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Th17 Cells in Multiple Sclerosis Express Higher Levels of JAK2, Which Increases Their Surface Expression of IFN- γ R2

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IFN- β inhibits the expansion of Th17 cells in active multiple sclerosis (AMS), and this might contribute to improve the clinical symptoms. The effectiveness of this inhibition, however, requires intact IFN- γ signaling in T cells. In this study, we report that both mRNA and cell surface expression of the signaling chain of the IFN- γ receptor (IFN- γ R2) and its cognate tyrosine kinase JAK2 are enhanced in peripheral blood Th17 cells and clones from patients with AMS compared with those with inactive multiple sclerosis (IMS) or healthy subjects (HS). IFN- γ decreased the frequency of Th17 peripheral cells and proliferation of Th17 clones from AMS patients. Stimulation of PBMCs from HS in Th17-polarizing conditions resulted in the enhancement of JAK2 expression and accumulation of cell surface IFN- γ R2. The role of JAK2 in the modulation of IFN- γ R2 was demonstrated as its transduction prevented rapid internalization and degradation of IFN- γ R2 in JAK2-deficient γ 2A cells. In conclusion, these data identify JAK2 as a critical factor that stabilizes IFN- γ R2 surface expression in Th17 cells from AMS patients, making them sensitive to IFN- γ . These data may have clinical implications for a better use of IFNs in multiple sclerosis and possibly other inflammatory diseases. *The Journal of Immunology*, 2012, 188: 1011–1018.

T helper 17 lymphocytes play a central role in the pathogenesis of many autoimmune disorders, such as multiple sclerosis (MS) and its murine model experimental autoimmune encephalomyelitis (EAE), psoriasis, and rheumatoid arthritis (1, 2).

The inhibition of Th17 cell expansion in active MS has been shown (1, 3) to be one of the mechanisms of action of IFN- β in this disease, and it could contribute to improve the clinical symptoms of MS. Not all MS patients, however, respond to IFN- β , and it would be useful to find markers to identify the responder patients before starting the treatment.

IFN- β inhibition of the Th17 cell response requires intact IFN- γ signaling (3), and thereby the mechanism by which IFN- γ signaling is regulated in Th17 cells from MS patients is of clinical interest. The biologic effects of IFN- γ in T cells require the cell surface expression of IFN- γ R, which is influenced by both intrinsic characteristics and environmental signals. IFN- γ R receptor consists of the ligand binding IFN- γ R1 chain and the IFN- γ R2 signaling chain. The IFN- γ /STAT1 pathway is predominantly activated in naive and Th2 cells expressing the IFN- γ R2 signaling chain. Those cells are thus sensitive to the antiproliferative effect of IFN- γ , whereas Th1 cells, which do not express this chain, are resistant (4).

In this study we have evaluated mRNA and cell surface expression of IFN- γ R2 in Th1 and Th17 peripheral cells and clones from untreated MS patients and from healthy subjects (HS). IFN- γ R2 was accumulated on the surface of Th17 cells from subjects with active MS (AMS) only. The expression of IFN- γ R2 correlated with suppressive IFN- γ responses and increased expression of mRNA and protein of the IFN- γ R2 cognate tyrosine kinase JAK2. The role of JAK2 in the stabilization of surface IFN- γ R2 was evaluated by using the JAK2-deficient cell line γ 2A. Ectopic expression of JAK2 stabilized IFN- γ R2 on the surface of Th17 cells making them susceptible to the antiproliferative effect of IFN- γ .

Materials and Methods

Human subjects

PBMCs were isolated from heparinized venous blood from patients with relapsing-remitting MS (5) at the San Luigi Gonzaga University MS center (Orbassano, Italy), and from age- and gender-matched volunteer HS. The

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Abbreviations used in this article: AMS, active multiple sclerosis; BFA, brefeldin A; CSIP, cell surface immunoprecipitate; EAE, experimental autoimmune encephalomyelitis; HS, healthy subject; IMS, inactive multiple sclerosis; IS, surface; IZ, internalized; MFI, mean fluorescence intensity; MRI, magnetic resonance imaging; MS, multiple sclerosis.

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institutional review boards of the participating center approved the study, and all subjects gave written informed consent. Patients with AMS had a clinically documented exacerbation within the last 10 d, but were not on corticosteroids. Patients with inactive MS (IMS) had no exacerbations in the last 3 mo, and no new PD/T2 or gadolinium-enhancing lesions on two subsequent magnetic resonance imaging (MRI) scans performed 3 mo apart. Brain MRI scans (24 contiguous axial slices, 5 mm thick, of conventional PD/T2 and T1 spin-echo, pre- and postcontrast sequences) were obtained using a predefined repositioning protocol (6). MRI evaluations were performed by investigators totally unaware of the patients' clinical characteristics and treatment.

