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Expression of CCR2, CCR5, and CXCR3 by CD4+ T cells is stable during a 2-year longitudinal study but varies widely between individuals

Pia Kivisäkk,[1] Corinna Trebst,[1] Jar-Chi Lee,[2] Barbara H Tucky,[1] Richard A Rudick,[1] James J Campbell,[3] and Richard M Ransohoff[1]

[1]*Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA;* [2]*Department of Biostatistics and Epidemiology, Cleveland Clinic Foundation, Cleveland, Ohio, USA; and* [3]*Joint Program in Transfusion Medicine, Children's Hospital and Harvard Medical School, Department of Pathology, Boston, Massachusetts, USA*

Blockade of chemokine receptors (CKRs) has recently emerged as a possible pathway for therapeutic intervention in disease. In the present report, the expression of CCR2, CCR5, and CXCR3, associated with migration of mononuclear cells to inflamed tissue, was determined on CD4+ T cells in a 2-year longitudinal study of healthy volunteers using flow cytometry. Large interindividual variations in the expression of these receptors on CD4+ T cells were observed, whereas levels remained remarkably stable over time within subjects. The expression of CCR2, CCR5, and CXCR3 on CD4+ T cells was directly proportional to percentages of CD45RO^{hi}/CD4+ T cells. In addition, highly significant associations between levels of CCR2, CCR5, and CXCR3 on CD4+ T cells were demonstrated in individual subjects, implying a common mechanism for regulating the expression of these CKRs on circulating T cells. These associations were not due to coexpression of CKRs on individual CD45RA−/CD4+ T cells. The results provide insight into the regulation of CKR expression on CD4+ T cells *in vivo*, and suggest that major fluctuations of CKR expression in individuals are uncommon. *Journal of NeuroVirology* (2003) **9,** 291–299.

Keywords: CD4+ T cells; chemokine receptors; healthy volunteers; longitudinal

Introduction

Considerable interest has recently been focused on mechanisms for leukocyte trafficking into inflamed tissue, a process for which chemokines and their receptors have been shown to play crucial roles. Chemokines produced in tissue can be transcytosed across endothelial cells and become immobilized in the vascular lumen, resulting in arrest of rolling leukocytes expressing cognate receptors, followed by transendothelial migration and tissue infiltration of the leukocytes (Luster, 1998; Murphy *et al*, 2000; Sallusto *et al*, 2000). In combination with adhesion molecules, chemokine receptors (CKRs) expressed on leukocytes and chemokines produced in tissue define the cellular composition of an inflammatory infiltrate (Springer, 1994).

Leukocytes expressing a variety of inflammatory CKRs, most consistently CCR2, CCR5, and CXCR3, have been identified in diverse inflammatory tissues, including multiple sclerosis (MS) brain lesions and synovial fluid from patients with rheumatoid arthritis (Balashov *et al*, 1999; Brühl *et al*, 2001; Kunkel *et al*, 2002; Mack *et al*, 1999; Qin *et al*, 1998; Simpson *et al*, 2000a, 2000b; Sørensen *et al*, 1999; Trebst *et al*, 2001). Even though the chemokine network is notorious for its ligand redundancy and receptor promiscuity *in vitro*, studies in CKR-knockout mice have shown that disruption of a single CKR gene can lead to impaired tissue infiltration of leukocytes. For instance, mice lacking CCR2 developed normally but failed to recruit macrophages in an experimental

Address correspondence to Dr. Richard M. Ransohoff, Department of Neurosciences, NC30, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA. E-mail: ransohr@ccf.org

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model of peritoneal inflammation (Boring *et al*, 1997). CCR2◯/◯ mice were furthermore resistant to experimental autoimmune encephalomyelitis (EAE), and showed a significant reduction in numbers of T cells and macrophages infiltrating the central nervous system (CNS) after immunization with myelin oligodendrocyte glycoprotein peptide 35–55 (Fife *et al*, 2000; Izikson *et al*, 2000). Interfering with leukocyte recruitment through blockade of CKRs may thus constitute a feasible way of reducing tissue damage in inflammatory diseases.

Several cross-sectional studies have approached the question whether individuals with inflammatory diseases have altered expression of CKRs on circulating leukocytes. In patients with MS, levels of CCR5+ T cells have rather consistently been demonstrated to be elevated compared to healthy individuals, whereas levels of CXCR3+ T cells were elevated in some, but not all, studies (Balashov *et al*, 1999; Calabresi *et al*, 1999; Misu *et al*, 2001; Sørensen *et al*, 1999, 2002; Strunk *et al*, 2000; Teleshova *et al*, 2002; Wu *et al*, 2000; Zang *et al*, 2000). Levels of circulating CCR5+ T cells were increased in six relapsing-remitting MS patients during relapse, compared to patients in remission, with normalized levels 3 weeks later during the recovery phase, suggesting an association between CCR5+ T cells and disease activity (Misu *et al*, 2001).

