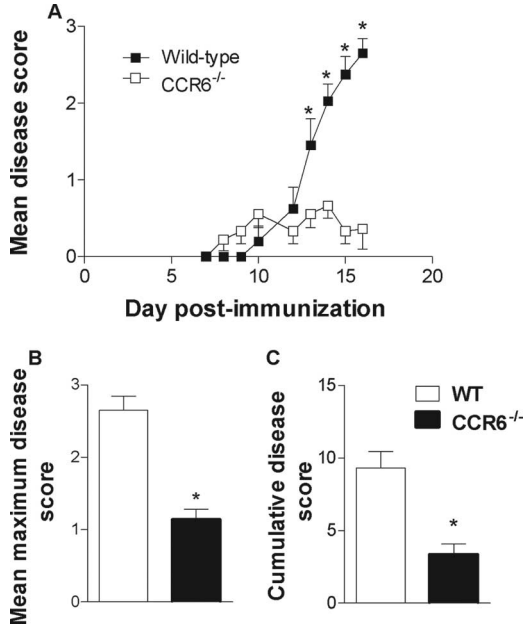

FIGURE 2. CCR6 has a pathogenic role in neuroinflammation. *A*, Wild-type ($n = 10$) and CCR6 knockout mice ($n = 9$) were immunized with 100 μg of MOG_{35-55} in CFA to induce EAE, and CCR6 knockout mice showed significantly lower disease scores during disease progression. *B* and *C*, Cumulative disease scores and mean maximum disease scores for the experiment shown in *A*. Results shown are means ± SEM. *, $p < 0.05$.

Probes) and incubated at 2.5×10^6 cells/ml with $PLP_{139-151}$ at a concentration of 5 or 50 μg/ml, or with 1.0 μg/ml Con A. After 4 days of culture, the cells were harvested, labeled for CD4-PE (BD Pharmingen), and analyzed by flow cytometry using a BD FACScan and CellQuest Pro software (BD Biosciences). Cell division (proliferation) was determined as a progressive halving in CFSE fluorescence intensity.

In vivo proliferation assays were performed using BrdU administration. BrdU (Sigma-Aldrich) was added to the drinking water (0.8 mg/ml) of the animals, starting at day 6 postimmunization until the day of experimental endpoint. Proliferation was analyzed ex vivo by paraformaldehyde permeabilization followed by FITC-labeled anti-BrdU (BD Immunocytometry Systems) and flow cytometry.

Quantitation of lymphocytes by flow cytometry

Blood was collected from day 9 EAE mice pretreated with $CCL20_{(6-70)}$ or $CCL2_{(4Ala)}$ by cardiac puncture, aliquoted into tubes containing heparin (50 U/ml, Sigma-Aldrich), and RBCs were lysed. The remaining whole blood was then double labeled with rat anti-mouse CCR6 (R&D Systems) followed by polyclonal PE-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories) and FITC-conjugated rat anti-mouse CD4 (BD Pharmingen) (26, 27). The percentage of double-positive lymphocytes was then determined by flow cytometric analysis using a BD FACSCanto and FACSDiva software (BD Biosciences). In other experiments, mice with EAE were sacrificed by asphyxiation, perfused with PBS through the left ventricle to remove circulating leukocytes, and spinal cord tissue was collected. Spinal cords were homogenized through 70-μm mesh filters and the cells separated from the myelin cake by density centrifugation using a Percoll gradient. RBCs were then lysed and cells were enumerated and labeled with PE-conjugated anti-CD4 (BD Pharmingen). The number of $CD4^+$ lymphocytes present in the spinal cords was then determined by flow cytometric analyses using a BD LSRII and FACSDiva software (BD Biosciences).

