nature immunology

C-C chemokine receptor 6–regulated entry of T_H-17 cells into the CNS through the choroid plexus is required for the initiation of EAE

Andrea Reboldi[1], Caroline Coisne[2], Dirk Baumjohann[1], Federica Benvenuto[3,4], Denise Bottinelli[1], Sergio Lira[5], Antonio Uccelli[3,4,6], Antonio Lanzavecchia[1], Britta Engelhardt[2] & Federica Sallusto[1]

Interleukin 17–producing T helper cells (T_H-17 cells) are important in experimental autoimmune encephalomyelitis, but their route of entry into the central nervous system (CNS) and their contribution relative to that of other effector T cells remain to be determined. Here we found that mice lacking CCR6, a chemokine receptor characteristic of T_H-17 cells, developed T_H-17 responses but were highly resistant to the induction of experimental autoimmune encephalomyelitis. Disease susceptibility was reconstituted by transfer of wild-type T cells that entered into the CNS before disease onset and triggered massive CCR6-independent recruitment of effector T cells across activated parenchymal vessels. The CCR6 ligand CCL20 was constitutively expressed in epithelial cells of choroid plexus in mice and humans. Our results identify distinct molecular requirements and ports of lymphocyte entry into uninflamed versus inflamed CNS and suggest that the CCR6-CCL20 axis in the choroid plexus controls immune surveillance of the CNS.

The identification of specialized subsets of effector $CD4^+$ T cells has provided a paradigm for understanding immunity and immunopathology. Interleukin 17 (IL-17)-producing T cells (T_H-17 cells) have been characterized in the mouse as a distinct lineage of $CD4^+$ T cells that can differentiate from uncommitted naive T cell precursors under the aegis of the transcription factors RORγt and RORα and the polarizing cytokines transforming growth factor-β, IL-6 and IL-23 (ref. 1–3). IL-17 can mediate protection against extracellular pathogens by promoting neutrophil recruitment but has also been shown to cause immunopathology in various models of autoimmunity[4,5].

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T cell–mediated disease of the central nervous system (CNS) that is used as a model of multiple sclerosis, a devastating inflammatory demyelinating disease of the human CNS. Several lines of evidence indicate that T_H-17 cells are involved in the onset and maintenance of EAE[6]. Thus, mice lacking RORγt, IL-17 or IL-23 as well as mice treated with IL-17-blocking antibodies are less susceptible to EAE than are wild-type or untreated mice[7–10]. In addition, IL-17^+ T cells have been found in lesions in brain tissues from patients with multiple sclerosis[11]. However, it has also been shown that T helper type 1 (T_H1) cells are present in lesions of EAE and in multiple sclerosis during the active phase of the disease and that mice lacking T-bet, the T_H1 'master' transcription factor, are resistant to the development of EAE[12]. EAE can be induced by transfer of either T_H-17 or T_H1 cells[13,14] and the T_H-17/T_H1 ratio of infiltrating cells determines where inflammation occurs in the CNS[15,16]. Together these studies suggest the possibility that T_H-17 and T_H1 cells may be involved in pathogenesis at different times or at different sites.

In the model of active EAE, autoreactive myelin-specific effector T cells are primed in peripheral lymph nodes and must migrate into uninflamed CNS to initiate tissue inflammation. The molecular determinants that control this initial step of cell migration, which is probably the same used for constitutive immune surveillance in the brain, remain to be determined. In contrast, the molecular requirements for lymphocyte rolling and adhesion to activated vessels of the inflamed blood-brain barrier have been intensively investigated in both active and passive models of EAE[17]. The integrin $\alpha_4\beta_1$ (VLA-4) serves a key function in controlling the entry of lymphocytes into the CNS by interacting with the adhesion molecule VCAM expressed by inflamed endothelial cells[18]. P-selectin does not seem to be necessary for the recruitment of inflammatory cells during active EAE[19] but can be expressed in small amounts on resting brain endothelium or can be rapidly induced on endothelial cells by inflammatory stimuli[20,21]. Chemokine receptors are required for the entry of lymphocytes into the CNS[21,22], but the nature of the receptors has not been identified and may vary depending on the inflammatory condition or location.

[1]Institute for Research in Biomedicine, Bellinzona, Switzerland. [2]Theodor Kocher Institute, University of Bern, Bern, Switzerland. [3]Neuroimmunology Unit and [4]Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy. [5]Mount Sinai School of Medicine, New York, New York, USA. [6]Advanced Biotechnology Center, Genoa, Italy. Correspondence should be addressed to F.S. (federica.sallusto@irb.unisi.ch).

Received 4 November 2008; accepted 10 February 2009; published online 22 March 2009; doi:10.1038/ni.1716

Lymphocytes can also enter into the CNS through the choroid plexus[23,24]. These plexuses are villous structures extending into the cerebrospinal fluid–filled ventricular spaces that establish a blood–cerebrospinal fluid barrier at the level of apical tight junctions between epithelial cells of the choroid plexus. The specific enrichment for memory T cells in the cerebrospinal fluid of healthy people[24] and patients with multiple sclerosis[25], as well as the regulated functional expression of adhesion molecules on the choroid plexus epithelium[26], suggest that T cells may use these structures to enter the cerebrospinal fluid and disseminate to the meningeal and perivascular spaces[27]. However, the homing determinants that regulate the entry of lymphocytes through the choroid plexus and the function of this port of entry relative to that of the blood-brain barrier remain to be defined.

It is well established that both in humans and mice, chemokine receptors have differences in expression on subsets of effector and memory T cells and provide specificity to cell trafficking both in the steady state and inflammation[28]. For example, CCR7 endows naive and central memory T cells with the ability to migrate into peripheral lymph nodes, whereas CCR9 and CCR4 direct the migration of memory T cells into the gut and skin, respectively. In addition, some receptors, such as CXCR3 and CCR5, are 'preferentially' expressed on T_H1 cells, whereas others, such as CCR3, CCR8 and CRTH2, are 'preferentially' expressed on T_H2 cells. The finding that in humans, CCR6 (A000629), the receptor for CCL20 (a chemokine expressed in the liver, lungs and Peyer's patches)[29], is expressed on IL-17-producing T cells (including some that also produce interferon-γ (IFN-γ))[30] prompted us to investigate the function of CCR6 in regulating T_H-17-mediated immune pathology.

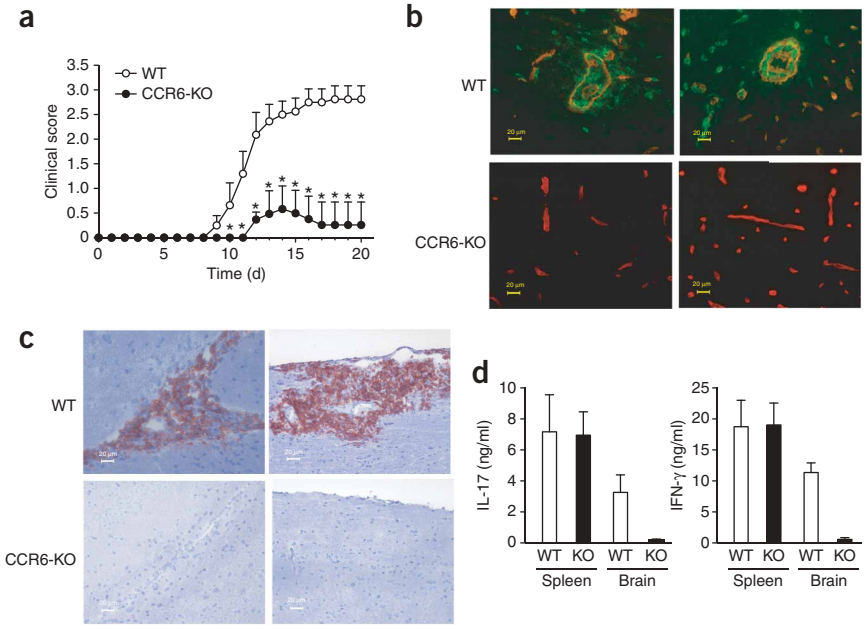

Figure 1 CCR6-deficient mice are resistant to EAE induction. (**a**) Clinical scores of wild-type mice (WT; $n = 5$) and CCR6-knockout mice (CCR6-KO; $n = 5$) at various times after immunization with MOG(33–55) in CFA. *, $P < 0.01$ (Student's *t*-test). Data are representative of six experiments (mean and s.d.). (**b**,**c**) Immunofluorescence and histology of cryosections of brains and spinal cords from wild-type and CCR6-knockout mice with clinical scores of 2 and 0, respectively, collected on day 13 after perfusion. (**b**) Staining with anti-laminin (red) and anti-CD45 (green). (**c**) Immunoperoxidase staining for CD45 with a hemalaun counterstain. Scale bars, 20 μm. Results are representative of three experiments. (**d**) IL-17 and IFN-γ in supernatants of total splenic cells (spleen; 2.5×10^6) and $CD45^+$ cells enriched from brains (brain; 0.5×10^6 to 1×10^6) obtained from MOG(35–55) immunized wild-type mice (clinical score, 3) and CCR6-knockout mice (KO; clinical score, 0), restimulated *in vitro* with MOG(33–55) and assessed at 72 h of culture. IL-17 and IFN-γ were not detected in supernatants of unstimulated cultures (data not shown). Data are representative of three independent experiments with three mice per group in each (mean and s.d.).

