

Chemokine receptors on infiltrating leucocytes in inflammatory pathologies of the central nervous system (CNS)

C. Trebst*, S. M. Staugaitis*, B. Tucky*, T. Wei*, K. Suzuki†, K. D. Aldape‡, C. A. Pardo§, J. Troncoso§, H. Lassmann¶ and R. M. Ransohoff**

*Department of Neurosciences, the Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH, USA,

†Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA, ‡Department of Pathology,

University of California, San Francisco, CA, USA, §Department of Neurology and Pathology, The Johns Hopkins University,

Baltimore, MD, USA, ¶Brain Research Institute, University of Vienna, Vienna, Austria, and **The Mellen Center for

Multiple Sclerosis Treatment and Research, Department of Neurology, The Cleveland Clinic Foundation, Cleveland, OH, USA

C. Trebst, S. M. Staugaitis, B. Tucky, T. Wei, K. Suzuki, K. D. Aldape, C. A. Pardo, J. Troncoso, H. Lassmann and R. M. Ransohoff (2003) *Neuropathology and Applied Neurobiology* 29, 584–595, doi: 10.1046/j.1365-2990.2003.0507.x

Chemokine receptors on infiltrating leucocytes in inflammatory pathologies of the central nervous system (CNS)

Haematogenous leucocytes enter the central nervous system (CNS) during diverse disorders of varied aetiologies. Understanding the trafficking cues that mediate CNS leucocyte infiltration might promote the development of flexible and selective means to modulate inflammation to achieve clinical benefit. The trafficking machinery of leucocytes has been elucidated during the past decade and consists of cell-surface adhesion molecules, chemoattractant cytokines (chemokines) and their receptors. Recent work in our laboratory characterized chemokine receptors found on T lymphocytes and monocytes in brain sections from subjects with one pathological subtype of multiple sclerosis (MS), an immune-mediated inflammatory demyelinating disease. In these tissues, the types 1 and 5 CC chemokine receptors (CCR1 and CCR5) were detected on perivascular monocytic cells whereas only CCR5 was present on parenchymal macrophages. The type 3 CXC

chemokine receptor (CXCR3) was present on virtually all CD3-positive T cells. In the current study, we evaluated the expression of these receptors on the infiltrating cells present in cases of other inflammatory CNS disorders including those of dysimmune, infectious, neoplastic, and vascular aetiology. Perivascular and parenchymal monocytic cells expressed CCR1 in all cases and CXCR3 was consistently present on a substantial proportion of CD3+ T cells. The occurrence of CCR5 on parenchymal macrophages was much less uniform across the varied disorders. These data implicate CCR1 in monocyte infiltration of the CNS and are consistent with reports of studies in CCR1-deficient mice. CXCR3 is also likely to play a role in accumulation of T cells in the inflamed CNS. By contrast, our findings suggest that regulation of CCR5 on phagocytic macrophages may be contingent on the lesion environment.

Keywords: chemokine receptors, chemokines, CNS immunopathology, monocytes, T-lymphocytes

Correspondence: Richard M. Ransohoff, Department of Neurosciences, Mail Code NC30, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA. Tel: +1 216 444 0627; Fax: +1 216 444 7927; E-mail: ransohr@ccf.org

Introduction

Leucocyte infiltrates of the central nervous system (CNS) are distinct from those observed in other organs in composition, and likely in mechanism. Because the CNS is

stringently intolerant to swelling, inflammation (except in transient responses to acute insults) appears to be heavily biased towards monocytes and T cells, lending a highly selective character to CNS infiltrates. Chemokines and their receptors govern physiological and pathological leucocyte trafficking, and may be particularly pertinent for haematogenous leucocyte entry into the CNS, given the exacting specificity with which the chemokine system regulates cellular migration events.

Circulating leucocytes cross endothelial monolayers in discrete steps [32]. In what is perhaps the best-characterized step, activated leucocyte integrins mediate arrest on the endothelial cell adhesion molecules (CAMs) or on the extracellular matrix components of the endothelial glycocalyx. Integrin activation is post-translational, and requires signalling through G α i-linked receptors, of which the most intensely studied are members of a superfamily of receptors for chemokines, formyl peptides and leucotrienes [2,3,28]. The roles of chemokines in leucocyte recirculation and inflammatory trafficking have been well established [6,17]. Recent findings support the possibility of extending the overall scheme to the CNS endothelial bed. In this regard, there appear to be organ-specific molecular details for leucocyte trafficking [17,23] and, therefore, each site of inflammation must be approached as a distinct system.

During the last decade, a significant amount of information about leucocyte entry into the inflamed CNS has emerged from studies conducted in one animal model, murine experimental autoimmune encephalomyelitis (EAE). Many of these experiments took advantage of gene-targeted mice that lacked chemokines or chemokine receptors, or function-blocking antibodies (reviewed in [33]). These studies consistently demonstrated salient roles for CCR1 and CCR2 in the accumulation of monocytes in the CNS during EAE.

More recently, chemokine receptors on the CNS infiltrates of multiple sclerosis (MS) lesions have been evaluated. The complexity of this research in human material was amplified by recent proposals indicating pathological heterogeneity in MS lesions [19]. Therefore, we confined one series of recent studies to a major pathological variant termed pattern II, in which tissue injury is linked to the presence of T cells, macrophages and complement components associated with degenerating myelin sheaths [19,31,34]. We characterized CCR1 and CCR5 on mononuclear phagocytes and CXCR3 on T cells in this homogeneous series of well-characterized MS lesions. We found

that CCR1 and CCR5 were co-expressed on perivascular round CD68+ cells, which were proposed to constitute infiltrating monocytes. In more advanced regions of these lesions populated by phagocytic macrophages, CCR1 was not detected. However, CCR5 was present on more than 80% of such cells. CXCR3 was consistently observed on more than 90% of CNS T cells in MS lesions, and its expression co-localized with the presence of one ligand, interferon-gamma inducible protein, 10 kDa (IP-10), which is now termed CXCL10 in a new systematic nomenclature.

The presence of CCR1 and CCR5 on haematogenous monocytes in MS lesions, and of CXCR3 on T cells, was in close agreement with the expression of these receptors on the corresponding populations of leucocytes in cerebrospinal fluid (CSF) [15,31,34].