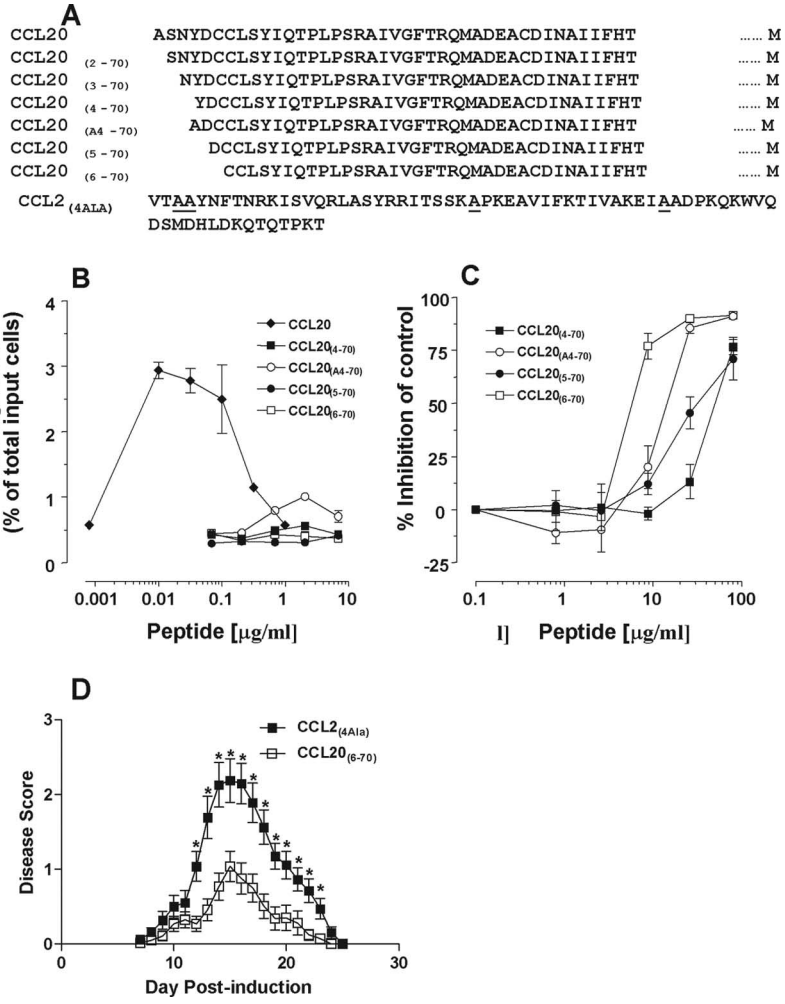

FIGURE 3. Development and characterization of a CCR6 antagonist. *A*, Amino acid sequence of wild-type and mutant forms of CCL20 used in this study. *B*, Dose-response curve of LPS-stimulated murine splenocytes to CCL20 and the synthetic truncated peptides $CCL20_{(4-70)}$, $CCL20_{(A4-70)}$, $CCL20_{(5-70)}$, and $CCL20_{(6-70)}$. *C*, The inhibition of CCL20-mediated chemotaxis of LPS-stimulated murine splenocytes (60 ng/ml) by synthetic truncated CCL20 peptides. Data are presented as means ± SEM (*B*, $n = 5$; *C*, $n = 6$). *D*, In two independent experiments, mice were injected i.p. with 100 μg of $CCL20_{(6-70)}$ or the control peptide $CCL2_{(4Ala)}$ on days 1, 3, 5, 7, 9, 11, 13, and 15 postinduction of EAE. Results were pooled from individual experiments to produce data points representing means ± SEM ($n = 18$). Individual experimental results are listed in Table I. *, $p < 0.05$.

Table I. *Effect of CCR6 inhibition on the development of clinical EAE*

	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	$CCL2_{(4Ala)}$	$CCL20_{(6-70)}$	$CCL2_{(4Ala)}$	$CCL20_{(6-70)}$	NRIgG	Anti-CCR6	NRIgG	Anti-CCR6
No. sick/total	8/8	4/9	10/10	8/10	7/7	7/7	6/6	7/7
Mean day of onset[a,b]	11.25 ± 0.56	12.25 ± 0.95	11.8 ± 0.42	13.4 ± 0.32*	6.3 ± 0.18	7.6 ± 0.43*	7.8 ± 0.5	8.3 ± 0.3
Mean day of recovery[a,b]	18.39 ± 1.4	17.50 ± 1.6	21.0 ± 0.33	18.0 ± 0.91*	20.1 ± 1.4	20.0 ± 1.9	22.8 ± 0.6	19.5 ± 2.1
Mean length of disease (days)[a,c]	7.8 ± 1.4	2.3 ± 1.2*	9.2 ± 0.5	3.7 ± 1.1*	13.9 ± 1.5	12.4 ± 2.0	15.8 ± 0.6	11.0 ± 2.0
Mean max. clinical score[a,c]	2.16 ± 0.24	0.83 ± 0.34*	1.3 ± 0.15	1.25 ± 0.28	2.1 ± 0.21	2.0 ± 0.29	3.1 ± 0.4	2.5 ± 0.5
Mean max. clinical score[a,b]	2.16 ± 0.24	1.88 ± 0.24	1.3 ± 0.15	1.6 ± 0.24	NA	NA	NA	NA
Cumulative disease score[a,c,d]	12.8 ± 7.0	3.0 ± 5.0*	10.3 ± 3.0	5.3 ± 6.0*	19.0 ± 7.0	16.1 ± 8.0	22.3 ± 5.0	12.1 ± 6.0*

[a] Mean ± SEM.
[b] Excluding asymptomatic mice.
[c] Including asymptomatic mice (mice that never showed clinical manifestation were classified as showing a disease length of 0 and a maximum clinical score of 0).
[d] Excluding mice that died during EAE development.
NA indicates not applicable. *, Significantly different from the control treatment group at $p < 0.05$.

Immunohistochemistry

Day 14 EAE mice were sacrificed and perfused with PBS before spinal cords were extracted from the spinal column and frozen in Tissue-Tek OCT (Sakura Finetek) (28). Six-micrometer cryostat sections were acetone-fixed, dipped in 1% BSA/PBS, and blocked with 0.3% H_2O_2/PBS for 10 min. Sections were washed and then blocked with 100 μg/ml mouse γ-globulin (Rockland) for 30 min. After washing, sections were then incubated with 10 μg/ml rat anti-mouse CD45 (BD Pharmingen) for 1 h at 4°C, and then incubated with donkey anti-rat IgG-HRP (Jackson ImmunoResearch Laboratories) for 1 h at 4°C. Sections were developed with diaminobenzidine for 15 min (Dako) as per the manufacturer's recommendations and counterstained with hematoxylin (Sigma-Aldrich). Slides were viewed and photographed using a Zeiss Axiphot microscope and using Photograb software (Magi Consulting) for image capture.

Statistical analysis

Unless otherwise stated, two-tailed Student's *t* tests were used for all statistical analyses. Results were considered significant if the *p* value was <0.05.

Results

Up-regulation of CCR6 and CCL20 during EAE

The results of previous experiments indicated up-regulation of CCL20 protein and mRNA in the LNs and spinal cords, respectively, of mice

