Distinct Properties of Circulating CD8+ T Cells in FTY720-Treated Patients With Multiple Sclerosis

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Objective: To define the capacity of peripheral blood CD8+ T cells from patients with multiple sclerosis (MS) receiving fingolimod (FTY720) to migrate in response to chemokines that contribute to trafficking into the central nervous system.

Results: In vitro addition of active (phosphorylated) FTY720 increased migration of CD8+ T cells from untreated patients to CXCL12 and CCL2. The CD8+ or CD8+ CCR7+ T cells from MS-FTY patients migrated less to CXCL12 and CCL2 compared with those from untreated donors. The proportion of CD8+ CCR7+ cells that express the CCL2 chemokine receptor, CCR2, was significantly reduced in the MS-FTY group. The CD8+ CCR7+ cells from the MS-FTY patients were enriched with CD27−CD28− (late effector) memory cells, a population with reduced expression of CCR2 compared with early (CD27+CD28+) effector memory cells.

Conclusions: Therapy with FTY720 results in a subset of CD8+ T cells with distinct functional migratory properties dominating the peripheral circulation. The expected forthcoming use of FTY720 as a sustained therapy for MS will clarify how this redistribution of lymphocyte populations affects the overall process of immune surveillance.

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Fingolimod (FTY720) is a sphingosine 1–phosphate receptor agonist with demonstrated efficacy in reducing inflammatory events in the central nervous system in multiple sclerosis (MS).1 Patients who receive this therapy demonstrate marked lymphopenia in the peripheral blood that reflects redistribution rather than destruction of this cell population.2 Such redistribution has been attributed to restricted reentry into the peripheral blood compartment of those lymphocyte populations that circulate through secondary lymphoid organs. These populations include naive and central memory (CCR7+) T cells and most B cells. The rapid onset (within days) of lymphopenia likely reflects that initial exposure to the agent also enhances initial lymphocyte trafficking into secondary lymphoid organs by sensitizing cells to chemokine cues.3 The central aim of this study was to define the properties of those lymphocyte populations that persist in the peripheral circulation in patients receiving long-term FTY720 therapy as a means to further our understanding of how this therapy affects overall immune surveillance capacity. Our previous studies have confirmed that CD8+ CCR7+ cells are the predominant population of T cells found in the circulation of FTY720-treated patients with MS (MS-FTY).4 The CD8+ CCR7+ effector memory cells can be further subdivided into early (CD27+CD28+), intermediate, and late (CD27−CD28−) subtypes based on expression of CD27 and CD28 surface molecules.5 As effector memory T cells progress along the differentiation pathway, they sequentially upregulate cytotoxic factors.6 The late effector memory cell population includes the CD45RA+ effector memory T cell population, a population previously noted to be increased in MS-FTY patients when compared with untreated individuals. Examination of cerebrospinal fluid from patients with relapsing-remitting MS has shown that there is relative enrichment of early rather than late CD8+ T cells in the

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cerebrospinal fluid compared with peripheral blood, suggesting that the former have a selective migratory advantage.7 In this study, we demonstrate that there is a relative increase in late effector memory cells (CD27+ CD28−) and a decrease in early effector memory cells (CD27+ CD28+) within the overall CD8+ CCR7+ population of MS-FTY patients, linking this finding to differences in the capacity of early and late effector memory cells to migrate to chemokine gradients (CXCL12 or CCL2) that regulate lymphocyte entry into tissue including the central nervous system compartment. Our results highlight how the selective migration patterns of lymphocyte populations in MS-FTY patients result in a functionally distinct subset of lymphocytes that compose only a minority population in untreated individuals.

**METHODS**

**DONORS**

Peripheral venous blood was obtained with informed consent from an untreated donor group and patients with MS receiving oral FTY720 (MS-FTY patients). The untreated group comprised untreated healthy donors or untreated patients with MS; no differences between these donors were observed in this series. The MS-FTY patients were part of extension phases of phase 2 and 3 clinical trials for relapsing-remitting MS and were treated with fingolimod (FTY720; Novartis Pharmaceuticals Corp, East Hanover, New Jersey), 1.25 mg once daily. The number of patients used for each experiment is indicated in individual figure legends. Patients used in the functional migration studies had been in the extension phase of FTY720 clinical trials for a mean of 6 years; those used for phenotyping studies included individuals receiving extension-phase therapy for 18 months. As no differences were noted, the data from the 2 cohorts were combined. All patients had Expanded Disability Status Scale scores less than 2.0. This study was approved by the institutional review board of McGill University, Montreal, Quebec, Canada.