Media

The culture medium was RPMI 1640 (BioWhittaker, Walkersville, MD) containing gentamicin (Schering-Plough, Milan, Italy) with 10% FBS (Invitrogen, Carlsbad, CA), designated medium. All in vitro cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere.

Generation of T cell clones from AMS and HS

The culture medium used was RPMI 1640 supplemented with 2 mmol/l L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 2×10^{-5} mol/l 2-ME (Invitrogen), and 10% FCS HyClone (Life Technologies Laboratories, Grand Island, NY) and recombinant human IL-2 (Eurocetus, Milan, Italy). Clones from HS were generated from PBMCs in Th1 (IL-12, 20 ng/ml, plus neutralizing anti-IL-4 mAb 10 μ g/ml) or Th17 (IL-23, 50 ng/ml, plus a mix of the neutralizing anti-IFN- γ R1 and anti-IFN- γ γ R99, γ R38, γ 123 ascites diluted 1:100 mAbs) polarizing conditions. Cells were seeded (0.3 cells/well) in microwells in the presence of 2×10^5 irradiated (5000 rad) PBMCs, PHA (0.5% vol/vol; Life Technologies Laboratories), and IL-2 (50 U/ml). At weekly intervals, 2×10^5 irradiated PBMCs and IL-2 were added to each microculture to maintain the expansion of growing clones (7). For AMS T cell clones, peripheral T cells were obtained from patients upon the approval of the local ethical committee. T cells were cloned under limiting dilution, as described above. In a few cases, Ag-specific T cell lines were generated by stimulating PBMCs from AMS patients with recombinant myelin oligodendrocyte glycoprotein for 7 d in the presence of IL-2 (50 U/ml). After two weeks, T cell blasts were cloned by limiting dilution as described above. To elicit cytokine production by T cell clones, T cell blasts were cultured at 10^6 /ml and cultured for 36 h in the presence of PMA (10 ng/ml) plus ionomycin (200 ng/ml). Cell-free supernatants were collected and assayed in duplicate for IFN- γ and IL-17 content by commercial ELISA assays (Bio-Source International, Camarillo, CA). Supernatants showing IFN- γ or IL-17 levels 5 SD over the mean levels in control supernatants derived from irradiated APC alone were regarded as positive. CD4⁺ T cells clones able to produce IFN- γ were categorized as Th1, clones able to produce IL-17 as Th17 (Supplemental Table I). Once established, T cell clones were analyzed by FACS for their intracellular expression of IFN- γ , IL-17, and for Tbet and ROR γ T (eBioscience, San Diego, CA; Supplemental Table I).

T lymphocyte activation and polarization

PBMCs from HS were activated with plate-coated anti-CD3 (10 μ g/ml) and soluble anti-CD28 mAbs (1 μ g/ml; BD Biosciences, San Diego, CA) for 5 d (day 0 to 4) in the presence of medium (nonpolarizing condition), IL-12 (20 ng/ml; Peprotech, Rock Hill, NJ), and neutralizing anti-IL-4 mAb (10 μ g/ml; 34019; R&D Systems, Minneapolis, MN; *Th1 condition*), or IL-23 (50 ng/ml, R&D Systems) plus a mix of neutralizing anti-IFN- γ R1 and anti-IFN- γ (γ R99, γ R38, γ 123 ascites diluted 1:100) (8) mAbs (*Th17 condition*).

FACS analysis

For intracellular cytokine detection, PBMCs, clones, or polarized Th cells were cultured for 5 h with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (BFA, 10 μ g/ml, Sigma-Aldrich, St. Louis, MO), and then stained extracellularly with PerCP-labeled anti-CD4 (BioLegend, San Diego, CA), with anti-IFN- γ R2 mAb, with anti-IFN- γ R1 (U.S. Biological, Swampscott, MA) or anti-JAK2 mAb (Cell Signaling Technology, Danvers, MA), followed by PE-conjugated goat anti-mouse Ig, and intracellularly with alexa fluor 647-conjugated anti-IL-17 and FITC-labeled anti-IFN- γ mAbs (all from BioLegend), as described previously (1, 9). IFN- γ R2 stability was analyzed by incubating JAK2-deficient γ 2a or JAK2-expressing γ 2aJ2 cells (10) with or without cycloheximide (50 μ g/ml, Sigma-Aldrich) at 37°C. At different time points, aliquots of cells were harvested and stained with anti-IFN- γ R2 mAb at 4°C for 30 min, followed by PE-conjugated rabbit anti-mouse Ig. IFN- γ R2 stability was calculated by dividing the mean fluorescence intensity (MFI) of the untreated cells by the MFI of cycloheximide-treated cells. To evaluate IFN- γ R2 internali-

