

Chemokine receptors as biomarkers in multiple sclerosis¹

Robert J. Fox^{a,d,*}, Pia Kivisäkk^{b,**}, Jar-Chi Lee^c, Barbara Tucky^b, Claudia Lucchinetti^e, Richard A. Rudick^{a,d} and Richard M. Ransohoff^{a,b,d}

^a*Department of Neurology, Mellen Center for Multiple Sclerosis Treatment and Research, Cleveland, OH, USA*

^b*Neuroinflammation Research Center, Cleveland Clinic Foundation, Cleveland OH, USA*

^c*Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA*

^d*Department of Quantitative Health Sciences, Cleveland Clinic Foundation, Cleveland, OH, USA*

^e*Cleveland Clinic Lerner School of Medicine at Case Western Reserve University, Cleveland, OH, USA*

^f*Department of Neurology, Mayo Clinic, Rochester, MN, USA*

Abstract. Leukocyte infiltrates characterize tissue inflammation and are thought to be integral in the pathogenesis of multiple sclerosis (MS). This attribute underlines the importance of understanding mechanisms of leukocyte migration. Chemokines are secreted proteins which govern leukocyte trafficking into targeted organs. Chemokine receptors (CKR) are differentially expressed on leukocytes and their modulation is a potential target for MS disease modifying therapies. Chemokines and their receptors are also potential biomarkers of both disease activity and response to treatment. We describe the fluctuations in CKR expression on peripheral leukocytes in a group of MS patients followed longitudinally for up to 36 months. We observed little fluctuation in CKR expression within each patient over time, despite considerable variability in CKR expression between patients. These observations suggest that individual patients have a CKR set point, and this set point varies from one patient to another. Evaluation of chemokines or chemokine receptors as biomarkers in MS will need to account for this individual variability in CKR expression.

An essential element in the pathology of multiple sclerosis (MS) is the accumulation and activation of mononuclear cells within the central nervous system (CNS). Mononuclear cells are hypothesized to have an effector role in the inflammatory cascade and subsequent tissue injury in MS. Chemokines and their receptors are a phylogenetically ancient intercellular communication network employed by organisms as ancient

as the jawless fish. In their original form, chemokines guide cell migration and organ formation throughout an organism's development. Chemokine networks have been adopted by the immune system to guide the migration of lymphocytes, monocytes, eosinophils, basophils, and neutrophils in relation to inflammation. Acting through specific high-affinity receptors, chemokines both attract leukocytes to migrate along concentration gradients and reversibly activate leukointegrins, which are involved in interactions with endothelial cells and other cellular components. Accordingly, chemokines may play a critical role in attracting mononuclear cells into areas of active inflammation and have potential usefulness in both understanding the pathogenesis of MS and treating the underlying disease.

Human studies evaluating the role of chemokines in MS have focused on flow cytometry of CSF and peripheral leukocytes as well as histologic staining of biopsy

¹This study was supported in part by grants from the National Institutes of Health (PO1 NS38667 to RMR; K23 NS 47211 to RJF; M01 RR018390 to RAR), the National Multiple Sclerosis Society (RG 3185 to CL and RMR) and the Nancy Davis Center Without Walls.

*Corresponding author: Robert J. Fox, Mellen Center for Multiple Sclerosis Treatment and Research, U-10, 9500 Euclid Avenue, Cleveland, OH 44122, USA.

**Current affiliation: Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA, USA.

and autopsy tissue. In studies of peripheral leukocytes of MS patients, the chemokine receptor (CKR) CCR5 has been variably found expressed on a greater proportion of T-cells than in controls, and in small preliminary studies, the number of cells expressing CCR5 was occasionally found to correlate with disease activity, defined as symptomatic relapse [1–4]. Similarly, some investigators (although not all) have found increased CXCR3 expression in circulating T-cells from MS patients, too. T-cells expressing CCR5 and CXCR3 are antigen-experienced memory cells. CXCR3+ cells in the circulation are polarized to the T-helper 1 cytokine profile, and secrete more pro-inflammatory interferon γ and tumor necrosis factor α [2,4]. In one report, T-cell expression of CCR5 was decreased in MS patients treated with interferon β -1a compared to untreated MS patients, and T-cells exposed *in vitro* to interferon β -1a showed decreased mRNA expression for CCL3, CCL5, and CCR5 [5].