The present study was undertaken to address the generality of these findings, and to examine the hypothesis that leucocyte chemokine receptor expression might suggest determinants of trafficking to the CNS. This research carries potential medical implications, as small-molecule drugs that block chemokine receptors have been examined in pilot clinical trials and might provide useful additions to the therapeutic armamentarium for inflammatory disorders including MS [7,14]. To provide initial insights into this process, we examined the distribution of CCR1, CCR5 and CXCR3 in inflammatory CNS lesions associated with a wide variety of clinical disorders of diverse aetiologies, as chemokine receptor expression is necessary (although it may not be sufficient) to direct chemotaxis towards specific chemokines. Quantitative immunohistochemistry on serial sections was used to establish relationships between lineage markers for leucocytes (CD3 for T cells; CD68 for mononuclear phagocytes) and chemokine receptors. The data supported hypotheses about determinants of trafficking to the CNS that were initially proposed during the course of studies of MS tissues. Further, the chemokine receptor CCR5, which can be expressed by resident activated microglia, was detected less uniformly, consistent with the notion that its expression is regulated in part by microenvironmental influences.

Materials and methods

Autopsy and biopsy material

Archival autopsy and biopsy material was collected at the Cleveland Clinic Foundation, University of California, San

Francisco, Johns Hopkins University, University of Vienna and University of North Carolina. All cases underwent routine gross examination and histopathological evaluation at the sites of collection. Diagnoses were assigned by an expert neuropathologist (S.M.S., K.S., K.D.A., J.T. or H.L.), based on comprehensive neuropathological evaluation and clinical history. A total of 36 tissue sections from 25 individual cases were included in our analysis (Table 1). Diagnoses were as follows: Rasmussen's encephalitis (1), neurosarcoidosis (2), acute disseminated encephalomyelitis (ADEM) (1), haemorrhagic necrotizing (Weston Hurst) leucoencephalitis (1), progressive multifocal leucoencephalopathy (PML) (1), Herpes simplex virus (HSV) encephalitis (3), rabies encephalitis (2), cerebral toxoplasmosis (2), CNS malaria (1), chronic encephalitis of unknown aetiology (2), primary CNS lymphoma (2), and ischaemic stroke (7).

Thirteen paraffin-embedded archival brain tissue sections from three individuals without known neurological, inflammatory or metastatic disorder were collected at the Cleveland Clinic Foundation and served as noninflammatory controls. All the three individuals died from sudden cardiac arrest.

This study was exempt from review under FDA and NIH guidelines, as determined by the Institutional Review Board (IRB) of the Cleveland Clinic Foundation.

Immunohistochemistry

Immunohistochemistry was performed as previously described [30,34]. In brief, 5 µm sections were placed on Superfrost slides. Paraffin-embedded tissue sections were deparaffinized with xylenes and rehydrated in ethanol. After antigen-retrieval by steaming in citrate buffer, slides were incubated overnight with primary antibody at 4°C, washed in phosphate-buffered saline (PBS), incubated with secondary antibody at room temperature for 40 min, washed, and incubated with avidin-biotin-horseradish peroxidase complex (Vectastain Elite; Vector Laboratories, Burlingame, CA). After development with 3,3'-diaminobenzidine (DAB) substrate (Sigma Chemical Co.), slides were dehydrated and mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Primary antibodies were omitted in controls. For analysis of co-localization of CCR1 and MRP14, sections were simultaneously labelled with primary antibodies, followed by incubation with species and isotype specific Texas Red- and fluorescein isothiocyanate-conjugated secondary antibodies (Southern

Biotechnology Associates, Inc., Birmingham, AL). In controls, primary antibodies were omitted, and tests for cross-reactivity by secondary antibodies were performed.

Antibodies

Richard Horuk (Berlex Biosciences, Richmond, CA) kindly provided a rabbit polyclonal anti-CCR1 antibody [12]. Murine monoclonal antihuman CCR5 (Clone 45549.111, mouse IgG_{2B}) was obtained from R&D Systems, murine monoclonal anti-CD68 (Clone KP1, mouse IgG₁) from DAKO Corporation (Carpinteria, CA, USA), murine monoclonal antihuman MRP14 (Clone S 36.48, mouse IgG₁) from Bachem Bioscience Inc. (King of Prussia, PA, USA). Murine monoclonal anti-CXCR3 (clone 1C6.2, mouse IgG₁) was generously provided by Walter Newman (LeukoSite, Cambridge, MA) and rat monoclonal anti-CD3 (clone CD3-12) was obtained from Serotec Inc. (Raleigh, NC, USA).

Quantification

The number of immunostained cells was determined in at least four standardized fields (146 200 µm², defined by a morphometric grid) from each region of pathology, which were localized in both white matter and grey matter (rabies encephalitis). Immunostained sections were photographed using a Leica DMR microscope (Leica Wetzlar, Heidelberg, Germany) and an Optronix Magnafire digital camera system and analysed using Image Pro[®] Plus (Media Cybernetics, Silver Spring, MD, USA).

Lineage and activation markers for leucocytes

Macrophage-related protein (MRP) 14 (termed S100A9 in systematic nomenclature) is a 14-kDa calcium-binding protein related to the S100 family, which is primarily expressed on circulating human neutrophils and monocytes [22]. *In vitro*, this antigen declines in expression during monocyte differentiation [9]. MRP14 can be expressed in isolation or complexed to a related protein MRP8 (S100A8), in heterodimers, which are detected by the monoclonal antibody 27E10 [8]. In the current study, as in our prior evaluations of human autopsy material, MRP14 was used as a marker of activated monocytes that had recently entered CNS tissues.