RESULTS

CCR6-knockout mice are highly resistant to EAE

To study the function of CCR6 in T_H-17-mediated immunopathology, we compared the susceptibility of wild-type and CCR6-knockout mice[31] to EAE induction. When immunized by subcutaneous injection of a peptide consisting of amino acids 35–55 of myelin oligodendrocyte glycoprotein (MOG(35–55)) in complete Freund's adjuvant (CFA) and pertussis toxin, wild-type mice developed a monophasic disease characterized by ascending paralysis 9–16 d after immunization and prominent leukocyte infiltration and microglial activation in the CNS (**Fig. 1a–c** and data not shown). Notably, most CCR6-knockout mice were completely resistant to the development of EAE and did not show leukocyte infiltration in the CNS, whereas a few (8 of 26) had only minimal disease (clinical score, 0.17 ± 0.28 (mean ± s.d.)) that developed with similar kinetics but had a much lower score (**Table 1**). On day 20, we detected MOG-specific T cells able to produce IL-17 and IFN-γ in the spleens of both wild-type and CCR6-knockout mice (**Fig. 1d**). In contrast, we detected MOG-specific T cells that produced IL-17 and IFN-γ only in the brains of wild-type diseased mice. These results indicate that in CCR6-knockout mice, MOG-reactive T_H-17 and T_H1 cells are primed in lymph nodes and enter the circulation but fail to migrate into the CNS and induce EAE.

CCR6 is not required for TH-17 priming

To rule out a possible contribution of CCR6 in the priming and differentiation of T cells, we assessed the ability of T cells from CCR6-knockout mice to polarize into effector T cells *in vitro*. We stimulated wild-type and CCR6-knockout $CD4^+$ naive T cells *in vitro* in T_H1-, T_H2- or T_H-17-polarizing conditions (**Supplementary Methods** online). Both wild-type and CCR6-knockout T cells showed a similar capacity to upregulate mRNA encoding the transcription factors T-bet, GATA-3 and RORγt and to produce IFN-γ, IL-4 and IL-17,

Table 1 EAE in wild-type and CCR6-knockout mice

Mouse genotype	Incidence	Day of onset (mean ± s.d.)	Maximum clinical score (mean ± s.d.)
Wild type	26 of 29 (89.65%)	16.67 (±0.81)	2.78 (±0.42)
CCR6-knockout	8 of 26 (30.77%)	16.33 (±0.52)	0.17 (±0.28)

Results are cumulative data from six different experiments.

respectively (**Supplementary Fig. 1a** online). As shown before in humans[30], CCR6 was selectively upregulated on wild-type T_H-17 cells but not T_H1 or T_H2 cells (**Supplementary Fig. 1b**). To address whether the lack of CCR6 affected T cell priming *in vivo*, for example, by affecting antigen presentation by dendritic cells that express CCR6 (ref. 32), we immunized wild-type and CCR6-knockout mice by subcutaneous injection of ovalbumin admixed with lipopolysaccharide–monophosphoryl lipid A or CFA (**Supplementary Methods**). The magnitude and quality of the ovalbumin-specific recall T cell responses were similar in wild-type and CCR6-knockout mice, with T_H1 responses prevailing in mice primed with lipopolysaccharide–monophosphoryl lipid A and T_H-17 responses prevailing in mice primed with CFA (**Supplementary Fig. 1c**). Furthermore, OT-II T cell antigen receptor (TCR)–transgenic T cells labeled with the cytosolic dye CFSE (carboxyfluorescein diacetate succinimidyl ester) and adoptively transferred into wild-type or CCR6-knockout mice proliferated and differentiated into T_H-17 cells to a similar extent in both types of mice after immunization with ovalbumin in CFA (**Supplementary Fig. 2** online). These results collectively indicate that CCR6 is selectively upregulated in developing mouse T_H-17 cells and that its expression is not required for T_H-17 priming and differentiation *in vitro* and *in vivo*.

CCR6-knockout mice given T cells are susceptible to EAE

To determine whether CCR6 was required only on T cells, we adoptively transferred green fluorescent protein–positive (GFP^+) MOG-specific naive 2D2-transgenic T cells into CCR6-knockout mice or wild-type control mice. After immunizing mice with MOG(35–55) in CFA, we found $CCR6^+$ IL-17-producing 2D2 T cells in similar proportions in the spleens of CCR6-knockout and wild-type mice (**Fig. 2a**,**b**), which indicated that in CCR6-knockout mice, MOG-reactive T cells differentiated into $CCR6^+$ T_H-17 effector cells. Notably, when given 2D2 T cells, both CCR6-knockout and wild-type mice developed EAE with same kinetics and with greater severity relative to that of mice that did not receive 2D2 T cells (**Fig. 2c**). Finally, CCR6-knockout mice given CCR6-deficient 2D2 T cells (obtained by crossing of 2D2 TCR–transgenic mice with CCR6-knockout mice) did not develop EAE (**Fig. 2c**). These results indicate that CCR6 expression on transferred T cells is both necessary and sufficient to reconstitute disease susceptibility in CCR6-knockout mice.

We further investigated the molecular requirements for the reconstitution of susceptibility to EAE induction in CCR6-knockout mice. Adoptive transfer of wild-type polyclonal naive $CD4^+$ T cells reconstituted disease susceptibility in CCR6-knockout mice, as did the transfer of naive $CD4^+$ T cells from CXCR3-knockout and IFN-γ-knockout mice (**Fig. 2d**). Transfer of naive $CD4^+$ T cells from CCR7-knockout mice induced the development of EAE, which was delayed approximately 2 weeks (**Fig. 2d**), possibly due to inefficient priming of CCR7-deficient naive T cells in lymph nodes. The results presented above collectively indicate that T cells able to reconstitute disease susceptibility in CCR6-knockout mice require CCR6, which is characteristic of T_H-17 cells, but not CXCR3 or IFN-γ, which are characteristic of T_H1 cells.

CCR6-knockout T cells enter the CNS during active EAE

We next analyzed the cells that infiltrated the CNS at the peak of the disease. We isolated $CD45^+$ cells from the perfused brains and spinal cords of CCR6-knockout and wild-type mice given 2D2 T cells and counted endogenous and transferred 2D2 T cells on the basis of the expression of GFP and CD45 congenic markers. Unexpectedly, in both wild-type and CCR6-knockout mice, GFP^+ 2D2 T cells were almost completely undetectable in the brain, and we detected only a few in the spinal cord on day 20 (**Fig. 3a**). Notably, the abundant cellular infiltrate in both CCR6-knockout and wild-type mice was mainly endogenous CCR6-knockout $CD4^+$ and $CD8^+$ T cells, B cells, neutrophils and inflammatory monocytes (**Fig. 3a**,**b** and data not shown). We confirmed those findings by immunohistology that showed only rare GFP^+ cells in inflammatory clusters and several inflammatory foci with apparently no infiltrating GFP^+ cells (**Fig. 3c**). After *in vitro* stimulation with MOG(35–55), brain-infiltrating endogenous $CD4^+$ T cells produced IL-17 and IFN-γ (**Fig. 3d**), which demonstrated that they were antigen specific and may have been T_H-17 cells, T_H1 cells and T cells producing both IL-17 and IFN-γ. The results reported above suggest that both CCR6-sufficient

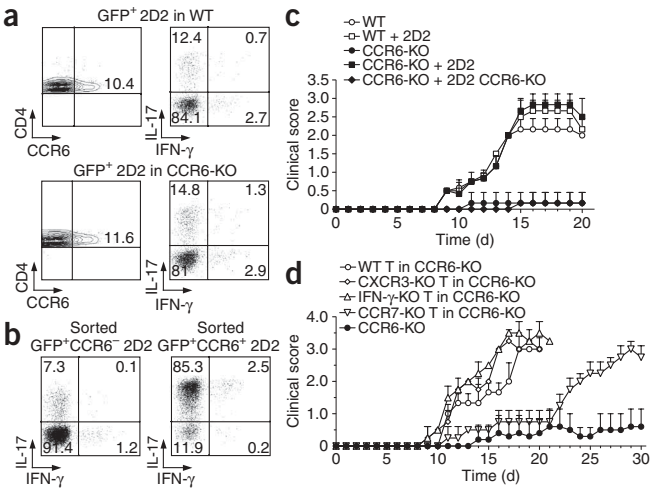

Figure 2 Transfer of wild-type 2D2 T cells reconstitutes EAE susceptibility in CCR6-knockout mice. Analysis of wild-type and CCR6-knockout mice given sham treatment (PBS) or adoptive transfer of naive GFP^+ $CD4^+$ 2D2 T cells or naive polyclonal $CD4^+$ T cells from wild-type mice or mice of various knockout strains, then immunized 16 h later with MOG(33–55) in CFA for EAE induction. (**a**) Expression of CCR6 (left) and production of IL-17 and IFN-γ (right) by GFP-gated 2D2 T cells transferred into wild-type and CCR6-knockout mice and primed 7 d earlier (left). Numbers in quadrants indicate percent $CCR6^+$ cells on GFP-gated 2D2 T cells (left) or percent cells in each (right). Data are representative of three different experiments. (**b**) Production of IL-17 and IFN-γ by sorted $CCR6^+$ and $CCR6^-$ 2D2 T cells primed in wild-type mice. Numbers in quadrants indicate percent cells in each. (**c**) Clinical scores of wild-type and CCR6-knockout mice given sham treatment or adoptive transfer of 2D2 T cells (+ 2D2) or with 2D2 × CXCR6-knockout T cells (+ 2D2 CXCR6-KO). (**d**) Clinical scores of CCR6-knockout mice given sham treatment or adoptive transfer of $CD4^+$ T cells (T) from wild-type, CXCR3-knockout, IFN-γ-knockout or CCR7-knockout mice. Data are representative of at least three different experiments with groups of four or five mice per condition (**b**,**c**; mean and s.d.).

