T-Helper 17 Cells Expand in Multiple Sclerosis and Are Inhibited by Interferon-β

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Objective: T-helper 1 (Th1) and Th17 lymphocytes are involved in experimental autoimmune encephalomyelitis, the model of multiple sclerosis (MS). We characterized the Th1/Th17 cell populations in peripheral blood (PB), their interferon (IFN) receptor expression sensitivity to IFN-β in MS patients.

Methods: In 30 untreated patients with active MS (AMS) and 32 with inactive MS (IMS), and in 22 healthy subjects, we measured intracellular cytokine expression, interleukin-17–producing myelin basic protein–stimulated PB lymphocytes, surface IFN type I receptor chain1 (IFN-αR1) expression, IFN-β-dependent signal transducer and activator of transcription 1 (STAT1) phosphorylation, and apoptosis of anti-CD3 monoclonal antibody–stimulated PB lymphocytes.

Results: Th17 cell percentage increased around sevenfold in AMS compared with IMS or healthy subjects, but there was no change in Th1 cells. Th17 cells in AMS were myelin basic protein specific. The longitudinal follow-up of 18 MS patients shifting between AMS and IMS showed that the percentage of Th17 but not Th1 cells always increased in AMS. IFN-αR1 expression, IFN-β–induced STAT1 activation, and apoptosis were significantly greater in Th17 than Th1 cells. IFN-αR1 expression and IFN-β–dependent STAT1 activation progressively increased in vitro with a highly significant positive correlation only in developing Th17 but not in Th0 or Th1 cells.

Interpretation: Evidence that an expansion of peripheral Th17 cells, a Th subset that can infiltrate brain parenchyma and damage cells, is associated with disease activity in MS. The greater IFN-αR1 level expressed by Th17 compared with Th1 cells might make them a selective target for IFN-β therapy.

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A self-reactive interferon (IFN)-γ–producing T-helper 1 (Th1) lymphocyte response plays a role in the pathogenesis of multiple sclerosis (MS).[1] Th1 cells have been found in central nervous system (CNS) perivascular infiltrates,[2,3] and IFN-γ is increased in the brain, cerebrospinal fluid, and peripheral blood (PB) of MS patients.[4–6] However, the pathogenic T-cell population involved in the MS animal model, experimental autoimmune encephalomyelitis (EAE), includes interleukin (IL)-17–producing cells (Th17).[7,8] IL-17 and the related cytokine, IL-23,[9,10] are increased in CNS lesions and in peripheral blood mononuclear cells (PBMCs) of MS patients.[11–13]

A number of immune abnormalities have been observed in the PB of MS patients, particularly disease activity correlates with decreased PB regulatory T-cell activity.[14,15] MS clinical exacerbations are associated with a flare of magnetic resonance imaging (MRI)–detectable CNS lesions and are preceded by a blood–brain barrier breakdown, manifesting as contrast enhancement of MRI lesions.[16] These correspond, at biopsy, to the perivascular infiltrates of immune cells.[17]

Both Th1 and Th17 cytokines could sustain tissue inflammation and damage cells,[4,8,18,19] and IFN-γ, which protects mice from EAE,[20] greatly worsens MS.[21] This uncertainty about the Th subset involved in MS pathogenesis prompted us to analyze the Th1/Th17 balance in patients with active or inactive MS.

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Finally, because IFN-β was the first drug approved for chronic MS treatment,[22] we have studied the expression of IFN receptors on Th1 and Th17 lymphocytes, and the sensitivity of these Th subsets to IFN-β.

Subjects and Methods

Subjects

The PB of outpatients affected with relapsing-remitting MS[23] from the San Luigi Gonzaga University Hospital MS center (Orbassano, Italy), and of age- and sex-matched healthy subjects (HS) was analyzed. No patients had used immunomodulatory therapy (IFN-β or glatiramer acetate) or immunosuppressive treatment for at least 2 years. Patients with active MS (AMS) had a clinically documented exacerbation within the last 10 days but were free from corticosteroid treatment. Patients with inactive MS (IMS) had no exacerbations in the last 3 months and no new proton density/T2 or gadolinium-enhancing lesions on two subsequent MRI scans performed 3 months apart. Brain MRI scans (24 contiguous axial slices, 5mm thick, of conventional PD/T2 and T1 spin-echo, precontrast and postcontrast sequences) were obtained using a predefined repositioning protocol.[24] MRI evaluations were performed by investigators totally unaware of patients' clinical characteristics and treatment. Neurological assessments, performed by MS-experienced investigators, were done at baseline; then for patients followed up longitudinally, assessments were repeated every 3 months, or in case of relapses; MRI scans and immunological studies were repeated every 6 months. Exacerbations, defined according to Poser's criteria,[25] were assessed within 7 days for objective confirmation and treated with intravenous methylprednisolone (1gm/day for 5–10 days). Some patients started chronic treatment with 250μg IFN-β-1b (Betaferon; Bayer Schering, Berlin, Germany) every other day 45 to 60 days after the exacerbation that defined their entry in the study. The institutional review boards of the participating centers approved the study, and all subjects gave written informed consent.

T-Lymphocyte Activation and Polarization

PBMCs were isolated by density gradient centrifugation. PBMCs from HS were activated with plate-coated anti-CD3 (10μg/ml) and soluble anti-CD28 monoclonal antibodies (MAbs) (1μg/ml; BD Biosciences, San Diego, CA) for 5 days (days 0–4) in the presence of the following: (1) medium alone *(Th0 polarizing condition);* (2) IL-12 (20ng/ml; Peprotech, Rock Hill, NJ) plus neutralizing anti–IL-4 MAb (10μg/ml; 34019; R&D Systems, Minneapolis, MN) *(Th1 polarizing condition);* or (3) IL-23 (50ng/ml; R&D Systems) plus a mix of neutralizing anti–IFN-γR1 (γR99, γR38 ascites diluted 1:100),[26] anti–IFN-γ (20μg/ml; B27; BD Biosciences), and anti–IL-4 MAbs *(Th17 polarizing condition)* (see Supplementary Fig 1). In some experiments, PBMCs were restimulated simultaneously with anti-CD3 MAb and increasing doses (0–1,000IU/ml) of IFN-β-1a (Rebif; Merck-Serono, Geneva, Switzerland) for 72 hours. PBMCs from MS patients were analyzed with flow cytometry or enzyme-linked immunosorbent spot (ELISPOT) either immediately after isolation or after 72-hour stimulation with anti-CD3 MAb and increasing doses (0–1,000IU/ml) of IFN-β-1a. PBMCs from MS patients analyzed longitudinally were frozen and then analyzed simultaneously to reduce experimental variability.