Large interindividual variations in CCR5 expression, together with small differences between different patient populations, have, however, made interpretation of such cross-sectional studies difficult. Apart from a small study of six healthy individuals in whom the expression of CCR5 and CXCR3 on memory T cells was followed serially over 3 weeks (Campbell *et al*, 2001), little is known regarding fluctuations over time in CKR expression in individual subjects. We conducted a longitudinal analysis of CCR2, CCR5, and CXCR3 expression on peripheral blood T cells in a group of healthy controls (HCs) using flow cytometry. Although large interindividual variations in the expression of CCR2, CCR5, and CXCR3 on CD4+ T cells were observed, levels within each individual subject remained remarkably stable over 2 years' follow-up. In addition, we observed that the expression of CCR2, CCR5, and CXCR3 was proportional to the expression of $CD45RO^{hi}$/CD4+ memory T cells in blood, suggesting that calculating the percentage of CKR-positive cells of the total population of CD4+ T cells in humans may primarily reflect levels of previously activated memory T cells in these individuals.

Results

Chemokine receptor expression on CD4+ T cells varies widely among individuals but is stable over time

Using flow cytometry, the expression of CCR2, CCR5, and CXCR3 on CD4+ T cells was quantified in peripheral blood from 26 HCs. In this group, 19.7% ◯6.5% (mean ◯SD) of all CD4 + T cells expressed CCR2, 24.9% ◯9.0% expressed CCR5, and 43.5% ◯8.4% expressed CXCR3. The majority of the subjects had levels of CCR2, CCR5, and CXCR3 that were closely distributed, with an interquartile range around 10 percentage units (Figure 1**A**). The total ranges of CCR2, CCR5, and CXCR3 levels were, however, considerable larger, especially for CCR5, and CXCR3, which were expressed by 5.0% to 40.2% and 26.9% to 68.5%, respectively, of all CD4+ T cells. This variability in CKR expression between individuals was not explained by differences in age or gender.

Two samples obtained with 12-month interval were obtained from 20 HCs. Unexpectedly, the fluctuation in CKR expression over time was remarkably low. In particular, the median change in levels of CD4+ T cells expressing CCR2, CCR5, or CXCR3 between these two time points in individual subjects varied between 1.8 and 2.5 percentage units (Figure 1**B**). A third sample was obtained 2 years after the initial sample in nine of the controls. As shown in Figure 1**C**, levels of CKR expression remained stable over the 2-year follow-up in individual subjects.

The expression of CCR2, CCR5, and CXCR3 on CD4+ T cells in individual subjects is tightly interrelated

Data from the longitudinal study suggested that the expression of CCR2, CCR5, and CXCR3 on CD4+ T cells was regulated in a coordinate fashion. That is, an increase in the expression of one receptor seemed to be associated with increases in the expression also of the other two receptors. Such an association was tested using correlation analysis, which demonstrated significant linear relationships between levels of CCR2, CCR5, and CXCR3 on CD4+ T cells (Figure 2**A**).

An association between levels of CCR2, CCR5, and CXCR3 in individual subjects was not specific for CD4+ T cells. In the CD8+ T-cell compartment, weak correlations between levels of CCR2 and CCR5 ($r = .49$, $P < .05$) as well as between levels of CCR5 and CXCR3 ($r = .50$, $P < .05$) were observed. In contrast, no relationship was identified between levels of CCR2 and CCR5 on CD14+ monocytes (data not shown).

Chemokine receptor expression on CD4+ T cells is closely related to the percentage of $CD45RO^{hi}$/CD4+ T cells

Inflammatory CKRs are predominantly expressed on activated effector/memory CD4+ T cells (Qin *et al*, 1998; Rabin *et al*, 1999). A potential explanation for a coordinate pattern of expression of CCR2, CCR5, and CXCR3 on CD4+ T cells could be that such expression reflects the general state of activation of these cells. Therefore, we established relationships