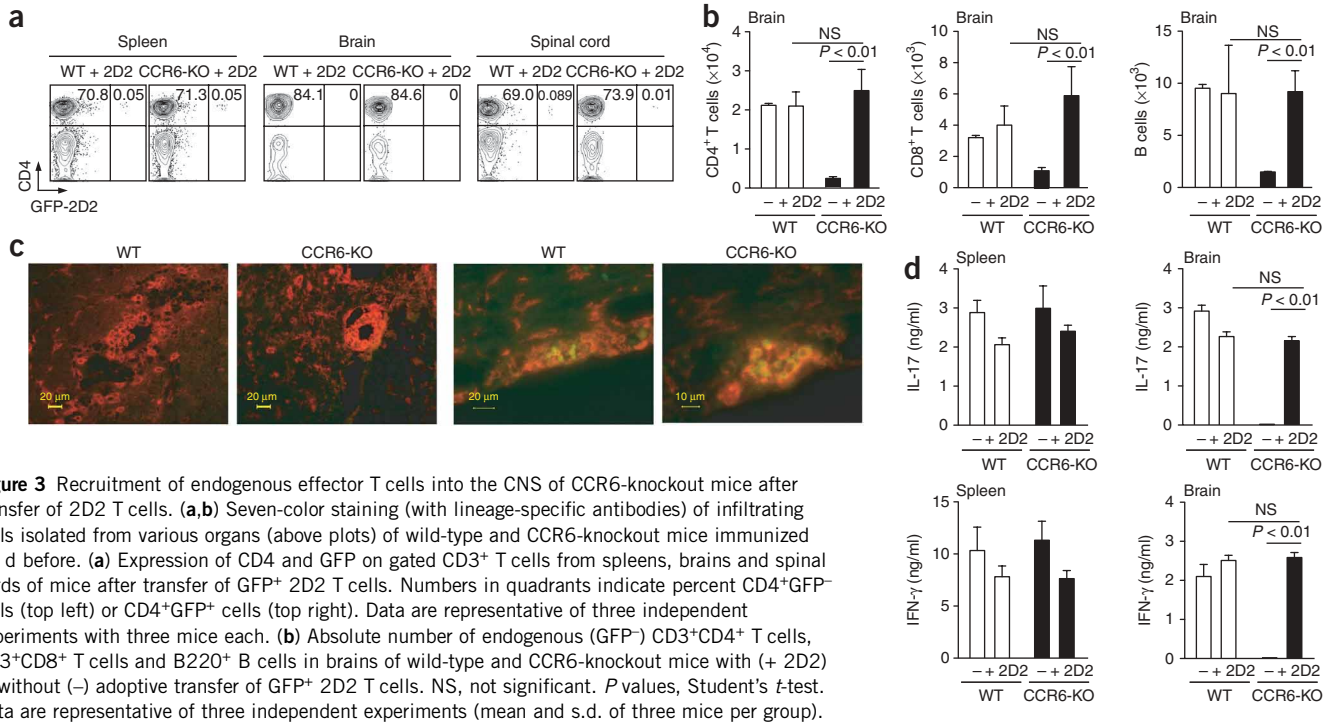

Figure 3 Recruitment of endogenous effector T cells into the CNS of CCR6-knockout mice after transfer of 2D2 T cells. (**a**,**b**) Seven-color staining (with lineage-specific antibodies) of infiltrating cells isolated from various organs (above plots) of wild-type and CCR6-knockout mice immunized 20 d before. (**a**) Expression of CD4 and GFP on gated $CD3^+$ T cells from spleens, brains and spinal cords of mice after transfer of GFP^+ 2D2 T cells. Numbers in quadrants indicate percent $CD4^+GFP^-$ cells (top left) or $CD4^+GFP^+$ cells (top right). Data are representative of three independent experiments with three mice each. (**b**) Absolute number of endogenous (GFP^-) $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $B220^+$ B cells in brains of wild-type and CCR6-knockout mice with (+ 2D2) or without (–) adoptive transfer of GFP^+ 2D2 T cells. NS, not significant. *P* values, Student's *t*-test. Data are representative of three independent experiments (mean and s.d. of three mice per group). (**c**) Immunofluorescence staining for CD45 (red) in spinal cords from a wild-type mouse and a CCR6-knockout mouse (two images from each) after adoptive transfer of GFP^+ 2D2 T cells at day 20 of EAE, showing inflammatory cuffs with or without GFP^+ 2D2 T cells. Scale bars, 20 μm. Results are representative of three independent experiments. (**d**) IL-17 and IFN-γ in supernatants of $CD45^+$ cells enriched from spleens and brains of immunized mice with (+ 2D2) or without (–) adoptive transfer of GFP^+ 2D2 T cells, and stimulated *in vitro* with MOG(33–55), assessed at 72 h of culture. IL-17 and IFN-γ were not detected in unstimulated cultures (data not shown). *P* values, Student's *t*-test. Data are representative of three separate experiments (mean and s.d.).

and CCR6-deficient T cells can enter the CNS in mice with active EAE and that at least some of these cells are MOG specific.

CCR6-knockout T cells roll and adhere to inflamed CNS venules

To visualize the interaction of effector T cells with CNS postcapillary venules, we injected *in vitro*–primed wild-type and CCR6-knockout T_H-17 cells into wild-type mice in which EAE had been induced 12 d before and measured cell rolling and adhesion by intravital microscopy. Activated wild-type and CCR6-knockout $CD4^+$ T cells rolled and adhered to spinal cord postcapillary venules to a similar extent (**Fig. 4a**). In addition, the fraction of permanently adhering T cells was similar and did not change over time (**Fig. 4b**), which suggested that the adhering cells began to enter the spinal cord during the time of observation. In contrast, when we injected T cells into CCR6-knockout mice that had been primed with MOG(35–55) and CFA 12 d before but did not develop disease, the cells failed to interact with endothelial cells (data not shown). Similarly, and consistent with a published report[21], in healthy mice, activated T cells of either wild-type or CCR6-knockout origin failed to show adhesive interaction with brain endothelium (data not shown). These results indicate that CCR6 is not sufficient to mediate adhesion of T_H-17 cells to uninflamed endothelial cells (even in CCR6-knockout mice immunized with CFA and pertussis toxin) and that it is not required for the entry of T cells into the CNS once tissue inflammation is established.

T cells migrate into the CNS in two waves

On the basis of the results reported above, we hypothesized that a first wave of T_H-17 cells migrating into the uninflamed CNS in a CCR6-dependent way is needed to trigger activation of parenchymal endothelial cells and the recruitment of a second wave of effector cells, composed of T_H-17 and T_H1 cells, which can migrate in a CCR6-independent way across the activated blood-brain barrier. To test our hypothesis, we transferred into wild-type mice a mixture of naive 2D2 T cells from wild-type and CCR6-knockout mice that could be identified by their expression of GFP and CD45 and, after immunizing the recipient mice with MOG(35–55), we measured the relative proportions of transferred cells in the brain during disease development (**Fig. 5a**). On day 10, wild-type T cells predominated over CCR6-knockout T cells. In contrast, on day 16, more cells of both populations were present in the brain in similar proportions.

We also adoptively transferred a mixture of *in vitro*–primed T_H-17 cells from wild-type mice (which had more than 60% $CCR6^+$ cells) and CCR6-knockout mice and, 48 h later, measured their migration into the spleens and brains of mice that developed EAE (**Fig. 5b**). We recovered wild-type and CCR6-knockout T cells in similar proportions from spleens at all time points tested. In contrast, in the brain, wild-type T cells were present at higher frequency than were CCR6-knockout T cells on day 7, but the proportion of CCR6-knockout cells steadily increased at later time points (days 10 and 16; **Fig. 5b**). These results are collectively consistent with the hypothesis that early migration of T cells in the brain is CCR6 dependent, whereas late migration can occur in a CCR6-independent way.