CD68 is a type 1 membrane glycoprotein, identical to the receptor for oxidized LDL and orthologous to the

Table 1. Quantification of CD3+, CXCR3+, CD68+, MRP14+, CCR1+ and CCR5+ cells in cases with a variety of neurological disorders and in control brain sections

Case number	Gender	Age	Diagnosis	Number of sections	Number of CD3+ cells/mm ²	Number of CXCR3+ cells/mm ²	Number of CD68+ cells/mm ²	Number of MRP14+ cells/mm ²	Number of CCR1+ cells/mm ²	Number of CCR5+ cells/mm ²
<i>Autoimmune/inflammatory</i>										
CCF01	F	13	Rasmussen's encephalitis	1	72	75	809	0	0	14
UCSF02	F	31	Neurosarcoidosis	2	Section 1 Section 2	445 908	715 347	101 46	72 2	156 216
JH06	F	26	Neurosarcoidosis	1	376	335	910	118	110	275
UV01	M	13	ADEM	1	508	496	1505	1289	1096	821
UNC01	F	13	Weston Hurst encephalitis	1	15	12	1024	636	545	299
<i>Viral</i>										
JH01	F	79	PML	1	87	48	1108	133	245	732
CCF06	F	22	HSV encephalitis	1	15	29	501	21	156	272
CCF07	M	69	HSV encephalitis	1	115	56	505	156	460	375
CCF08	M	26	HSV encephalitis	4	679 359 367 946 617 221 130	67 200 269 431 207 89 139	951 749 744 588 937 1501 580	1006 39 248 299 489 1654 445	990 24 520 135 640 1616 373	1749 200 393 130 653 1200 115
JH02	M	10	Rabies encephalitis	1	221	89	1501	1654	1616	1200
JH03	M	8	Rabies encephalitis	1	130	139	580	445	373	115
<i>Infectious/nonviral</i>										
CCF11	M	62	Cerebral toxoplasmosis and embolic infarction in a case of lymphoma	2	Section 1 Section 2	151 528	563 1625	800 109	1047 799	523 1955
CCF02	F	30	Cerebral toxoplasmosis	2	Section 1 Section 2	5 9	963 402	3 168	0 120	36 14
JH05	M	39	Cerebral malaria	1	0	0	246	3	31	2
<i>Other</i>										
CCF05	F	64	Chronic encephalitis of undetermined aetiology	2	Section 1 Section 2	368 457	788 891	0 9	0 3	436 617
CCF13	M	52	Chronic encephalitis of undetermined aetiology	1	216	251	347	0	0	132

CCF03	F	87	Cerebral lymphoma	2	Section 1 Section 2	55 614	70 662	528 409	0 0	0 0	43 153
CCF12	F	36	Cerebral lymphoma complicated by acute and subacute infarction because of embolus	1		171	135	1059	92	0	171
<i>Vascular</i>											
CCF19	M	48	Ischaemia stroke	2	Section 1 Section 2	0 8.6	0 0	1684 2096	1385 749	438 686	251 499
CCF21	F	76	Ischaemia stroke	1		245	164	1298	7	68	322
CCF25	F	74	Ischaemia stroke	2	Section 1 Section 2	29 92	27 46	1399 915	17 677	10 118	286 149
CCF31	M	66	Ischaemia stroke	1		0	0	1076	983	621	123
CCF33	M	75	Ischaemia stroke	1		39	0	1120	831	573	227
CCF34	M	45	Ischaemia stroke	2	Section 1 Section 2	56 30	15 21	869 819	200 3	292 29	26 1069
CCF35	F	81	Ischaemia stroke	1		50	48	2144	754	1204	1423
<i>Controls</i>											
CCF14	F	74	Control	5	Section 1 Section 2 Section 3	4 2 7	2 0 14	131 196 186	0 2 0	2 0 0	9 4 5
CCF16	M	72	Control	4	Section 4 Section 5 Section 1 Section 2 Section 3 Section 4	7 28 7 5	11 11 2 2	121 121 130 100	0 0 0 0	4 0 0 0	7 0 32 0
CCF17	F	42	Control	4	Section 1 Section 2 Section 3 Section 4	0 0 16 9	0 0 2 12	114 117 138 112	0 0 0 0	2 0 0 0	0 0 4 0
					Section 2 Section 3 Section 4	5 5	7 2	117 98	2 0	0 0	0 21

CCF, Cleveland Clinic Foundation; UCSF, University of California, San Francisco; UV, University of Vienna; ADEM, acute disseminated encephalomyelitis; UNC, University of North Carolina; JH, Johns Hopkins; PML, progressive multifocal leucoencephalopathy; HSV, herpes simplex virus.

murine protein macrosialin [13,25–27]. The KP1 monoclonal antibody detects CD68 on monocytes and macrophages, expressed both within the cytoplasm and at the plasma membrane [24]. In the current studies, CD68 is used to denominate mononuclear phagocyte lineage cells expressing chemokine receptors of interest. This population includes both monocytes and macrophages. CD68 is expressed by macrophages derived both from monocytes and from microglia. Most but not all mononuclear phagocytes in CNS tissues express CD68; however, a subpopulation of activated microglial cells remains CD68-negative (Corinna Trebst & Richard M. Ransohoff, unpub. obs.).

CD3 is a multimeric complex associated with the T-cell receptor and essential for signalling after engagement by cognate antigen [35]. In these studies, we used CD3 immunoreactivity to quantify T lymphocytes within tissue.

Results

Expression of the monocyte-activation marker MRP14 and the chemokine receptor CCR1 are closely related in inflammatory CNS pathologies

In the current study, cells that were immunoreactive for either MRP14 or CCR1 exhibited markedly similar morphology and distribution. In particular, MRP14 and CCR1 were expressed predominantly on round, nonprocess bearing cells, morphologically consistent with monocytes. MRP14- or CCR1-immunoreactive cells were localized in perivascular accumulations and dispersed throughout the parenchyma. MRP14+ and CCR1+ cells were consistently numerous in cases of rabies encephalitis (Figure 1), in cases with HSV encephalitis, in a case of ADEM and in the case of haemorrhagic necrotizing (Weston Hurst) leucoencephalitis. In these cases, the numbers of cells expressing MRP14 and CCR1 approached the levels of CD68+ cells, suggesting that a majority of mononuclear phagocytes in these tissues were newly recruited monocytes.

Furthermore, where MRP14+ cells were either very few or not detected, CCR1 immunoreactivity was also absent. This pattern was observed in two cases of chronic encephalitis, in two cases of cerebral lymphoma and in one case of Rasmussen's encephalitis (Table 1). It is clear that the absence of MRP14+/CCR1+ cells in these three cases must be interpreted cautiously, as the

timing of entry of monocytes may not have coincided with the acquisition of tissue. However, the presence of substantial numbers of T cells (average $> 250/\text{mm}^2$ in chronic encephalitis; $75/\text{mm}^2$ in Rasmussen's encephalitis) suggests that active inflammation was taking place within these lesions. The absence of both MRP14- and CCR1-immunoreactivity from these lesions is consistent with the hypothesis that these markers are co-expressed on infiltrating monocytes.