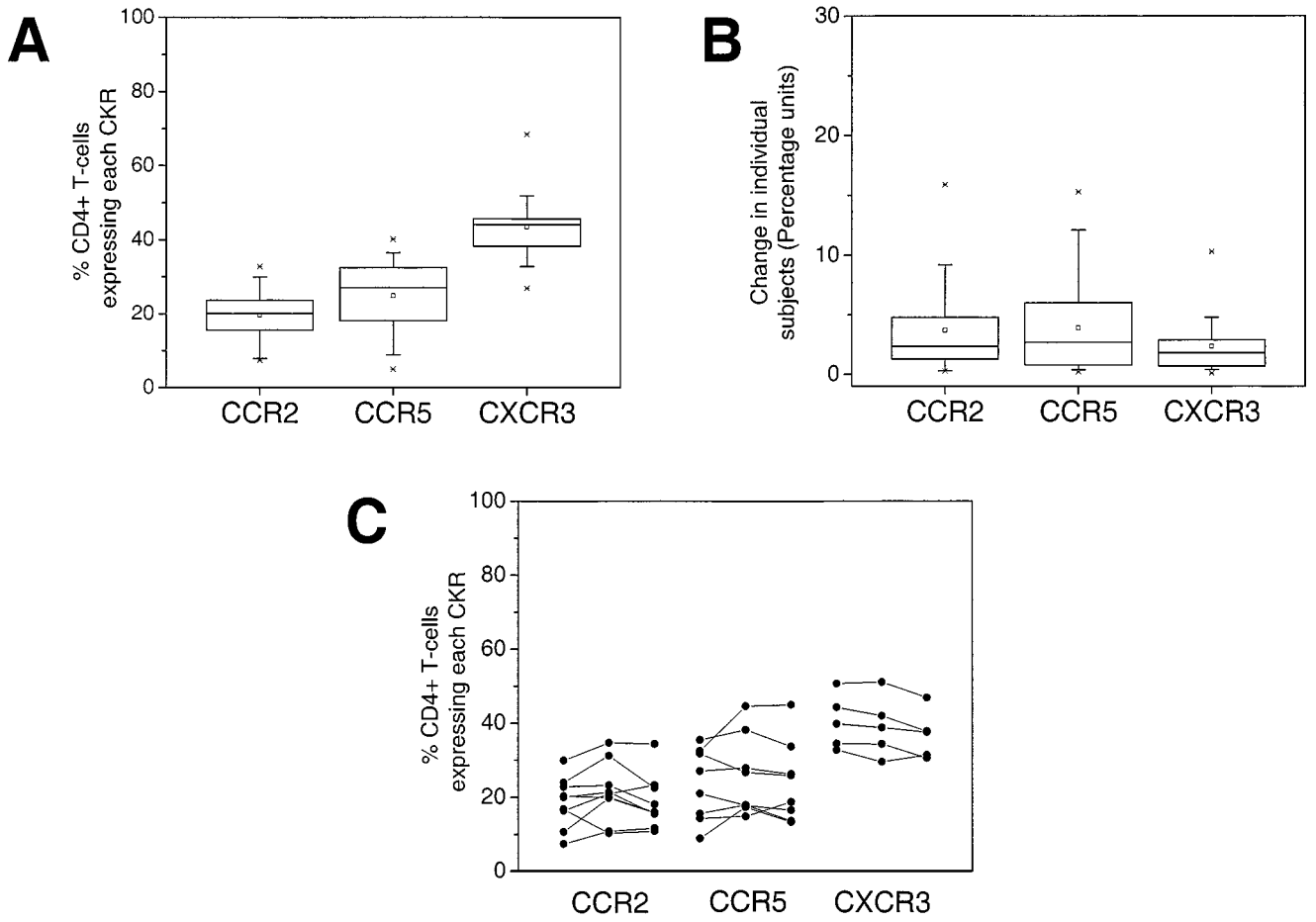

Figure 1 The expression of CCR2, CCR5, and CXCR3 on CD4+ T cells was determined in a group of HCs using flow cytometry. (**A**) Levels of CKR expression on CD4+ T cells from 26 HCs examined at the time of entry into the study. Box and whisker plots depict the median level of each group (horizontal line in the box), the 25th and 75th percentiles (upper and lower edges of box), the 5th and 95th percentiles (lines extending above and below box), and maximal and minimal values (short horizontal lines). (**B**) The changes in levels of CKR expression between the time of entry into the study and 12 months follow-up were calculated in 20 subjects. The figure shows absolute changes expressed in percentage units. (**C**) Three consecutive samples obtained with 12 months interval were available from nine HCs. Results from individual subjects are connected with solid lines.

between CKR expression and activation markers on CD4+ T cells.

We found that CCR2, CCR5, and CXCR3 were expressed selectively by the subpopulation of $CD45RO^{hi}$/CD4+ T cells (Figure 2**B**), whereas the expression of these three receptors was not higher on CD4+ T cells expressing CD25 or CD26 compared to the total population of CD4+ T cells. CD69 was only expressed by a negligible fraction of CD4+ T cells from peripheral blood. The expression of CCR5 and CXCR3 was increased on CD4+ T cells expressing human leukocyte antigen (HLA)-DR, but a large proportion of chemokine receptor expressing cells was HLA-DR negative.

