**Context:** T-lymphocyte migration through the blood-brain barrier is a central event in the process of lesion formation in multiple sclerosis (MS).

**Objectives:** To assess the ability of lymphocytes derived from the peripheral blood of patients with clinically active and inactive MS to migrate across an artificial model of the blood-brain barrier and to elucidate the molecular mechanisms involved in such a process.

**Design:** We developed an in vitro model of lymphocyte migration using a Boyden chamber coated with a monolayer of human brain microvascular endothelial cells.

**Results:** The rates of migration of lymphocytes obtained from patients with acutely relapsing and active secondary progressive MS was significantly increased compared with those obtained from healthy controls and patients with inactive secondary progressive disease. Ribonuclease protection assays and enzyme-linked immunosorbent assays indicated that monocyte chemoattractant protein 1 and interleukin 8 were the major chemokines produced by brain endothelial cells grown under the culture conditions used for the migration assays. The rate of migration of the MS lymphocytes could be inhibited by 60% with an antimonocyte chemoattractant protein 1 monoclonal antibody, indicating a functional role for this chemokine in the migration process. In agreement with previous reports, we found that the tissue inhibitor of metalloproteinase 1, a matrix metalloproteinase inhibitor, also reduced migration of MS lymphocytes by 50%.

**Conclusions:** The results demonstrate an increased migration rate of MS T lymphocytes across the brain endothelium barrier and that such migration is dependent on chemokine monocyte chemoattractant protein 1 and on matrix metalloproteinases.

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**MULTIPLE SCLEROSIS (MS)** is a disease of the central nervous system characterized by multifocal infiltration of autoreactive T lymphocytes from the systemic immune system across the blood-brain barrier. Brain endothelial cells (ECs) are the first cells of the blood-brain barrier encountered by such leukocytes and are thought to restrict immune cell access to the central nervous system. The sequence of cellular events underlying migration include the processes of lymphocyte-EC adhesion, chemotaxis, and proteolysis of the compact basal membrane surrounding the blood-brain barrier by matrix metalloproteinases (MMP).

In previous studies, we used a Boyden chamber coated with fibronectin as an in vitro model of leukocyte migration. This assay primarily models the interaction of lymphocytes with the extracellular matrix proteins. We found that lymphocytes derived from patients with relapsing-remitting (RR) disease who were in remission or in acute relapse (AR), as well as patients with secondary progressive (SP) disease, migrated faster than did cells from healthy donors, and that treatment with interferon (IFN) β or glatiramer acetate inhibited migration. The tissue inhibitor of metalloproteinase (TIMP)-1, an MMP-9 inhibitor, was a partial but significant antagonist of lymphocyte migration in this assay, suggesting that this enhanced migration of MS T lymphocytes across fibronectin was dependent on the up-regulation of MMP-9 in MS. Several investigators have used human umbilical vein ECs or peripheral organ ECs to study lymphocyte interaction and migration across an EC barrier. Techniques have recently been developed to isolate and use human brain–derived microvascular ECs (HBECs), providing a model that more closely reflects lymphocyte-EC interactions and lymphocyte migration across the human blood-brain barrier. We have used HBECs grown on fibronectin-coated membranes of Boyden chambers for our migration studies.
PATIENTS AND METHODS

PATIENTS

The ex vivo lymphocyte studies were conducted on groups of patients with clinically confirmed MS31 presented in the Table. Patients were selected from the databases of our MS clinics (Montréal Neurological Hospital, Montréal, Québec, and Notre-Dame Hospital, Montreal) based on their clinical profiles and having been classified as having RR or SP MS disease with or without intermixed relapses. Patients with RR MS were selected on the basis of disease activity: patients with new onset of neurological symptoms (consistent with a new relapse) and an increase in the Expanded Disability Status Scale score of at least 1.0 were classified having AR MS (n=5). Patients with RR MS with no new neurological symptoms for at least 6 months were classified as having remitting MS (n=7). Patients with SP MS were further selected on the basis of disease activity as determined by changes in disability scores (>0.5) or the occurrence of relapses in the previous 18 months (Table). Patients who showed either no signs of progression or no relapse during this period were considered to have clinically inactive disease. The active SP group was younger than the inactive SP group (51±8 and 56±3 years, respectively) and had a shorter disease duration (10.5±2.5 and 17±3.8 years, respectively). The RR MS group was younger (31±3 years) and had a shorter disease duration (5.6±1.2 years) than both SP MS groups. The control group included healthy donors, patients with epilepsy, and individuals affected with the influenza virus; the ages of these groups were 29±4, 32±4, and 32±3 years, respectively. None of the patients used in this study were receiving immunosuppressive/ modulatory therapy. For patients with AR and patients affected with the influenza virus, blood was drawn at the time of symptoms.