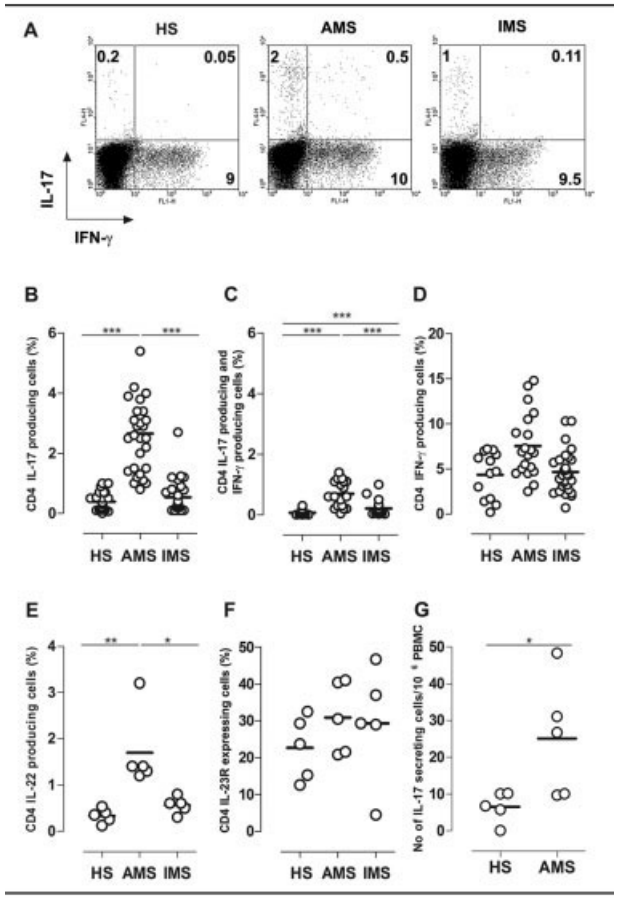

Fig 1. T-helper 17 (Th17) but not Th1 lymphocytes are increased in the peripheral blood (PB) of clinically active multiple sclerosis (AMS) patients. Fluorescence-activated cell sorting (FACS) analysis of interleukin (IL)-17 or interferon (IFN)-γ production from $CD4^+$ $CD45RO^+$ lymphocytes of healthy figures (HS), and AMS or clinically and magnetic resonance imaging inactive MS (IMS) patients stimulated for 5 hours with phorbol myristate acetate and ionomycin in the presence of brefeldin A. (A) Dot plots of a representative subject for each group. (B–D) Graphs of the percentage of $CD4^+$ $CD45RO^+$ IL-17–producing, IL-17/IFN-γ–producing, and IFN-γ-producing cells from HS, AMS, and IMS patients. (E) Graph of the percentage of $CD4^+$ IL-22–producing cells from HS, AMS, and IMS patients. (F) Graph of the percentage of $CD4^+$ IL-23R–expressing cells from HS, AMS, and IMS patients. (G) Evaluation of IL-17–producing cells in MBP-stimulated PBMCs from HS and AMS patients by enzyme-linked immunosorbent spot (ELISPOT). Data represent the number of spots after background subtraction of unstimulated PBMC. Each circle *represents a single subject;* horizontal bars *indicate means.* $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0001$, *Mann–Whitney* U *test.*

Flow Cytometry (Fluorescence-Activated Cell Sorting)

Stained PBMCs were acquired on a FACSCalibur and analyzed with CellQuest (BD Biosciences). To evaluate the ki-

netics of type I IFN receptor (IFN-αR) chains surface expression, we harvested cells daily on activation days 0 to 4 and stained with anti–IFN-αR1 (clone 64G12)[27] or anti–IFN-αR2 (PBL Biomedical Laboratories, New Brunswick, NJ) MAbs, followed by goat anti–mouse biotin-conjugated secondary antibody and phycoerythrin (PE)-conjugated streptavidin (Jackson Laboratory, Newmarket, United Kingdom) as described previously.[28] For intracellular cytokine detection, in vitro activated, fresh, or thawed PBMCs were cultured for 5 hours with phorbol 12-myristate 13-acetate (50ng/ml), ionomycin (500ng/ml), and brefeldin A (10μg/ml; Sigma-Aldrich, St. Louis, MO), and then stained extracellularly with anti–IFN-αR1, biotin-conjugated anti–IL-23 receptor (IL-23R) (R&D Systems), or PE-conjugated anti-CD45RO (BD Biosciences) MAbs. Cells then were intracellularly stained using Alexa fluor 647–conjugated anti–IL-17 (eBioscience, San Diego, CA), fluorescein isothiocyanate–labeled anti–IFN-γ, or PE-labeled anti–IL-22 and peridinin-chlorophyll-protein complex–labeled anti-CD4 or -CD8 MAbs (BD Biosciences). To analyze IFN-β–induced signal transducer and activator of transcription 1 (STAT1) phosphorylation, we harvested cells on activation day 4, cultured them for 5 hours with phorbol 12-myristate 13-acetate, ionomycin, and brefeldin A, treated with or without IFN-β-1a (100IU/ml) for the last 15 minutes, and then intracellularly stained them using anti–IL-17, anti–IFN-γ, anti-CD4, and PE-labeled anti–phospho-Tyr (701) STAT1 (BD Biosciences) MAbs. For cell cycle analysis, cells were harvested on activation day 4 and restimulated with anti-CD3 MAb and IFN-β-1a (100IU/ml) for 24, 48, and 72 hours. At each time point, aliquots of cells were fixed in ethanol, and incubated with 100μg/ml RNase A and 50μg/ml propidium iodide (Sigma-Aldrich) for 30 minutes. Propidium iodide staining is used to detect cell apoptosis based on the measurement of cell DNA content.[29] The percentage of cells in the various phases of the cell cycle was calculated by using both CellQuest and ModFit LT (Verity Software House, Topsham, ME) software. To evaluate IFN-β–induced apoptosis, we harvested Th1 and Th17 polarized cells on day 4, then stimulated with anti-CD3 MAb with or without IFN-β-1a (100IU/ml) for 72 hours, and then stained with PE-conjugated annexin V and 7-amino-actinomycin D (7-AAD; BD Biosciences).

Assessment of Antigenic Specificity of IL-17–Producing Cells by Enzyme-Linked Immunosorbent Spot

IL-17–producing cells were assessed using a specific ELISPOT kit (eBioscience). In brief, 96-well plates (Millipore, Bedford, MA) were coated with anti–IL-17 MAb, and PBMCs were plated at 2×10^5 cells/well in triplicate and stimulated with myelin basic protein (MBP; 40μg/ml), or with purified protein derivative of tuberculin (PPD; 50μg/ml; Sigma-Aldrich) or complete medium. After incubation for 48 hours at 37°C, the ELISPOT was performed according to the manufacturer's protocol. The spots corresponding to IL-17–secreting cells were counted by computer-assisted image analysis (Transtec 1300 ELISpot Reader; AMI Bioline, Buttigliera Alta, Italy). Median values for the triplicates, adjusted as the number of IL-17–producing cells/10^6 PBMCs, were used. To obtain the MBP-specific net secretion, we subtracted the number of spontaneously IL-17–secreting cells from the number of MBP-stimulated cells.