Based on these results, we studied the expression of CCR2, CCR5, and CXCR3 in relation to the expression of CD45RO on CD4+ T cells in 31 HCs. The results showed that levels of CCR2, CCR5, and CXCR3 on CD4+ T cells were directly proportional to the percentage of $CD45RO^{hi}$/CD4+ T cells in blood (Figure 2**C**).

The interrelationships of CCR2, CCR5, and CXCR3 on $CD45RO^{hi}$/CD4+ T cells are not explained by coexpression on individual cells

Five-color analysis was performed in four HCs to determine the colocalization of CCR2, CCR5, and CXCR3 on individual CD45RA◯/CD4+ T cells. Although a third of all CD45RA◯/CD4+ cells lacked the expression of any of these three CKRs, the pattern of expression on the remaining cells was heterogeneous (Figure 3). There was a large overlap in the expression of CCR2, CCR5, and CXCR3 on individual CD45RA◯/CD4+ cells, with almost two thirds of all CKR-positive cells expressing more than one receptor. In the CKR-positive population, most cells expressed CXCR3 (mean: 81%). The majority of all CCR2- and CCR5-positive cells were contained

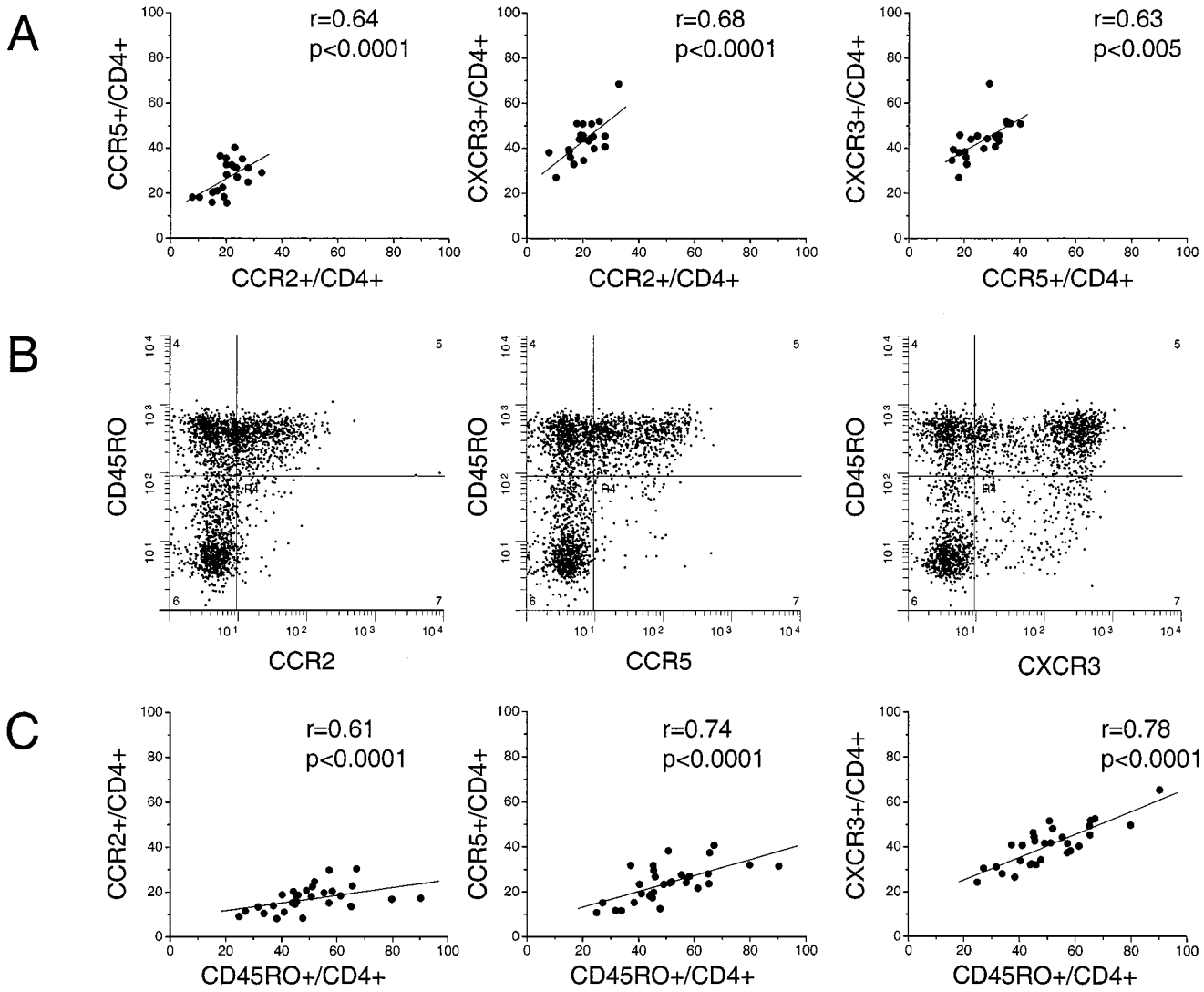

Figure 2 Relationships between individual CKRs, and between CKRs and CD45RO on CD4+ T cells. (**A**) Pearson correlation was used to establish relationships between levels of CCR2, CCR5, and CXCR3 on CD4+ T cells in individual subjects. (**B**) CCR2, CCR5, and CXCR3 are predominantly expressed by $CD45RO^{hi}$/CD4+ T cells. (**C**) Levels of CCR2, CCR5, and CXCR3 on CD4+ T cells were proportional to the percentage of $CD45RO^{hi}$/CD4+ T cells.