In CSF studies of MS patients, CXCL10 (IP-10) is reproducibly elevated and CCL5 (RANTES) is less reliably elevated, as compared to non-inflammatory disease controls, but surprisingly, CCL2 (MCP-2) is reproducibly decreased, not only in MS but also in other chronic neuroinflammations including Behcet's disease [1]. The reason for this reduction remains uncertain, as CCL2 is highly expressed within parenchymal inflammatory lesions of MS (see below) and is typically elevated in acute neuroinflammation as exemplified by viral meningitis. CCL2 and CCL5 mRNA expression by CSF mononuclear cells was similar in MS and other inflammatory disorders, suggesting that the major source of CSF chemokines was parenchymal cells, during periods of inflammation [6,7]. CSF T-cells are highly enriched for CXCR3, with over 90% of T-cells expressing this chemokine receptor, compared to about 40% in the periphery [1]. CSF is also enriched with CCR5-expressing T-cells, although not to the same extent as CXCR3, and this enrichment was later found simply to reflect the preferential accumulation of memory cells in CSF. Virtually all CSF monocytes express both CCR1 and CCR5 and are enriched in CSF (>70%) compared to the periphery (<15%). As with lymphocytes, this enrichment is seen in both inflammatory and non-inflammatory settings.

Studies of brain tissue allow a direct evaluation of the presence of chemokines and CKR in MS inflammation. Similar to the CSF, CXCR3 is expressed on virtually all perivascular and parenchymal lymphocytes in MS brain tissue, and CCR5 is expressed on a subset of these perivascular lymphocytes [1,2,8]. Despite the abun-

dant expression of CXCR3 and CCR5 on CSF lymphocytes in healthy controls, these chemokines are rarely observed in brains of controls that died without neuroinflammatory disease, because of the paucity of lymphocytes in the tissue. The relevance of CXCR3- and CCR5-expressing lymphocytes in perivascular brain parenchyma is suggested by the presence of appropriate receptor ligands. Specifically, increased expression of CXCL10 is observed on MS astrocytes, which may retain CXCR3-expressing cells and prevent their recirculation, or may deliver signals for effector/survival function to infiltrating cells [9].

Brain parenchymal mononuclear phagocytes also express chemokines, including CCR1, CCR5, and to a much lesser extent, CCR2 [1,2,10]. Similar to that seen with chemokine expression on lymphocytes, CCR1+/CCR5+ mononuclear phagocytes are observed in brain perivascular regions only in inflammatory states and not in control tissues [11]. This observation correlates to the paucity of hematogenous inflammatory cells within brain parenchyma under physiological conditions. Bespeaking the pertinence of monocyte chemokine receptor expression in neuroinflammation, CCL3 and CCL4 are expressed on parenchymal inflammatory cells (macrophages and microglia), and CCL3 is also found on activated neuroglial cells [12–14]. Astrocyte and inflammatory cell expression of CCL2, CCL7, and CCL8 is seen within inflammatory MS lesions [15,16].

The identification of different MS pathology subtypes has provided a framework within which to evaluate chemokine and CKR involvement in MS pathogenesis [17]. Pattern II lesions are characterized by focal demyelination, perivascular lymphocyte aggregates, antibody deposition on degenerating myelin sheaths, and complement activation. CCR1+ mononuclear phagocytes expressing MRP14 localize to lesion edges at sites of active demyelination, and CCR1 is down regulated as monocytes transform to macrophages [11]. In contrast, CCR5 is maintained on macrophages and increased on microglia as inflammatory demyelination proceeds. Pattern III lesions are characterized by less discrete demyelination and diffuse lymphocyte infiltration, and show no antibody deposition or complement activation. In contrast to Pattern II, CCR1 and CCR5 are co-expressed within Pattern III lesions and do not shift their expression throughout lesion maturation. These characteristics might reflect the hypoxic character of tissue injury in pattern III lesions and a different cytokine environment from that seen in pattern II [18].