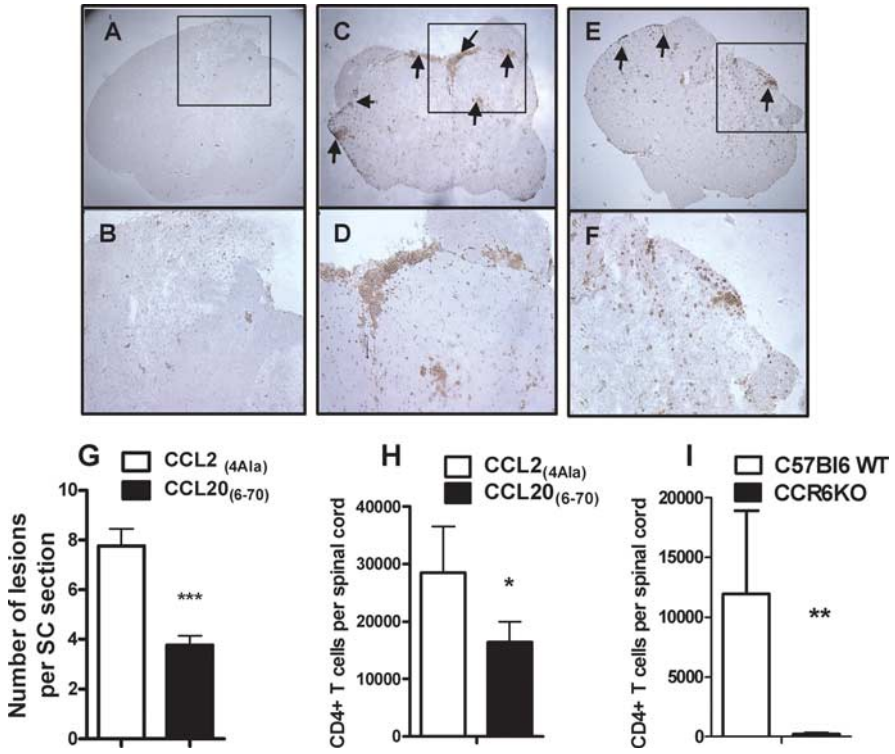

FIGURE 4. Treatment with $CCL20_{(6-70)}$ reduces the histopathology in the CNS, and treatment with $CCL20_{(6-70)}$ or CCR6 deletion reduces infiltration of $CD4^+$ T cells into the spinal cord during EAE. Representative transverse spinal cord sections labeled for CD45 (diaminobenzidine) with hematoxylin counterstain from naive mice (*A* and *B*), EAE mice pretreated with $CCL2_{(4Ala)}$ (*C* and *D*), and EAE mice pretreated with $CCL20_{(6-70)}$ (*E* and *F*). Spinal cords were collected on day 14 postinduction of EAE. These sections are from mice with disease scores of 2.25 (*C* and *D*) and 0.5 (*E* and *F*). Magnification ×25 (*A*, *C*, and *F*), ×100 (*B*, *D*, and *F*). The data are representative of 10 sections each from three mice. The number of lesions (see arrows in *C* and *E*) visible from each section was quantified (*G*). Ten sections from three mice were analyzed and the results shown are the means ± SEM. ***, $p < 0.0001$. Lesions were defined as accumulations of >10 $CD45^+$ cells. The number of $CD4^+$ T cells recovered from the spinal cord of SJL/J mice on day 12 of $PLP_{139-151}$-induced EAE pretreated with $CCL2_{(4Ala)}$ or $CCL20_{(6-70)}$ were enumerated by flow cytometry (*H*). Data shown are the mean numbers of $CD4^+$ T cells ± SEM. *, $p < 0.05$. The number of $CD4^+$ T cells recovered from the spinal cords of $CCR6^{-/-}$ or wild-type C57BL/6 mice on day 16 of MOG_{35-55}-induced EAE were quantified by flow cytometry (*I*). Data shown are the mean numbers of $CD4^+$ T cells ± SEM. **, $p < 0.01$.

immunized with mouse spinal cord homogenate (18). In the present study, this was confirmed using $PLP_{139-151}$ as the neuroimmunogen, and the level of expression of CCR6 was examined. According to RT-PCR, the immunization procedure caused an up-regulation of CCL20 mRNA expression in the draining (inguinal) LNs, which was accompanied by an up-regulation of CCL20 mRNA and CCR6 mRNA in the spinal cord in PLP-immunized mice only (data not shown).

Expression of CCR6 mRNA during priming in the inguinal LN appeared stable at the whole organ level; however, purified $CD4^+$ T cells restimulated in vitro for 4 days with $PLP_{139-151}$ showed significant up-regulation of CCR6 mRNA expression and increased ability to migrate in response to CCL20 in vitro (data not shown), prompting a closer examination of CCR6 expression during priming.

The expression of CCR6 on $CD4^+$ T cells was therefore investigated by flow cytometry. To evaluate expression on actively dividing cells, mice were fed BrdU in their drinking water before assessment of CCR6 expression. A large proportion of actively dividing $CD4^+$ T cells expressed CCR6 after immunization with $PLP_{139-151}$. Accumulation of divided $CD4^+$ T cells was observed in the inguinal LN and spinal cord during the disease course (Fig. 1).

CCR6 has a pathogenic function during EAE

The temporal expression patterns of CCR6 and CCL20 are consistent with a role for the chemokine receptor-ligand pair during neuroinflammation in EAE. To determine whether the observed pattern is causative rather than correlative, EAE was induced in CCR6-deficient mice with MOG_{35-55}. Compared with wild-type mice, CCR6 knockout mice showed reduced disease with a cumulative disease score and mean maximum disease score less than half that of wild-type mice (Fig. 2). This demonstrates a role for CCR6 in the pathogenesis of EAE, with the caveat that loss of CCR6 may distort leukocyte development and lymphocyte differentiation (13, 16, 17).

$CCL20_{(6-70)}$ inhibits CCR6-mediated chemotaxis and EAE

To circumvent this consistent problem with knockout mouse models, interventionist approaches were developed that reduced the function of CCR6 in wild-type mice. The first interventionist approach taken to inhibit CCR6 function was that of receptor antagonism. To develop an antagonist, a series of truncation mutants of CCL20 were synthesized (Fig. 3*A*) and tested for their ability to directly induce chemotaxis and/or inhibit the ability of wild-type CCL20 to induce chemotaxis of lymphocytes. Wild-type CCL20 induced migration in a typical bell-shaped dose-dependent manner, with peak migration observed at 0.01–0.1 μg/ml CCL20 (Fig. 3*B*). The truncation mutants of CCL20 were tested for their ability to induce migration of lymphocytes and it was found that $CCL20_{(2-70)}$ and $CCL20_{(3-70)}$ strongly induced migration of lymphocytes with an order of potency of CCL20 > $CCL20_{(2-70)}$ > $CCL20_{(3-70)}$ (data not shown). In contrast, the remaining mutants with the sole exception of $CCL20_{(A4-70)}$ failed to induce chemotaxis of lymphocytes (Fig. 3*B*). These molecules were then tested for their ability to antagonize wild-type CCL20-mediated chemotaxis. The most potent in vitro antagonism was seen with $CCL20_{(6-70)}$, which significantly inhibited in vitro chemotaxis toward CCL20 at concentrations of 10–80 μg/ml (Fig. 3*C*) and also inhibited CCL20-mediated recruitment of CCR6-positive cells in an in vivo air pouch model (data not shown), while showing poor direct chemotactic properties (Fig. 3*B*). The control peptide $CCL2_{(4Ala)}$ (Fig. 3*A*) was unable to induce or inhibit chemotaxis (data not shown).