within the CXCR3+ population. Approximately 25% of all CCR2- or CCR5-positive cells were, however, CXCR3 negative. The overlap between the expression of CCR2 and CCR5 on CD45RA◯/CD4+ cells was limited, because only 40% of all CD45RA◯/CD4+ cells expressing either CCR2 or CCR5 were positive for both receptors.

Discussion

As blockade of CKR has emerged as a possible pathway for therapeutic intervention in inflammatory diseases (Kivisäkk *et al*, 2001; Ransohoff and Bacon, 2000), information regarding CKR expression patterns on circulating leukocytes is of fundamental importance for designing treatment strategies. Here we used a longitudinal approach to determine the extent of fluctuation over time in the expression of CCR2, CCR5, and CXCR3 on CD4+ T cells from healthy volunteers. In addition, the relationships between the expression of these three receptors, as well as their associations to activation markers, were analyzed *in vivo*.

The results showed that levels of CCR2, CCR5, and CXCR3 expression on CD4+ T cells in healthy subjects were remarkably stable over time, with fluctuations of only a few percentage units over a follow-up of 2 years. Stable levels of CXCR3 and CCR5 expression on CD4+ T cells have previously been demonstrated in a short-term study of six healthy donors analyzed every second day during 1 week (days 1, 3, and 5) plus after 21 days (Campbell *et al*, 2001). In contrast, levels of CCR2, CCR5, and CXCR3 varied

