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Reduction of the Peripheral Blood CD56<sup>bright</sup> NK Lymphocyte Subset in FTY720-Treated Multiple Sclerosis Patients

Trina A. Johnson,*‡ Barbara L. Evans,* Bryce A. Durafourt,* Manon Blain,† Yves Lapierre,‡ Amit Bar-Or,*‡,† and Jack P. Antel*‡

FTY720 (fingolimod) treatment of multiple sclerosis (MS) results in lymphopenia due to increased recruitment into and decreased egress from secondary lymphoid organs of CCR7<sup>+</sup> lymphocytes. Although absolute numbers of NK lymphocytes were reported as being unaltered in FTY720-treated MS patients (MS-FTY), such analyses did not detect a change in a minor subset. Because expression of CCR7 has been described on CD56<sup>bright</sup> NK cells, a minority population of NK cells, we investigated the effect of FTY720 treatment on the phenotype and function of human NK cells in the peripheral circulation of MS patients. MS-FTY patients displayed a decreased proportion of peripheral CD56<sup>bright</sup>CD56<sup>dim</sup>CCR7<sup>+</sup> NK cells compared with untreated MS and healthy donors. In vitro treatment with FTY720-P increased migration of untreated donor NK cells to CXCL12 while reducing the response to CX3CL1 with similar migration responses seen in NK cells from MS-FTY patients. FTY720-P inhibited sphingosine 1-phosphate–directed migration of CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells subsets from untreated healthy donors. IL-12– and IL-15– stimulated NK cells from MS-FTY patients displayed similar capacity to produce IFN-γ, TNF, IL-10, and MIP-1α cytokines/chemokines compared with NK cells from untreated healthy donors and displayed comparable levels of degranulation in response to K562 tumor cells compared with untreated donors. Subset alterations and function of NK cell populations will need to be considered as part of assessing overall immunosurveillance capacity of patients with MS who will receive sustained FTY720 therapy. The Journal of Immunology, 2011, 187: 570–579.

FTY720 is a sphingosine 1-phosphate (S1P) receptor agonist with demonstrated efficacy in reducing inflammatory events in the CNS of multiple sclerosis (MS) patients (1). MS patients receiving FTY720 oral therapy (MS-FTY) demonstrate marked peripheral blood lymphopenia that reflects redistribution rather than destruction of this cell population (2) (3). The rapid onset (within days) of the lymphopenia in MS-FTY patients likely reflects that initial exposure to the agent enhances initial lymphocyte trafficking into secondary lymphoid organs (SLOs) by sensitizing cells to chemokine cues (4). Entry into SLOs primarily involves CCR7-expressing cells responding to the CCR7 ligands (5). Restricted re-entry to the circulation of CCR7<sup>+</sup> lymphocyte populations (naive and central memory T cells, B cells) is attributed to FTY720-mediated internalization of S1P1 on lymphocytes (6). Entry of Ag-reactive T cells into the circulation following influenza vaccination appears unimpaired in FTY720-treated MS patients, suggesting that re-entry restriction is not universal (7).

To further define the impact of long-term FTY720 therapy on immunosurveillance capacity, we addressed how FTY720 treatment of MS patients alters the distribution of NK cell subsets and what effect it has on the functional capacity of the NK cells circulating in these patients. NK cells are constituents of the innate immune system that act to control microbial infections and cancer and comprise ~10% of the circulating lymphocyte pool in peripheral blood (8). Human NK cells are CD3<sup>+</sup> lymphocytes that express a series of distinct molecules, including CD16, Nkp46, and CD56; CD56 is not expressed by murine NK cells (9). NK cell precursors arise from the bone marrow and undergo maturation processes that lead to an increase in effector functions and alterations in the expression of both integrins and chemotactic receptors. This allows their redistribution from bone marrow to lymphoid and nonlymphoid compartments. Whereas murine NK cells have been classified as CD27<sup>+</sup> (mature tissue or blood-resident effector NK cells) and as CD11b<sup>+</sup> (lymphoid-resident, immature NK cells), human NK cells can be divided into two main corresponding subsets based on levels of CD56 expression, that is, CD56<sup>dim</sup> and CD56<sup>bright</sup>, which differ in their functional and homing properties (10). CD56<sup>dim</sup> NK cells constitute ~90% of peripheral blood NK cells, express perforin, and are considered to be cytotoxic effectors. CD56<sup>bright</sup> NK cells, which make up the remaining 10% of peripheral blood NK cells, lack perforin but can, upon stimulation, including with IL-12/IL-15, produce a range of regulatory and effector cytokines (11). Recent studies indicate that the CD56<sup>dim</sup> NK cell subset can also be a source of proinflammatory cytokines (12, 13). Alterations in peripheral blood NK cell subset numbers and functions have been linked to disease activity in MS (14, 15). Treatment of MS patients with daclizumab (IL-2Rα mAb therapy) leads to expansion of the IL-10–producing CD56<sup>bright</sup>...
As such, the highest expression of S1P5 is seen in human CD56dim cell maturation is associated with upregulated expression of S1P5. NK from the blood, spleen, and lung in S1P5-deficient mice and accumulation in lymph nodes (18). Additionally, CD62L is expressed at high levels on CD56bright NK cells found in lymph nodes (19) and has been shown to be required for NK cell entry into lymph nodes (20).