The process of chemoattraction to the human central nervous system is dependent on interactions between chemokines produced by brain ECs or by central nervous system parenchymal cells and chemokine receptors on the surface of lymphocytes. Monocyte chemoattractant protein (MCP) 1 is a C-C chemokine that has preferential affinity for the chemokine receptors CCR2 and CCR4.20,21 Monocyte chemoattractant protein 1 has been shown to increase transendothelial migration of α/β (CD45 and CD8) and γ/δ lymphocytes in chemotaxis assays using human umbilical vein ECs.3,9,22,23 Immuno- histochemical examination of central nervous system tissue derived from patients with MS and from animals with experimental allergic encephalomyelitis (EAE) has demonstrated increased expression of MCP-1 as well as macrophage inflammatory protein (MIP) 1α, interferon γ inducible protein 10 (IP-10), and RANTES in astrocytes and perivascular cells located in and around inflammatory plaques.24–28 Karpus and Kennedy29 showed that blocking MCP-1 could reduce the severity of relapsing EAE. Recently, a sequence polymorphism identified in the gene encoding MCP-1 was proposed as a candidate for the locus EAE7, a locus known to control susceptibility to monophasic EAE in the mouse,30 suggesting a causative role for MCP-1 in EAE.

We report that migration rates of lymphocytes derived from patients with RR and SP MS with active disease through HBEC monolayers are increased compared with patients with inactive disease and controls that include healthy donors, patients with epilepsy, and individuals with acute systemic viral infections. This increased migration could be inhibited, at least partially, by a blocking antibody directed at MCP-1, one of the major chemokines expressed by HBECs. Migration was also partially inhibited by the MMP inhibitor TIMP-1.

ISOLATION AND CHARACTERIZATION OF LYMPHOCYTES

Mononuclear cells were isolated from peripheral blood samples from patients or controls using a Ficoll density gradient (Amersham-Pharmacia-Biotech, Bâle D’Urfée, Quebec). Mononuclear cells were depleted of monocytes by a 1-hour culture at 37°C in an Rosewell Park Memorial Institute medium plus 10% fetal bovine serum in a 75-cm² plastic dish as previously described.31 Anti-CD3, -CD4, -CD8, -CD14, -CD56, -CD45RO, -CD45RA antibody staining and fluorescent-activated cell sorter analysis was done to evaluate the lymphocyte populations used in the migration assay.

ADHESION MOLECULES

Lymphocytes were also assessed for expression of adhesion molecules leukocyte function antigen 1 (LFA-1) and very late antigen 4 (VLA-4). Anti-LFA-1 and anti-VLA-4 monoclonal antibodies conjugated with fluorescein isothiocyanate or immunoglobulin (Ig) G1 isotype were added to 1 × 10^6 lymphocytes/50 μL for 30 minutes at 4°C. Cells were washed twice in phosphate-buffered isotonic sodium chloride solution/FCS 2% and fluorescence intensity was acquired on a fluorescein-activated cell sorter machine (Becton Dickinson, Mississauga, Ontario) and analyzed by a WinMDI program (Scripps, La Jolla, Calif).

EC ISOLATION AND CULTURE

Human brain–derived microvascular ECs were isolated and cultured as previously described31,3,31,3 from temporal lobe specimens resected from young adults undergoing epilepsy surgery. Tissue was minced and homogenized in phosphate-buffered isotonic sodium chloride solution, filtered through a 350-µm Nitex mesh, and then twice through a 112-µm mesh. Filtrate was collected, spun down, and digested for 10 minutes with collagenase type IV at 37°C.