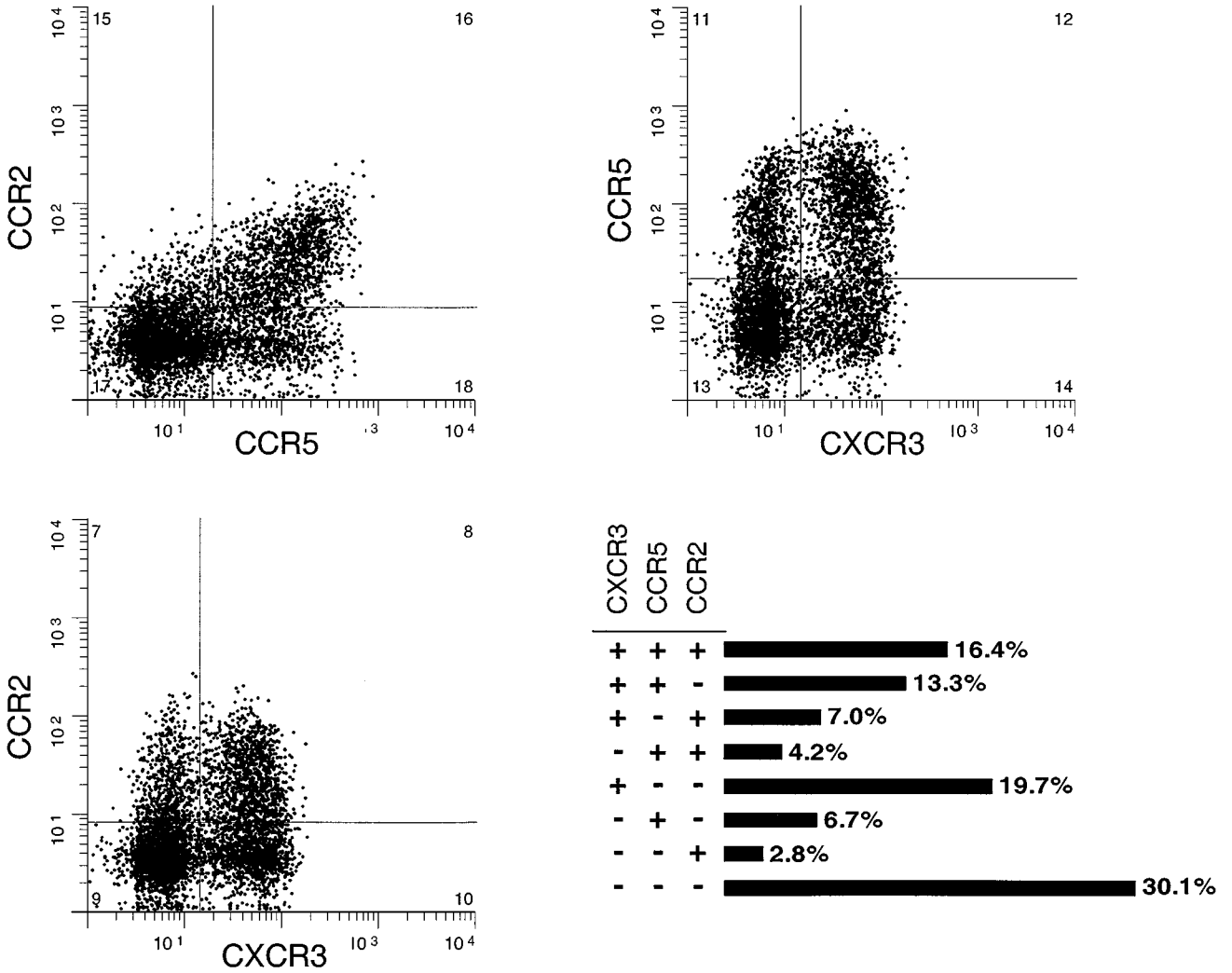

Figure 3 Colocalization of CCR2, CCR5, and CXCR3 on individual CD45RA◯/CD4+ T cells. (Left and right upper panels) Five-color flow cytometry was performed to determine the colocalization of CCR2, CCR5, and CXCR3 on individual CD45RA◯/CD4+ T cells. (Lower right panel) Percent CD45RA◯/CD4+ T cells expressing different combinations of CCR2, CCR5, and CXCR3. Results showed as mean of four experiments.

considerably between individuals. Especially the expression of CCR5 on CD4+ T cells showed a significant variability, with an eightfold difference from lowest to highest levels (total range: 5.0% to 40.2%). We have not analyzed if any of the HCs included in the study carried the CCR5 Δ32 mutation, which encodes a truncated protein not expressed on the cell surface (Liu *et al*, 1996; Wu *et al*, 1997). The lack of individuals completely devoid of CCR5+ T cells argues, however, against the inclusion of a CCR5 Δ32 homozygote. Further, the tight correlations between the expression of CCR5 and CCR2/CXCR3 on CD4+ T cells render it unlikely that CCR5 Δ32 heterozygosity played a determining role in the observations reported here.

Consequently, it seems that the expression of CKRs on circulating CD4+ T cells is a stable characteristic of each individual, set at variable levels between individuals, both at a short- and long-term perspective. This information is of importance when interpreting studies of CKR expression *in vivo*. First, the marked variability between individuals may require large sample sizes to identify differences in CKR expression between different populations. Further, the biological relevance of small differences between two populations of a few percentage units in CKR expression may be questionable. In the light of anticipated treatment trials, it will also be of interest to define whether subjects with different levels of CKR expression will need individually adjusted dosages of CKR antagonists for adequate coverage of targeted receptors. Secondly, the remarkably stable levels of CKR expression within individuals over time suggest that longitudinal approaches may be preferred to identify effects on CKR expression by therapeutic agents, or to define relations between CKR expression and disease activity in various conditions such as MS.

Levels of CCR2+/ and CCR5+/CD4+ T cells were distinctly higher in the present study (19.7% and 24.9%, respectively), compared to several earlier studies, which reported levels not exceeding 5% among healthy donors (Misu *et al*, 2001; Sørensen *et al*, 1999; Sørensen and Sellebjerg, 2001; Wu *et al*, 2000; Zang *et al*, 2000). It is clear that detection of CCR2 and CCR5 on T cells using flow cytometry can be profoundly altered by processing of blood samples. Further, different monoclonal antibodies (mAbs) can also give significantly different results (Lee *et al*, 1999). Because we have noted a selective reduction in surface detection of certain CKRs during isolation of peripheral blood mononuclear cells through centrifugation on Ficoll (Kivisäkk *et al*, 2002a, 2002b), lysed whole blood was used for the stainings in the present study.