Lymphoid cell egress from lymph nodes depends on essential chemotactic cues from S1P. Murine studies show that, whereas egress from lymph nodes in response to S1P for lymphocytes is regulated via S1P1 (21), egress of NK cells is regulated through both S1P1 and S1P5 (9, 22), the dominant SIPR expressed by the majority of this cell type, CD27dim NK cells (9). NK cells are lost from the blood, spleen, and lung in S1P5-deficient mice and accumulate in bone marrow and lymph node (9). S1P1-deficient mice show a small accumulation of NK cells in lymph nodes (22). NK cell maturation is associated with upregulated expression of S1P5. As such, the highest expression of S1P5 is seen in human CD56dim NK cells, with lower expression evident in CD56bright NK cells (9).

Additional chemokine receptors involved in the trafficking of NK cells include CXCR4 and CX3CR1, responsive to migration-directing cues delivered by their ligands CXCL12 and CXC3L1 (fractalkine), respectively (23). CX3CL1 has been shown to attract NK cells to CNS lesions in the experimental autoimmune encephalomyelitis model (24–26). CXCL12 contributes to NK cell homing to bone marrow and lymph nodes, as well as to inflammatory sites (27). It has been suggested that initial retention of NK cells in bone marrow is the result of CXCR4/CXCL12 interactions, and S1P1/S1P5/SIP-mediated signals are needed to overcome this interaction and allow egress from bone marrow (22).

Although the relative proportion of NK cells within the overall circulating lymphoid population is increased in FTY720-treated MS patients, previous studies have reported that absolute numbers of NK lymphocytes in FTY720-treated mice and humans are unaltered (9, 28). In the present study we investigated the relationship of NK cells given that these cells have increased sensitivity to chemokine ligands that promote ingress into lymph nodes. Because CD56bright NK cells are reported to have a relative overexpression of S1P1 compared with S1P5, we determined their relative response to S1P itself and whether their response was inhibited by FTY720. The functional properties of the NK cells remaining in the circulation of FTY720-treated patients was measured in terms of 1) sensitivity to effects of FTY720 on subsequent chemokine-directed migration in vitro using CXCL12, CX3CL1, or S1P, 2) IL-12– and IL-15–induced production of cytokines and chemokines, IFN-γ, TNF-α, IL-10, and MIP-1α, and 3) degranulation in response to tumor target cells.

Materials and Methods

Donors

Peripheral venous blood was obtained with informed consent from untreated healthy donors (UNTX), untreated MS patients (UNTX MS), and MS-FTY patients. MS-FTY patients were participants in the extension phases of phase II and III clinical trials for relapsing-remitting multiple sclerosis (1.25 mg fingolimod [FTY720] once daily with subsequent decrease to 0.5 mg daily). The number of patients contributing to each experiment is indicated in individual figure legends. Patients participating in the extension phases of fingolimod clinical trials were treated for between 2 and 6 y. All had expanded disability status scores scores of <2.0. This study was approved by the Institutional Review Board of McGill University.

Cell isolation

PBMCs were isolated from venous blood samples using a Ficoll density gradient (GE Healthcare, Baie d’Urfe, QC, Canada). For most studies, NK cells were isolated by depleting CD3+ lymphocytes using CD3 MACS (Miltenyi Biotec, Auburn, CA) followed by S1P5 selection for NK cells. For CD107 mobilization assays, NK cells were isolated using the NK cell negative isolation kit as per the manufacturer’s instructions (Miltenyi Biotec). All experiments were performed in RPMI 1640 complete medium (containing 10% FCS with antibiotics and glutamine; all from Invitrogen, Carlsbad, CA) except for migration experiments, which were performed in RPMI 1640 complete medium supplemented as indicated below. FTY720-P was a gift from Novartis Pharma, and vehicle control consisted of DMSO containing 50 mM HCl.