RESULTS

MS-DERIVED T-LYMPHOCYTE MIGRATION ACROSS THE HBEC MONOLAYER

Migratory behavior of T lymphocytes obtained from patients with MS and control donors was assessed in a Boyden chamber coated with HBECs grown under basal culture conditions. As shown in Figure 1, T cells obtained from AR, RR, and active SP MS groups had significantly
Cells were collected by centrifugation and seeded on a 0.5% gelatin-coated flask, in M199 (GIBCO-BRL, Burlington, Ontario) supplemented with 10% FCS, 20% human normal serum, insulin-transferin-selenium, and EC growth supplement (all from Sigma-Aldrich, Oakville, Ontario). On day 10, visible colonies were collected and expanded in fresh media. As previously demonstrated, these cells express factor VIII–related antigen, Ulex Agglutinin European 1 binding sites, and HT-7 antigen. Immuno-reactivity for α-myosin and glial fibrillary acidic protein could not be detected on the HBECs, suggesting the absence of contaminating astrocytes and smooth muscle cells. We estimated the percentage of contaminating cells in our cultures to be less than 1% after 3 passages.

TRANSENDOTHelial MIGRATION

All migration assays were conducted in Boyden chambers (3-µm pore-size membranes) precoated with fibronectin (Collaborative Biomedical Products, Bedford, Mass). Human brain–derived microvascular ECs (20000 cells) were seeded on the membrane 3 days prior to the migration experiment in EC culture media. On the day of the experiment, permeability of the monolayer was confirmed by adding fluorescein isocyanate–labelled albumin to the upper chamber and monitoring levels of fluorescence in the lower chamber. Chambers that allowed more than 5% of albumine diffusion after 6 hours were discarded. In uncoated chambers, levels of fluorescence equilibrated between compartments within 30 minutes. For migration, 10⁶ lymphocytes suspended in 1 mL of RPMI plus 2.5% FBS were added to the top chamber. After 18 hours at 37°C, the contents of the bottom chamber were collected and the number of cells present were determined by counting aliquots under the microscope. In some migration experiments, neutralizing monoclonal antibody (5 µg/mL), anti–IL-8 neutralizing antibody (5 µg/mL), and IgG1 isotype control were added to the upper chamber 10 minutes prior to the T cells. A similar procedure was used with TIMP-1 (15 ng/mL).

RIBONUCLEASE PROTECTION ASSAY AND ENZYME-LINKED IMMUNOSORBENT ASSAY FOR CHEMOKINE AND CHEMOKINE RECEPTORS

To detect chemokines produced by HBECs, ribonuclease (RNASE) protection assays and enzyme-linked immunosorbent assays (ELISAs) were performed using confluent monolayers of cells grown in 75-cm² flasks. Cells were either treated or not treated with IFN-γ (100 U/mL) and tumor necrosis factor (TNF-α) (100 U/mL) for 24 hours. RNA from HBEC monolayers was collected in Trizol (GIBCO-BRL; Invitrogen Life Technologies, Carlsbad, Calif) and 8 µg of total RNA was used for the chemokine–ribonuclease protection assay (kit hCR-5; Pharmingen, Mississauga, Ontario) as described in the manufacturer’s instructions. Culture supernatants were collected and assayed for the presence of IL-8, RANTES, IP-10, and MCP-1 by ELISA as described in the manufacturer’s instructions. Expression of chemokine receptors by T lymphocytes derived from patients with MS or control donors was also assessed by ribonuclease protection assay. RNA from T lymphocytes was collected in Trizol (GIBCO-BRL) and 10 µg of total RNA was used for the chemokine–ribonuclease protection assay (kit hCR-5, 6 and 8; Pharmingen) as described in the manufacturer’s instructions.

STATISTICS

Data are given as means±SEM unless otherwise indicated. For each donor, lymphocyte migration was performed in duplicate. Results are presented as column scatter plots, with the mean for each subgroup. Chemokine determination by ELISA was done in duplicate in 3 individual experiments using 3 different HBEC primary cell strains. Statistical comparison between the groups was performed using an analysis of variance test and a Bonferroni post-test.

higher migration rates compared with cells from healthy controls or patients with epilepsy (P<.05, P<.01, respectively). The migration rates of the active SP MS group were also significantly increased compared with the inactive SP MS group (P<.05). Donors affected with the influenza virus did not show an increased migration rate compared with healthy donors and patients with epilepsy.