Epithelial cells of the choroid plexus express CCL20

To identify the initial port of entry of $CCR6^+$ T cells, we analyzed in the CNS of healthy wild-type and CCR6-knockout mice and diseased wild-type mice expression of the CCR6 ligand CCL20, which is expressed in liver and Peyer's patches[29]. We found CCL20 on scattered

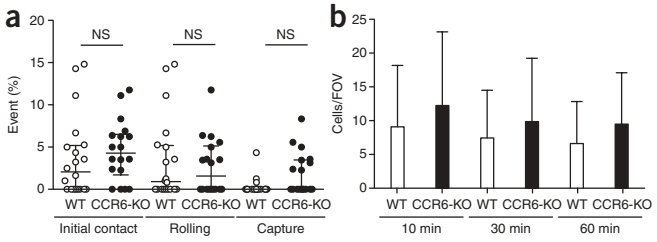

Figure 4 CCR6 is not required for T cell rolling and the adhesion of T cells to inflamed brain endothelia. Intravital microscopy (epi-illumination) of *in vitro*–primed T_H-17 cells obtained from wild-type and CCR6-knockout mice, labeled with CellTracker green and injected into the catheterized right carotid arteries of wild-type mice (with a clinical score of 0.5–1) that had undergone laminectomy from C2–C7 and removal of the dura. (**a**) Initial contact, rolling and capture of T cells in postcapillary venules, among total T cells passing through a given venule during a 1-min observation period. Each dot represents one postcapillary venule; small horizontal lines indicate the median with the interquartile range. Statistical analysis, Mann-Whitney U-test. Data are representative of three experiments analyzing 20 venules in five mice that received wild-type T_H-17 cells and 18 venules in four mice that received CCR6-knockout T_H-17 cells. (**b**) Adherent and plugging T cells, presented as cell number per field of view (Cells/FOV), normalized to the number of fields of view. Data are representative of three experiments analyzing four to six fields of view per mouse (mean and s.d. of five mice that received wild-type T_H-17 cells and four mice that received CCR6-knockout T_H-17 cells).

cells in several regions of the brain but not on normal or inflamed endothelial cells of the brain (data not shown). Notably, however, we found very high and uniform expression of CCL20 in epithelial cells of the choroid plexus of healthy wild-type and CCR6-knockout mice and of wild-type mice that developed EAE (**Fig. 5c** and data not shown). There was no staining of the choroid plexus parenchyma (data not shown), which suggested accumulation of CCL20 in and around choroid plexus epithelium. We detected CCL20 mRNA in liver and Peyer's patches, as expected, as well as comparable expression in choroid plexuses of healthy and EAE mice, whereas we did not detect it in the brain parenchyma (**Supplementary Methods** and **Supplementary Fig. 3** online).

The findings reported above suggested that CCL20 might be required for the initial entry of $CCR6^+$ cells into uninflamed CNS through the choroid plexus and cerebrospinal fluid and that the same pathway might be used for the constitutive migration of lymphocytes for immunosurveillance of the CNS. That hypothesis is consistent with two observations. First, the choroid plexus of MOG-immunized CCR6-knockout mice had an accumulation of $CD45^+$ cells greater than that of wild-type mice (**Fig. 5d**), which supports the idea that the epithelial layer of the choroid plexus is the barrier that EAE-initiating T cells must pass through. In addition, $CD45^+$ cells accumulated in the choroid plexus in CCR6-deficient mice in the parenchyma between laminin-positive endothelial and epithelial basement membranes (data not shown). Finally, in the steady state, CCR6-knockout mice had significantly fewer CNS-associated $CD4^+$ T cells, $CD8^+$ T cells and B cells than did wild-type mice (**Fig. 5e**).

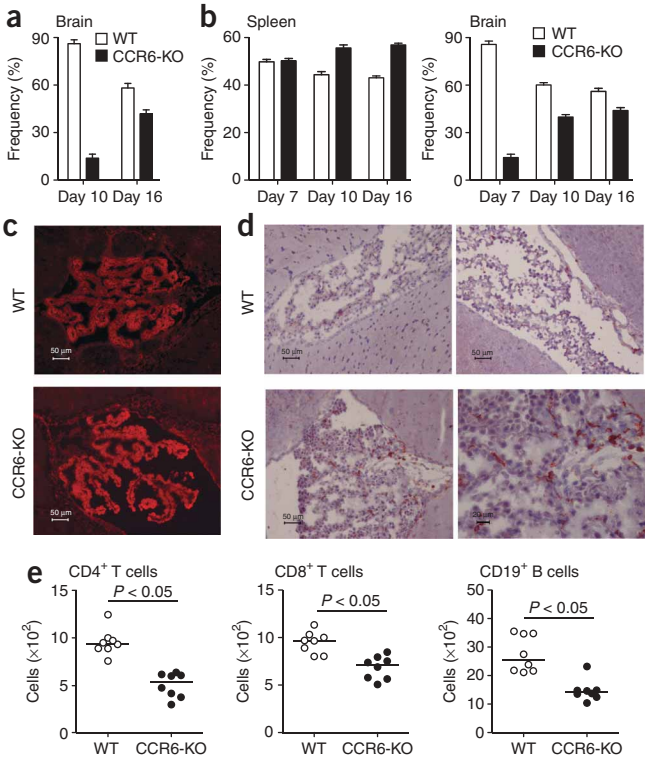

Figure 5 CCR6 is required for the migration of T cells into the CNS through CCL20-expressing epithelial cells of the choroid plexus in the steady state and at early time points of EAE. (**a**,**b**) Recruitment of wild-type and CCR6-knockout T cells in developing EAE. (**a**) Recovery of naive $CD4^+$ T cells (2.5×10^6) sorted from wild-type (GFP^+) mice and CCR6-knockout ($CD45.1^+$) mice, mixed at a ratio of 1:1 and transferred into wild-type $CD45.2^+$ mice, which were then immunized with MOG(33–55) in CFA, presented as the proportion of transferred wild-type and CCR6-knockout T cells recovered from the brain 10 d or 16 d after immunization. (**b**) Recovery of naive $CD4^+$ T cells sorted from wild-type (GFP^+) and CCR6-knockout ($CD45.1^+$) mice, stimulated *in vitro* in T_H-17 conditions, mixed at a ratio of 1:1 and transferred into $CD45.2^+$ wild-type mice in which EAE was induced 7, 10 or 16 d earlier, presented as the proportion of transferred T cells recovered from the spleen and brain at 48 h after transfer. Data are representative of three separate experiments (mean and s.d.). (**c**) Immunofluorescence of CCL20 staining of cryosections of brains from wild-type and CCR6-knockout mice collected after perfusion. Scale bars, 50 μm. Results are representative of three experiments. (**d**) Immunoperoxidase staining and hemalaun counterstaining of $CD45^+$ cells in the choroid plexus parenchyma of wild-type and CCR6-knockout mice in which EAE was induced 13 d earlier. Scale bars, 50 μm (top row and bottom left) or 20 μm (bottom, right). Results are representative of at least three separate experiments. (**e**) Absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells in brains of wild-type and CCR6-knockout mice in the steady state. Each symbol represents an individual mouse; small horizontal lines indicate the median. *P* values, Student's *t*-test. Data are representative of two experiments.

CCR6 and CCL20 in human cerebrospinal fluid and brain

To determine whether the findings reported above obtained with the mouse model could be extended to the human disease of multiple sclerosis, we analyzed CCR6 expression in T cells from patients with multiple sclerosis and analyzed CCL20 expression in the brains of controls and patients with multiple sclerosis. In eight patients with an initial demyelinating event (the first clinical episode of multiple sclerosis), $CCR6^+CD25^-CD4^+$ inflammatory T cells were present at significantly higher frequencies in the cerebrospinal fluid than in peripheral blood (**Fig. 6a**). This finding demonstrates that many cells detectable in the cerebrospinal fluid at the earliest clinical demyelinating event express CCR6.