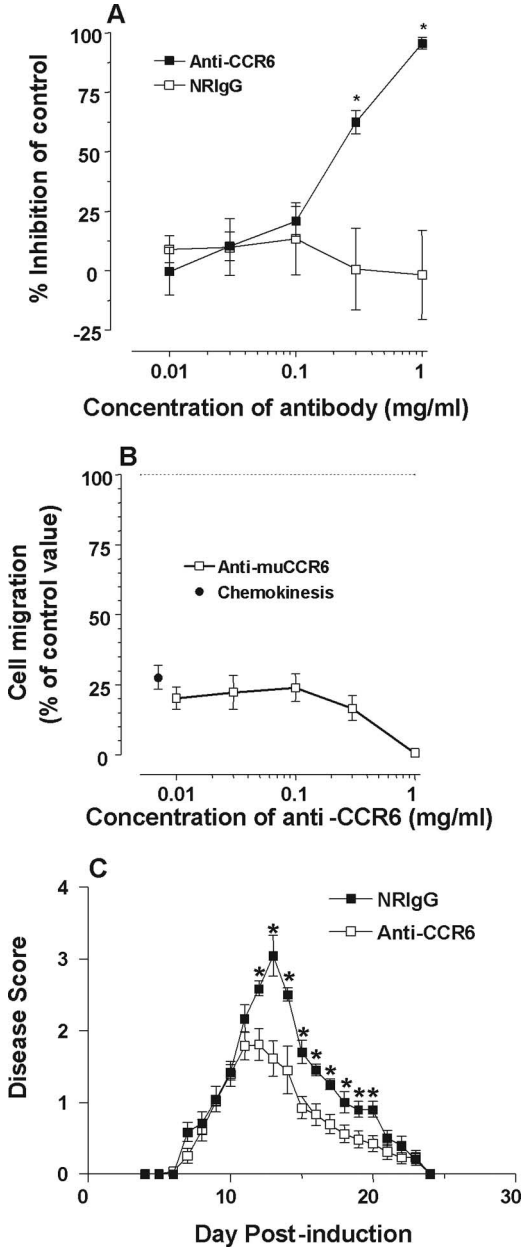

FIGURE 5. Characterization of neutralizing anti-CCR6 Abs and their effect on the development of EAE. *A*, Inhibition of CCL20-mediated chemotaxis by rabbit anti-CCR6 Abs but not NRIgG. *B*, Rabbit anti-murine CCR6 Abs do not induce cellular migration in vitro compared with random chemokinesis. *A*, $n = 16$ for anti-CCR6 Abs, $n = 5$ for NRIgG; *B*, $n = 4$. *C*, In two independent experiments, mice were injected with 100 μg of affinity-purified rabbit anti-mouse CCR6 Abs or NRIgG 24 h before disease induction. Results were pooled from individual experiments to produce data points representing means ± SEM ($n = 13$). *, $p < 0.05$. Individual experimental results are listed in Table I.

To determine whether antagonism of CCR6 altered the pathogenesis of EAE, mice were treated with either 100 μg of $CCL20_{(6-70)}$ or control peptide $CCL2_{(4Ala)}$ on days 1, 3, 5, 7, 9, 11, 13, and 15 postinduction with $PLP_{139-151}$ in two independent experiments. This dosing strategy was selected from the results of previous studies using a similar approach (29, 30), and was capable of inhibiting CCL20-mediated chemotaxis in vivo in an air-pouch chemotaxis model (data not shown). The effect of CCR6 antagonism on EAE development is shown in Fig. 3*D* (pooled results, $n = 18$ mice) and in Table I (individual experimental results). Mice treated with

$CCL20_{(6-70)}$ showed lower disease scores compared with $CCL2_{(4Ala)}$-treated mice. Additionally, $CCL20_{(6-70)}$ treatment demonstrated a trend toward a reduction in the mean length of disease (including delayed onset and enhanced recovery) and the mean maximum clinical score. Fewer $CCL20_{(6-70)}$-treated mice developed disease, while all $CCL2_{(4Ala)}$-treated mice were affected. The average cumulative disease score was significantly reduced by the $CCL20_{(6-70)}$ treatment.

As an independent assessment of pathology, tissue sections of lower thoracic spinal cord from naive or $CCL20_{(6-70)}$- and $CCL2_{(4Ala)}$-treated EAE mice at day 14 postinduction were examined for $CD45^+$ leukocyte infiltration by immunohistochemistry. No $CD45^+$ cells were observed in the spinal cords of naive animals (Fig. 4, *A* and *B*); however, $CD45^+$ cell clusters were observed in those from EAE mice, with a significantly greater number of $CD45^+$ cells present in sections from $CCL2_{(4Ala)}$-treated mice (Fig. 4, *C* and *D*) compared with those from $CCL20_{(6-70)}$-treated animals (Fig. 4, *E* and *F*). The number of lesions where extensive leukocyte infiltration was apparent was significantly reduced with $CCL2_{(4Ala)}$ treatment (Fig. 4*G*). Additionally, flow cytometric analysis also revealed that $CD4^+$ T cell infiltration into spinal cord tissue during EAE was significantly reduced at peak disease of EAE with $CCL2_{(4Ala)}$ treatment (Fig. 4*H*). The observed effect of CCR6 antagonism was similar to that observed in CCR6-deficienct mice in this respect (Fig. 4*I*), eliminating the caveat of developmental defects, and indicating that the pathogenic CCR6 function is sensitive to antagonist-mediated intervention.