Western Blotting

IFN-β–induced STAT1 phosphorylation was evaluated in PBMCs from HS activated under Th0, Th1, and Th17 polarizing conditions. Cells were cultured in serum-free medium for 1 hour and then treated with or without IFN-β-1a for 15 minutes. In the time-course experiments, cells were harvested daily from activation days 0 to 4 and treated with or without 100IU/ml IFN-β-1a. In the dose–response experiments, cells were harvested on day 4 and treated with 0 to 1,000IU/ml IFN-β-1a. STAT1 phosphorylation was analyzed as described previously,[28] with anti–phospho-Tyr (701)-STAT1, anti-STAT1 (Cell Signaling, Beverly, MA), and anti-actin (Sigma-Aldrich) polyclonal antibodies. Actin was used as a control for equal protein loading. Blots were scanned with ProXPRESS 2D (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA). The fold induction of IFN-β–dependent STAT1 phosphorylation was calculated as the ratio between the band intensities of IFN-β–treated and untreated cells, quantified after normalization with actin.

Statistical Analysis

Statistical analysis was performed with SPSS 14.0 (SPSS, Chicago, IL). To test the differences between groups, we applied the χ^2 or the Fisher's exact test for dichotomous outcomes, and parametric or nonparametric tests for continuous outcomes, according to the distribution of variables; p values ≤ 0.05 were considered statistically significant. One-way analysis of variance for repeated measures was used to test data from the longitudinal follow-up and dose–response curves. Bonferroni adjustment for repeated measurements was used when needed.

Results

Th17 but Not Th1 Cells Are Increased in Active Multiple Sclerosis

We analyzed the PB of 22 HS and 62 MS patients (30 AMS and 32 IMS). In addition, 18 of these patients were prospectively followed up for 6 to 15 months, of them before and after starting chronic subcutaneous IFN-β-1b. Patient demographic data are shown in the Table.

The PBMC Th17 cell percentage was low in HS and IMS patients, whereas it increased by about sevenfold in AMS patients (Fig 1A, B). The very low percentage of $CD4^+$ lymphocytes simultaneously producing IL-17 and IFN-γ (Th1-17) detected in HS was increased 9.3-fold in AMS and 4.2-fold in IMS (see Fig 1A, C) . In contrast, the Th1 cell percentage was not significantly increased in AMS or IMS compared with HS (see Fig 1A, D). Most Th1 and all Th17 cells had a memory $CD45RO^+$ phenotype (see Supplementary Fig 2). The few Th1-17 cells were included thereafter in the Th17 population.

Table. **Baseline Clinical and Demographic Characteristics of Study Groups**

Characteristics	HS (n = 22)	AMS (n = 30)	IMS (n = 32)
Mean age ± SD, yr	34.5 ± 4.8	33.0 ± 9.2	37.5 ± 8.4
Women, %	77.3	79.3	79.3
Mean disease duration ± SD, yr	—	1.4 ± 1.6	3.9 ± 4.9[a]
Mean baseline EDSS score ± SD	—	1.5 ± 1.0	1.1 ± 1.0
Mean baseline yearly exacerbation rate ± SD	—	0.7 ± 0.4	0.7 ± 0.5

Exacerbation rate is the total number of relapses divided by the period of observation expressed in years. Means refer to the 2 years before baseline.
[a]Clinically active multiple sclerosis (AMS) patients had a significantly lower disease duration ($p < 0.05$, two-tailed Student's t test) compared with clinically and magnetic resonance imaging inactive (IMS) patients. No other significant differences were observed.
HS = healthy subjects; SD = standard deviation; $EDSS^{45}$ = expanded disability status scale.

The expression of the Th17-related molecules, IL-22 and IL-23 receptor (IL-23R), was also evaluated in five HS, AMS, and IMS patients. The PBMC percentage of IL-22–producing T $CD4^+$ cells was low in HS and IMS, whereas it was increased fivefold in AMS (see Fig 1D). By contrast, IL-23R expression on $CD4^+$ cells from HS was similar to that observed in AMS and IMS (see Fig 1F).

To ascertain whether the Th17 cell population identified in AMS patients contained myelin antigen–specific cells, we stimulated PBMCs from five HS and five AMS with MBP for 48 hours, and the number of IL-17–producing cells were evaluated by ELISPOT. The number of IL-17–producing cells detected in MBP-stimulated PBMC was very low in HS, whereas it was significantly increased in AMS (see Fig 1G). In con-