Immunohistology of normal healthy tissues showed that CCL20 was expressed in the liver, mainly in Kupffer cells, and in scattered cells

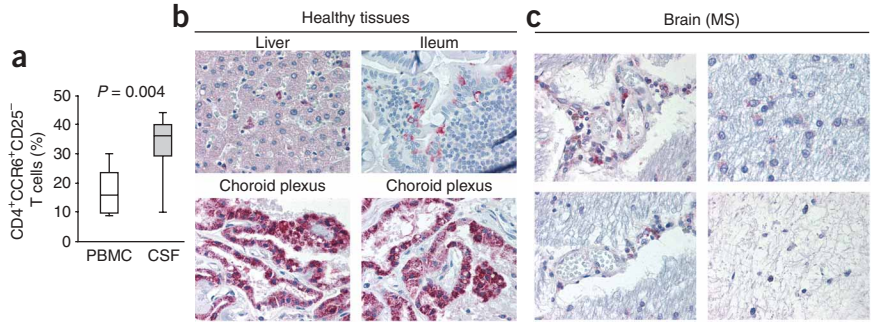

Figure 6 Expression of CCL20 and CCR6 in human normal tissue and multiple sclerosis tissues. (**a**) CCR6$^+$ cells among gated CD4$^+$ CD25$^-$ T cells from matched samples of peripheral blood (PBMC) and cerebrospinal fluid (CSF) obtained from eight patients with multiple sclerosis. *P* value, Mann-Whitney U-test. Data are representative of eight experiments. (**b**,**c**) CCL20 expression on normal human tissues (**b**) and in various areas of the brains of patients with multiple sclerosis (MS; **c**), assessed by immunohistochemistry of formalin-fixed, paraffin-embedded sections with a CCL20-specific antibody. Original magnification, ×40 (**b**, all images; **c**, top left and bottom row) or ×60 (**c**, top right). Results are representative of two separate experiments on two different tissue samples.

in the crypts of the ileum (**Fig. 6b**). Notably, there was strong and uniform staining for CCL20 in epithelial cells of the choroid plexus in the brain (**Fig. 6b**), whereas normal brain parenchyma contained some cells that stained faintly for CCL20, including astrocytes and cells with neuronal and microglial morphology (data not shown). In tissues from patients with multiple sclerosis, we detected high CCL20 expression in inflamed areas, in astrocytes positive for glial fibrillary acidic protein and in the choroid plexus (**Fig. 6c** and data not shown). Accordingly, we detected some CD3$^+$CCR6$^+$ T cells in inflamed parenchyma near astrocytes that were CCL20$^+$ (data not shown). These findings suggest that in humans, recruitment of CCR6$^+$ cells into the CNS may occur initially through epithelial cells of the choroid plexus that constitutively express CCL20, whereas at a later stage, CCL20 production by activated astrocytes may contribute to the recruitment of CCR6$^+$ T cells into the brain parenchyma. Thus, CCR6 and CCL20 may represent an evolutionary conserved axis that regulates the CNS entry and dissemination of T cells in the steady state and during inflammation.

DISCUSSION

We have shown here that the CCR6 serves an essential function in the initiation of EAE by controlling the migration of a first wave of autoreactive T_H-17 cells in the uninflamed CNS. The entry of CCR6$^+$ T cells into the CNS probably occurs through the blood–cerebrospinal fluid barrier, as epithelial cells of the choroid plexus constitutively expressed the CCR6 ligand CCL20. The first wave of migratory T cells was required for the recruitment of a second wave of T cells that entered the CNS parenchyma in a CCR6-independent way through activated parenchymal postcapillary venules.

The conclusions above were based on three main findings. First, when immunized by a standard protocol MOG(35–55) in CFA plus pertussis toxin, CCR6-knockout mice developed T_H-17 responses but failed to develop EAE. In particular, we did not recover MOG-reactive effector T cells from the CNS of these mice and their parenchymal venules did not support the extravasation of adoptively transferred T cell blasts. Second, the transfer of wild-type naive T cells into CCR6-knockout mice was sufficient to reconstitute disease susceptibility. However, wild-type CCR6$^+$ T cells predominated in the CNS only at an initial asymptomatic stage of the disease, whereas during active disease, the cellular infiltrate in the CNS parenchyma was composed of endogenous CCR6-knockout T cells, including MOG-reactive T_H-17 and T_H1 cells, that efficiently adhered to activated CNS endothelial cells. Third, the CCR6 ligand CCL20 had high and constitutive expression by epithelial cells of the choroid plexus but not by parenchymal endothelial cells. In addition, in CCR6-knockout mice, T cells seemed to be trapped between the endothelial and epithelial basement membranes of the choroid plexus.

Our findings provide a molecular and anatomical basis for distinguishing between constitutive and inflammatory pathways of T cell entry into the CNS and support a two-step model of EAE pathogenesis in which a first wave of CCR6$^+$ T_H-17 cells leads to the CCR6-independent recruitment in the CNS of a second wave of T cells, including T_H1 cells, and inflammatory leukocytes. The two-step model of EAE pathogenesis is consistent with the finding that in the early phases of EAE, CD4$^+$ T cells accumulate first in the subarachnoid space and subsequently appear in the CNS parenchyma[33,34]. In those studies, the nature of the T cells that initially infiltrate the subarachnoid space and their port of entry were not defined, although it was suggested that they might enter directly through the meningeal vessels or disseminate through the cerebrospinal fluid after crossing the epithelial cell layer of the choroid plexus. The model is also consistent with the published description of 'pioneer' lymphocytes that migrate into uninflamed CNS through the choroid plexus in a P-selectin-dependent way[20] and with studies emphasizing the importance of the activation state and antigenic specificity of T cells that migrate in the CNS in the initial phases of EAE[35,36]. A two-wave migration of T_H-17 and T_H1 cells into tissues may apply to other autoimmune diseases such as collagen-induced arthritis[37]. In addition, in a model of *Mycobacterium tuberculosis* infection, it has been shown that vaccination induces IL-17-producing CD4$^+$ T cells that populate the lung and, after challenge, trigger the production of chemokines that recruit CD4$^+$ T cells that produce IFN-γ, which ultimately restrict bacterial growth[38].

The findings that CCR6 is essential for the entry of T cells into the CNS in the early phase of EAE and the expression of CCL20 in epithelial cells of the choroid plexus provide a molecular determinant and an anatomical site for the first triggering step in EAE pathogenesis. It remains to be established which adhesion molecules are needed to T cells to cross the blood–cerebrospinal fluid barrier and whether chemokines other than CCL20 might be expressed on epithelial cells of the choroid plexus. Consistent with our finding that CXCR3 was not needed to mediate the entry of T cells in the initial phase of EAE, we found that its ligands, CXCL9 and CXC10, were not expressed in the choroid plexus of C57BL/6 mice (D.B., unpublished data), which also do not express CXCL11 because of a genetic defect.

An implication of our findings is that one function of the first wave of CCR6$^+$ T_H-17 cell is to activate the postcapillary venules in the CNS parenchyma, which in normal circumstances are inefficient in sustaining rolling and adhesion of activated leukocytes and hence the entry of cells into the CNS parenchyma[21]. It is conceivable that once they have entered through the choroid plexus into the cerebrospinal fluid, T_H-17 cells may disseminate at the pial surface and in the enlarged perivascular Virchow-Robin spaces, where they may recognize self antigens displayed on resident antigen-presenting cells. Once activated, T_H-17 cells produce cytokines and chemokines that act locally to trigger activation of the blood-brain barrier and to initiate the influx of large

numbers of inflammatory cells, including T_H-17 and T_H1 cells, neutrophils and inflammatory monocytes, that build up the lesions characteristic of EAE. Although we have shown that the T cell infiltrate was composed of MOG-reactive T cells, it is likely that antigen-nonspecific bystander T cells are also recruited through the blood-brain barrier and may constitute a large fraction of the total cellular infiltrate, as has been shown in delayed-type hypersensitivity reactions as well as several autoimmune diseases[39].

It is worth noting that in the experimental conditions of active EAE used in our studies, effector T cells were induced in peripheral lymph nodes by subcutaneous immunization and had to enter uninflamed CNS, as the inflammatory stimuli were limited to the local effect of CFA and the systemic effect of pertussis toxin administered at the time of immunization. It can be anticipated that the requirement for the CCR6-dependent entry of autoreactive T cells through the choroid plexus, which seems to be rate-limiting in steady-state conditions, may be bypassed whenever the CNS vasculature is activated by local or systemic inflammatory stimuli[40] or in models of passive EAE induced by the injection of highly activated T cell blasts.

An open issue relates to the nature of the cytokines produced by the first wave of T_H-17 cells needed for the initiation of EAE. T_H-17 cells produce IL-17, IL-22, tumor necrosis factor and, in some cases, IFN-γ, which can act on microglial cells and endothelial cells[41]. We found that IFN-γ expression was not required in the $CCR6^+$ T cells that initiated EAE, and we have preliminary evidence that IL-17A may also be dispensable (A.R., unpublished data). In addition, studies have shown that IL-17A and IL-17F, as well as IL-22, may not be required for the development of EAE[42,43]; such findings would be consistent with either a redundant function of these cytokines in disease development or with an essential involvement of another functional property (cytokine or chemokine production; chemokine receptor expression) of T_H-17-lineage cells. Experiments with CCR6-knockout mice given adoptive transfer of CCR6-sufficient T cells carrying selective genetic defects will help to resolve this issue.

We have shown here that once EAE was triggered, effector T cells of both wild-type and CCR6-knockout origin efficiently rolled and adhered to inflamed CNS postcapillary venules and migrated with similar efficiency into the CNS. These findings indicate that at a late stage, the CCR6-CCL20 axis is dispensable and the recruitment of inflammatory cells into the CNS parenchyma can be mediated by other chemokine receptors[44]. Several inflammatory chemokines are upregulated in the CNS during EAE[45–47], and some chemokine receptors, such as CCR1, CCR2 and CXCR3, have been shown to be involved in EAE[48–52]. In addition, a CCR5 receptor antagonist and antibodies to CCL20 have been reported to diminish disease severity[53,54]. Such findings are consistent with the presence of multiple redundant mechanisms that regulate the entry of leukocytes into the CNS once inflammation has been established.