Anti-CD3, -CD4, -CD8, -CD14, -CD56, -CD45RO, and -CD45RA antibody staining and fluorescent-activated cell sorter analysis revealed that 93% of the leukocytes used in the migration assays were CD3+, 61% were CD4+, 29% were CD8+, less than 3% were CD14+, and less than 3% were CD56+. We could not observe a difference in CD45RA/R0 staining between the control and each MS group (controls: CD45RA+49%±6%, CD45RO+42%±8%; overall MS groups: CD45RA+56%±8%, CD45RO+42%±4%).

ADHESION MOLECULE EXPRESSION ON LYMPHOCYTES

We analyzed the expression of VLA-4 and LFA-1 on MS and control T cells before migration, using immunostaining and flow cytometry. We found no difference in the proportion of CD3+ cells expressing these antigens when cells obtained from patients with AR, RR, and SP MS were compared with control T cells (LFA-1: 81%±2%, 84%±2%, 83%±3%, and 82%±2%, respectively; VLA-4: 77%±7%, 79%±6%, 77%±4%, and 79%±8%, respectively).
Characteristics and Demographics of Patients Enrolled in the Study

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Patients</th>
<th>Age, Mean (Range), y</th>
<th>Sex Ratio, F/M, Range</th>
<th>Disease Duration, Mean (Range), y</th>
<th>EDSS, Mean (Range)</th>
<th>Δ EDSS Score, Mean (Range) During 18 mo</th>
<th>Relapses, Mean (Range) During 18 mo</th>
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</thead>
<tbody>
<tr>
<td>MS RR</td>
<td>7</td>
<td>31 (28-36)</td>
<td>4/3</td>
<td>5.6 (4.5-6.6)</td>
<td>2.3 (0-3.5)</td>
<td>0.7 (0-1.5)</td>
<td>1.5 (0-3)</td>
</tr>
<tr>
<td>MS AR</td>
<td>5</td>
<td>32 (26-36)</td>
<td>3/2</td>
<td>4.1 (2.0-6.1)</td>
<td>4 (3-5)</td>
<td>2.5 (1.5-4.0)</td>
<td>2.0 (1-4)</td>
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<tr>
<td>MS SP inactive</td>
<td>6</td>
<td>56 (48-59)</td>
<td>2/1</td>
<td>17 (10.9-25.7)</td>
<td>5.9 (4-6.5)</td>
<td>0.2 (0-0.5)</td>
<td>0</td>
</tr>
<tr>
<td>MS SP active</td>
<td>8</td>
<td>51 (28-61)</td>
<td>3/5</td>
<td>10.5 (8.5-15)</td>
<td>4.9 (3.5-5.5)</td>
<td>1.4 (0.5-2)</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>Controls</td>
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<td>29 (22-50)</td>
<td>3/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Epilepsy</td>
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<td>2/5</td>
<td>14.1 (2-21)</td>
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<tr>
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<td>32 (28-34)</td>
<td>1/1</td>
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</table>

*EDSS indicates Expanded Disability Status Scale; MS, multiple sclerosis; RR, relapsing-remitting; AR, acutely relapsing; SP, secondary progressive; and ellipses, no data.

Figure 1. Lymphocyte migration across human brain endothelial cells. Ex vivo lymphocytes derived from the peripheral blood of healthy controls (n=12), patients with epilepsy (n=7), and controls affected by influenza virus (n=4) or relapsing-remitting (RR) (n=7), acutely relapsing (AR) (n=5), secondary progressive (SP) (n=8), and inactive SP (n=6) patients with multiple sclerosis were subjected to migration for 18 hours in a Boyden chamber coated with a human brain endothelial cell monolayer. Asterisk indicates AR and RR compared with controls, P<.05; dagger, active SP compared with controls, P<.01; and double dagger, inactive SP compared with active SP, P<.05.