Flow cytometry

PBMCs were analyzed for expression of surface markers using flow cytometry, according to standard procedures for staining. The following anti-human Abs were used in various combinations: CD16-FITC, CD56-PE or Alexa 488, CR7-PE or Alexa Fluor 647, NKp46-allophycocyanin, CD3-PerCP, CD262L-PE, CXCR4-PE, CX3CR1-PE, or the appropriate isotype control (all from BD Biosciences). After staining, cells were washed twice and fixed in 1% formaldehyde containing PBS. Results were analyzed with FlowJo software (Tree Star, Ashland, OR). To analyze CD56 expression, CD56CD3− isolated NK cells were stimulated for 1 or 18 h in vitro in RPMI 1640 complete media with 1 μM FTY720-P, followed by surface CD56 and CD3 staining and flow cytometry.

Migration assay

CD56CD3− NK cells were pretreated for 30 min in vitro with either vehicle or FTY720-P (1 μM) in RPMI 1640 complete media (containing antibodies and glutamine) supplemented with either 2.5% FCS (CXCL12/CX3CL1 migrations) or 0.1% BSA (SIP1 migrations) and seeded at 250,000 per well on top of 5-μm ChemTx migration chambers (Neuro Probe, Gaithersburg, MD). For CXCL12/CX3CL1 migrations, wells below the filter were filled with either RPMI 1640 (2.5% FCS) media alone or media with chemokine, 10 ng/ml CXCL12 (SDF-1α) (Sigma-Aldrich, St. Louis, MO), or 10 ng/ml CX3CL1 (fractalkine). For SIP1 migrations, wells below the filter were filled with either RPMI 1640 (0.1% BS) media alone or media with 1 or 10 nM S1P (Sigma-Aldrich). NK cells were allowed to migrate for 4 h in a humidified incubator (37°C, 5% CO2). Cells were removed from individual chambers and stained with combinations of Abs, including anti-human CD56-Alexa 488, CD3-PerCP, and NKp46-allophycocyanin (BD Biosciences). Cells were washed three times before analysis on a flow cytometer (FACSCalibur; BD Biosciences). Quantification of cell number was assessed by 30-s timed acquisition of total cell number in a fixed volume of 1% formaldehyde in PBS. Each condition was performed in triplicate for at least three donors. Migration is expressed either as 1) migration index, which is a fold migration over control consisted of DMSO containing 50 mM HCl.

Cytokine production by intracellular cytokine staining

CD56CD3− NK cells were isolated from either MS-FTY or untreated donors and treated in vitro for 24 h with a combination of IL-12 (10 ng/ml) and IL-15 (100 ng/ml). Monensin (2 μM) was added 5 h prior to harvesting (Sigma-Aldrich). Cells were stained with anti-CD56 and anti-CD3 Abs. Intracellular cytokine staining for IFN-γ (IFN-γ-PE), TNF-α (TNF-α-PE), IL-10 (IL-10-PE), or MIP-1α (CCL3, MIP-1α-PE) (BD Biosciences) was then performed following paraformaldehyde/saponin fixation and permeabilization. Cells were gated on CD56CD5− NK cells.

Degranulation (CD107a mobilization) assay

The CD107a mobilization assay was done as previously described (29). NK cells (effectors) isolated by negative selection were preincubated for 30 min in media, vehicle, or 1 μM FTY720-P. Following treatment, monensin
(10 μM) was added and NK effector cells (200,000 cells) were plated onto a confluent layer of K562 targets cells (American Type Culture Collection) incubated in RPMI 1640 at a 1:1 E:T ratio. Five microliters of PE-conjugated CD107a Abs or concentration matched PE-conjugated IgG1 isotype control was added to each well. Cells were mixed and spun at 900 rpm for 1 min and incubated for 4 h at 37°C and 5% CO₂. Cells were washed, surface stained with NKp46-allophycocyanin, and CD16-FITC and analyzed by flow cytometry. Actively degranulating cells were identified as NKp46⁺CD16⁺CD107a⁺ NK cells.