**CELL ISOLATION**

Peripheral blood mononuclear cells were isolated from venous blood samples using a Ficoll density gradient (GE Healthcare, Baie d’Urfe, Quebec). Total CD8+ T lymphocytes were isolated by positive magnetic bead–assisted cell separation as per the manufacturer’s instructions (Miltenyi Biotec, Auburn, California). All experiments were performed in RPMI (Roswell Park Memorial Institute) media containing 2.5% fetal calf serum with antibiotics and glutamine (all from Invitrogen Corp, Carlsbad, California). Active (phosphorylated) FTY720 (FTY720P) was provided by Novartis Pharmaceuticals Corp, and the vehicle control consisted of dimethyl sulfoxide containing 50mM hydrochloric acid.

**MIGRATION ASSAY**

The CD8+ T cells were pretreated for 30 minutes in vitro with either vehicle or FTY720P (1µM) and were seeded at 250 000/well on top of 3-µm ChemoTx migration chambers (Neuro Probe, Inc, Gaithersburg, Maryland). Wells below the filter were filled with either RPMI media (with 2.5% fetal calf serum) alone or media with chemokine. The concentration of CCL2 (monocyte chemotactic protein–1) (PeproTech, Inc, Rocky Hill, New Jersey) was 0.25 µg/mL, and the concentration of CXCL12 (stromal cell–derived factor 1α) (Sigma-Aldrich Co, St Louis, Mis-

**RESULTS**

Peripheral blood mononuclear cells were analyzed for expression of surface markers using flow cytometry according to standard procedures for staining. The following antihuman antibodies were used in various combinations: CD8–fluorescein isothiocyanate, CCR7–phycoerythrin or CCR7–Alexa Fluor 647, CD27–fluorescein isothiocyanate, CD28–peridinin chlorophyll protein or CD28–phycoerythrin (all from BD Biosciences, Minneapolis, Minnesota), or the appropriate isotype control. After staining, cells were washed twice and fixed in 1% formaldehyde containing phosphate-buffered saline. Results were analyzed with FlowJo software (Tree Star, Inc, Ashland, Oregon).

**FLOW CYTOMETRY**

Peripheral blood mononuclear cells were isolated from either MS-FTY patients or untreated donors and were treated in vitro for 4 hours with a combination of phorbol-12-myristate-13-acetate (20 ng/mL), ionomycin (1 µg/mL), and Brefeldin A (5 µg/mL) (Sigma-Aldrich Co). Cells were first surface stained with anti-CD8 and anti-CCR7 antibodies. Intracellular cytokine staining for interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (BD Biosciences) was then performed following paraformaldehyde and saponin fixation and permeabilization.

**CYTOKINE PRODUCTION BY INTRACELLULAR CYTOKINE STAINING**

Peripheral blood mononuclear cells were isolated from either MS-FTY patients or untreated donors and were treated in vitro for 30 minutes prior to migration either toward media alone or to chemokines CXCL12 or CCL2. A 1µM concentration of FTY720P was chosen as the dose for treatment in comparative studies as up to micromolar concentrations of FTY720 have been measured in the sera of FTY720-treated patients.8 Based on initial dose-response studies, we elected to use CXCL12 at a concentration of 10 ng/mL and CCL2 at a concentration of 1 µg/mL. After in vitro FTY720P exposure, total CD8+ T cells from untreated donors migrated toward CXCL12 to a greater degree than vehicle-treated CD8+ T cells (mean [SEM], 7138 [527] vs 5194 [514] cells, respectively; P < .001) (Figure 1A). In vitro FTY720P-

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We first compared migration of the total CD8\(^+\) T cells to CXCL12 and CCL2 chemokines. The CD8\(^+\) T cells isolated from untreated donors were treated with vehicle or 1\(\mu\)M FTY720P before migration to CXCL12 (10 ng/mL; n=8) (A) or CCL2 (0.25 \(\mu\)g/mL; n=5) (B) chemokine. Results are reported as total number of cells migrated as quantified by flow cytometry. The spontaneous migration rate for each group reflects the vehicle-treated cells that migrated in the absence of chemokine. In vitro migration results represent mean (SEM) values.