In cases of cerebral ischaemia ($n = 7$), MRP14 and CCR1 were frequently expressed on mononuclear phagocytes within ischaemic areas (Figure 1). An average of 42% of all CD68+ cells within these lesions expressed MRP14, and about 28% expressed CCR1 (Table 1). Consistent with prior reports, neither MRP14 nor CCR1 expression was present in 13 tissue sections of 3 control individuals [20] (Table 1). Spearman Rank correlations of numbers of MRP14+ and CCR1+ cells on serial sections underlined the robust relationship between the two antigens ($r = 0.88$; 95% confidence interval: 0.77–0.94; $P < 0.0001$) (Figure 2). In one PML lesion (JH01, lesion 2) MRP14+ cells were infrequent and outnumbered by CCR1+ cells. Such minor discrepancies are proposed to result from the limitations of quantification in serial sections. Taken together with previous findings from our laboratory and others [4,34], these observations support the hypothesis that CCR1 and MRP14 co-expression mark haematogenous monocytes in CNS inflammatory infiltrates.

CD3+ lymphocytes in CNS inflammatory pathologies are not invariably CXCR3+

To establish a more general relationship between CXCR3 and the presence of CNS lymphocytes, we analysed CD3 and CXCR3 expression by immunohistochemistry in non-MS inflammatory CNS pathologies (Table 1). CD3+ cells were observed to a variable degree in all cases except in a case with cerebral toxoplasmosis (case CCF02) and in cerebral malaria, where CD3+ cells were sparse or absent. In most cases, the majority of CD3+ cells were localized to the perivascular space, with variable dispersion of CD3+ cells throughout the parenchyma. CXCR3+ immunoreactivity was only detected in those cases that also showed CD3+ cells. Furthermore, where both were present, the distribution patterns of CXCR3 and CD3 immunoreactivity on serial sections were strikingly similar (Figure 3).