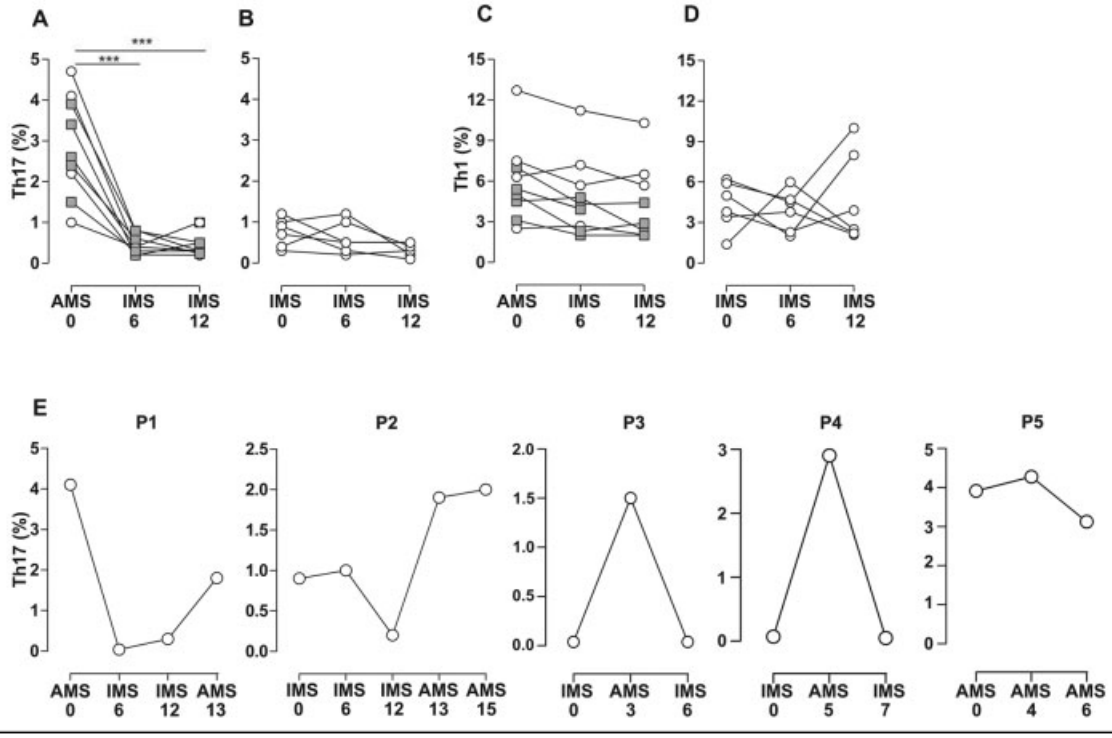

Fig 2. Longitudinal analysis of the percentage of T-helper 17 (Th17) (A, B, E) and Th1 (C, D) lymphocytes in multiple sclerosis (MS) patients followed up prospectively. Gray squares *represent patients treated with subcutaneous interferon (IFN)-β (IFN-β treatment started 45–60 days after the exacerbation that defined the entry into the study). (A, C) Graphs show Th percentages of nine patients entering the study during a clinical relapse (clinically active MS [AMS]) and shifting to clinically and magnetic resonance imaging inactive MS (IMS). ***$p < 0.0001$; one-way analysis of variance for repeated measures. (B, D) Th percentages of six patients entering the study in IMS phase and remaining in IMS for the whole follow-up. (E) Th17 percentages of five patients shifting several times from AMS to IMS or vice versa, or had multiple subsequent relapses (data of Patients P1 and P2 are also presented in [A] or [B]). Numbers on the abscissas indicate the months of follow-up.*

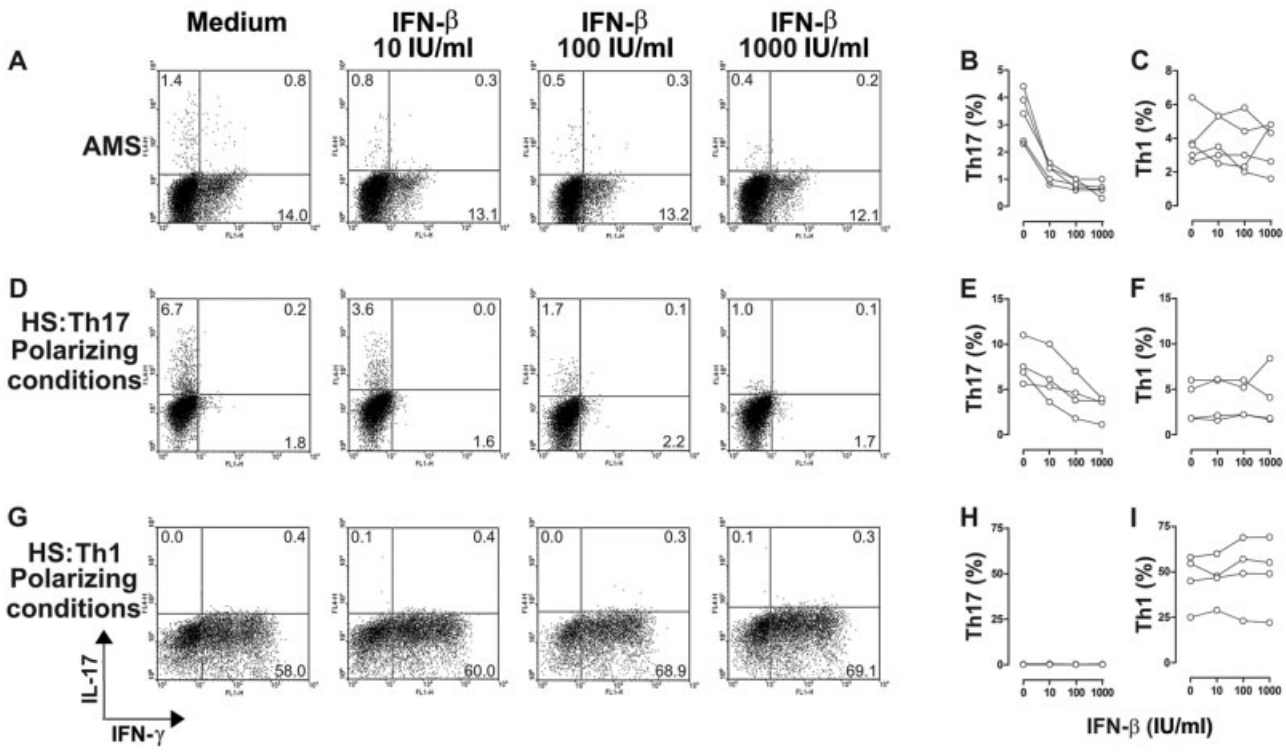

Fig 3. Interferon (IFN)-β inhibits T-helper 17 (Th17) but not Th1 lymphocytes in multiple sclerosis (MS) patients and healthy subjects (HS). (A–C) Fluorescence-activated cell sorting (FACS) analysis of interleukin (IL)-17 or IFN-γ production from $CD4^+$ $CD45RO^+$ lymphocytes of clinically active MS (AMS) patients stimulated for 3 days with anti-CD3 monoclonal antibody and treated with increasing doses of IFN-β-1a. Dot plots (A) show one representative AMS patient out of five; graphs show the percentage of Th17 (B) or Th1 (C) cells. One-way analysis of variance (ANOVA) for repeated measures showed that IFN-β reduced in a dose-dependent manner the percentage of Th17 ($F = 36.6$; $p < 0.00001$) but not Th1 cells. (D–I) FACS analysis of IL-17 and IFN-γ production by $CD4^+$ $CD45RO^+$ lymphocytes of HS activated for 5 days under Th17 (D–F) or Th1 (G–I) polarizing conditions. (D, G) Dot plots show one representative experiment out of four; (E–I) graphs show the percentage of Th17 (E,H) and Th1 (F,I) cells. In dot plots, numbers in quadrants indicate the percentage of cells. In graphs, each circle *represents a single experiment. One-way ANOVA for repeated measures showed that IFN-β reduced in a dose-dependent manner the percentage of Th17 ($F = 12.9$; $p = 0.001$) but not that of Th1 cells.*

trast, the number of IL-17–producing cells detected in response to the MS-unrelated antigen PPD was very low or absent in both HS and AMS (data not shown).

Th17 Lymphocyte Percentage Closely Matched the Changes in Disease Activity

Because the AMS and IMS patients we compared had different disease duration (see the Table), this could explain the difference in Th17 cell percentage we observed. Th17 and Th1 cells were, therefore, analyzed longitudinally in 18 MS patients followed up for 6 to 15 months.