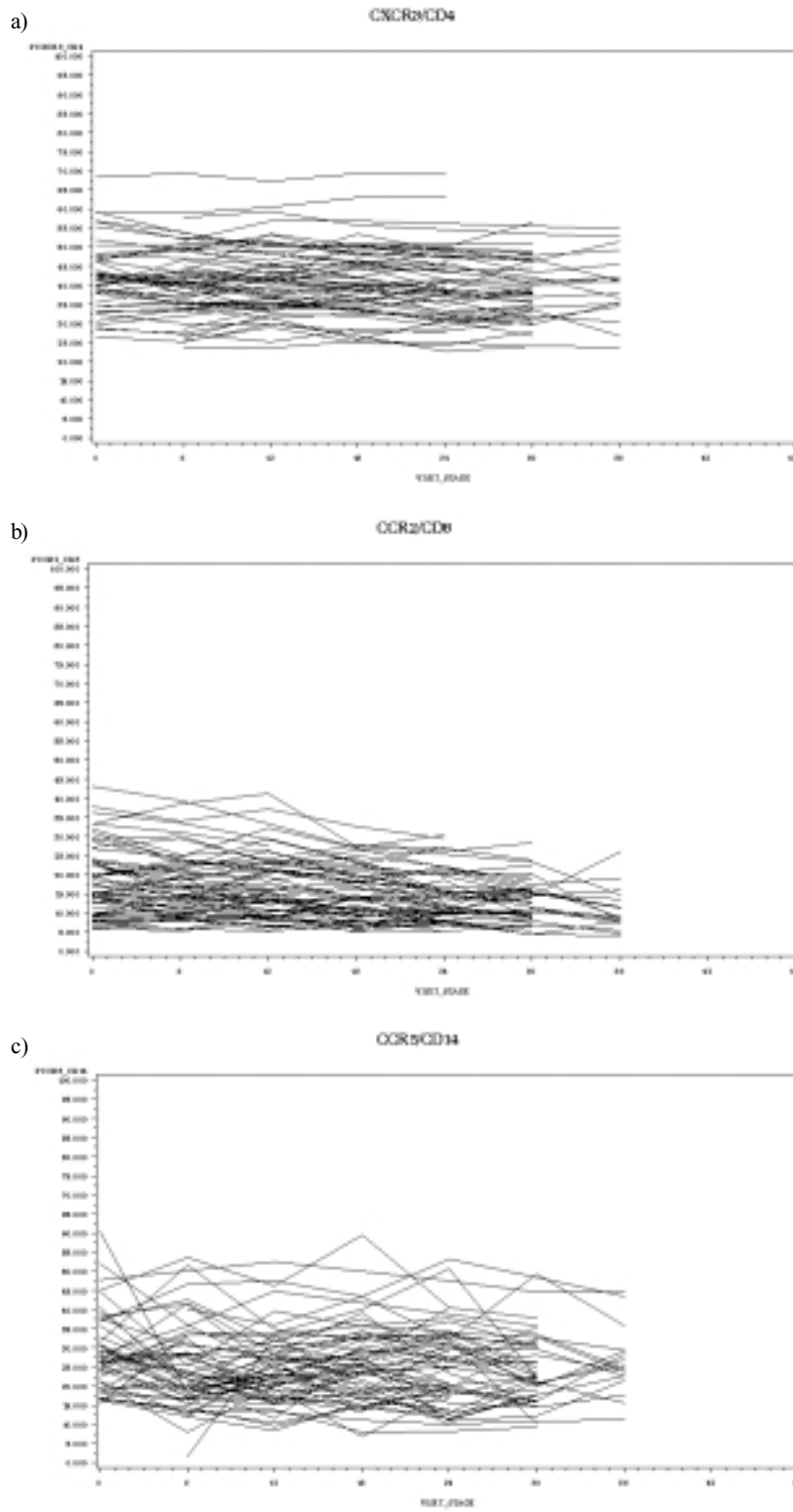


Fig. 1. Example plots of individual patient CKR over time. a) Percent CD4+ cells expressing CXCR3; b) Percent CD8+ cells expressing CCR2; c) Percent CD14+ cells expressing CCR5.