As expected, CD8\(^+\) T cells isolated from untreated donors were treated with vehicle or 1\(\mu\)M FTY720P before migration to CXCL12 (n=8 untreated donors, n=4 MS-FTY patients) (A) or CCL2 (n=5 untreated donors, n=3 MS-FTY patients) (B) chemokine. Fold migration was calculated as the number of cells migrating to the chemokine divided by the spontaneous migration rate of each group in the absence of chemokine. *P<.001. †P<.05.

MIGRATION OF CD8\(^+\) T CELLS FROM MS-FTY PATIENTS TO CXCL12 AND CCL2

We first compared migration of the total CD8\(^+\) T-cell population isolated from either MS-FTY patients or untreated donors toward CXCL12 and CCL2 chemokines. As shown in Figure 2A and B, CD8\(^+\) T cells isolated from MS-FTY patients exhibited significantly less migration to CXCL12 and CCL2 compared with CD8\(^+\) T cells isolated from untreated donors (P<.001).

As expected, CD8\(^+\) T cells from MS-FTY patients were mostly CCR7\(^-\) (mean [SEM], 98.2% [0.8%]) as compared with those from the untreated donors (mean [SEM], 63.9% [4.9%]) (P=.001) (Figure 2C). We directly compared the migratory capacity of CD8\(^+\) CCR7\(^-\) cells from these groups in the presence of either CXCL12 or CCL2 (Figure 2D and E). We identified CD8\(^+\) CCR7\(^-\) T cells within the total migrated CD8\(^+\) T-cell population by use of CCR7 immunofluorescent staining. The CD8\(^+\) CCR7\(^-\) T cells from MS-FTY patients displayed significantly less migration to CXCL12 (mean [SEM], 1.69 [0.20] cells) compared with those from untreated donors (mean [SEM], 3.46 [0.43] cells) (P=.01). Similarly, cells from MS-FTY patients displayed significantly less migration to CCL2 (mean [SEM], 1.09 [0.04] cells) compared with cells from untreated donors (mean [SEM], 1.31 [0.07] cells) (P=.02).
To address whether the mechanism for the altered chemokine-directed migratory capacity of CD8+ T cells in MS-FTY patients could be attributed to expression of chemokine receptors, we performed immunostaining using combinations of antibodies for the CCL2 chemokine receptor CCR2 and CD8+ T-cell subsets. As shown for representative donors in Figure 3A and for the overall groups in Figure 3B, CCR2 expression within the overall CD8+ CCR7− effector memory T-cell population was significantly less for the MS-FTY patients (mean [SEM], 9.0% [2.7%]; n=8) compared with untreated donors (mean [SEM], 17.5% [1.8%]; n=12) (P=0.03). We further analyzed the CD8+ population with regard to early (CD27+ CD28+), intermediate (CD27− CD28+), and late (CD27− CD28−) effector memory T-cell subsets. The relative proportions of these subsets are depicted for a representative untreated donor and an MS-FTY patient in Figure 3C. Overall, MS-FTY patients showed a relative decrease in the proportion of early (CD27+ CD28+) effector memory CD8+ T cells, with a mean (SEM) of 10.3% (5.3%) of their CD8+ CCR7− T cells coexpressing both CD27 and CD28, as compared with a mean (SEM) of 46.6%
A CD27− CD28− effector memory T-cell subset has a representative untreated donor in Figure 3E, we compared untreated donors (n=3) or MS-FTY patients (n=3), the proportion of early vs late effector memory cells that migrated. Although the numbers of migrating early and late effector memory cells increase in response to the chemokine signals, there is a relative increase in the proportion of early vs late effector memory cells that have migrated.