RNA isolation, reverse transcription, and real-time PCR
Total RNA isolation from CD56⁺CD3⁻ MACs-isolated NK cells, cDNA synthesis, and real-time PCR were carried out as previously described (30). Expression levels for S1PR1 (encoding for S1P1) and S1PR5 (S1P5) and 18S for all cell types were determined by real-time PCR using Assays-on-Demand primers and probe sets (Applied Biosystems, Foster City, CA). For studies comparing S1PR expression in NK cells from MS patients receiving FTY720 and controls, relative expression levels were expressed as arbitrary units derived by interpolation of the threshold cycle of each sample to a standard curve generated from 10-fold serial dilutions of mRNA derived from healthy donor PBMCs (S1P1and S1P5). Relative quantities of 18S mRNA derived from standard curves were also determined from the same cell types and used to normalize the data.

Statistical analyses
Results represent data from at least three independent biological experiments. After ANOVA, a Student t test was used. Statistical analyses were performed using Prism 5.0 (GraphPad Software). Probability values of <0.05 were considered to represent statistically significant differences. Figures show means ± SE.

Results
Phenotypic properties of NK cells found in the circulation of FTY720-treated MS patients
As expected, the total unfractionated lymphocyte count was markedly reduced in the FTY720-treated patients. The average lymphocyte count for patients on FTY720 therapy was 0.44 × 10⁹ cells/l (±2 SD), whereas the lower limit of normal was 0.8 × 10⁹ cells/l. As illustrated in Fig. 1A, MS-FTY patients feature a decrease in the CD3⁺ T cell subset in PBMCs and a relative increase in the proportion of NK (CD3⁻, NKp46⁺) cells compared with an untreated control. As shown for the overall cohort of donors in Fig. 1B, PBMCs from MS-FTY patients contained a significantly higher frequency of cells that expressed the NK cell markers CD16 (47.55 ± 5.46% SE, n = 8) compared with PBMCs from UNTX patients (18.11 ± 2.51% SE, n = 8, p = 0.0007) or UNTX MS patients (15.36 ± 2.58% SE, n = 5, p = 0.0043) and NKp46 (32.84 ± 3.54% SE, n = 8) compared with UNTX (12.60 ± 2.05% SE, n = 11, p = 0.0015) or UNTX MS patients (14.41 ± 2.23% SE, n = 5, p = 0.0031), as well as CD56 (38.74 ± 3.33% SE, n = 9) compared with UNTX (16.15 ± 2.29% SE, n = 12, p = 0.0004) or UNTX MS patients (13.25 ± 1.75% SE, n = 5, p = 0.001).

As shown for representative donors in Fig. 2A and the overall cohort of MS-FTY patients and untreated donors in Fig. 2B, within the CD56⁺ NK cell population (gated on CD56⁻CD3⁻ cells), the proportion of CD56bright NK cells was substantially lower for MS-FTY patients (1.58 ± 0.34%, n = 10) compared with UNTX (6.13 ± 1.26%, n = 12, p = 0.0009) or UNTX MS patients (6.38 ± 1.68%, n = 8, p = 0.0048), resulting in a relative increase in the proportion of CD56dim NK cells for MS-FTY patients. As shown in Fig. 2C for representative donors and as an overlay in Fig. 2D, the loss of CD56bright NK cells results in the concurrent loss of CD56⁺NKp46⁺CD62Lhi NK cells.

We confirmed that CD56bright NK cells from control donors can be distinguished from CD56dim NK cells by expression of CCR7 (Fig. 3A). For the untreated control, ~60% of CD56bright NK cells express CCR7 whereas <20% of CD56dim NK cells express CCR7.

CCL7. As FTY720 redistributes CCR7⁺ cells, we confirmed that the FTY720-treated MS patients had a significant loss of CCR7 expressing NK cells (Fig. 3B, left). As shown for a representative donor in Fig. 3B (right), the remaining CD56bright NK cells in MS-FTY patients display similar mean fluorescence intensity (MFI) for CCR7 as do the NK cells in control donors.

To assess further phenotypic and functional characteristics of peripheral blood NK cells in MS-FTY patients compared with untreated donors, we isolated CD3⁻CD56⁺ NK cells by immunomagnetic bead positive selection. As shown in Fig. 3C for representative donors and averaged for the overall cohort in Fig. 3D, the loss in the relative proportion of CD56bright NK cells was maintained in the population isolated by positive selection. These cells were used for further assays and were ≥90% CD56⁻CD3⁻.