Among the patients followed up for 12 months, the Th17 cell percentage decreased in nine patients whose disease status changed from AMS to IMS (Fig 2A) but remained stable in six IMS patients whose disease status did not change (see Fig 2B). In contrast, the Th1 cell percentage did not decrease significantly in patients whose disease status changed from AMS to IMS (see Fig 2C), and changed randomly in IMS patients with stable disease (see Fig 2D).

The association between a greater Th17 cell percentage and clinical disease activity was further confirmed by the data from five patients that changed disease status from AMS to IMS or from IMS to AMS several times or had multiple relapses (see Fig 2E). The Th17 cell percentage was always greater in AMS, lower or undetectable in IMS, and increased again during a subsequent relapse. The Th17 cell percentage persistently remained high in the patient who had several subsequent relapses (P5 of Fig 2E). By contrast, the Th1 cell percentage fluctuated randomly during the changes of disease activity (data not shown).

Interferon-β Inhibited Th17, but not Th1, Cells in Both Multiple Sclerosis Patients and Healthy Subjects

This analysis included five MS patients and four HS studied in vitro, and five MS patients followed up pro-

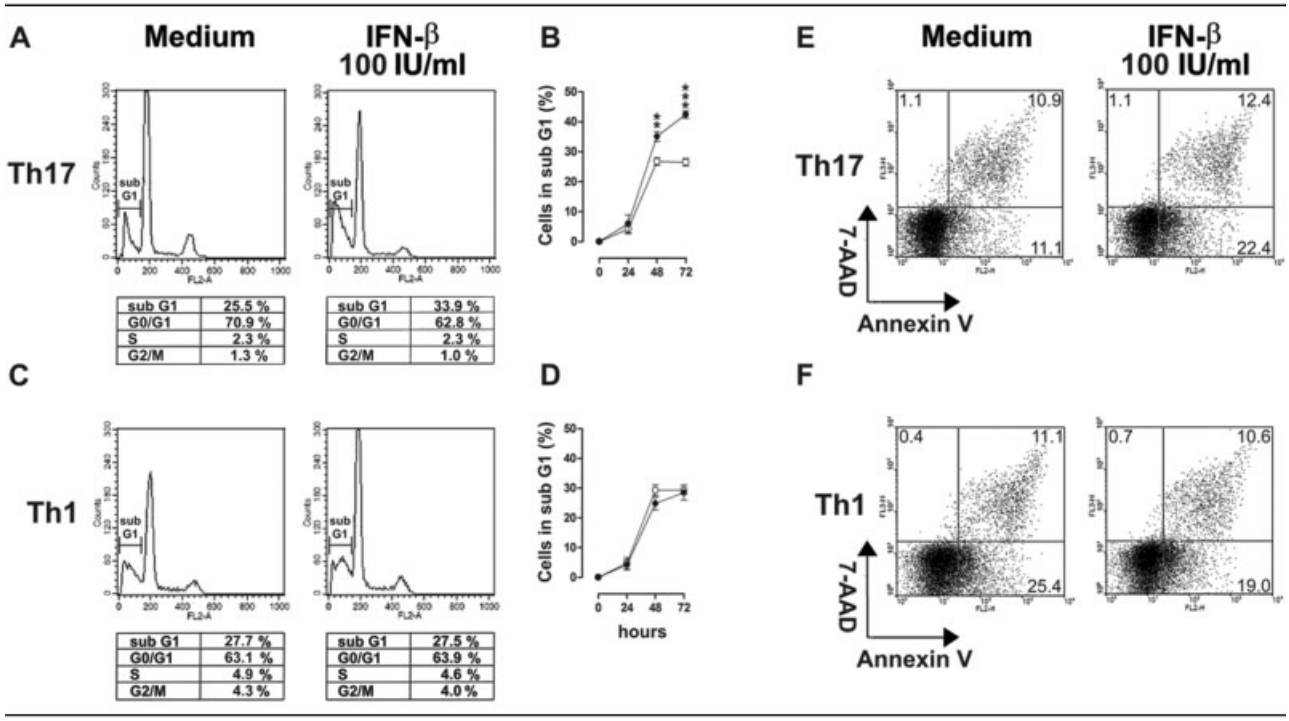

Fig 4. Interferon (IFN)-β enhances in vitro apoptosis of T-helper 17 (Th17) but not that of Th1 lymphocytes generated from the peripheral blood mononuclear cells of healthy subjects. (A–D) Cell-cycle analysis by propidium iodide staining of 5-day polarized Th17 (A, B) and Th1 (C, D) cells restimulated with anti-CD3 monoclonal antibody with (black squares) *or without* (white squares) *IFN-β-1a (100IU/ml). (A, C) Histograms show 72-hour-treated cells from one representative experiment out of four. The percentage of cells in the various phases of the cell cycle (sub-G1, G0/G1, S, and G2/M) is indicated in the boxes below each histogram; (B, D) graphs show the mean ± standard deviation of the percentage of hypodiploid cells (sub-G1 compartment) of four independent experiments. **$p < 0.01$; ***$p < 0.001$, Mann–Whitney test. (E, F) Annexin V/7-amino-actinomycin D (7-AAD) double staining of Th17 (E) and Th1 (F) cells activated and treated as in (A–D). Dot plots show 72-hour-treated cells from one experiment of four independently performed. In each dot plot, numbers in quadrants indicate the percentage of cells.*

spectively after starting IFN-β treatment. In vitro, in anti-CD3 MAb-stimulated $CD4^+$ cells from AMS patients, IFN-β reduced in a dose-dependent manner (on average, by 2.6-fold at 10IU/ml, 4.0-fold at 100IU/ml, and 5.1-fold at 1000IU/ml) the percentage of Th17 cells (Fig 3A, B) but did not significantly affect that of Th1 cells (see Fig 3A, C).

In vitro induced Th subsets were generated from PBMCs from four HS. Under the Th17 polarizing condition, IFN-β reduced in a dose-dependent manner the percentage of induced Th17 cells (see Fig 3D, E) but did not affect that of Th1 cells (see Fig 3D, F). IFN-β was also unable to reduce the percentage of Th1 cells generated under the Th1 polarizing condition (see Fig 3G, I).

Activated T cells polarized under Th0, Th1, or Th17 conditions consisted in more than 97% of $CD3^+$ cells as evaluated by fluorescence-activated cell sorting (data not shown). All the described experiments have been done on $CD4^+$-gated lymphocytes, and in the polarizing culture, most IL-17–producing cells were $CD4^+$ (see Supplementary Fig 1). We observed, however, a low percentage of $CD8^+$ lymphocytes producing IL-17 in the polarizing cultures (see Supplementary Fig 1).