(12.2%) of corresponding CD8+ CCR7− T cells in untreated donors (P=.009) (Figure 3D). As shown for a representative untreated donor in Figure 3E, we confirm a previous report that the CD8+ CCR7− late (CD27− CD28+) effector memory T-cell subset has a smaller proportion of CCR2+ cells as compared with their early (CD27+ CD28+) effector memory cell counterparts.9 We also observed a reduction in the proportion of CD8+ CCR7+ T-cells expressing CCR5 in the MS-FTY group compared with that in untreated donors (data not shown).

We directly compared migration to CXCL12 and CCL2 of CCR7+ early (CD27+ CD28+) and CCR7− late (CD27− CD28+) effector memory cells obtained from untreated donors only as MS-FTY patients have too-limited numbers of early (CD27+ CD28+) effector memory cells to allow such an analysis. As illustrated for a representative donor in Figure 4, although the chemokines induced an increase in both early (CCR7− CD27+ CD28+) and late (CCR7− CD27− CD28+) effector memory cells that migrated, there was a significantly increased proportion of early memory effector cells in the chemokine-directed migrated cell fraction.

IFN-γ AND TNF PRODUCTION BY CD8+ T LYMPHOCYTES FROM MS-FTY PATIENTS

As illustrated with representative donors in Figure 5A, IFN-γ and TNF production by CD8+ T cells in response to phorbol-12-myristate-13-acetate and ionomycin (PMA/I) were surface stained for CD8 and CCR7 before intracellular cytokine staining for IFN-γ and TNF. A, Representative dot plots for CD8+ CCR7+ (early) and late (CD27− CD28−) effector memory cells to CXCL12 and CCL2 chemokines (B). I indicates intermediate; E, early; and L, late.

Figure 5. Peripheral CD8+ T lymphocytes from patients with multiple sclerosis (MS) treated with FTY720 (MS-FTY) express relatively more interferon-γ (IFN-γ) and tumor necrosis factor (TNF) compared with untreated donors. Peripheral blood mononuclear cells stimulated with phorbol-12-myristate-13-acetate and ionomycin (PMA/I) were surface stained for CD8 and CCR7 before intracellular cytokine staining for IFN-γ and TNF. A, Representative dot plots for CD8+ CCR7+ (early) and late (CD27+ CD28−) effector memory cells. B, IFN-γ and TNF production by CD8+ T lymphocytes from MS-FTY patients compared with untreated donors. Results represent mean (SEM) values.
pared with untreated donors. The CCR7− population was the predominant source of each cytokine. When directly compared, cytokine production by CCR7− cells was comparable between the MS-FTY patients and the untreated donors (Figure 5B).

This study focused on characterizing the effect of FTY720 therapy on the migratory capacity of peripheral blood CD8+ T cells. Our initial in vitro studies showed that a single exposure of CD8+ T cells from untreated donors (whether untreated patients with MS or healthy donors) to FTY720P sensitizes these cells to migration toward a CXCL12 or CCL2 gradient, consistent with conclusions reached from prior in vitro studies of total CD3+ T cells derived from untreated animals. Honig et al. described a mechanism whereby FTY720P binds sphingosine 1–phosphate receptors to affect the multitransporter ABC1 and ABC2 as well as 5-lipoxygenase, leading to cytochalasin-leukotriene C4 production. Cytochalasin-leukotriene C4 binds its cognate receptor, cysLT1, increasing chemokine receptor responsiveness. The chemokine CXCL12 is expressed in high endothelial venules of lymph nodes and directs migration of naïve and CCR7− central memory effector T cells to the lymph node. The chemokines CCL2 and CXCL12 are both expressed by inflamed tissues and are chemotactants for activated or effector memory T lymphocytes.11,12 Thus, FTY720 exposure would enhance chemokine-directed migration of T cells bearing the corresponding chemokine receptor.