Neutralizing anti-CCR6 Abs inhibit the pathogenesis of EAE

To further verify effects of CCR6 inhibition, a neutralizing anti-CCR6 Ab was developed. Rabbit polyclonal Abs were raised against the N-terminal extracellular region of CCR6 and evaluated for their ability to inhibit CCL20-induced chemotaxis in vitro. The affinity-purified Abs inhibited migration toward 0.6 μg/ml CCL20 in a dose-dependent manner (Fig. 5*A*) and were not capable of directly inducing chemotaxis of splenocytes (Fig. 5*B*). The possibility that lymphocyte chemotaxis reduction was due to cellular aggregation of $CCR6^+$ cells induced by the anti-CCR6 Abs was excluded through microscopic analysis (data not shown). The neutralizing CCR6 Abs were then used in vivo, with treatment of 100 μg 24 h before EAE induction. As observed with CCR6 antagonism, CCR6 neutralization reduced the severity and duration of the disease in two independent experiments (Fig. 5*C* and Table I). This effect was not due to depletion of $CCR6^+$ cells, as the same mitigation in EAE severity was observed when using anti-CCR6 Fab fragments (data not shown).

CCR6 is not involved in the effector phase of EAE

The cellular and tissue distributions of CCR6 and CCL20 suggest two distinct mechanisms through which CCR6 could have a pathogenic function during EAE. First, CCL20 was up-regulated in the draining LN during the early priming phase and may contribute to the accumulation of Ag-loaded DC and/or activated neuropeptide-reactive T cells in the nodes. Second, CCL20 was up-regulated in the spinal cord on day 6, followed by accumulation of CCR6-expressing $CD4^+$ T cells and peak disease, suggesting a role in the migration of effector cells to the nervous tissue.

To determine whether treatment with $CCL20_{(6-70)}$ inhibited the effector phase of the immune response, passive transfer experiments were conducted. PLP-specific lymphocytes were generated through in vivo immunization and ex vivo restimulation, and they were passively transferred into naive hosts that were treated with either $CCL20_{(6-70)}$ or $CCL2_{(4Ala)}$. $CCL20_{(6-70)}$ treatment did not alter the development of passive EAE (Fig. 6*A*). Likewise, treatment with neutralizing anti-CCR6 Abs showed no effect (Fig. 6*B*), suggesting that CCR6 does not play a role in the effector phase of EAE.

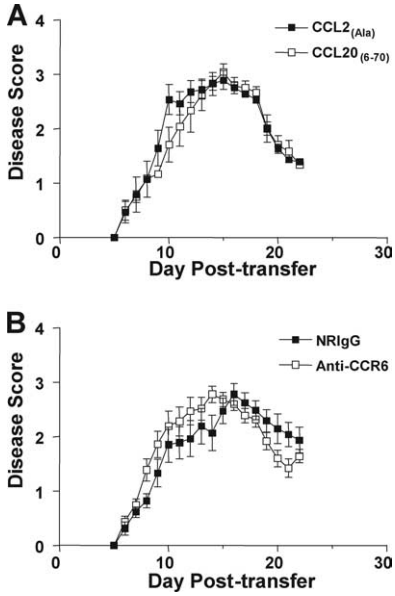

FIGURE 6. Lack of effect of CCR6 inhibition on adoptive transfer of EAE. Donor mice were immunized with $PLP_{139-151}$. Ten days postimmunization, draining LN cells were restimulated for 4 days in vitro with 50 μg/ml $PLP_{139-151}$, and 5×10^7 cells were transferred into naive recipients that had been treated with 100 μg of either (*A*) $CCL20_{(6-70)}$ or the control peptide $CCL2_{(4Ala)}$ on days 1, 3, 5, 7, 9, 11, 13, and 15 posttransfer or (*B*) affinity-purified rabbit anti-mouse CCR6 Abs or NRIgG 24 h before transfer (pooled from two independent experiments). Data are presented as mean clinical disease scores ± SEM ($n = 6$ for each experimental group in *A*, 12 in *B*).

CCR6 is involved in the sensitization phase of EAE and lymphocyte egress from the draining LN

The effect of inhibition of CCR6 activity on the sensitization phase of the immune response was determined by examining the ability of lymphocytes from immunized mice to proliferate in response to Ag restimulation in vitro. Mice were immunized with $PLP_{139-151}$ and treated with either $CCL20_{(6-70)}$ or $CCL2_{(4Ala)}$ as above, and the draining LNs were harvested on day 9. CFSE labeling and restimulation in vitro with $PLP_{139-151}$ was used to measure the

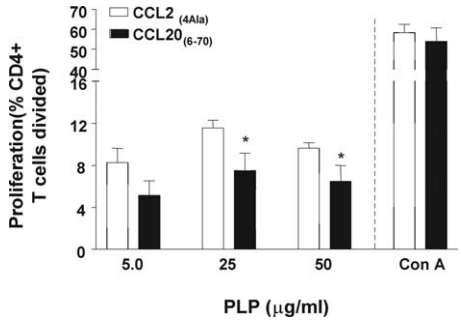

FIGURE 7. Effect of $CCL20_{(6-70)}$ treatment on sensitization of T cells to encephalitogenic peptides. Mice were immunized to induce EAE and treated i.p. with 100 μg of $CCL20_{(6-70)}$ or $CCL2_{(4Ala)}$ on days 1, 3, 5, 7, 9, and 11 postinduction. On day 12, lymphocytes from the draining LN were labeled with CFSE and assayed for proliferation upon restimulation in vitro with $PLP_{139-151}$ or 1.5 μg/ml Con A. Cells were labeled with PE-conjugated anti-CD4 Abs before analysis.