CD56 expression is unaltered by FTY720 treatment in vitro
To address whether the decreased numbers of CD56bright NK cells in the periphery of MS-FTY patients could result from FTY720 downregulating expression of CD56 in NK cell subsets, we treated isolated CD56⁺CD3⁻ NK cells from healthy donors in culture for 1 or 18 h to either vehicle or 1 μM FTY720-P. Levels of CD56.
expression at these time points were analyzed by flow cytometry gating for CD56^bright NK cell populations. As shown in Fig. 4A for a representative untreated donor, CD56 expression was not modulated after 1 or 18 h treatment in either media alone (UNTX), vehicle (VEH), or 1 μM FTY720-P (FTY). The proportion of CD56^bright CD62L^hi NK cells from the gated CD3^-NKp46^+ population in MS-FTY patients. B. Percentage of NKp46^+ NK cells from UNTX or UNTX MS donors or MS-FTY patients that express a CD56^bright phenotype (n = 12 UNTX, n = 8 for UNTX MS, and n = 10 for MS-FTY patients). ***p < 0.001. Error bars represent SE. C. Representative FACS profile of a UNTX control and MS-FTY patient, depicting a decreased frequency of CD56^bright CD62L^hi NK cells from the gated CD3^-NKp46^+ population in MS-FTY patients. D. Overlay histogram of CD56^bright CD62L^hi cells (gated with box in C) from a representative UNTX donor and MS-FTY patient showing loss of CD62L^hi CD56^bright NK cells in MS-FTY patients. FSc, forward scatter.

Functional properties of NK cells exposed to FTY720 in vitro or derived from FTY720 treated patients: chemokine-directed migration in response to CXCL12 and CX3CL1

The previously reported main functional effect of FTY720 is to increase the sensitivity of lymphocytes to chemokine cues for migration (4). As shown in Fig. 5A and 5B for a representative untreated donor, both CD56^dim and CD56^bright NK cells express the chemokine receptors CXCR4 and CX3CR1 for the chemokine ligands CXCL12 and CX3CL1, respectively.

Fig. 5C shows the results of in vitro treatment of CD3^- CD56^- NK cells from untreated donors or MS-FTY patients in vitro with vehicle or 1 μM FTY720-P and assessing chemokine-directed migration through microchemotaxis chambers to CXCL12. NK cells from untreated donors that were treated in vitro with 1 μM FTY720-P displayed a significant increase in migration to
CXCL12 of 68.1 ± 6.6% (n = 4) over baseline migration to media alone compared with vehicle-treated NK cells from the same donors (50.4 ± 6.6%, n = 4) over baseline (p = 0.027). The percentage increase in migration over baseline of NK cells derived from MS-FTY patients either after vehicle treatment (60.2 ± 14.6%, n = 3) or treatment with 1 μM FTY720-P (69.0 ± 11.5%, n = 3) was not significantly different from migration results observed for the untreated donors.

Fig. 5D shows the results of in vitro treatment of CD56 bright CD3− NK cells from untreated donors or MS-FTY patients in vitro with vehicle or 1 μM FTY720-P and assessing chemokine-directed migration to CX3CL1. NK cells from untreated donors that were treated in vitro with 1 μM FTY720-P displayed reduced response to CX3CL1 (14.0 ± 5.1% increase in migration over baseline, n = 3) compared with vehicle-treated NK cells from the same donors (36.6 ± 6.0%, n = 3, p = 0.016). Percentage migration over baseline of NK cells derived from MS-FTY patients displayed a similar pattern with a significantly reduced response after treatment with 1 μM FTY720-P (13.4 ± 4.8%, n = 3) compared with the vehicle treatment (40.2 ± 10.8%, n = 3, p = 0.004).

Human NK cells express S1P1 and S1P5 and display increased migration in the presence of the S1P1 agonist SEW2871

In Fig. 6A, we confirm that human CD56 bright CD3− NK cells taken from both healthy donors and MS-FTY patients express mRNA transcripts for the S1P receptors S1P1 and S1P5. To assess the effect of S1P1-specific agonism on migration to CXCL12, we exposed CD56 bright CD3− NK cells from healthy controls to vehicle or the S1P1 agonist SEW2871, then assessed migration toward media alone or CXCL12 in vitro. As shown in Fig. 6B, total CD56 bright CD3− NK cells from healthy controls migrated significantly more to CXCL12 when treated with 100 nM SEW2871 (2.43 ± 0.21, n = 4) than with vehicle (1.98 ± 0.18, n = 8, p = 0.004). NK cells from each condition that had migrated were immunostained with CD56 fluorescent Abs and quantified for the number of CD56 bright NK cells; dim, CD56 dim NK cells; FTY-P 1 μM FTY720-P; VEH, vehicle.
treated with 100 nM SEW2871 (1.77 ± 0.09, \( n = 4 \)) versus vehicle (1.41 ± 0.06, \( n = 4 \), \( p = 0.001 \)) (Fig. 6D).