We observed that the expression of CCR2, CCR5, and CXCR3 was proportional to the expression of $CD45RO^{hi}$/CD4+ memory T cells in blood, whereas there was no clear association with other markers of acute or chronic activation, including CD25, CD26, CD69, and HLA-DR. Increased percentages of CD45RO+/CD4+ T cells have been reported in some cross-sectional studies of patients with inflammatory diseases, including MS, with higher levels during MS attacks compared to stable disease, (Crucian *et al*, 1995; Porrini *et al*, 1992; Wu *et al*, 2000). Consequently, calculating the percentage of CKR-positive cells of the total population of CD4+ T cells in patients with inflammatory diseases may primarily reflect levels of CD45RO+/CD4+ T cells in these conditions. Indeed, a recent paper demonstrated that methylprednisolone treatment of patients with MS resulted in a significant increase in the percentage of CCR5+/CD4+ T cells in peripheral blood on day 3. This increase was, however, fully explained by a relative increase in numbers of CD45RO+ T cells at this time point (Martinez-Caceres *et al*, 2002). Similarly, although increased levels of CCR5 + CD4+ T cells have been reported repeatedly in MS patients compared to HCs (Misu *et al*, 2001; Sørensen and Sellebjerg, 2001; Zang *et al*, 2000), levels of CCR5+/CD45RO+/CD4+ T cells did not differ between these two groups (Wu *et al*, 2000).

In vitro, the expression of CCR2, CCR5, and CXCR3 is associated with cell activation. As first shown for CCR2 and later extended to CCR5 and CXCR3, long-term cultivation of T cells in the presence of interleukin (IL)-2 results in reversible up-regulation of receptor expression and accentuated ligand-induced T-cell migration (Bleul *et al*, 1997; Frade *et al*, 1997; Loetscher *et al*, 1996a, 1996b, 1998; Qin *et al*, 1998; Wu *et al*, 1997). Further, stimulation through the T-cell receptor using immobilized anti-CD3 antibodies, alone or in combination with soluble anti-CD28 antibodies, rapidly inhibited the expression of CCR2, CCR5, and CXCR3 (Loetscher *et al*, 1996b, 1998; Qin *et al*, 1998; Sallusto *et al*, 1999). In contrast, short-term activation of peripheral blood T cells with OKT3 for 3 days resulted in increased expression of CXCR3 on all T-cell populations, including naïve cells, whereas a moderate increase in CCR2 and CCR5 expression was restricted to memory subsets (Rabin *et al*, 1999). These results suggested that CXCR3 and its ligands may play a selective role in the early stages of T-cell activation, whereas CCR2 and CCR5 functions are more significant for fully differentiated cell populations.

Less is known about the factors that influence the expression of CCR2, CCR5, and CXCR3 *in vivo*. It has been demonstrated that peripheral blood T cells bearing acute activation markers such as CD25 and CD69 are CXCR3+/CCR5◯, whereas T cells that display markers of previous cell activation such as CD26 and CD95 express CCR5 (Bleul *et al*, 1997; Qin *et al*, 1998; Wu *et al*, 1997). Similarly, freshly isolated naïve T cells did not express CCR2 or CCR5, but stained weakly for CXCR3 (Rabin *et al*, 1999), consistent with an association between CXCR3 and early phases of T-cell activation. Using correlation analysis, we detected clear linear relationships between the expression of CCR2, CCR5, and CXCR3 on CD4+ T cells, suggesting that the regulation of these three CKRs in peripheral blood is coordinated in healthy individuals.

Because the focus of this report was to provide a baseline for future studies regarding changes in CKR expression in health and disease, we have not addressed molecular mechanisms behind the coordinated levels of CKR expression. It has, however, been demonstrated that many CKR genes are closely related and may have developed through relatively recent gene duplications. For instance, CCR2 and CCR5 genes are highly homologous and are colocalized in a CKR gene cluster on chromosome 3p21 only 14 kb apart (Dean *et al*, 1996; Samson *et al*, 1996). It is plausible that this close genetic relationship accounts for the observed correlation between levels of CCR2 and CCR5 expression.

Because most T cells express more than one CKR at a defined time point (Kim *et al*, 2001; Kunkel *et al*, 2002; Qin *et al*, 1998), we used five-color flow cytometry to determine if the observed correlations between the expression of CCR2, CCR5, and CXCR3 on CD4+ T cells were due to coexpression on individual cells. Approximately 70% of all CD45RA◯/CD4+ T cells expressed at least one inflammatory CKR. Of these cells, 25% coexpressed all three receptors, whereas 40% expressed only one CKR. CXCR3 was expressed by the largest percentage of cells, with the majority of CCR2+ and CCR5+ cells contained within this population. In contrast to a previous report (Qin *et al*, 1998), we could, however, identify distinct populations of CCR2+/CXCR3◯ and CCR5+/CXCR3◯ cells. Only 40% of all CD45RA◯/CD4+ cells expressing either CCR2 or CCR5 were positive for both receptors, indicating that the observed associations could not fully be explained by coexpression on individual cells.