In the context of its function in migration into the CNS, CCR6 expression on activated and memory T cells and other cell types deserves mention. We have shown that CCR6 was selectively upregulated in developing mouse T_H-17 cells but not T_H1 or T_H2 cells both *in vitro* and *in vivo*. In addition, we have shown that in mice, CCR6 was selectively induced by CFA, the adjuvant typically used for EAE induction. It is notable that in humans, CCR6 is expressed not only on T_H-17 cells, where it is expressed together with CCR4, but also on cells that produce both IL-17 and IFN-γ, as well as on a subset of T_H1 cells characterized by CXCR3 expression[30]. It remains to be determined whether CCR6 can be induced on mouse T_H1 cells in certain conditions, a possibility that may explain the finding that T_H1 cells can enter uninflamed brain[40], although in that case[40], the port of entry into the CNS was not identified. CCR6 is also expressed on B cells, which have been linked to the pathogenesis of multiple sclerosis[55], and on a subset of regulatory T cells[56]. Indeed, it has been shown that CCR6 is important in regulating the recruitment of T_H-17 cells as well as regulatory T cells into inflammatory tissues[57]. Thus, it is possible that CCR6 is used by a variety of cells to enter the brain through the choroid plexus and participate in immunoregulatory circuits.

Our findings help address a controversial aspect of the relative functions of T_H1 and T_H-17 cells in brain inflammation. Although studies have demonstrated a requirement for IL-17-producing T cells in EAE[10], it was puzzling that T_H-17 cells often accounted for a minor fraction of infiltrating T cells, at least at the peak of the disease. Indeed, subsequent studies have led to a reevaluation of the function of T_H1 cells[58]. The two-step model of EAE pathogenesis provides a way to reconcile those findings with the following considerations. First, the proportion of T_H1 and T_H-17 cells may be highly variable depending on the priming conditions. Second, a few autoreactive $CCR6^+$ T cells may be able to promote the vascular recruitment of large numbers of inflammatory cells. Third, CCR6 is expressed in subsets of T_H1 cells in humans and possibly in mice.

It is well appreciated that chemokines and their receptors, together with adhesion molecules, control the constitutive homing of lymphocytes to lymphoid and nonlymphoid tissues to accomplish their immunosurveillance function. For example, CCR9 defines a subset of gut-homing lymphocytes, whereas CCR4 and CCR10 direct skin-tropic T-cell trafficking[59]. Similar selective mechanisms that target T cells to the CNS are thought to exist[60], but they have not been identified. On the basis of our findings, we propose that CCR6 is a brain-specific determinant for the constitutive trafficking of patrolling T cells and B cells in the CNS. The rapid distribution of T cells throughout the choroid plexus and the cerebrospinal fluid into the meningeal space seems to be instrumental for broad surveillance over the entire surface of the CNS, thus maximizing the chance that antigen-bearing antigen-presenting cells will be detected at superficial sites while at the same time the entry of inflammatory cells into the parenchyma, where this is needed, will be elicited. The cytokine-mediated activation of endothelial cells of the blood-brain barrier executed by patrolling T cells might provide a means for targeting large numbers of effector cells to a precise region of the CNS parenchyma.

Some aspects of the mouse model also apply to humans. These include the fact that in humans, CCR6 is expressed by T_H-17 cells[30], as well as the enrichment for $CCR6^+$ cells in the cerebrospinal fluid of patients presenting with the first clinical symptom of multiple sclerosis, a condition that may parallel the earliest phase of EAE. Moreover, in noninflammatory conditions in humans, CCL20 is expressed almost exclusively by epithelial cells of the choroid plexus, whereas in multiple sclerosis tissues, CCL20 can also be expressed by astrocytes positive for glial fibrillary acidic protein (A.U., unpublished data), which suggests that in multiple sclerosis, selective recruitment of $CCR6^+$ cells may occur through the choroid plexus in the early phase of disease, whereas at a later stage, astrocytes may contribute to the recruitment of $CCR6^+$ T cells in brain parenchyma. These findings indicate involvement of the CCR6-CCL20 axis in initiating brain inflammation in humans and possibly an evolutionary conserved mechanism of immune surveillance in the CNS. The relapsing-remitting form of human multiple sclerosis and the anatomical distribution of multiple sclerosis lesions may be consistent with either distinct waves of migration or asynchronous activation of already resident $CCR6^+$ T cells. Distinguishing between these possibilities is relevant to understanding the potential therapeutic use of CCR6-blocking drugs in human multiple sclerosis.

METHODS

Mice. C57BL/6 mice were from Harlan; 2D2 TCR–transgenic (006912), UBC-GFP (004353) and *Ifng*$^{-/-}$ (002287) mice were from the Jackson Laboratory. OT-II TCR–transgenic mice (provided by J. Kirberg) were bred onto backgrounds of various *Cd45* alleles in the animal facility of the Institute for Research in Biomedicine. *Ccr6*$^{-/-}$ mice have been described[31], *Cxcr3*$^{-/-}$ mice were provided by C. Gerard, and *Ccr7*$^{-/-}$ mice were provided by M. Lipp. Mice were treated in accordance with guidelines of the Swiss Federal Veterinary Office and experiments were approved by the Dipartimento della Sanità e Socialità.

EAE model. Groups of female C57BL/6 mice 8–10 weeks of age were immunized subcutaneously on day 0 with 100 μg MOG(35–55) (MEVG WYRSPFSRVVHLYRNGK; Servei de Proteòmica, Pompeu Fabra University, Barcelona) emulsified in CFA (Difco). Pertussis toxin in 100 μl saline was injected intravenously twice, on days 0 and 2 or days 1 and 3. Disease severity was assigned scores on the following scale: 0, no disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness or paralysis; 5, moribund or dead. In some EAE experiments, naive T cells (1×10^5) from GFP$^+$ 2D2-transgenic mice or 2D2 × CCR6-knockout mice or total CD4$^+$ T cells (10×10^6) from wild-type, CXCR3-knockout, IFN-γ-knockout or CCR7-knockout mice were transferred intravenously into mice 16 h before immunization. In some experiments, a mixture of GFP$^+$ 2D2-transgenic T cells (2.5×10^6) and 2D2 × CCR6-knockout T cells (at a ratio of 1:1) were transferred intravenously into mice 16 h before immunization. Alternatively, effector T_H-17 cells were generated *in vitro* from GFP$^+$ 2D2-transgenic T cells (2.5×10^6) and 2D2 × CCR6-knockout T cells (at a ratio of 1:1) and were transferred intravenously in wild-type mice at various times after EAE induction. For the preparation of CNS lymphocytes, mice were perfused through the left cardiac ventricle with cold PBS. The forebrain and cerebellum were dissected, were cut into pieces and were digested for 45 min at 37 °C with collagenase D (1 mg/ml; Roche Diagnostics) and DNaseI (1 mg/ml; Sigma). CD45$^+$ cells were isolated by passage of the tissue through a cell strainer (70 μm), followed by incubation with beads coated with antibody to CD45 (anti-CD45; 130-052-301; Milteny). After passage through the column, CD45$^+$ cells were washed and resuspended in culture medium for further analysis.

Flow cytometry analysis and *in vitro* stimulation. For analysis of mouse phenotypes, the following monoclonal antibodies were used: anti-L-selectin (MEL14), anti-CD44 (IM7), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD3 (145-2C11), anti-CD28 (37.51), anti-B220 (RA3-6B2), anti-CD19 (1D3), anti-CD11b (M1/70), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD127 (A7R34) and anti-IFN-γ (XMG1.2; all from eBiosciences); and anti-IL-17A (TC11-18H10) and anti-CCR6 (140706; both from BD Biosciences). For intracellular cytokine staining, cells were stimulated for 4 h with phorbol 12-myristate 13-acetate (100 nM; Sigma) and ionomycin (1 μg/ml; Sigma), with the final 2 h of culture in the presence of brefeldin A (10 μg/ml; Sigma). Labeled antibodies were used after cells were fixed in 4% (wt/vol) paraformaldehyde and made permeable with 0.5% (wt/vol) saponin (Sigma-Aldrich). Six-color staining of the cell surface was done with the appropriate combinations of antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, peridinine chlorophyll protein complex, phycoerythrin-indotricarbocyanine, allophycocyanin, allophycocyanin-indotricarbocyanine or biotin, and with streptavidin labeled with phycoerythrin-indotricarbocyanine or allophycocyanin-indotricarbocyanine (BD Biosciences). Samples were acquired on a FACSCanto (BD Biosciences) and were analyzed with FlowJo software (TreeStar).