Since these pathology patterns are described by the investigator team as being homogeneous within individual patients (although this observation is not universally accepted), such observations suggest that chemokine-targeted therapies might need to be tailored to individual patients, testifying to the need for biomarkers of lesion pattern without biopsy. Ongoing research is attempting to identify pathology type in MS patients using imaging or biochemical/immunological constituents of blood or CSF, and these tools may help tailor chemokine-targeted therapies to the specific pathophysiology of each MS patient.

The purported role of chemokines in MS pathophysiology raises the question: could chemokines or their receptors provide useful biomarkers of either the MS disease course or the clinical response to therapeutic interventions? Clinical study outcomes in MS are complicated by widely disparate neurologic symptoms as well as the long disease time course. Objective clinical examinations have been developed to assist clinical investigators, but they only capture a small fraction of the disease activity seen on brain MRI and do not accurately reflect the degree of tissue destruction inflicted by the disease. Magnetic resonance imaging provides additional insights into inflammatory tissue injury and destruction, but is expensive to perform and analyze, and current techniques only measure the end-organ injury arising from the disease and not the disease itself.

The chemokine network is among a group of immunologic measures that may have utility in measuring MS disease. Chemokine secretion attracts lymphocytes, and so their levels in the peripheral blood or certain organs may indicate the inflammatory status of a particular organ system. Paradoxically, although chemokine blood levels may reflect organ-specific inflammation, high levels of chemokines themselves may, in the circulation, down-regulate leukocyte CKRs and thus provide anti-inflammatory effects. CKR may be altered on lymphocytes in circulating blood or their target organ (i.e. central nervous system), and these alterations may be an indicator of ongoing inflammation. Individual differences in CKR expression may predispose to MS, and CKR expression may change in response to immunomodulatory therapies.

Importantly, there may be biologic variability in CKR expression unrelated to inflammation, and this variation may impair our ability to use CKR as a biomarker of either MS disease activity or response to therapy. We sought to evaluate this biologic variability by measuring expression of three CKRs on different types of peripheral mononuclear leukocytes in a group of MS patients over time.

1. Methods

We evaluated CKR expression from peripheral blood mononuclear cells in a group of 59 MS patients every six months for up to 36 months as part of an ongoing natural history study. MS patients were recruited from our general MS clinical practice and were receiving treatment with a variety of long-term MS therapies. Relapses were typically treated with a course of intravenous methylprednisolone as clinically indicated.

The proportion of cells expressing CCR2, CCR5, and CXCR3 on CD4+ and CD8+ lymphocytes and CCR2 or CCR5 on CD14+ monocytes were measured by flow cytometry as previously described [19]. In brief, blood samples were drawn every six months and lymphocytes were evaluated directly from whole, fresh peripheral blood. One hundred microliters of venous blood was blocked with mouse immunoglobulin, stained with directly conjugated monoclonal antibodies against CKR and CD4, CD8 or CD14, lysed to remove erythrocytes, and then washed. Acquisition of stained samples was performed on a Becton Dickinson LSR1 flow cytometer and analysis done by a single investigator (PK) using WinList software (Verity Software House, Topsham, ME).

Within-patient variability was calculated by deriving the standard deviation within each patient for each CKR expression over time and then averaging these standard deviations among all patients. Between-patient standard deviations were calculated for each CKR at each time point using all patient measures for that time point.

2. Results

Complete demographic description of studied patients is listed in Table 1. Most patients (85%) received interferon therapy sometime over the course of the study, and a small proportion (25%) of patients received a variety of other therapies. Median follow-up was 30 months, with a median of six CKR assays performed on each patient.