Five patients started IFN-β treatment some weeks after the relapse. At the next evaluation of Th1 and Th17 cells, after 6 to 12 months of subcutaneous IFN-β therapy and when all the patients had inactive MS, the percentage of Th17 cells was reduced compared with baseline, reaching the level found in HS (see Fig 2A, gray squares). In contrast, the Th1 cell percentage was almost unchanged (see Fig 2C, gray squares).

Interferon-β Enhanced Cell Apoptosis In Vitro in Th17 Cells, But Not in Th1 Cells

IFN-β–induced apoptosis of Th17 and Th1 cells generated from HS PBMCs was studied after they had been polarized. Cell-cycle analysis showed that the percentage of hypodiploid cells (sub-G1 compartment) was significantly increased by IFN-β at 48 and 72 hours in the Th17 (Fig 4A, B) but not in the Th1 polarizing condition (see Fig 4C, D). In the Th17 polarizing condition, the mean percentage of sub-G1 cells was 26% without IFN-β and increased by almost

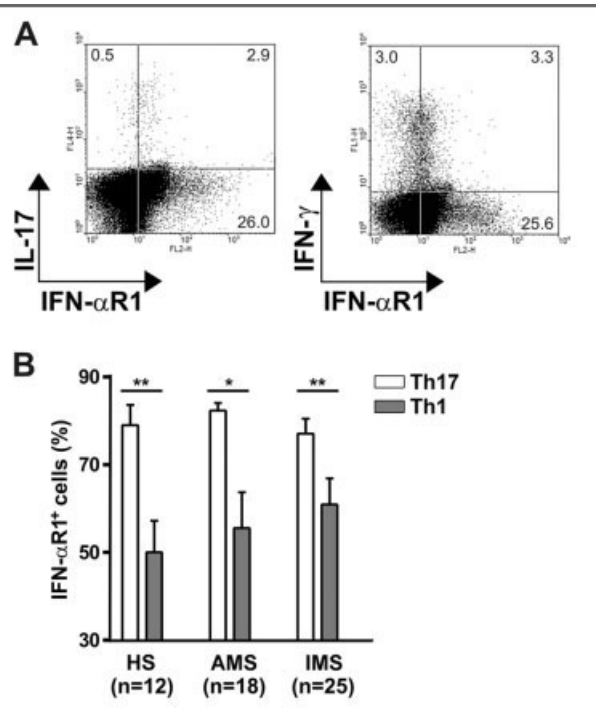

Fig 5. The percentage of cells expressing type I interferon receptor chain 1 (IFN-αR1) was significantly greater in T-helper 17 (Th17) than in Th1 lymphocytes. Peripheral blood mononuclear cells of healthy subjects (HS) and clinically active multiple sclerosis (AMS) or clinically and magnetic resonance imaging inactive MS (IMS) patients were stimulated as in Figure 1, and analyzed by fluorescence-activated cell sorting (FACS) for IFN-αR1 surface expression and IFN-γ and interleukin (IL)-17 intracellular production. (A) Dot plots show representative IFN-γ/IFN-αR1 or IL-17/IFN-αR1 staining on $CD4^+$ cells from one AMS patient. The quadrants were selected to include in the lower left quadrants more than 98% of cells stained with Alexa Fluor 647–conjugated IgG1-negative control and with mouse IgG1 followed by biotin-conjugated goat anti–mouse antibody followed by phycoerythrin conjugated streptavidin. (B) Bars show the means ± standard deviations of the percentage of Th17 (white bars) *or Th1* (gray bars) *cells expressing IFN-αR1 in HS, AMS patients, and IMS patients. The percentage was calculated using this formula: % upper right quadrant cells/(% upper left quadrant cells + % upper right quadrant cells) × 100.* $^*p < 0.05$; $^{**}p < 0.01$, *Mann–Whitney* U *test.*

2-fold (41%) with IFN-β (see Fig 4B). Annexin V and 7-AAD double staining confirmed that IFN-β increased the percentage of early apoptotic (annexin V^+ 7-AAD^-) cells in the Th17 (see Fig 4E) but not in the Th1 polarizing condition (see Fig 4F).

More Th17 Compared with Th1 Cells Expressed the R1 Chain of the Type I Interferon Receptor

To investigate the sensitivity of Th17 and Th1 cells to IFN-β, we investigated the surface expression of the IFN-αR1 chain in PBMCs from 12 HS and 43 MS patients (18 AMS and 25 IMS). In all three groups, the percentage of cells expressing IFN-αR1 was significantly greater in Th17 than in Th1 cells, without any difference among study groups (Fig 5A, B). The longitudinal follow-up of 18 MS patients also showed the higher expression of IFN-αR1 on Th17 cells (data not shown).

Th17 Compared with Th1 Cells Expressed More Interferon-αR1 during Their Differentiation, and This Expression Correlated with Their Higher Sensitivity to Interferon-β

To account for the different response of Th1 and Th17 cells to IFN-β, we studied in parallel on PBMCs from four HS the kinetics of the surface expression of IFN-αR1 and IFN-αR2, and the IFN-β–dependent STAT1 phosphorylation. PBMCs activated under Th0, Th1, and Th17 conditions were harvested daily and divided into two aliquots, one for IFN-αR expression and the other for STAT1 phosphorylation. IFN-αR2 was highly expressed under all polarizing conditions (Fig 6A). In contrast, IFN-αR1, which was highly expressed on resting T cells, decreased just after activation under all polarizing conditions. Thereafter, IFN-αR1 increased on developing Th17 cells starting on day 2 and remained highly expressed until day 4, unlike under Th0 and Th1 polarizing conditions where its expression remained at a significantly lower level (see Fig 6B, C).

Every day polarized cells were treated or not with 100 IU/ml of IFN-α for 15 minutes. Developing Th17 cells displayed a progressively increasing IFN-β–dependent STAT1 response that peaked at day 4 (see Fig 6D, black squares). IFN-β–dependent STAT1 phosphorylation was higher in developing Th17 than in developing Th0 or Th1 cells, and showed a significant positive correlation with the IFN-αR1 expression ($R = 0.78$; $p < 0.00001$, Spearman's rank-order correlation) (see Fig 6B, black squares). At day 4 (see Fig 6D, E), the Th17 IFN-β–dependent STAT1 response was twofold greater than that shown by developing Th0 and Th1 cells. This was also confirmed by fluorescence-activated cell sorting analysis of 5-days polarized Th17 and Th1 cells treated or not with IFN-β (see Fig 6F). Dose–response analysis of STAT1 phosphorylation showed that Th17 cells were sensitive to much lower doses of IFN-β than Th1 cells. In fact, the Th1 cell response started from 100IU/ml IFN-β compared with that of Th17 cells, which started from 0.1IU/ml IFN-β (see Fig 6G).