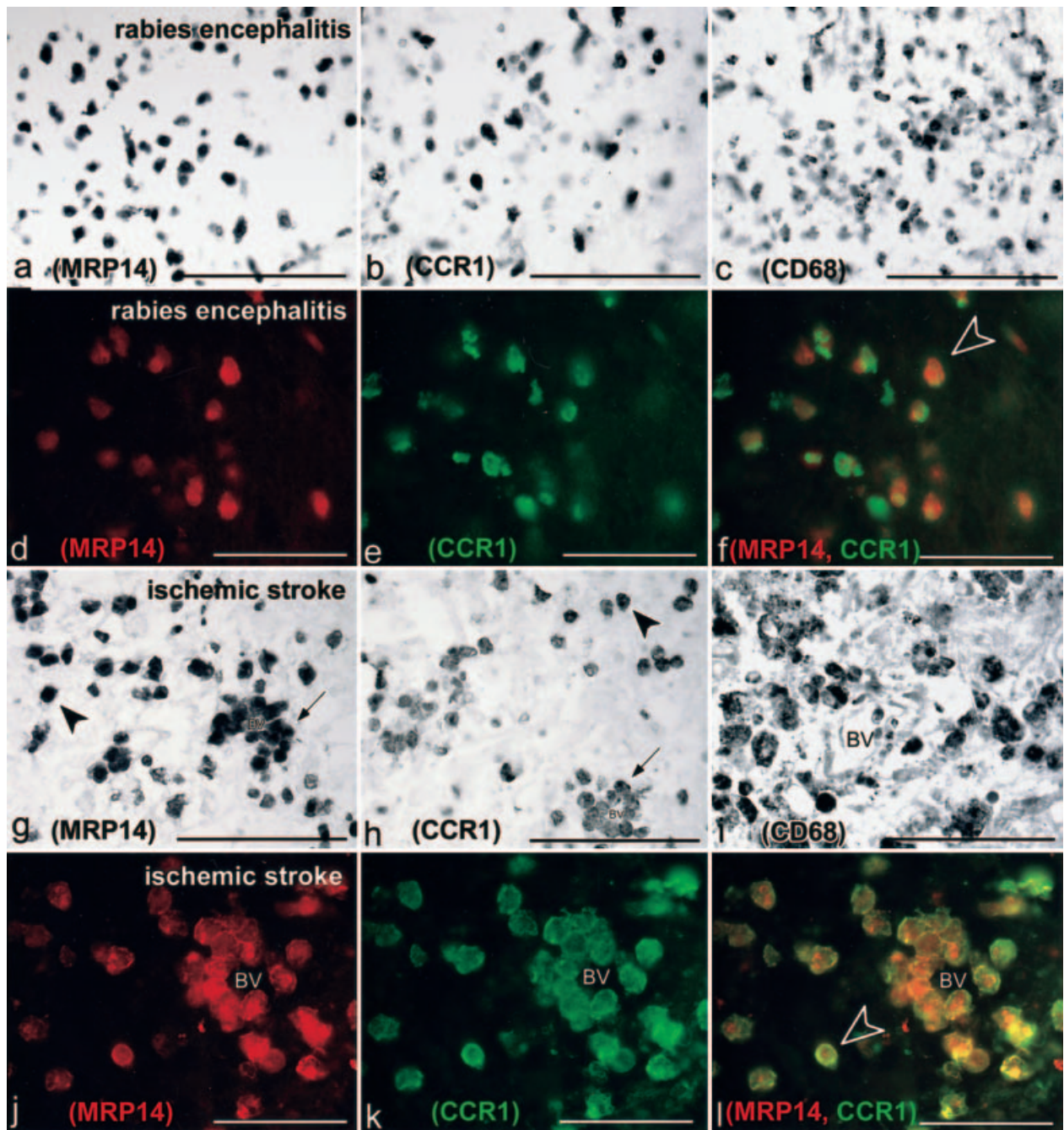


Figure 1. Expression of MRP14 and CCR1 in rabies encephalitis and ischaemic stroke. Immunohistochemistry for CD68 (c,i), MRP14 (a,g) and CCR1 (b,h) was performed on serial sections in a case with rabies encephalitis (case JH02, a–f) and in a case with ischaemic stroke (case CCF33, g–i). Both MRP14 and CCR1 were expressed on small round, nonprocess bearing cells, morphologically consistent with monocytes in perivascular cell accumulations (g,h, arrows) and dispersed into the parenchyma (g,h, arrowheads). Immunohistochemistry was markedly similar for MRP14 and CCR1. Co-localization was confirmed by double-fluorescence microscopy in the same cases (d–f case JH02 and j–l case CCF33). MRP14 immunoreactivity is shown in red (d,j); green indicates CCR1-immunoreactivity (e,k); merged images are shown in f and l. As indicated by an arrowhead in f and l, MRP14 and CCR1 were expressed on the same cells. BV, blood vessel. Bars in a–c and g–i = 100 µm, bars in d–f and j–l = 50 µm.

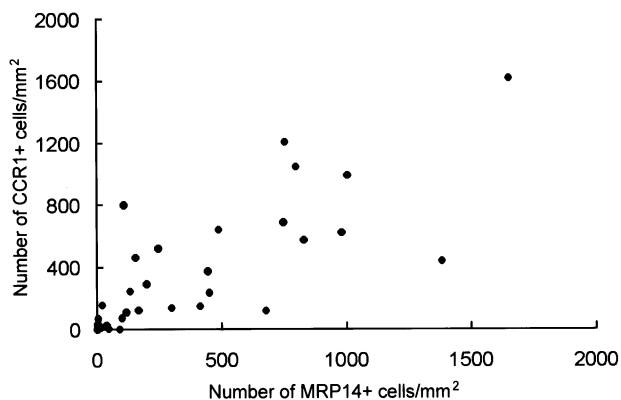


Figure 2. Correlation of numbers of MRP14+ cells and CCR1+ cells. Spearman rank correlations of numbers of MRP14+ and CCR1+ cells on serial sections underlined the robust relationship between the two antigens ($r = 0.88$; 95% confidence interval: 0.77–0.94; $P < 0.0001$). Individual data points of quantification of numbers of MRP14+ and CCR1+ cells in a total of 36 tissue sections of 25 individual cases are depicted.

Quantitative immunohistochemistry was performed to analyse the relationship between CD3+ and CXCR3+ cells. In most cases the majority of CD3+ cells expressed CXCR3. In particular, in 31 sections that contained more than 10 CD3+ cells/mm² (range 15–970) in only two cases (one of toxoplasmosis and one of HSV encephalitis) were the numbers of CXCR3+ cells in serial sections less than 40% of the total. The most robust and invariant relationship between CXCR3 and CD3 expression was apparent in cases with presumed autoimmune or inflammatory pathogenesis (Table 2). In several cases of CNS parenchymal infection (HSV encephalitis, cerebral toxoplasmosis and rabies encephalitis) only a minority of CD3+ cells expressed CXCR3 (Table 2).

CD3 and CXCR3 immunoreactivity were also analysed in 7 cases of ischaemic stroke. CD3+ cells were observed to a variable degree in stroke lesions; where present, CD3+ cells were dispersed in the parenchyma within the