S1P-dependent migration of NK cell subsets

As shown in Fig. 7A, total NK cells (CD56\(^+\)CD3\(^-\)) migrate to 1 nM S1P. Subset analysis of the overall migrated cell population indicated that there was an increase in the migration index for both CD56\(^{dim}\) cells (Fig. 7B) and CD56\(^{bright}\) NK cells (Fig. 7C) in response to 1 nM S1P. This effect was abolished by pretreatment of these cells with 1 \( \mu \)M FTY720-P.

Cytokine production by IL-12/IL-15–stimulated NK cells

We treated CD56\(^+\)CD3\(^-\) cells from control donors in vitro with IL-12 and IL-15 cytokine for 24 h and performed intracellular staining for TNF-\( \alpha \), IFN-\( \gamma \), IL-10, and MIP-1\( \alpha \). Cells were also immunostained for CD56 expression. The addition of IL-12 or IL-15 alone results in very little production of any cytokine/chemokine, whereas the addition of IL-12 and IL-15 significantly increased cytokine/chemokine production.

As shown in Fig. 8, there was no significant difference in the overall production of any of the cytokines/chemokine tested in total CD56\(^+\)CD3\(^-\) NK cells isolated from MS-FTY patients compared with untreated healthy donors, suggesting the relative overabundance of CD56\(^{dim}\) NK cells in both groups masks any small changes that may be seen due to the loss of the minority population of CD56\(^{bright}\) NK cells in MS-FTY patients.

Cytotoxicity of NK cells from MS-FTY patients

In the peripheral circulation, CD56\(^{dim}\) NK cells are the cytolytic subset of NK cells. To determine whether long-term FTY720 treatment alters the ability of this population to degranulate in response to target cells, we examined CD107a mobilization as a degranulation indicator in response to the K562 tumor target cell line. NK cells isolated by negative selection from untreated or MS-FTY patients were incubated in the absence or presence of target K562 cells, and CD107a and CD16 expression were assessed on NKp46\(^+\) cells. Degranulating cells were defined as CD16\(^+\)/CD107a\(^+\) cells. In Fig. 9A, representative plots from one untreated individual (UNTX) and one MS-FTY patient show that...
NK cells from an untreated individual in the absence of K562 target demonstrate minimal spontaneous degranulation (2.0%) and no effect by vehicle (1.8%) or FTY720-P (1.7%) acute treatment. In the presence of K562 target cells, NK cells from a representative donor displayed similar degranulation profiles for untreated (12.6%), vehicle (12.5%), and FTY720-P–treated (12.1%) conditions. NK cells from MS-FTY patients displayed comparable levels of degranulation whether they were not pretreated (16.6%), pretreated with vehicle (16.4%), or pretreated with FTY720-P (14.8%). As expected, the MFI for CD16 decreases as degranulation occurs.

In Fig. 9B, for the overall cohort of untreated control, the effect of no treatment, vehicle, or 1 μM FTY720-P on degranulation (CD107a+ cells) is shown. No significant difference in CD107a+ cells was noted for untreated control NK cells in contact with K562 target cells either in the presence of no treatment (no tx, 16.3 ± 1.3%, n = 4), pretreatment with vehicle (VEH, 15.7 ± 1.3%, n = 4), or FTY720-P (FTY, 15.0 ± 1.3%, n = 4). Additionally, rates of spontaneous degranulation (absence of K562 target cells) were not significantly different in all conditions. Fig. 9C demonstrates the overall cohort for both untreated controls and MS-FTY patients, which were averaged showing comparable levels of degranulation between groups. NK cells, not pretreated, showed similar degranulation in the presence of K562 target cells for the untreated controls (16.3 ± 1.3%, n = 4) and the MS-FTY patients (16.4 ± 1.2%, n = 3). As with the untreated controls, MS-FTY patients showed similar spontaneous degranulation in the absence of K562 cells.

Discussion

This study focused on the phenotypic and functional properties of NK cell lymphocyte populations isolated from the circulation of MS-FTY patients and the short-term in vitro effects of FTY720 on NK cells isolated from MS-FTY and untreated donors. Daily oral FTY720 therapy is demonstrated to produce a significant reduction in T and B cells in the systemic circulation resulting from a redistribution of these cells (3, 31). FTY720 retains CCR7+ lymphocytes in secondary lymphoid compartments, resulting in an increased relative proportion of CCR7+ populations in the blood of MS-FTY patients (3). Our recent analysis of the residual circulating CD8+ lymphocyte populations in FTY720–treated patients indicated persistence of cells (late effector memory CCR7+ CD27+ CD28−) with a reduced capacity to respond to chemokine signals that would contribute to recruitment of these cells into tissues, including the CNS (32). Such changes in the adaptive immune system may imply an increased reliance on the innate immune system for immunosurveillance in FTY720–treated patients.