In conclusion, our results demonstrate that levels of CCR2, CCR5, and CXCR3 on CD4+ T cells were surprisingly stable over a follow-up of 2 years in individual subjects, whereas interindividual variations were large. Consequently, it seems that the expression of CKRs on circulating CD4+ T cells is a stable characteristic of each individual, set at variable levels between individuals. Further, we observed that the expression of CCR2, CCR5, and CXCR3 was proportional to the expression of $CD45RO^{hi}$/CD4+ memory T cells in blood, suggesting that calculating the percentage of CKR-positive cells of the total population of CD4+ T cells in patients with inflammatory diseases may primarily reflect levels of previously activated memory T cells in these conditions. Knowledge regarding baseline CKR expression patterns in healthy individuals is of importance for understanding studies delineating differences in CKR expression among patient populations, and is essential for the optimal design of future therapeutic interventions aimed at manipulating with the chemokine system.

Materials and methods

Study design
Blood samples were obtained from 26 HCs (17 women) who were included as controls in a longitudinal study of brain atrophy in patients with MS. The controls were recruited from the spouses of the patients followed in the study. Exclusion criteria were a history of neurological disease, hypertension requiring chronic therapy, heart disease, chronic pulmonary disease, chronic renal insufficiency, or diabetes. None of the controls had any known autoimmune or inflammatory disease requiring daily medication. Several individuals used medications for allergies or mild asthma. One individual became diabetic during follow-up. Repeated blood sampling was performed after 12 months in 20 of the subjects, and after 48 months in 9 subjects. In addition, blood samples were obtained from 16 healthy volunteers (12 women) recruited from personnel in the lab. None of the volunteers had either acute or chronic disease requiring regular daily medication. The age of the donors was 19 to 56 years (mean 38.2 years). The study was approved by the Institutional Review Board of the Cleveland Clinic Foundation and informed consent was obtained from all subjects.

Immunostaining for detection of chemokine receptor expression
One hundred microliter of heparinized venous blood was blocked with 0.2 mg/ml normal mouse immunoglobulin G (IgG) (Caltag Laboratories, Burlingame, CA) for 15 min at room temperature (RT) to reduce nonspecific FcR binding, and subsequently stained with directly conjugated mAbs for 15 min at RT. After staining, cells were incubated with 2 ml of fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences, San Jose, CA) for 10 min at RT to lyse erythrocytes. Cells were centrifuged, washed twice with ice cold FACS buffer (phosphate-buffered saline [PBS] containing 2% fetal calf serum [FCS] and 0.1% sodium azide), and fixed with 1% paraformaldehyde (PFA) for 5 min.

For studies of colocalization of CKRs on individual cells, peripheral blood was incubated with nonconjugated anti-CKR mAbs, followed by fluorochrome-labeled isotype-specific secondary Abs and subsequent addition of directly conjugated mAbs against cell-surface markers. Each step was incubated for 15 min at RT and followed by two washes with FACS buffer containing 10% FCS. Erythrocyte lysis and fixation was performed after the last staining step. There was no cross-reactivity between the isotype-specific secondary Abs when tested in preliminary experiments.

mAbs used
The mAbs used were CCR2 unlabeled/PE (48607.211) from R&D Systems (Minneapolis, MN); CCR5 unlabeled/PE (2D7), CXCR3 unlabeled/PE (1C6), CD25 PE (MA251), CD45RO FITC/APC (UCHL1), CD69 PE (FN50), HLA-DR FITC (G466) from BD PharMingen (San Diego, CA); CD4 PerCP (SK3), CD8 PerCP (SK1), CD14 PerCP (MøP9), and CD26 PE (L272) from BD Biosciences; CD45RA PETR (2H4) from Beckman Coulter (Fullerton, CA); polyclonal anti-mouse IgG1 fluorescein isothiocyanate (FITC), IgG2a biotin, and IgG2b PE from Southern Biotechnology Associates (Birmingham, AL). Streptavidin PECy7 was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). Isotype-matched control mAbs were purchased from BD Biosciences and Southern Biotechnology.

Flow cytometric analysis
Two-color stainings were acquired on a FACScan flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences). Five-color stainings were acquired on a MoFlo flow cytometer (Cytomation, Fort Collins, CO) and analyzed with WinList software (Verity Software House, Topsham, ME). Cells were gated according to forward and side light-scattering properties, and were positively selected for CD4, CD8, or CD14 expression. Isotype-matched control mAbs were used for defining background fluorescence.

Statistical analysis
Levels of CKR expression on different subsets of T cells and in male and female donors were compared using Student's t test. Correlations among the expression of various CKRs as well as between CKR expression and age of the donors were assessed by Pearson correlation coefficient. Reported P values are two-tailed and considered statistically significant at $P < .05$.

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