We observed very little variability in CKR expression within individual patients over time. Example plots of CKR expression from all MS patients over time are shown in Fig. 1. Except for CCR2 on CD14+ cells, the mean within-patient standard deviation of CKR expression varied between 2.44 and 5.87 (Fig. 2). CCR2 was expressed on 95–100% of CD14+ cells in almost all samples, and this ceiling restricted the standard deviation of CKR expression over time within the same pa-

Table 1
Demographic description of MS patient population

Total MS patients studied, n	59
Age, mean years(SD)	43.5 (9.0)
Duration of disease, mean years (SD)	11.6 (8.1)
Sex, n Female (%)	43 (73%)
Race, n Caucasian, Black, Other (%)	54 (91.5%), 4 (6.8%), 1 (1.7%)
Disease type, n RRMS, SPMS (%)	38 (64.4%), 21 (35.6%)
EDSS, Mean(SD), Median	3.6(2.2), 3.5
Received Interferon during study	49 (83%)
Received other MS therapy during study, including Glatiramer acetate, methotrexate, azathioprine, cyclophosphamide, pulse methylprednisolone	15 (25%)

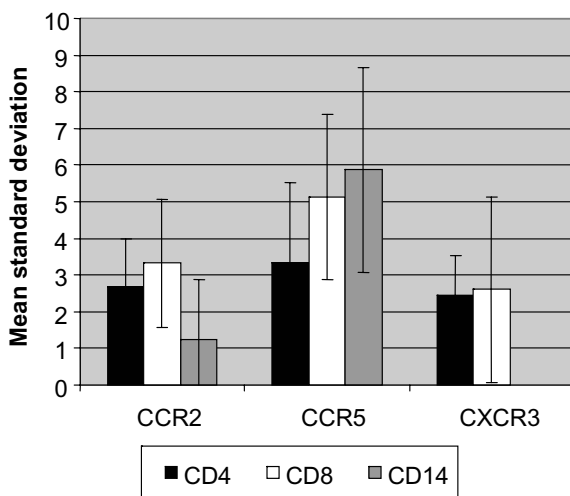


Fig. 2. Mean within-subject standard deviation of CKR expression over time. Error bars represent standard deviation of mean standard deviation.

tient. In general, there was greater variability in CCR5 expression compared to CCR2 and CXCR3. CD8+ T-cells demonstrated greater variability of CKR expression than CD4+ T-cells.

In contrast, there was significantly greater variability in CKR expression between patients than within patients (Fig. 3). Except for CCR2 on CD14+ cells, standard deviations for CKR expression varied between 4.58 and 15.67. CCR2+/CD14+ cells showed the same restricted variability as in the within-subject analysis because of a ceiling effect. Similar to the within-patient variability, the between patient variability was greater for CCR5 expression compared to CCR2 expression, while the between-patient variability was greater for CD4+ T-cells compared to CD8+ T-cells. Despite the ceiling expression of CCR2 on CD14+ cells, between subject variability was consistently greater than the mean within-subject variability.

We recently performed analysis of these chemokine receptors in a second cohort of MS patients enrolled

in a longitudinal clinical/immunological/MRI analysis study being conducted by the MS Lesion Project investigators, and found precisely comparable inter-individual variability, with intra-individual stability in expression of CKRs on circulating CD4+, CD8+ and CD14+ mononuclear leukocytes (data not shown).

3. Discussion

Immunologic measures are attractive biomarkers because of their purported process specificity in relation to MS disease pathogenesis and their relative ease of measurement in the laboratory. Markers of immunologic activation are expected to fluctuate throughout the relapsing remitting course of the disease, and alterations in these measures may provide useful information regarding the disease course and response to therapeutic interventions.

We show here that variability in CKR receptor expression within patients is much less than the variability of CKR expression between patients. Based on this data, individual patients appear to have a CKR set-point, and this set-point is different from patient to patient. This observation raises the possibility that variation in the physiologic set-points of immunologic markers may be a biomarker of disease activity or therapeutic response. Alternatively, the set-point levels for individual patients might relate to their outcomes during the MS disease process. Notably, the chemokine receptor set-points we describe here were also found in healthy individuals followed similarly, and are likely characteristic of patients prior to acquiring the MS diagnosis [19]. Chemokine receptor set-points on CD4+ lymphocytes are closely related to the numbers of CD45RO+ memory cells.