Discussion

We provide evidence that an increase of PB Th17 lymphocytes is associated with disease activity in MS. Th17 lymphocytes, barely detectable in PBMCs from HS and IMS patients, increased in patients with a clin-

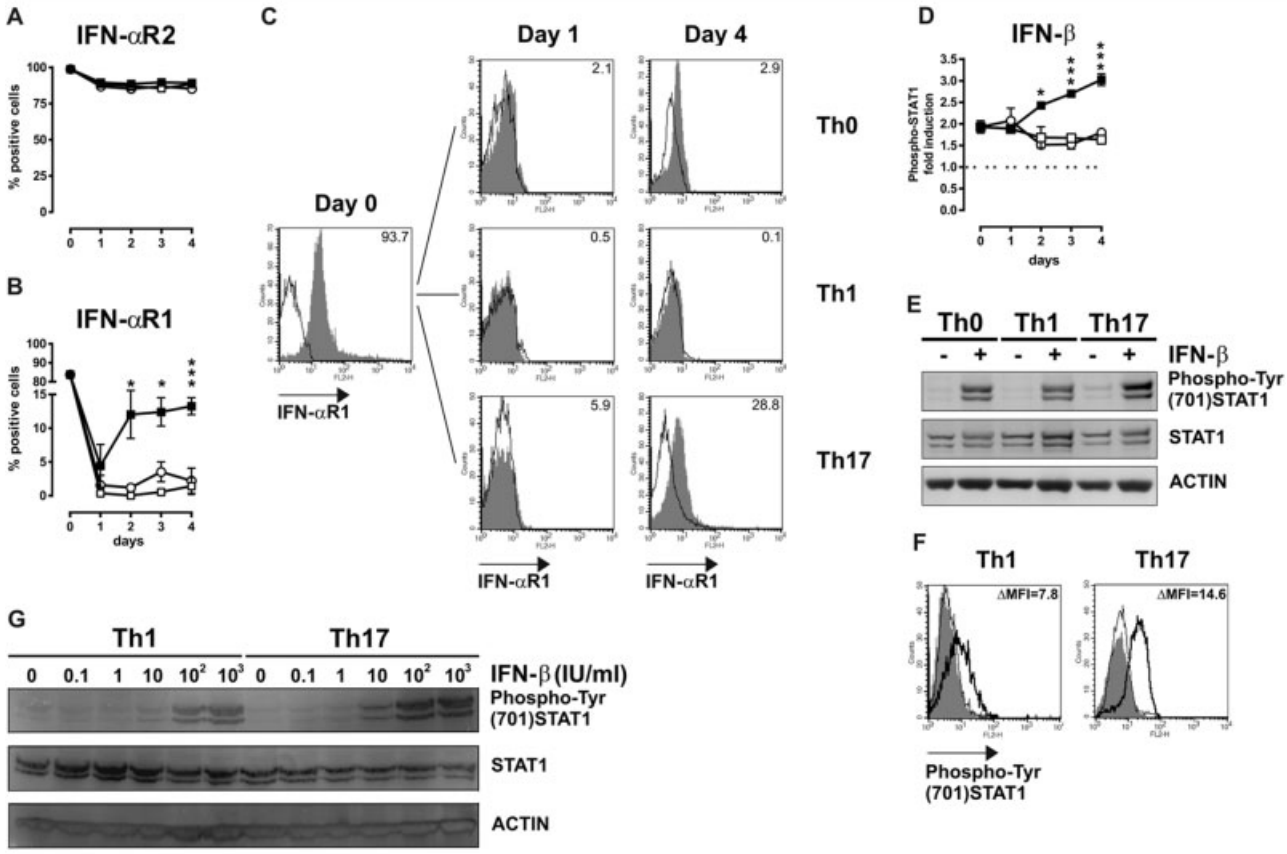

Fig 6. T-helper 17 (Th17) compared with Th1 cells express more type I interferon receptor chain 1 (IFN-αR1) during their differentiation, and this expression correlates with their higher sensitivity to IFN-β. Peripheral blood mononuclear cells of healthy subjects (HS) were activated for 5 days under Th0, Th1, or Th17 polarizing conditions. (A–C) Fluorescence-activated cell sorting (FACS) analysis of the kinetics of (A) IFN-αR chain 2 (IFN-αR2)– or (B) IFN-αR1–positive cell percentage (means ± standard deviations) in Th0 (white circles), *Th1* (white squares), *and Th17* (black squares) *lymphocytes from four experiments. (C)* Open histograms *represent the background of mouse IgG1-negative control;* gray histograms *represent the IFN-αR1 expression;* numbers *indicate the percentage of positive cells. (D, E) Kinetics of IFN-β–induced signal transducer and activator of transcription 1 (STAT1) phosphorylation studied with Western blot in polarized Th0* (white circles), *Th1* (white squares), *and Th17* (black squares) *treated with or without IFN-β-1a. (D) Means ± standard deviations of the fold induction of IFN-β-1a induced STAT1 phosphorylation from four blots. (E) One representative blot at day 4. (F) FACS analysis of STAT1 phosphorylation in 5-day polarized Th1 ($CD4^+$ IFN-gamma gated cells, left) and Th17 ($CD4^+$ IL-17 gated cells, right) treated with or without IFN-β-1a.* Gray histograms *represent the negative control background;* open histograms with thin line *represent the phospho-STAT1 expression untreated;* open histograms with thick line *represent the phospho-STAT1 expression in IFN-β–treated cells from one experiment out of three. Numbers in quadrants indicate mean specific fluorescence intensity (ΔMFI) of IFN-β–induced STAT1 phosphorylation, calculated by subtracting the MFI of untreated cells from that of IFN-β–treated cells. (G) Dose response of STAT1 phosphorylation in Th1 and Th17 lymphocytes treated on day 4 with increasing doses of IFN-β-1a. One representative Western blot out of three is shown.* $^*p < 0.05$; $^{**}p < 0.001$; *Mann–Whitney U test.*

ical exacerbation. In AMS patients, the number of $CD4^+$ cells that simultaneously produce IL-17 and IFN-γ also increases. This subset has been considered as an intermediate population in the differentiation from Th17 to Th1.[30] No data are currently available to clarify their function. Repeated longitudinal analysis of each patient showed that Th17 lymphocyte percentage always paralleled disease activity changes. In contrast with Th17 cells, Th1 cells, present in PBMCs at a greater percentage, did not increase in patients with AMS or IMS.