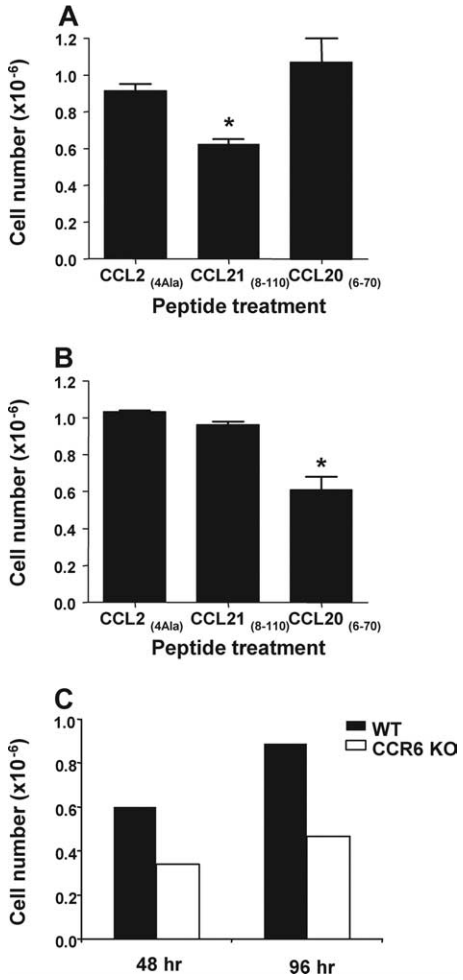

FIGURE 8. Effect of $CCL20_{(6-70)}$ treatment or CCR6 deficiency on the migration of DCs to draining LNs during EAE induction. Mice were injected with either $CCL20_{(6-70)}$, $CCL21_{(8-110)}$, or $CCL2_{(4Ala)}$ i.p. 1 h before immunization with CFA/PLP s.c. in each flank. At 48 (*A*) and 96 h (*B*) postimmunization, inguinal LNs were removed, single-cell suspensions prepared, and $CD11c^+$ DCs positively selected and enumerated. Data are presented as means ± SEM ($n = 14$ for each experimental group). *, $p < 0.05$. *C*, The same approach was used to evaluate the migration of $CD11C^+$ DCs to the draining LN in $CCR6^{-/-}$ mice compared with wild-type controls. Data represent pooled values from five individual mice.

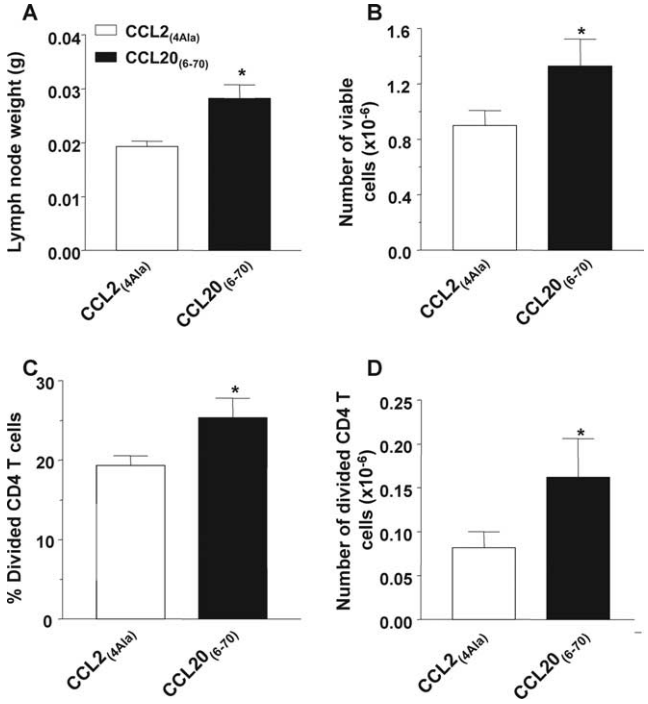

FIGURE 9. Effect of $CCL20_{(6-70)}$ treatment on characteristics of the draining LN. Mice were immunized to induce EAE and treated i.p. with 100 μg of $CCL20_{(6-70)}$ or $CCL2_{(4Ala)}$ on days 1, 3, 5, 7, 9, and 11 postinduction. On day 12, the LNs were collected *A*, LN weight. *B*, Number of viable cells in the LN. Alternatively, mice were fed BrdU in their drinking water and the LNs were collected for further analysis. *C*, The percentage of divided $CD4^+$ T cells present in the draining LN. *D*, The absolute number of divided $CD4^+$ cells in the draining LN. Data are presented as means ± SEM (*A* and *B*, $n = 7$; *C* and *D*, $n = 5$).

proliferative response. The results of these experiments showed that treatment with $CCL20_{(6-70)}$ significantly reduced subsequent proliferative responses to the stimulating Ag, but not toward the nonspecific stimulus Con A (Fig. 7), indicating reduced levels of priming during the sensitization phase.

The documented role of CCR6 and CCL20 in the recruitment of immature DC to sites of immune challenge (6, 31) suggests that CCL20 and CCR6 may influence the extent of priming within a draining LN due to effects on the maintenance of DC recruitment to the s.c. Ag depot to provide a continual supply of DCs to the LN during the chronic Ag exposure. To investigate whether CCR6 plays a role in the accumulation of mature DCs in the draining LN during the induction of EAE, two approaches were taken. First, mice were injected i.p. with either $CCL20_{(6-70)}$ or control peptide $CCL2_{(4Ala)}$ 1 h before s.c. injection of $CFA/PLP_{139-151}$ in each flank. In these experiments, mice were also treated with $CCL21_{(8-110)}$, a CCR7 antagonist, to compare CCR7- and CCR6-dependent DC recruitment. Second, $CCR6^{-/-}$ or wild-type mice were immunized with CFA/MOG_{35-55} in each flank. Inguinal LNs were removed either 48 or 96 h after immunization and DC numbers were compared between each group.

At 48 h postimmunization, animals that were treated with the CCR7 antagonist $CCL21_{(8-110)}$ showed significantly fewer DCs in the draining LNs compared with $CCL2_{(4Ala)}$-treated animals, whereas DC numbers from those treated with $CCL20_{(6-70)}$ were comparable to controls (Fig. 8*A*). At 96 h, however, the initial effect of $CCL21_{(8-110)}$ had abated, with DC numbers from these animals comparable with $CCL2_{(4Ala)}$-treated mice, while the number of DCs recovered from mice treated with $CCL20_{(6-70)}$ dropped 40% (Fig. 8*B*). Consistent with this observation, migration of DCs to the draining LN was also reduced in $CCR6^{-/-}$ mice compared with wild-type mice (Fig. 8*C*).