Further studies will be needed to evaluate the utility of chemokine secretion and CKR expression as a biomarker for MS. Definitive demonstration of their

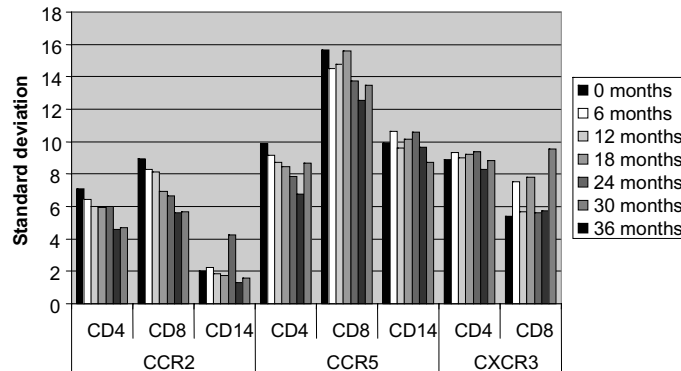


Fig. 3. Between-subject variation (standard deviation) in chemokine receptor expression at each time point.

role in MS pathogenesis has not been established, but there is growing evidence to support their role in the immune cascade that leads to demyelination and cell loss. A recent report described alterations in CKR expression in MS patients treated with the immunomodulating therapy glatiramer acetate [20]. Compared to baseline, patients treated with glatiramer acetate for 1 year showed a reduction in CXCR3 and CCR5 expression on glatiramer acetate-reactive and myelin-reactive CD4+ T-cells. There was also an increase in CCR7 expression on the same cells. This CKR alteration adds further evidence in support of a role for chemokine networks in the pathogenesis of MS and the effectiveness of MS therapeutics.

There are several challenges to using chemokines and CKR as biomarkers of MS disease activity. Chemokines are secreted locally to traffic leukocytes into focal areas of inflammation, and so measuring them from peripheral blood may be quite difficult. Chemokine secretion may be transient, so frequent assessments may be necessary to adequately characterize chemokine secretion. Chemokines are also variably bound by the Duffy antigen receptor for chemokines (DARC) on erythrocytes, providing an enormous bound reservoir of intravascular chemokine in equilibrium with free peptide.

Although we show here that CKR expression in peripheral blood is relatively stable within MS patients over time, we have also recently observed that lymphocytes migrating through an artificial blood brain barrier downregulate some but not all CKRs upon migration in response to ligand. We hypothesize that a similar modulation of CKR expression might occur as CKR-expressing lymphocytes migrate from the periphery into the inflamed CNS (either through the blood-CSF barrier or the blood-brain-barrier). This type of modulation suggests that assessments of CKR expression

will need to account for cells moving from one compartment to another. This type of accounting assumes that some chemokine receptor-bearing cells recirculate from compartments such as CSF and return to the circulation, which has yet to be conclusively demonstrated. This difficulty is clearly less pertinent for effector cells localized within inflamed tissue.

Chemokines and their receptors are attractive candidate biomarkers for assessing MS disease activity and the effects of immunomodulating therapies. An underlying necessity to using chemokines and their receptors as biomarkers in MS is their correlation with clinical and radiologic measures of inflammation and tissue damage, and to date this has not been demonstrated. Chemokine modulation is an attractive therapeutic strategy, and several clinical trials targeting CCR2 are in the planning stages or already underway [21]. Success in these studies would provide further motivation for characterizing CKRs or chemokines as MS biomarkers. Recognition of individual patient set-points of CKR expression in peripheral blood will be useful in understanding how CKR expression relates to disease activity and its response to therapies.

Acknowledgements

We thank Kathy Schemo from the Lerner Research Institute Flow Cytometry Core Laboratory for technical assistance, and Dr. Elizabeth Fisher for her comments and suggestions on this manuscript.

References

- [1] T.L. Sorensen et al., Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients, *The Journal of Clinical Investigation* **103**(6) (1999), 807–815.