ANTIMIGRATORY EFFECTS OF ANTI–MCP-1 NEUTRALIZING ANTIBODY AND TIMP-1

To evaluate the contribution of MCP-1, IL-8, and MMPs in the transendothelial migration of healthy control- and MS-derived lymphocytes, we added anti–MCP-1 or anti–IL-8 neutralizing antibody (5 µg/mL), or TIMP-1 (15 ng/mL) to our assay system. As shown in Figure 4A-C, the addition of either anti–MCP-1 neutralizing monoclonal antibody (5 µg/mL) or TIMP-1 (15 ng/mL) significantly inhibited the migration of lymphocytes derived from patients with both active and inactive SP MS as well as those obtained from patients with AR MS (either treatment compared with untreated cells, P<.05). Anti–MCP-1 antibody and TIMP-1 treatment also reduced healthy control T-cell migration (Figure 4A and B), although the data did not reach statistical significance (anti–MCP-1 compared with untreated cells, P> .30; TIMP-1 compared with untreated cells, P> .20). Treatment with the appropriate isotype control (IgG1) or with anti–IL-8 antibody did not affect healthy control- or MS-derived lymphocyte migration in this assay.

CHEMOKINE RECEPTOR EXPRESSION BY MS LYMPHOCYTES

To evaluate the expression of specific chemokine receptors for IL-8 and MCP-1 by lymphocytes obtained from patients with MS and healthy donors, we performed ribonuclease protection assay analysis using RNA samples extracted from lymphocytes from each of these donor groups. As shown in Figure 3, lymphocytes did not express mRNA for either the IL-8 chemokine receptors CXCR1 and CXCR2, or the MCP-1 receptor CCR2A and CCR2B. We could not detect differences in the mRNA expression of the MCP-1 receptor CCR4 between patients with MS and controls. While CXCR4 mRNA was equally expressed in healthy donors and MS-derived lymphocytes, levels of CXCR3 mRNA were higher in MS-derived samples, as previously reported.33 Messenger RNA levels of the orphan chemokine receptor GPR15 were also elevated in MS lymphocytes compared with controls.

COMMENT

We have previously studied lymphocyte migration in patients with MS, using a Boyden chamber in which fibronectin was the only barrier to migrating cells. We have shown that lymphocytes derived from patients with MS with relapsing and actively progressive disease migrated faster than healthy control cells.1 This increased migration of MS lymphocytes was found to be inhibited by the pretreatment of cells with IFN-β and TIMP-1, an inhibitor of MMPs.3,4

In the present study, we have used HBECs grown on Boyden chamber membranes to evaluate transendothelial lymphocyte migration in MS. We used lymphocytes obtained from patients with active MS, either RR or SP, and from patients with inactive MS. These groups were defined based on data recorded at our MS clinics on relapses and disability scores (EDSS) during a 2-year period. Results presented here show that T cells obtained from patients with RR and active SP MS migrated faster through HBECs than cells derived from controls or patients with inactive MS. We have previously shown
that patients with active and inactive SP MS differ in another in vitro immune assay, namely, functional suppressor activity assay. Using our HBEC-based migration assay, we also demonstrated that the migration rate of lymphocytes through brain endothelium is not solely dependent on the activation state of the peripheral immune system since cells obtained from donors affected with the influenza virus migrated at the same rate as those obtained from healthy donors. This observation does not, however, allow us to conclude that our findings are specific for MS since other organ-specific inflammatory diseases were not included.

The role of adhesion molecules, such as VLA-4 and LFA-1 ligation to vascular cell adhesion molecule and intercellular adhesion molecule, is known to be critical in leukocyte adhesion and migration through the endothelium. We did not detect differences in expression of VLA-4 and LFA-1 on lymphocytes between lymphocytes of patients with MS and those of control donors, consistent with previous reports. We could also not correlate differences in rates of migration in our assay with levels of VLA-4 or LFA-1 expressed on lymphocytes.