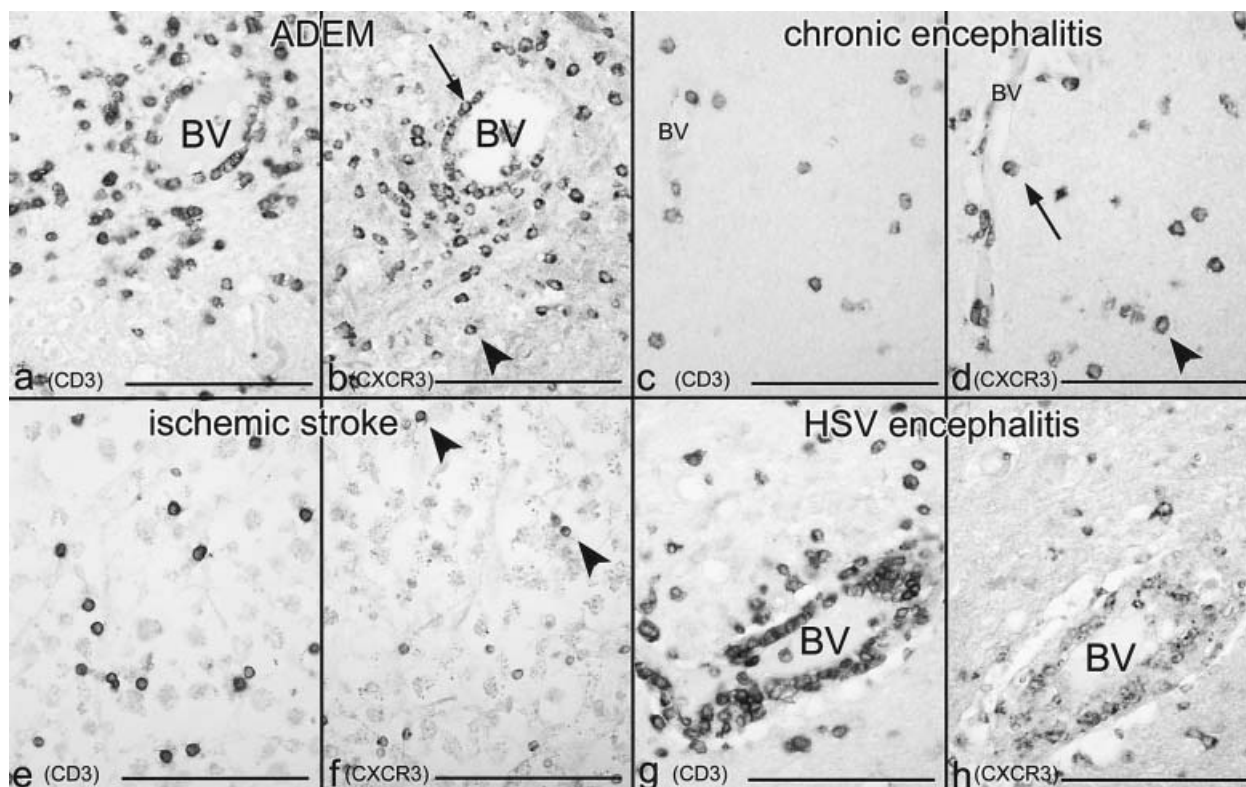


Figure 3. Expression of CD3 and CXCR3 in different inflammatory pathologies of the central nervous system (CNS). Immunohistochemistry for CD3 (a,c,e,g) and CXCR3 (b,d,f,h) was performed on serial sections in a case with acute disseminated encephalomyelitis (ADEM, case UV01, a,b), a case with chronic encephalitis of undetermined aetiology (case CCF05, c,d), a case with ischaemia stroke (case CCF34, e,f) and in a case with herpes simplex virus (HSV) encephalitis (case CCF08, g,h). CXCR3 was expressed on lymphocytes in perivascular cell accumulations (b,d, arrows; h) and dispersed throughout the parenchyma (b,d,f, arrowheads). Immunohistochemistry was markedly similar for CD3 and CXCR3 in all cases. BV, blood vessel. Bars = 100 μ m.

Table 2. Numbers of CD3+ and CXCR3+ cells were quantitated in serial sections as described in Materials and Methods and these values were used to calculate the percent of CD3+ cells that were also CXCR3+. The columns indicate a listing of cases in which the percent of CXCR3+/CD3+ cells was either less than (left column) or greater than (right column) 50%. CCF, Cleveland Clinic Foundation; UCSF, University of California, San Francisco; UV, University of Vienna; ADEM, acute disseminated encephalomyelitis; UNC, University of North Carolina; JH, Johns Hopkins University; PML, progressive multifocal leukoencephalopathy; HSV, herpes simplex virus.

<i>The minority of CD3+cells express CXCR3</i>			<i>The majority of CD3+cells express CXCR3</i>		
<i>Case number</i>	<i>Case pathology</i>	<i>Percentage of CXCR3+ cells</i>	<i>Case number</i>	<i>Case pathology</i>	<i>Percentage of CXCR3+ cells</i>
CCF11	Cerebral toxoplasmosis	38	CCF01	Rasmussen's encephalitis	100
CCF06	HSV encephalitis	49	UCSF02	Neurosarcoidosis	74
CCF07	HSV encephalitis	10	JH06	Neurosarcoidosis	89
CCF08	HSV encephalitis	51	UV01	ADEM	98
JH02	Rabies encephalitis	40	UNC01	Weston Hurst encephalitis	78
			JH01	PML	121
			JH03	Rabies encephalitis	100
			CCF05	Chronic encephalitis	90
			CCF13	Chronic encephalitis	116
			CCF03	Cerebral lymphoma	118
			CCF12	Cerebral lymphoma	79

ischaemic area (Figure 3). CXCR3 and CD3 immunoreactivity tended to co-localize in stroke lesions and quantitative analysis revealed that, on average, about 50% of CD3+ cells expressed CXCR3. This relationship was consistently observed regardless of the absolute number of infiltrating CD3+ T cells, or the ratio of CD3+ T cells to CD68+ mononuclear phagocytes (ranging from 1 : 100 in haemorrhagic necrotizing leucoencephalitis to 3 : 1 in one case of neurosarcoidosis).

In control tissue sections CD3 and CXCR3 immunoreactivity were only occasionally found and localized to the perivascular space. In these control sections, we commonly found less than 10 CD3+ cells/mm², and there were frequent dissociations between CD3 and CXCR3 expression, very likely indicating the limitations of comparing serial sections to establish relationships between markers on small numbers of cells.

CCR5 is expressed on lymphocytes and mononuclear phagocytes in CNS inflammatory pathologies

In the current series of inflammatory CNS pathologies, we found CCR5 expression in all cases (Table 1). CCR5 expression was associated both with lymphocytes and mononuclear phagocytes (Figure 4). In stroke cases, CCR5 was

strongly expressed on mononuclear phagocytes within ischaemic areas (Figure 4). CCR5 was infrequently found in control brain sections (Table 1), on widely scattered perivascular cells with the morphology of monocytes (Figure 4).

Discussion

These studies were undertaken with the goal of determining whether chemokine receptor expression profiles found in pattern II lesions of MS could be generated in other inflammatory pathologies of the human CNS. We found significant similarities, with regard to receptors that are considered likely trafficking determinants and/or activation markers for marrow derived cells in the human CNS. In particular, CCR1 expression was closely associated with MRP14+ monocytic cells, as previously shown in pattern II MS lesions. Additionally, CD3 and CXCR3 were closely related on infiltrating lymphocytes. The expression of CCR5 is positively modulated on resident microglia during maturation of pattern II MS lesions, and is the only one, of three receptors analysed here, that is strongly expressed by a major population of intrinsic CNS cells. Therefore, CCR5 immunoreactivity may be more reflective of the environment associated with a specific tissue reaction than infiltration by CCR5+ cells.