For studies of human cell phenotypes, peripheral blood and cerebrospinal fluid were obtained from eight patients (after informed consent was provided) who presented with a first demyelinating event suggestive of multiple sclerosis and underwent venipuncture and lumbar puncture for diagnostic purposes. The study was approved by the Ethical Committee and Board of the Department of Neurosciences, Ophthalmology and Genetics, University of Genoa. Peripheral blood mononuclear cells were isolated with Ficoll and cerebrospinal fluid leukocytes were collected after centrifugation, then these cells were incubated for 30 min with the following monoclonal antibodies: fluorescein isothiocyanate–conjugated anti-CD4 (RPA-T4), phycoerythrin-conjugated anti-CCR6 (11A9), phycoerythrin-indodicarbocyanine–conjugated anti-CD25 (M-A251) and allophycocyanin-conjugated anti-CD45RO (UCHL1; all from BD Biosciences). At the end, cells were washed with PBS, were resuspended, were counterstained with propidium iodide (1 μg/ml; Sigma Aldrich) and were analyzed by flow cytometry of the propidium iodide–negative population. A FACSCanto (BD Biosciences) was used for all flow cytometry and data were analyzed with FACSDiva & FloJo software.

For antigen-specific restimulation of mouse T cells, 5×10^6 splenocytes or 2.5×10^6 lymph node cells were cultured for 3 d in presence of MOG(35–55). Cells purified from the brain were cultured for 3 d in presence of fixed splenocytes loaded with MOG(35–55). For fixation, spleens were removed from naive C57BL/6 mice and erythrocytes were lysed. Splenocytes were incubated for 90 min at 37 °C with MOG(35–55) (50 μg/ml), then were washed in 1% (vol/vol) FCS in PBS and were fixed for 30 s at 20 °C in 0.05% (vol/vol) glutaraldehyde (Merck). An equal volume of a solution of glycine (0.2 M; Sigma-Aldrich) was added for 30 s and then cells were washed and used for coculture. Cytokines in culture supernatants were measured by enzyme-linked immunosorbent assay according to the manufacturer's protocols (BD Biosciences). Data were analyzed with the SoftMax program.

Immunohistology and immunofluorescence. Mice were perfused through the left cardiac ventricle with 1% (vol/vol) formaldehyde (Grogg Chemie) in PBS. Brains and spinal cords were removed, were embedded in Tissue-Tek optimum cutting temperature compound (Haslab) and were 'snap-frozen' in a bath of dry ice and isopentane (Grogg Chemie). Cryostat sections 6 μm in thickness were air-dried overnight, were fixed in acetone and were stained for immunohistology with a three-step immunoperoxidase staining kit according to the manufacturer's protocol (Vectastain; Reactolab). For immunofluorescence staining, sections were blocked for 20 min with skim milk and then were incubated for 1 h each with primary and secondary antibody diluted in skim milk, with washing steps of Tris-buffered saline between incubations. After a final wash in Tris-buffered saline, sections were mounted in Mowiol solution (Calbiochem). Antibodies used were as follows: fluorescein isothiocyanate–conjugated anti-CD45 (30F11; BD Biosciences), anti-CCL20 (AB9829; Abcam), anti-laminin (Z0097; Dako), anti-PECAM-1 (Mec13.3; BD Biosciences) and anti–'pan cytokeratin' (C2562; Sigma).

LifeSpan Biosciences analyzed CCL20 expression in healthy and diseased human tissues. Formalin-fixed, paraffin-embedded tissues were stained with rabbit polyclonal anti-CCL20 (15 μg/ml; AB9829; Abcam). The detection system consisted of anti-rabbit secondary antibody (BA-1000; Vector) and an ABC-AP kit (avidin–biotinylated enzyme complex–alkaline phosphatase; AK-5000; Vector) with a Red substrate kit (SK-5100; Vector), which was used to produce a fuchsia-colored deposit. Only tissues that were positive for staining of CD31 and vimentin were used. The negative control consisted of immunohistochemistry of adjacent sections in the absence of primary antibody. Slides were imaged with a DVC 1310C digital camera coupled to a Nikon microscope.

Intravital microscopy. Active EAE was induced by immunization with MOG(35–55) in CFA. Mice with a score of 0.5 (limp tail) to 1 (hind leg weakness) were used for intravital fluorescence videomicroscopy experiments. The spinal cord window was created as described in the **Supplementary Methods**. Normal body temperature was maintained throughout the entire experiment. Epi-illumination techniques were used for intravital fluorescence videomicroscopy with an IVM500 microscope (Mikron Instruments) coupled to a 50-Watt mercury lamp (HBO 50 microscope illuminator; Zeiss) and combined with blue filter blocks (exciter, 455DF70; dichroic, 515DRLP; emitter, 515ALP) and green filter blocks (exciter, 525DF45; dichroic, 560DRLP; emitter, 565ALP). Observations were made with 4×, 10× and 20× long-distance working objectives (Zeiss). All experiments were recorded by means of a low-light silicon-intensified target video camera with an optional image intensifier for weak fluorescence (Dage-MTI of Michigan City). Data were transferred to a digital video system for later offline analysis of the interaction of cells with CNS microvessels. The injection of 1% (vol/vol) tetramethylrhodamine isothiocyanate–conjugated dextran in 0.9% (wt/vol) NaCl allowed visualization of the spinal cord microvasculature. In parallel, mouse T cells were stained for 45 min at 37 °C with 2.5 μM CellTracker green (Molecular Probes) and were injected into the carotid artery (4×10^6 T cells in three

0.1-ml aliquots). Injection into this catheter resulted in direct transport into observed vessels, where interactions were recorded by digital video for subsequent offline analysis. Several adjacent areas were scanned in a 'stepwise' way for recording of permanently adhering T cells at 10 min, 30 min and 1 h after injection. Recorded videos were subsequently analyzed offline as described (**Supplementary Methods**).

Statistics. Differences between data sets were analyzed by Student's *t*-test or a Mann-Whitney U-test.

Accession code. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A000629

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank D. Jarrossay for cell sorting; T. Périnat and S. Minghelli for immunohistology analysis; E. Mira Catò and L. Perlini for technical assistance; B. Becher (University Hospital Zurich), C. Gerard (Harvard Medical School), J. Kirberg (Max-Plank Institute of Immunobiology) and M. Lipp (Max Delbruck Center) for mouse strains; L. Sallusto for discussions and support; and A. Almeida, A. Martín-Fontecha, G. Napolitani, U. Schenk and M. Uguccioni for discussions. Supported by the Swiss National Science Foundation (31-101962 to F.S.), the European Commission Sixth Framework Programme (LSB-CT-2005-518167 IINOCHEM and LSHG-CT-2005-005203 MUGEN), the US National Multiple Sclerosis Society (B.E. and C.C.), the Swiss Multiple Sclerosis Society, the Italian Foundation for Multiple Sclerosis (Stem Cell Project 2007-2009), the European Union-funded International Graduate Program in Molecular Medicine (A.R.), Boehringer Ingelheim Fonds (D.B.) and the Helmut Horten Foundation (to The Institute for Research in Biomedicine).

AUTHOR CONTRIBUTIONS

A.R. did most of the experiments and contributed to experimental design; C.C., D. Baumjohann, F.B. and D. Bottinelli did experiments; S.L. generated the CCR6-knockout mice and provided intellectual input; B.E. and A.U. interpreted data, provided intellectual input and contributed to writing the manuscript; A.L. provided intellectual input and wrote the manuscript; and F.S. conceived the study, interpreted the data and wrote the manuscript.