Interestingly, antagonism of CCR6 also consistently resulted in an increase in both LN weight and the number of viable cells recovered from the inguinal LNs (Fig. 9, *A* and *B*). BrdU labeling indicated that this increase in cellularity was associated with a 20% increase in $CD4^+$ cells that had divided over the previous 4 days (Fig. 9*C*), and a 100% increase in the absolute number of divided $CD4^+$ cells in the draining LN (Fig. 9*D*). The contrast between the reduced priming of Ag-specific T cells (see Fig. 7) and the increased number of actively dividing $CD4^+$ T cells within the draining LNs of EAE mice treated with $CCL20_{(6-70)}$ suggested that CCR6 may be instrumental in the traffic of autoreactive T cells from the draining LNs into the efferent lymphatics before disease onset. To test this hypothesis, peripheral blood was collected at day 9 postinduction of EAE by cardiac puncture from mice treated with either $CCL20_{(6-70)}$ or $CCL2_{(4Ala)}$, and cells were double-stained for CD4 and CCR6 before quantitation by flow cytometric

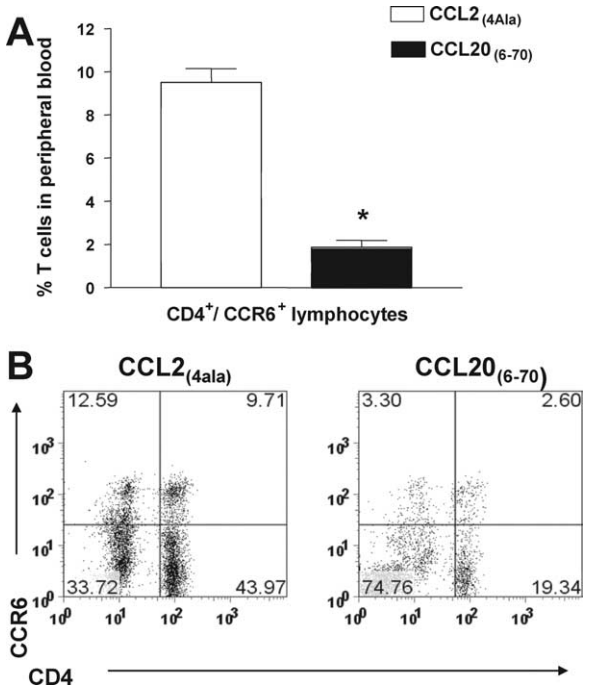

FIGURE 10. Treatment with $CCL20_{(6-70)}$ results in fewer circulating $CD4^+/CCR6^+$ lymphocytes after Ag priming, before disease onset. Blood was collected from day 9 EAE mice pretreated with either $CCL20_{(6-70)}$ or $CCL2_{(4Ala)}$, and the percentage of circulating $CD4^+/CCR6^+$ lymphocytes was determined by flow cytometric analysis. *A*, Data are presented as mean percentage of blood ± SEM ($n = 6$ for each experimental group). *, $p < 0.0001$. A representative dot plot showing CD4 and CCR6 staining on lymphocyte-gated peripheral blood cells is shown in *B*.

analysis. Mice treated with $CCL20_{(6-70)}$ had significantly fewer circulating $CD4^+/CCR6^+$ T lymphocytes (1.9%) compared with $CCL2_{(4Ala)}$-treated mice (9.5%), as shown in Fig. 10.

Discussion

Due to involvement in DC and effector T cell migration, the chemokine receptor CCR6 has been hypothesized to play an important role in both the priming and effector phases of the peripheral immune response. While evidence exists for a role in the effector phase (16), there is a paucity of information regarding a role in immune priming. In this study we have analyzed the role of CCR6 in both phases of the immune response using EAE as a model and have identified several novel aspects with respect to CCR6 biology. First, using a combination of CCR6 knockout mice as well as two independent approaches to inhibit CCR6 function in wild-type mice using novel reagents, we have determined a role for CCR6 in the priming phase of EAE. Second, we provide evidence indicating that CCR6-dependent recruitment of immature DCs to the tissue is a limiting factor for T cell priming during chronic immune stimulation. Third, we have identified a novel function for CCR6 in the regulation of lymphocyte egress from peripheral LNs during an active immune response.

Our initial data indicated increased expression of both CCR6 and its only known chemokine ligand CCL20 in the draining LNs and the spinal cord during the pathogenesis of EAE, potentially implicating these molecules in both the priming and effector phases of the disease. Ag-dependent activation of T lymphocytes leads to an alteration in chemokine receptor profile that appears to correlate closely with altered trafficking patterns and effector cell function (32–34). This has been best documented in the case of CXCR3 and CCR5 on Th1 cells and CCR3, CCR4, and CCR8 on Th2 cells. However, we have also observed up-regulation of CCR6 on $CD4^+$ T cells during the in vitro MLR (27).

To directly test the hypothesis that CCR6 plays a role in the neuroinflammatory response in EAE, the effect of genetic deletion of CCR6 on the induction of EAE was examined. The delayed onset and reduced disease severity we observed in $CCR6^{-/-}$ mice compared with wild-type mice indicate that CCR6 plays a pathogenic role in EAE. However, because it has previously been demonstrated that the distribution of $CD11c^+$ DCs and numbers and distribution of lymphocyte subsets are abnormal in CCR6 knockout mice (13, 16, 17), and this or other as yet unidentified developmental abnormalities could have caused the observed effect, novel reagents were developed to inhibit CCR6 in an interventionist approach in wild-type mice. Previous studies have indicated that mutated chemokine ligands can function effectively as chemokine receptor antagonists both in vitro and in vivo and can thus be used to probe the role of chemokines and chemokine receptors in the immune system (16, 29, 30, 35, 36). We therefore designed, synthesized, and tested a series of truncation mutants of CCL20 to antagonize CCR6. By a number of criteria, $CCL20_{(6-70)}$ was shown to be a highly specific antagonist for CCR6 function in vitro and in vivo. Additionally, we developed and characterized neutralizing anti-CCR6 Abs.