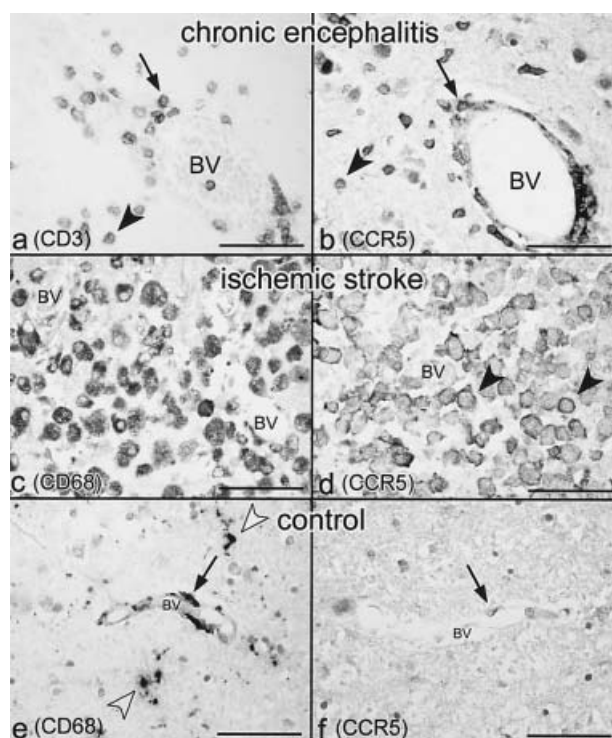


Figure 4. Expression of CCR5 in a case with chronic encephalitis, a case with ischaemia stroke and in a normal control brain. Immunohistochemistry for CD3 (a), CD68 (c,e) and CCR5 (b,d,f) was performed on serial sections in a case with chronic encephalitis of undetermined aetiology (case CCF05, a,b), a case with ischaemia stroke (case CCF19, c,d) and in a normal control brain (case CCF16, e,f). CCR5 was expressed on lymphocytes in perivascular cell accumulations (a,b, arrows) and dispersed in the parenchyma (a,b, arrowheads) in the case with chronic encephalitis. Within ischaemia stroke CCR5 was strongly expressed on mononuclear phagocytes in areas of ischaemia (d, arrowheads). Within normal control brains CD68+ cells were either found in perivascular localizations (e, arrow) or as processes-bearing microglial cells in the parenchyma (e, arrowhead). Only occasionally CCR5 immunoreactivity was found on perivascular cells (f, arrow). BV, blood vessel. Bars = 100 μ m.

CCR1 was the first chemokine receptor to be isolated and characterized by molecular cloning. The human receptor binds at least nine distinct chemokines (CCL3, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23), most of which are associated with inflammation. CCR1 is highly expressed by circulating monocytes, but found in very low levels on CD4+/CD45RO+ T cells [21].

In MS lesions, MRP14 expression is associated with the earliest stage of macrophage-mediated demyelinating activity [5]. Co-expression of CCR1 and MRP14 has previously been interpreted as identifying a population of newly recruited haematogenous monocytes in MS tissue sections

[36]. In the absence of CNS pathology, MRP14 immunoreactivity is virtually undetectable in human brain sections [20]. In pathological states, MRP14 expression varies widely, being present on microglia in some cases of cerebral malaria [29] but not detected in acute or chronic lesions of HTLV I-associated myelopathy [1].

CXCR3 selectively responds to three chemokines (CXCL9, CXCL10, CXCL11), all of which are inducible by interferon (IFN)-gamma. Because it is expressed at high levels on activated and memory T lymphocytes, CXCR3 has been designated CD183. Cells bearing CXCR3 are very frequently detected in inflammatory infiltrates, and corresponding ligands are typically expressed by the resident cells of the inflamed organ. Both *in vitro* and *in vivo*, CXCR3 expression is associated with type 1 helper (Th1) T cells. It has been proposed that the elaboration of IFN- γ by these cells elicits CXCR3 ligands and provides an amplification loop for Th1 immune responses. We previously described the expression of CD3 and CXCR3 in brain sections of patients with the major pattern II subtype of MS lesion, and found that the majority of CD3+ cells in perivascular cuffs and demyelinating lesions expressed CXCR3 [30]. Using both serial-section and dual-label immunofluorescence analyses, we demonstrated a strong positive relationship between the distribution of CXCR3 on T cells near vessels and one ligand, CXCL10, on the processes of astrocytes of the perivascular glia limitans [31]. These observations correlated well with the finding that CXCR3 was highly enriched on CD45RO+/CD4+ CSF T cells, when compared with the corresponding population in blood [15]. Other chemokine receptors, including CCR1, CCR2, CCR5, and CCR6 were expressed equally or at lower levels by CSF CD45RO+/CD4+ T cells, as compared with those in the circulation [15]. Overall, these data supported the concept that tissue-infiltrating T cells, including those found in the CNS parenchyma, are likely to express CXCR3 [16].

The chemokine receptor CCR5 is highly expressed on memory T cells, dendritic cells and monocytes [18]. Furthermore, activated CNS microglial cells express CCR5, an attribute that correlates with their infectivity by human immunodeficiency virus (HIV)-1 [11]. The expression of CCR5 on monocytes *in vitro* is dependent on the activation and differentiation state of the cells [10]. In studies of human blood and CSF cells, we found that a small minority of circulating CD14+ monocytes expressed CCR5, while virtually all CSF monocytes were CCR5+ [34]. On human T cells, CCR5 is associated with commitment to

the Th1 pattern of cytokine expression, particularly when co-expressed with CXCR3. Interestingly, we found that the population of CCR5+/CXCR3- T cells in blood was virtually absent from CSF [15]. These results suggested that trafficking from blood to CSF is positively associated with CCR5 expression on monocytes, but that lymphocyte expression of CCR5 was not sufficient for cells to gain entry to this compartment.

In the pattern II variant of MS tissue pathology [19], CCR5 expression was observed on perivascular lymphocytes, monocytes and on macrophages within demyelinated lesions [30,34]. CCR5 expression on mononuclear phagocytes increased during MS lesion evolution, a finding that was interpreted as indicating up-regulation of CCR5 on resident microglial cells and haematogenous monocytes upon activation [34].

Despite examining a wide range of pathologies involving both white matter and cortex, we identified several consistent features. In particular, CCR1 was routinely associated with MRP14 immunoreactivity. This observation was interpreted to indicate expression of these markers by infiltrating monocytes. CXCR3 was highly, but not invariably, associated with CD3 immunoreactive cells, suggesting the presence of this receptor on most T cells. CCR5 expression was strikingly variable, suggesting that its presence is governed by lesion-specific micro-environmental influences. These observations provide additional activation markers for leucocytes and microglial within CNS tissues and might also indicate useful directions for further research on haematogenous cell trafficking into the nervous system.

Acknowledgements

This research was supported by the National Institutes of Health (PO1 NS38667 to RMR) and by a fellowship of the Deutsche Forschungsgemeinschaft, Germany (TR463/1-1 to CT) and by the European Union (Project QLG3-CT-2002-00612 to HL). We gratefully acknowledge the MS Women's Committee of Cleveland, OH, and the Nancy Davis MS Center Without Walls for providing support to purchase an imaging station and associated software.