These data are supportive of a prominent role of Th17 compared with Th1 lymphocytes associated with MS exacerbations. Moreover, we found that a proportion of Th17 cells in AMS were MBP specific, suggesting that, like in EAE, Th17 cells can be encephalitogenic in MS. The frequency of MPB-specific IL-17–producing cells was in the range reported for MPB-specific IFN-γ–producing cells in MS patients.[31] In addition, the increased percentage of IL-22–producing $CD4^+$ T cells in AMS strengthens the association between Th17 and MS exacerbation, as this cytokine is

related to the Th17 subset.[8] Conversely, the expression of IL-23R, which did not vary significantly among HS, AMS patients, and IMS patients, was similar in Th17 and Th1 (not shown) cells, in accordance with Acosta-Rodriguez and colleagues'[32] report. It appears, therefore, that IL-23R is not a Th17-related molecule in MS. The Th17 cell subset was present at a low percentage in HS (0.2–1%) but increased more than ninefold in AMS. Other studies in Crohn's disease or uveitis have confirmed the clinical relevance of this small T-cell subset.[33,34] Consistent with our data are the reports of increased IL-17 messenger RNA in PBMCs of patients with MS and in CNS lesions,[11–13] and of an expansion of Th17 lymphocytes in EAE.[7–10]

Because IFNs play important roles in T-lymphocyte homeostasis[35,36] and IFN-β was the first drug approved for chronic MS treatment,[22] we also studied the sensitivity of these T-lymphocyte subsets to IFN-β. In T cell receptor-stimulated PBMCs from AMS patients, in vitro IFN-β decreased the Th17 cell number but did not affect that of Th1 cells. Th17, but not Th1, cells polarized from PBMCs of HS were also sensitive to the effect of IFN-β. This is likely due to the induction of Th17 cell apoptosis, because IFN-β directly induces in Th17 cells the activation of STAT1, a pro-apoptotic transcription factor.[36] Because IFN-β sends survival signals to T cells,[37] future research will aim to elucidate the signaling events of apoptosis or survival, and this should help to clarify the different effects of IFN-β in specific Th subsets.

Because IFN-γ has different effects on T lymphocytes depending on the surface expression of IFN-γR2 chain,[36] we hypothesized that the different sensitivity of these two Th subsets to IFN-β could be ascribed to a different expression of their cell surface receptor. We, therefore, analyzed type I IFN receptor (IFN-αR) expression on Th17 and Th1 lymphocytes from HS and MS patients. IFN-αR is made of two subunits, IFN-αR1 and IFN-αR2.[38] Because our data showed that IFN-αR2 is not modulated in $CD4^+$ T lymphocytes, we hypothesize that their response to type I IFNs is mainly dependent on the surface expression of IFN-αR1. Our data showed that Th17 cells express a greater level of IFN-αR1 than Th1 cells. In addition, Th17 cells, unlike Th1 cells, are sensitive to the inhibitory effect of IFN-β. Because IFN-αR1 is needed for type I IFN signal transduction,[38] the level of IFN-αR1 on Th17 cell surface might account for their higher sensitivity to IFN-β. We have shown that resting T cells expressed high IFN-αR1 surface levels, which rapidly declined on cell activation under the Th0, Th1, and Th17 polarizing conditions, and increased again only during Th17 differentiation. IFN-αR1 downregulation on activation is presumably due to its internalization.[35] The progressive increase of IFN-αR1 on Th17 cells suggests that a perturbation of its internalization, probably induced by IL-23 or other cytokines involved in Th17 differentiation, takes place during their development.

In addition, the sensitivity to the IFN-β/STAT1 pathway of Th17 polarized cells progressively increased during their differentiation, whereas Th1 polarized cells displayed a lower sensitivity to IFN-β/STAT1 signaling, which remained unchanged during their development. During Th17 polarization, a positive correlation exists between the increase of IFN-αR1 reexpression and the IFN-β–induced STAT1 phosphorylation. The lower expression of IFN-αR1 on Th0 and Th1 allowed a limited IFN-β–induced STAT1 phosphorylation and did not trigger apoptosis. In contrast, the higher expression of IFN-αR1 on Th17 cells resulted in a stronger IFN-β–induced STAT1 phosphorylation, leading to apoptosis.[36] Compared with Th1, Th17 cell sensitivity to IFN-β–induced STAT1 phosphorylation was up to 10- to 20-fold greater and already 2-fold at the dose of 100IU/ml, which equates to the serum level found during chronic IFN-β MS treatment.[39] Finally, because the high level of IFN-αR1 expression on Th17 was observed in HS, AMS patients, and IMS patients, we hypothesize that this is a constitutive feature of this lymphocyte subset, independent of disease state or activity.

IFN-β was the first drug approved for chronic MS treatment. It reduces both clinical and MRI signs of disease activity.[22] However, the mechanism of action of IFN-β for MS has not yet been fully elucidated. IFN-β may affect T-lymphocyte cytokine production and resistance to apoptotic signals, or might inhibit matrix metalloproteinase production, adhesion molecule, and chemokine expression.[18,40] The inhibition of Th17 lymphocytes, associated with EAE development[7–10] and with MS exacerbations, as we have shown here, has added a further item to the long list of the pleomorphic effects of IFN-β in this disease. It is, therefore, of interest that Th17 cells from MS patients cross in vitro the blood–brain barrier more easily than Th1 cells[41] and that, in EAE, Th17 cells infiltrate the brain parenchyma only when Th17 cells are increased compared with Th1 cells.[42] IFN-β reduces lymphocyte migration in vitro;[18,40] therefore, the reduction in T-lymphocyte migration into the brain and the induction of Th17 cell apoptosis could be key mechanisms explaining the efficacy of IFN-β in counteracting MS disease activity.

However, our results do not rule out both the contribution of Th1 lymphocytes to MS pathology and an antigen-presenting cell-mediated effect of IFN-β on Th17 lymphocytes. The importance of Th1 in MS is confirmed by the dramatic effect of administering IFN-γ to MS patients,[21] and the ability of IFN-β to indirectly inhibit Th17 by activating antigen-presenting cells has been shown in EAE.[43,44]

Although all of our experiments were done on gated $CD4^+$ T lymphocytes, we, as well as others,[13] also observed a few IL-17–producing $CD8^+$ T lymphocytes. Because $CD8^+$ lymphocytes are an important part of the immune repertoire and predominate in damaging axons in CNS MS lesions,[18] this subset certainly deserves further investigation. In conclusion, because both Th1 and Th17 lymphocytes have been shown to be encephalitogenic in EAE,[19] possibly in different phases of the disease, further study is needed to investigate the interplay between these Th cell subsets in CNS tissue damage, their distribution, and that of IFN-αR1 in active brain lesions of MS patients. In addition, the pleomorphic effects of IFN-β on lymphocytes, macrophages, and microglia need to be investigated in MS patients before and after starting IFN-β treatment.

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