Published online at http://www.nature.com/natureimmunology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. McGeachy, M.J. & Cua, D.J. Th17 cell differentiation: the long and winding road. *Immunity* **28**, 445–453 (2008).
2. Bettelli, E., Korn, T., Oukka, M. & Kuchroo, V.K. Induction and effector functions of T_H17 cells. *Nature* **453**, 1051–1057 (2008).
3. Dong, C. T_H17 cells in development: an updated view of their molecular identity and genetic programming. *Nat. Rev. Immunol.* **8**, 337–348 (2008).
4. Weaver, C.T., Hatton, R.D., Mangan, P.R. & Harrington, L.E. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* **25**, 821–852 (2007).
5. Gaffen, S.L. An overview of IL-17 function and signaling. *Cytokine* **43**, 402–407 (2008).
6. Steinman, L. A brief history of T_H17, the first major revision in the T_H1/T_H2 hypothesis of T cell-mediated tissue damage. *Nat. Med.* **13**, 139–145 (2007).
7. Ivanov, I.I. *et al.* The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells. *Cell* **126**, 1121–1133 (2006).
8. Komiyama, Y. *et al.* IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* **177**, 566–573 (2006).
9. Hofstetter, H.H. *et al.* Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cell. Immunol.* **237**, 123–130 (2005).
10. Langrish, C.L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233–240 (2005).
11. Tzartos, J.S. *et al.* Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am. J. Pathol.* **172**, 146–155 (2008).
12. Bettelli, E. *et al.* Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **200**, 79–87 (2004).
13. Luger, D. *et al.* Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J. Exp. Med.* **205**, 799–810 (2008).
14. Kroenke, M.A., Carlson, T.J., Andjelkovic, A.V. & Segal, B.M. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J. Exp. Med.* **205**, 1535–1541 (2008).
15. Stromnes, I.M., Cerretti, L.M., Liggitt, D., Harris, R.A. & Goverman, J.M. Differential regulation of central nervous system autoimmunity by T_H1 and T_H17 cells. *Nat. Med.* **14**, 337–342 (2008).
16. Lees, J.R., Golumbek, P.T., Sim, J., Dorsey, D. & Russell, J.H. Regional CNS responses to IFN-γ determine lesion localization patterns during EAE pathogenesis. *J. Exp. Med.* **205**, 2633–2642 (2008).
17. Engelhardt, B. & Ransohoff, R.M. The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends Immunol.* **26**, 485–495 (2005).
18. Yednock, T.A. *et al.* Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha_4\beta_1$ integrin. *Nature* **356**, 63–66 (1992).
19. Engelhardt, B., Vestweber, D., Hallmann, R. & Schulz, M. E- and P-selectin are not involved in the recruitment of inflammatory cells across the blood-brain barrier in experimental autoimmune encephalomyelitis. *Blood* **90**, 4459–4472 (1997).
20. Carrithers, M.D., Visintin, I., Kang, S.J. & Janeway, C.A. Jr. Differential adhesion molecule requirements for immune surveillance and inflammatory recruitment. *Brain* **123**, 1092–1101 (2000).
21. Piccio, L. *et al.* Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G_i-linked receptors. *J. Immunol.* **168**, 1940–1949 (2002).
22. Vajkoczy, P., Laschinger, M. & Engelhardt, B. α4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. *J. Clin. Invest.* **108**, 557–565 (2001).
23. Engelhardt, B., Wolburg-Buchholz, K. & Wolburg, H. Involvement of the choroid plexus in central nervous system inflammation. *Microsc. Res. Tech.* **52**, 112–129 (2001).
24. Ransohoff, R.M., Kivisakk, P. & Kidd, G. Three or more routes for leukocyte migration into the central nervous system. *Nat. Rev. Immunol.* **3**, 569–581 (2003).
25. Giunti, D. *et al.* Phenotypic and functional analysis of T cells homing into the CSF of subjects with inflammatory diseases of the CNS. *J. Leukoc. Biol.* **73**, 584–590 (2003).
26. Steffen, B.J., Breier, G., Butcher, E.C., Schulz, M. & Engelhardt, B. ICAM-1, VCAM-1, and MAdCAM-1 are expressed on choroid plexus epithelium but not endothelium and mediate binding of lymphocytes in vitro. *Am. J. Pathol.* **148**, 1819–1838 (1996).
27. Pedemonte, E. *et al.* Mechanisms of the adaptive immune response inside the central nervous system during inflammatory and autoimmune diseases. *Pharmacol. Ther.* **111**, 555–566 (2006).
28. Bromley, S.K., Mempel, T.R. & Luster, A.D. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat. Immunol.* **9**, 970–980 (2008).
29. Rossi, D.L., Vicari, A.P., Franz-Bacon, K., McClanahan, T.K. & Zlotnik, A. Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3α and MIP-3β. *J. Immunol.* **158**, 1033–1036 (1997).
30. Acosta-Rodriguez, E.V. *et al.* Surface phenotype and antigenic specificity of human interleukin 17–producing T helper memory cells. *Nat. Immunol.* **8**, 639–646 (2007).
31. Cook, D.N. *et al.* CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* **12**, 495–503 (2000).
32. Greaves, D.R. *et al.* CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3α and is highly expressed in human dendritic cells. *J. Exp. Med.* **186**, 837–844 (1997).
33. Brown, D.A. & Sawchenko, P.E. Time course and distribution of inflammatory and neurodegenerative events suggest structural bases for the pathogenesis of experimental autoimmune encephalomyelitis. *J. Comp. Neurol.* **502**, 236–260 (2007).
34. Kivisakk, P. *et al.* Localizing central nervous system immune surveillance: Meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. *Ann. Neurol.* published online, doi:10.1002/ana.21379 (21 May 2008).
35. Hickey, W.F., Hsu, B.L. & Kimura, H. T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* **28**, 254–260 (1991).
36. Flugel, A. *et al.* Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity* **14**, 547–560 (2001).
37. Hirota, K. *et al.* Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J. Exp. Med.* **204**, 2803–2812 (2007).
38. Khader, S.A. *et al.* IL-23 and IL-17 in the establishment of protective pulmonary CD4$^+$ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat. Immunol.* **8**, 369–377 (2007).
39. Steinman, L. A few autoreactive cells in an autoimmune infiltrate control a vast population of nonspecific cells: a tale of smart bombs and the infantry. *Proc. Natl. Acad. Sci. USA* **93**, 2253–2256 (1996).
40. O'Connor, R.A. *et al.* Cutting Edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J. Immunol.* **181**, 3750–3754 (2008).
41. Kebir, H. *et al.* Human T_H17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat. Med.* **13**, 1173–1175 (2007).
42. Kreymborg, K. *et al.* IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. *J. Immunol.* **179**, 8098–8104 (2007).
43. Haak, S. *et al.* IL-17A and IL-17F do not contribute vitally to autoimmune neuroinflammation in mice. *J. Clin. Invest.* **119**, 61–69 (2009).
44. Charo, I.F. & Ransohoff, R.M. The many roles of chemokines and chemokine receptors in inflammation. *N. Engl. J. Med.* **354**, 610–621 (2006).
45. Balashov, K.E., Rottman, J.B., Weiner, H.L. & Hancock, W.W. CCR5$^+$ and CXCR3$^+$ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. *Proc. Natl. Acad. Sci. USA* **96**, 6873–6878 (1999).

46. Ambrosini, E., Columba-Cabezas, S., Serafini, B., Muscella, A. & Aloisi, F. Astrocytes are the major intracerebral source of macrophage inflammatory protein-3α/CCL20 in relapsing experimental autoimmune encephalomyelitis and in vitro. *Glia* **41**, 290–300 (2003).
47. Columba-Cabezas, S., Serafini, B., Ambrosini, E. & Aloisi, F. Lymphoid chemokines CCL19 and CCL21 are expressed in the central nervous system during experimental autoimmune encephalomyelitis: implications for the maintenance of chronic neuroinflammation. *Brain Pathol.* **13**, 38–51 (2003).
48. Rottman, J.B. *et al.* Leukocyte recruitment during onset of experimental allergic encephalomyelitis is CCR1 dependent. *Eur. J. Immunol.* **30**, 2372–2377 (2000).
49. Muller, M. *et al.* CXCR3 signaling reduces the severity of experimental autoimmune encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system. *J. Immunol.* **179**, 2774–2786 (2007).
50. Liu, L. *et al.* Severe disease, unaltered leukocyte migration, and reduced IFN-γ production in CXCR3$^{-/-}$ mice with experimental autoimmune encephalomyelitis. *J. Immunol.* **176**, 4399–4409 (2006).
51. Gaupp, S., Pitt, D., Kuziel, W.A., Cannella, B. & Raine, C.S. Experimental autoimmune encephalomyelitis (EAE) in CCR2$^{-/-}$ mice: susceptibility in multiple strains. *Am. J. Pathol.* **162**, 139–150 (2003).
52. Eltayeb, S. *et al.* Temporal expression and cellular origin of CC chemokine receptors CCR1, CCR2 and CCR5 in the central nervous system: insight into mechanisms of MOG-induced EAE. *J. Neuroinflammation* **4**, 14 (2007).
53. Matsui, M. *et al.* Treatment of experimental autoimmune encephalomyelitis with the chemokine receptor antagonist Met-RANTES. *J. Neuroimmunol.* **128**, 16–22 (2002).
54. Kohler, R.E., Caon, A.C., Willenborg, D.O., Clark-Lewis, I. & McColl, S.R. A role for macrophage inflammatory protein-3α/CC chemokine ligand 20 in immune priming during T cell-mediated inflammation of the central nervous system. *J. Immunol.* **170**, 6298–6306 (2003).
55. Franciotta, D., Salvetti, M., Lolli, F., Serafini, B. & Aloisi, F. B cells and multiple sclerosis. *Lancet Neurol.* **7**, 852–858 (2008).
56. Kleinewietfeld, M. *et al.* CCR6 expression defines regulatory effector/memory-like cells within the CD25^{+}CD4^{+} T-cell subset. *Blood* **105**, 2877–2886 (2005).
57. Yamazaki, T. *et al.* CCR6 regulates the migration of inflammatory and regulatory T cells. *J. Immunol.* **181**, 8391–8401 (2008).
58. Steinman, L. A rush to judgment on Th17. *J. Exp. Med.* **205**, 1517–1522 (2008).
59. Sigmundsdottir, H. & Butcher, E.C. Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. *Nat. Immunol.* **9**, 981–987 (2008).
60. Hickey, W.F. Basic principles of immunological surveillance of the normal central nervous system. *Glia* **36**, 118–124 (2001).