References

- 1 Abe M, Umehara F, Kubota R, Moritoyo T, Izumo S, Osame M. Activation of macrophages/microglia with

- the calcium-binding proteins MRP14 and MRP8 is related to the lesional activities in the spinal cord of HTLV-I associated myelopathy. *J Neurol* 1999; **246**: 358-64
- 2 Bacon KB, Harrison JK. Chemokines and their receptors in neurobiology: perspectives in physiology and homeostasis. *J Neuroimmunol* 2000; **104**: 92-7
- 3 Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998; **392**: 565-8
- 4 Bruck W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, Lassmann H. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol* 1995; **38**: 788-96
- 5 Brück W, Sommermeier N, Bergmann M, Zettl U, Goebel HH, Kretzschmar HA, Lassmann H. Macrophages in multiple sclerosis. *Immunobiology* 1996; **195**: 588-600
- 6 Cyster JG. Chemokines and cell migration in secondary lymphoid organs. *Science* 1999; **286**: 2098-102
- 7 Elices MJ. BX-471 Berlex. *Curr Opin Investig Drugs* 2002; **3**: 865-9
- 8 Eue I, Pietz B, Storck J, Klempt M, Sorg C. Transendothelial migration of 27E10+ human monocytes. *Int Immunol* 2000; **12**: 1593-604
- 9 Goebeler M, Roth J, Henseleit U, Sunderkotter C, Sorg C. Expression and complex assembly of calcium-binding proteins MRP8 and MRP14 during differentiation of murine myelomonocytic cells. *J Leukoc Biol* 1993; **53**: 11-18
- 10 Hariharan D, Douglas SD, Lee B, Lai JP, Campbell DE, Ho WZ. Interferon-gamma upregulates CCR5 expression in cord and adult blood mononuclear phagocytes 53. *Blood* 1999; **93**: 1137-44
- 11 He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, Gabuzda D. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* 1997; **385**: 645-9
- 12 Hesselgesser J, Halks-Miller M, DelVecchio V, Peiper SC, Hoxie J, Kolson DL, Taub D, Horuk R. CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. *Curr Biol* 1997; **7**: 112-21
- 13 Holness CL, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood* 1993; **81**: 1607-13
- 14 Kivisakk P, Trebst C, Eckstein DJ, Kerza-Kwiatecki AP, Ransohoff RM. Chemokine-based therapies for MS: how do we get there from here? *Trends Immunol* 2001; **22**: 591-3
- 15 Kivisakk P, Trebst C, Liu Z, Tucky BH, Sorensen TL, Rudick RA, Mack M, Ransohoff RM. T-cells in the cerebrospinal fluid express a similar repertoire of inflammatory chemokine receptors in the absence or presence of CNS inflammation: implications for CNS trafficking. *Clin Exp Immunol* 2002; **129**: 510-18
- 16 Kunkel EJ, Boisvert J, Murphy K, Viera MA, Genovese MC, Wardlaw AJ, Greenberg HB, Hodge MR, Wu L,

- Butcher EC, Campbell JJ. Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am J Pathol* 2002; **160**: 347–55
- 17 Kunkel EJ, Butcher EC. Chemokines and the tissue-specific migration of lymphocytes. *Immunity* 2002; **16**: 1–4
- 18 Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proc Natl Acad Sci USA* 1999; **96**: 5215–20
- 19 Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions, implications for the pathogenesis of demyelination. *Ann Neurol* 2000; **47**: 707–17
- 20 Mittelbronn M, Dietz K, Schluesener HJ, Meyermann R. Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude. *Acta Neuropathol (Berl)* 2001; **101**: 249–55
- 21 Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000; **52**: 145–76
- 22 Odink K, Cerletti N, Bruggen J, Clerc RG, Tarcsay L, Zwadlo G, Gerhards G, Schlegel R, Sorg C. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* 1987; **330**: 80–2
- 23 Piccio L, Rossi B, Scarpini E, Laudanna C, Giagulli C, Issekutz AC, Vestweber D, Butcher EC, Constantin G. 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* 2002; **168**: 1940–9
- 24 Pulford KA, Rigney EM, Micklem KJ, Jones M, Stross WP, Gatter KC, Mason DY. KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections. *J Clin Pathol* 1989; **42**: 414–21
- 25 Pulford KA, Sipos A, Cordell JL, Stross WP, Mason DY. Distribution of the CD68 macrophage/myeloid associated antigen. *Int Immunol* 1990; **2**: 973–80
- 26 Rabinowitz SS, Gordon S. Macrosialin, a macrophage-restricted membrane sialoprotein differentially glycosylated in response to inflammatory stimuli. *J Exp Med* 1991; **174**: 827–36
- 27 Ramprasad MP, Terpstra V, Kondratenko N, Quehenberger O, Steinberg D. Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. *Proceedings of the Natl Acad Sci USA*, 1996: 14833–8
- 28 Ransohoff RM, Bacon KB. Chemokine receptor antagonism as a new therapy for multiple sclerosis. *Expert Opin Invest Drugs* 2000; **9**: 1079–97
- 29 Schluesener HJ, Kreamsner PG, Meyermann R. Widespread expression of MRP8 and MRP14 in human cerebral malaria by microglial cells. *Acta Neuropathol (Berl)* 1998; **96**: 575–80
- 30 Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, Qin S, Rottman J, Sellebjerg F, Strieter RM, Frederiksen JL, Ransohoff RM. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* 1999; **103**: 807–15
- 31 Sorensen TL, Trebst C, Kivisakk P, Klaege KL, Majmudar A, Ravid R, Lassmann H, Olsen DB, Strieter RM, Ransohoff RM, Sellebjerg F. Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. *J Neuroimmunol* 2002; **127**: 59–68
- 32 Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; **76**: 301–14
- 33 Trebst C, Ransohoff RM. Investigating chemokines and chemokine receptors in patients with multiple sclerosis: opportunities and challenges. *Arch Neurol* 2001; **58**: 1975–80
- 34 Trebst C, Sorensen TL, Kivisakk P, Cathcart MK, Hesselgesser J, Horuk R, Sellebjerg F, Lassmann H, Ransohoff RM. CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. *Am J Pathol* 2001; **159**: 1701–10
- 35 Werlen G, Palmer E. The T-cell receptor signalosome: a dynamic structure with expanding complexity. *Curr Opin Immunol* 2002; **14**: 299–305

Received 7 January 2003

Accepted after revision 11 June 2003