For our phenotypic analysis of NK cells, we focused on expression of NKp46, CD56, and CD16. Whereas NKp46 is expressed solely on NK cells, CD56 and CD16 are expressed additionally on other subsets (CD56+ NKT cells CD16+ monocytes) (33). In our experiments, NK cells were identified as NKp46+ or CD56+ CD3− cells. We confirmed the previously reported relative increase in the proportion of NK cells in the peripheral circulation of MS-FTY patients (28). We took advantage of CD56+ being a marker for the regulatory subset of NK cells in humans to demonstrate redistribution of NK cell subsets that would not have been evident in animal studies (9) or previous human studies (28) that did not take into account the minor circulating subset of CD56+ NK cells. We now document a loss of CD56+ NK cells (CD62L hi) and a relative increase in the proportion of CD56– NK cells in the periphery of MS-FTY patients. Such a finding could reflect either a downregulation of CD56 from the surface of the CD56+ NK cells, or a redistribution of the CD56+ NK cell population by action of the drug, or both. We directly demonstrate that short-term FTY720 treatment does alter expression of CD56 in vitro, thus supporting our hypothesis that the decrease in the CD56+ NK cell population in the circulation of MS-FTY patients reflects a redistribution of the CD56– NK cells.

As previously reported, CCR7 is detected on 60% of human CD56+ NK cells and only a minority of CD56– NK cells (34, 35). It is the CCR7-expressing population that is depleted from the systemic circulation of the MS-FTY group. We have previously shown that CCR7 expression underlies the redistribution of CD8+ T lymphocyte subsets in MS-FTY patients (3, 32). T lymphocyte egress from lymphoid organs is mainly dependent on S1P1 with S1PR1 (36–38). Recent animal based studies have identified S1P5 as being required for NK cell exit from bone marrow and spleen.

![Image](http://www.jimmunol.org/)

**FIGURE 8.** NK cells from MS-FTY display similar IFN-γ, TNF, IL-10, or MIP-1α production after stimulation with IL-12 and IL-15 in vitro versus untreated controls. A, Mean percentage of CD56+CD3− NK cells from UNTX donors or MS-FTY patients that are IFN-γ+ after no treatment or IL-12 with or without IL-15 stimulation for 24 h in vitro (n = 7 UNTX, n = 10 MS-FTY). B, Mean percentage of CD56+CD3− NK cells from UNTX donors or MS-FTY patients that are TNF+ after no treatment or IL-12 with or without IL-15 stimulation for 24 h in vitro (n = 8 UNTX, n = 11 MS-FTY). C, Mean percentage of CD56+CD3− NK cells from UNTX donors or MS-FTY patients that are IL-10+ after no treatment or IL-12 with or without IL-15 stimulation for 24 h in vitro (n = 3 UNTX, n = 5 MS-FTY). D, Mean percentage of CD56+CD3− NK cells from UNTX donors or MS-FTY patients that are MIP-1α+ after no treatment or IL-12 with or without IL-15 stimulation for 24 h in vitro (n = 3 UNTX, n = 5 MS-FTY). Error bars represent SE. no tx, no treatment.
as well as for allowing their circulation in the blood, spleen, and lung (9). Animal-based studies report that S1P5 is resistant to FTY720-induced downmodulation; FTY720 treatment of animals continues to allow NK cell egress (9). As mentioned, CD56\textsuperscript{bright} NK cells, unlike CD56\textsuperscript{dim} NK cells, do not show preferential expression of S1P5. Our data indicate that both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells respond to S1P; pretreatment with FTY720-P inhibits this migration.