The CCL20 antagonist and Ab-mediated neutralization approaches both reproduced the inhibition of EAE that was observed in the CCR6 knockout mouse. The interventionist approach also allowed us to dissect the role of CCR6 into the priming and effector phases, and the results indicate a role for CCR6 in both lymphocyte priming and lymphocyte egress from the draining LN. These data implicating CCR6 in immune system priming are in contrast to previous studies using CCR6 knockout mice in allergic pulmonary inflammation (37) and allogeneic immune responses (16). Those studies indicated that while CCR6 knockout mice showed reduced immune responses, Ag-dependent lymphocyte priming responses were normal. In further contrast to the data generated by Varona et al., using a delayed-type hypersensitivity model in CCR6 knockout mice (16), we did not observe a role for CCR6 in the effector phase of the immune response in EAE. This difference could be indicative of the altered importance of immune mediators in the immunoprivileged nervous tissue, or it could be due to developmental defects in knockout mice influencing the outcome of previous studies.

Examination of the mechanism by which CCR6 inhibition inhibits EAE implicated CCR6 in the priming phase of the immune response through two distinct mechanisms: first, a decrease in sensitization of T cells to Ag; and second, an inhibition or delay in the release of Ag-specific T cells from the draining LN. With respect to the first mechanism, the observed inhibition of sensitization to Ag is consistent with an important role for CCR6 and CCL20 in the migration of immature DCs. Previous data have indicated a role for CCR6 and CCL20 in maintaining the level of surveillance DCs in the tissue during immune homeostasis (6) and in the recruitment of immature DCs to the peripheral tissues following CCL20 up-regulation during an immune response (31, 38). In support, homeostatic migration of $CD11c^+$ DCs to mucosal surfaces is compromised in CCR6 knockout mice (13, 16). In the present study, it is demonstrated that both systemic inhibition of CCR6 function and CCR6 deletion resulted in a sharp decline in the number of mature DCs arriving in the draining LN from the Ag depot, consistent with poor recruitment of immature DCs to the inflamed tissue. As Ag presentation by DCs in the peripheral LNs has previously been shown to be necessary for stimulation of myelin-specific T cells during EAE (39), the inhibition of DC migration

may be responsible for the reduced efficiency of priming of PLP-specific T cells, thereby inhibiting disease pathogenesis, as observed.

With respect to the second mechanism, inhibition of CCR6 function gave the counterintuitive observations of a decrease in Ag-specific T cell proliferation, indicating a reduction of priming to the Ag, and an increased number of divided $CD4^+$ T cells in the draining LNs, accompanied by a substantial enlargement in the overall size of LNs. Corresponding with the increased number of divided $CD4^+$ T cells in the draining LNs, inhibition of CCR6 caused a sharp decline in the number of $CCR6^+$ $CD4^+$ T cells in circulation. Taken together, these observations implicate CCR6 in the release of activated $CD4^+$ T cells from the draining LN. This would account for the observation that despite a decrease in Ag-specific sensitization, inhibition of CCR6 resulted in increased numbers of divided $CD4^+$ T cells in the LN. In this context, it is of interest that this progressive increase of LN size has also been observed in mice treated with neutralizing anti-CCL20 Abs upon induction of EAE (18), and in CCR6-deficient mice infected with *Leishmania major*, where the draining LNs are enlarged even after the infection had been resolved (H. Korner, unpublished results). The retention of neuropathogenic T cells in the LNs by CCR6 inhibition is consistent with the delayed onset of disease observed in the present study after treatment with the CCR6 antagonist and in $CCR6^{-/-}$ mice.

The mechanism by which CCR6 regulates lymphocyte egress is unclear and requires further investigation. However, of potential relevance, recent data have demonstrated a crucial role for sphingosine-1-phosphate (S1P) and the G protein-coupled receptor $S1P_1$ in lymphocyte egress from the LN (40). Deletion of $S1P_1$ inhibits the release of mature lymphocytes from the thymus, and inhibition of S1P function using the immunosuppressive drug FTY720 blocks lymphocyte egress from LNs under both homeostatic conditions and during induction of the immune response (40, 41). In contrast, in the present study inhibition of lymphocyte egress was only observed during induction of the immune response, suggesting that induced expression of CCL20 and acquisition of CCR6 on lymphocytes are required for egress of activated lymphocytes only. The retention of $CD4^+$ cells in the LN induced by blocking CCR6 function did not appear to be related to alterations in the expression of other chemokine receptors or adhesion molecules, as the expression of CXCR3, CCR7, or CD62L by $CD4^+$ T cells in draining LNs following immunization was not affected in $CCR6^{-/-}$ mice or in animals treated with the CCR6 antagonist (data not shown). The results of a recent study indicated that microvascular endothelial cells of lymph channels secrete CCL20 into the lumen of the efferent lymphatics of LN in response to inflammatory cytokines such as TNF-α and IL-1β (42), supporting a role of CCL20/CCR6 in transendothelial migration of $CCR6^+$ cells into the efferent lymphatics. Whatever the mechanism, this role of CCR6 appears to be more subtle than that of $S1P/S1P_1$, as CCR6 knockout mice do not appear to demonstrate a global defect in lymphocyte recirculation, although there is clearly a lymphocyte trafficking defect in the Peyer's patches (13).

In summary, the results of this study provide novel insights into the pathobiology of the chemokine receptor ligand pair CCR6/CCL20. They demonstrate an important role for CCR6 in the priming phase of EAE and indicate novel activities of CCR6 in the accumulation of DCs to draining LNs and in the egress of activated $CD4^+$ T cells from the nodes. Finally, this study has led to the development of a novel CCL20 antagonist that can be used to further probe the role of CCL20 and CCR6 in the immune response in normal mice.

Acknowledgments

The coauthors dedicate this manuscript to the memory of Professor Ian Clark-Lewis, who passed away during the completion of this work.

Disclosures

The authors have no financial conflicts of interest.

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