When we examined CD8+ T cells derived from the peripheral blood of MS-FTY patients, we found that these cells, which as expected were almost exclusively CCR7−, migrated less to both CXCL12 and CCL2 compared with CD8+ T cells from untreated donors, which are mostly CCR7+ and CCR7− cells. Our subsequent phenotypic and functional studies comparing the CD8+ CCR7− T cells of MS-FTY patients with those of untreated donors provided a basis for the observed differences in their chemokine migration responses. By using an immunostaining step that distinguishes CCR7+ and CCR7− cells within the total migrating CD8+ population, we could directly compare CCR7+ populations from MS-FTY patients and untreated donors. The immuno–magnetic bead separation technique yielded cells from MS-FTY patients and untreated donors with similar CD8 fluorescence intensities (data not shown), and populations of similar CD8 intensities were chosen for further phenotypic analysis along with gating for CCR7− cells. We found that the migration rates of CD8+ T cells from MS-FTY patients to CCL2 and CXCL12 were still reduced compared with migration rates of CCR7− cells from untreated donors. We also show that the CD8+ CCR7− cells from MS-FTY patients have a lower proportion of CCR2-expressing cells compared with the corresponding population from untreated donors. Our results involving CD27 and CD28 as markers for early and late effector memory cells indicate a relative increase in late vs early effector memory cells (CD27− CD28 vs CD27+ CD28−, respectively) within the CCR7− population in MS-FTY patients compared with untreated individuals. Our direct comparison of the CCR7− late (CD27− CD28−) and CCR7− early (CD27+ CD28+) effector memory cell populations derived from untreated donors demonstrates that late effector memory cells have reduced migration to CCL2 and that a reduced proportion of their cells express CCR2, the receptor for CCL2.

Our combined functional migration and phenotypic studies of the circulating CD8+ T-cell population in MS-FTY patients and untreated individuals provide insights into the mechanisms whereby in vivo FTY720 therapy leads to a redistribution of distinct effector memory CD8+ T-cell populations between tissue and blood compartments based on expression of CCR2. We postulate that initial FTY720 therapy, as with short-term in vitro exposure, enhances migration of effector memory cells capable of responding to chemokines into tissues and that this is not replaced by cells emanating from regional lymph nodes or thymus. This results in a unique residual population of effector memory cells in the circulation that are less capable of responding to chemokine gradients from target tissues. An alternate explanation would be that FTY720 induces an actual downregulation of chemokine receptors. However, treatment with FTY720 was reported to not have an effect on chemokine receptor expression in whole lymphocytes or lymph nodes, at least following brief exposure.13 We also did not observe changes in CCR2 expression after brief exposure to FTY720P (data not shown).2

In our study, we provide data that the CD8+ T-cell population that dominates the peripheral circulation in MS-FTY patients has distinct cytokine properties. Initial studies had shown that the peripheral lymphocytes of MS-FTY patients retain their ability to proliferate and produce cytokines.5 Using intracellular cytokine detection assays, we show that total CD8+ T cells from the MS-FTY patients produce higher amounts of IFN-γ and TNF compared with CD8+ T cells from untreated individuals. This greater overall production of both cytokines reflects the overrepresentation of CD8+ CCR7− cells in the periphery of MS-FTY patients as compared with the CCR7+ and CCR7− population in untreated individuals. When CD8+ CCR7− populations from both groups are compared, effector memory CD8+ cells from MS-FTY patients are not altered in their ability to produce either cytokine, suggesting that long-term FTY720 therapy does not modify cytokine production in these cells.

The distinct population of CD8+ T cells that predominates in the circulation of MS-FTY individuals composes only a minor fraction of the T lymphocytes circulating in untreated individuals. The expected forthcoming use of FTY720 as a sustained therapy in MS will clarify how the observed redistribution and function of lymphocyte populations affect the overall process of immune surveillance.

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Study concept and design: Johnson, Bar-Or, and Antel. Acquisition of data: Johnson and Lapierre. Analysis and interpretation of data: Johnson, Bar-Or, and Antel. Drafting of the manuscript: Johnson and Antel. Critical revision of the manuscript for important intellectual content: Johnson, Lapierre, Bar-Or, and Antel. Obtained funding: Antel. Administrative, technical, and material support: Lapierre and Bar-Or. Study supervision: Antel.

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