To evaluate the potential contribution of chemokines produced by HBECs to the lymphocyte migration process, we determined which of these molecules were expressed by HBECs under the culture conditions in which we performed our migration assay. Our findings demonstrate that HBECs grown under these basal culture conditions produce IL-8 and MCP-1. When exposed to IFN-γ and TNF-α, HBECs also up-regulate additional chemokines, IP-10 and RANTES. While IL-8 is known for its ability to attract primarily neutrophils, MCP-1 is a monocyte-lymphocyte chemoattractant molecule. Monocyte chemoattractant protein 1 immunoreactivity has been demonstrated in reactive astrocytes and macrophages in and around inflammatory plaques in MS and EAE. Monocyte chemoattractant protein 1 immunoreactivity can be detected in the brain and specifically in brain ECs in EAE at the onset of inflammation and prior to clinical expression of the disease. Anti-MCP-1 antibodies can reduce the severity of relapsing EAE. Furthermore, CCR2 (MCP-1 receptor) knock-out mice did not develop clinical or pathologic features of EAE. In our migration assay, the addition of anti-MCP-1 neutralizing antibody but not anti-IL-8, significantly reduced MS lymphocyte migration. Anti-MCP-1 antibodies have also been shown to inhibit monocyte migration across human umbilical vein ECs. Our finding that TIMP-1 inhibits MS lymphocyte migration supports previous reports showing that MMP inhibitors can reduce lesion formation and clinical scores in EAE.

To evaluate the chemokine receptor by which MCP-1 could modulate lymphocyte migration across HBECs, we...
performed ribonuclease protection analysis of chemokine receptor mRNA expression by lymphocytes obtained from controls and patients with MS. CCR2 mRNA and CCR4 mRNA are the chemokine receptors that are established to bind to MCP-1. CCR2 mRNA was not detected in MS and control donor lymphocytes. CCR4 mRNA was detected at comparable levels. We showed an up-regulation of the recently identified GPR15 receptor in MS-derived lymphocytes. Since the chemotactic ligands for GPR molecules (orphan receptors) have not yet been identified, it is possible that MCP-1 exerts its chemotactic activity through such a receptor. This hypothesis remains to be tested. We also confirmed a previous report showing increased expression of the IP-10 receptor CXCR3 on T lymphocytes in MS.33 In our hands, lymphocytes derived either from patients with MS or healthy controls did not express mRNA for CXCR1 and CXCR2, the known receptors for IL-8.

In this study, we present functional data that show increased migration of T lymphocytes derived from patients with active MS across a human brain-endothelium barrier. The migration of MS-derived T cells could not be attributed to a differential expression of VLA-4 and LFA-1 on the surface of MS T lymphocytes. We showed that brain ECs grown under basal culture conditions used to assess migration produce the chemokine MCP-1 and that MCP-1 chemoattraction contributed to the migratory process. Additional experiments will be needed to define the migratory behavior of MS T cells across activated HBECS and the role of IP-10 and RANTES in such a process.

Figure 3. Chemokine receptor expression by multiple sclerosis (MS) lymphocytes. Ribonucllease protection assay (10 µg of total RNA per lane) revealed that while CCR4 messenger RNA (mRNA) is expressed at similar levels by MS- and control-derived lymphocytes, CCR2α+b, CXCR1, and CXCR2 mRNA are not expressed. Both CXCR3 and GPR15 mRNA were found to be elevated in acutely relapsing (AR) and secondary progressive (SP) MS-derived lymphocytes and low in healthy donor lymphocytes. These gels are representative of 3 independent experiments using lymphocytes derived from 12 patients with MS and 10 healthy controls. GAPDH indicates chloroplast glyceraldehyde-3-phosphate dehydrogenase.

Figure 4. Antimigratory effects of anti–monocyte chemoattractant protein 1 (MCP-1) antibodies and tissue inhibitor of metalloproteinase (TIMP-1). A, Ex vivo peripheral blood lymphocytes from patients with inactive secondary progressive (SP) multiple sclerosis (MS) (n=5) as well as from control donors (n=4) were subjected to migration for 18 hours in a Boyden chamber coated with a human brain endothelial cell monolayer in the presence or absence of antihuman–MCP-1 neutralizing antibody (5 µg/mL) or TIMP-1 (15 ng/mL). B and C, Identical experiments were conducted using T lymphocytes obtained from patients with active SP (n=5) and acutely relapsing (AR), (n=4) MS and compared with independent groups of healthy donors. IL indicates interleukin; asterisk, anti–MCP-1 and TIMP-1 compared with untreated cells; P<.05. Horizontal bars represent the mean of number of cells recovered from the lower chamber.

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