- [2] K.E. Balashov et al., CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions, *Proceedings of the National Academy of Sciences of the United States of America* **96**(12) (1999), 6873–6878.
- [3] P.A. Calabresi, R. Martin and S. Jacobson, Chemokines in chronic progressive neurological diseases: HTLV-1 associated myelopathy and multiple sclerosis, *Journal of Neurovirology* **5**(1) (1999), 102–108.
- [4] T. Strunk et al., Increased numbers of CCR5+ interferon-gamma- and tumor necrosis factor-alpha-secreting T lymphocytes in multiple sclerosis patients, *Annals of Neurology* **47**(2) (2000), 269–273.
- [5] Y.C. Zang et al., Regulation of chemokine receptor CCR5 and production of RANTES and MIP-1alpha by interferon-beta, *Journal of Neuroimmunology* **112**(1–2) (2001), 174–180.
- [6] P. Kivisäkk et al., No evidence for elevated numbers of mononuclear cells expressing MCP-1 and RANTES mRNA in blood and CSF in multiple sclerosis, *Journal of Neuroimmunology* **91**(1–2) (1998), 108–112.
- [7] D. Franciotta et al., Serum and CSF levels of MCP-1 and IP-10 in multiple sclerosis patients with acute and stable disease and undergoing immunomodulatory therapies, *Journal of Neuroimmunology* **115**(1–2) (2001), 192–198.
- [8] J.E. Simpson et al., Expression of the interferon-gamma-inducible chemokines IP-10 and Mig and their receptor, CXCR3, in multiple sclerosis lesions, *Neuropathology and Applied Neurobiology* **26**(2) (2000), 133–142.
- [9] C. Trebst and R.M. Ransohoff, Investigating chemokines and chemokine receptors in patients with multiple sclerosis: opportunities and challenges, *Archives of Neurology* **58**(12) (2001), 1975–1980.
- [10] J. Simpson et al., Expression of the beta-chemokine receptors CCR2, CCR3 and CCR5 in multiple sclerosis central nervous system tissue, *Journal of Neuroimmunology* **108**(1–2) (2000), 192–200.
- [11] C. Trebst et al., CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis, *The American Journal of Pathology* **159**(5) (2001), 1701–1710.
- [12] J.E. Simpson et al., Expression of monocyte chemoattractant protein-1 and other beta-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions, *Journal of Neuroimmunology* **84**(2) (1998), 238–249.
- [13] N. Woodrooffe et al., The role of chemokines in the pathogenesis of multiple sclerosis, *Advances in Experimental Medicine and Biology* (1999), 468.
- [14] L.A. Boven et al., Macrophage inflammatory protein-1alpha (MIP-1alpha), MIP-1beta, and RANTES mRNA semiquantification and protein expression in active demyelinating multiple sclerosis (MS) lesions, *Clinical and Experimental Immunology* **122**(2) (2000), 257–263.
- [15] P. Van Der Voorn et al., Expression of MCP-1 by reactive astrocytes in demyelinating multiple sclerosis lesions, *The American Journal of Pathology* **154**(1) (1999), 45–51.
- [16] C. McManus et al., MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study, *Journal of Neuroimmunology* **86**(1) (1998), 20–29.
- [17] C. Lucchinetti et al., Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination, *Annals of Neurology* **47**(6) (2000), 707–717.
- [18] D.J. Mahad et al., Expression of chemokine receptors CCR1 and CCR5 reflects differential activation of mononuclear phagocytes in pattern II and pattern III multiple sclerosis lesions, *Journal of Neuropathology and Experimental Neurology* **63**(3) (2004), 262–273.
- [19] P. Kivisäkk et al., Expression of CCR2, CCR5, and CXCR3 by CD4+ T cells is stable during a 2-year longitudinal study but varies widely between individuals, *Journal of Neurovirology* **9**(3) (2003), 291–299.
- [20] R. Allie et al., Bystander modulation of chemokine receptor expression on peripheral blood T lymphocytes mediated by glatiramer therapy, *Archives of Neurology* **62**(6) (2005), 889–894.
- [21] R.J. Fox and R.M. Ransohoff, New directions in MS therapeutics: vehicles of hope, *Trends in Immunology* **25**(12) (2004), 632–636.