We and others have previously investigated the mechanism of action for FTY720 to alter lymphocyte sensitivity to chemokine signals. Our previous studies of in vitro treatment with FTY720-P highlighted a role for FTY720 in increasing the sensitivity of effector memory CD8\textsuperscript{+} lymphocytes to the chemokines CXCL12 and CCL2 when modeled using microchemotaxis assays (32). NK cells express the chemokine receptors CXCR4 and CX3CR1, which respond to their cognate ligand chemokines CXCL12 (SDF-1\textalpha) and CX3CL1 (fractalkine) with increased expression on the CD56\textsuperscript{bright} NK cells compared with the CD56\textsuperscript{dim} NK cells. In vitro treatment with FTY720-P promoted migration of untreated donor NK cells to CXCL12, an effect also observed with human CD8\textsuperscript{+} T cells (32). We postulate that the lack of increased response in the MS-FTY patient-derived NK cells reflects either the effects of chronic FTY720 therapy or a relative lack of response by CD56\textsuperscript{dim} NK cells. Our observation that this chemokine-directed migration was enhanced by the S1P1 agonist SEW2871 suggests that the FTY720-P effect was mediated via S1P1. We found that in vitro treatment with FTY720-P reduced the migration response of NK cells to CX3CL1, with such an effect being maintained even on cells derived from patients on chronic therapy. Our data on the differential effects of FTY720 on individual chemokine signals parallel studies in mice that showed FTY720 promotes lymph node homing and in vitro migration to some chemokines, including CXCL12, but has no effect on others, including CX3CL1 (39).

Although not present in large numbers in the peripheral circulation, activated CD56\textsuperscript{bright} NK cells are a major contributor to cytokine production. Using an intracellular staining method, we stimulated NK cells with the monokines IL-12 and IL-15, a stimulus known to induce cytokine production from NK cells (11). As previously reported we note that both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells are capable of producing cytokines, and thus any loss of cytokine production that may have been expected by the loss of CD56\textsuperscript{bright} NK cells from the overall NK population is masked by the continued production of cytokine from CD56\textsuperscript{dim} NK cells, and no overall differences in cytokine production are evident by comparing the percentages of total NK cells producing cytokine.

With regard to cytotoxic functions, CD56\textsuperscript{dim} resting NK cells are cytotoxic, whereas CD56\textsuperscript{bright} NK cells require activation by IL-2 to become cytolytic (40). Because the numbers of CD56\textsuperscript{bright} NK cells in the periphery are altered, we sought to confirm whether this has any outcome on cytotoxic function of the remaining CD56\textsuperscript{dim} population. As reviewed by Segal (15), clinical or radiological disease activity in patients with MS has been linked to the frequency and functional competence of circulating NK cells. These early experiments did not specifically isolate NK cells but, rather, they examined the cytotoxic potential of PBLs; as such, they take into account the differential cytotoxic capabilities of CD56\textsuperscript{dim} and CD56\textsuperscript{bright} subsets.

We opted to perform CD107a mobility assays to determine the degranulation capabilities of total NKp46\textsuperscript{+} NK cells isolated from either untreated donors or MS-FTY patients in the presence or absence of FTY720. In a recent study, it was shown that S1P can protect K562 cells from NK cell-mediated cell lysis by acting through S1P1 on the tumor target cells. FTY720, acting on the tumor cells, can reverse this protection, suggesting that FTY720 may promote tumor targeting (41). The authors implicated an effect on the target cell rather than on the NK cell. Our results indicate that CD56\textsuperscript{dim} NK cells isolated from MS-FTY patients have retained the ability to directly target foreign cells and thus retain their immunosurveillance capabilities.

The overall consequence of a redistribution of CCR7\textsuperscript{+}CD56\textsuperscript{bright} NK cells to the lymph node may be minimal since most CD56\textsuperscript{bright} NK cells already act within regional lymph nodes in their regulatory capacity (42, 43). As mentioned, treatment of MS patients with daclizumab (IL-2R\alpha mAb therapy) leads to expansion of the...
IL-10–producing CD56<sup>bright</sup> subset of NK cells, which may act to regulate the proliferation of potentially autoreactive T lymphocytes. In the context of inflammation within target tissues, however, loss of circulating CD56<sup>bright</sup> NK cells may have consequences. In the periphery, most NK cells (~90%) are CD56<sup>dim</sup> CD16<sup>+</sup>, whereas at sites of inflammation CD56<sup>bright</sup>CD16<sup>+</sup> NK cells predominate (10) and can synergize with monocytes to promote a proinflammatory environment (45). The presence of NK cells at inflamed sites, including the CNS, has been shown to be important for controlling experimental autoimmune encephalomyelitis (45, 46). With the recent U.S. Food and Drug Administration approval for the use of FTY720 as a sustained therapy for MS patients, the impact of the observed redistribution and function of NK cell populations on immunosurveillance and disease susceptibility, as revealed in larger treatment cohorts, may soon be determined.

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