zation, γ 2a and γ 2aJ2 cells were labeled with anti-IFN- γ R2 mAb at 4°C, and then incubated for the indicated times at 37°C, rapidly washed and incubated at 4°C for 30 min with PE-conjugated rabbit anti-mouse Ig. Internalization was calculated as follows: surface (IS)/internalized (IZ) = $(MFI_{t=0} - MFI_{t=n})/MFI_{t=n}$, where $MFI_{t=0}$ stands for surface expression at time 0 and $MFI_{t=n}$ stands for surface expression at each time point. All experiments were performed with a FACSCalibur flow cytometer (Becton Dickinson). Each plot represents the results from 50,000 events.

Microarray analysis

CD4⁺ Th17 cell clones were generated from AMS patients and HS peripheral blood, and microarrays were performed as described (11). RNA from different samples was amplified and labeled with Digoxigenin-UTP (Applied Biosystems); 10 μ g of Digoxigenin-labeled cRNA was hybridized to the Human Genome Survey Microarray and read with a 1700 Chemiluminescent Microarray Analyzer (Applied Biosystems). Expression Array System software (Applied Biosystems) was used to analyze the microarray images, and microarrays with a normalized signal intensity >5000 and a median background <600 were analyzed and normalized with Spotfire and Intergomics software (Spotfire). Class comparisons expressed as Benjamini-Hochberg false discovery rates were obtained by using parametric tests (LIMMA) after log transformation. Each sample was analyzed three times. Microarray data are available from the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) database under accession number GSE22642.

Real-time PCR

PBMCs activated under Th17 and Th1-polarizing conditions were recovered and purified at d 2 and d 4 with the IL-17 or IFN- γ Secretion Assay kit (Miltenyi Biotec, Bergish Gladbach, Germany), respectively, according to the manufacturer's instructions. Total RNA was isolated from the recovered cells or from PMA plus ionomycin stimulated T cells clones with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized by using an oligo-dT primer (dT15; Invitrogen, Carlsbad, CA). Transcripts were quantified by real-time PCR on the I-Cycler (Biorad) with the SYBR Green qPCR Detection System (Invitrogen). The following primers were purchased from Primm (Milan, Italy): JAK2: GCTCAGTGGCGGCATGAT-fw and CACTGCCATCCCAAGACATTC-rw; IFN- γ R2: ACAAGTCCAGGCACAACACTGCTTTG-fw and TTCAGGACCAGGAAGAAACAGGCT-rw; b2-microglobulin: CATTCTGAAGCTGACAGCATTC-fw and TGCTGGATGAGCTGAGTAAACC-rw. For each sample, mRNA abundance was normalized to the amount of the housekeeping gene β 2-microglobulin.

Western blots

To analyze IFN- γ R2 expression, total proteins were extracted from Th0, Th1, and Th17 cells harvested on d 2 and 4 from activation and analyzed as described (12) with anti-IFN- γ R2 mAb (Usbiological, Swampscott, MA). Actin was used as a control for equal protein loading. Cell surface immunoprecipitates (CSIP) of IFN- γ R2 were performed by incubating 5×10^6 γ 2a or γ 2aJ2 cells with anti-IFN- γ R2 mAb in PBS at 4°C for 3 h. The cells were washed three times with cold PBS and lysed in 1 ml triton lysis buffer. The lysates were clarified by centrifugation, and the supernatants were incubated with Protein G beads for 1 h; 20 ml of the immunoprecipitates or the respective lysates were subjected to Western blot. The membrane was probed with anti-IFN- γ R2 mAb, stripped, and reprobed with anti-JAK2 Ab (Upstate Biotechnology, Lake Placid, NY).

Cell proliferation

To analyze IFN- γ antiproliferative effects, Th1 and Th17 clones from AMS were cultured in triplicate in round-bottom 96-well plates in the presence or absence of 1000 U/ml IFN- γ . After 48 h, the cultures were pulsed with 1 μ Ci [³H]TdR (Amersham, Milan, Italy). After 6 h, the cells were harvested and [³H]TdR uptake was evaluated by TopCount Microplate reader (Packard Instrument, Wellesley, MA).

Statistical analysis

Pearson *t* test (GraphPad Prism 3, GraphPad Software) and parametric or nonparametric tests were used.

Results

Analysis of IFN- γ R2 expression on Th17 cells from MS patients

Effective inhibition of the Th17 cell response by IFN- β requires the cooperation of an intact IFN- γ signaling in CD4⁺ cells (3). We

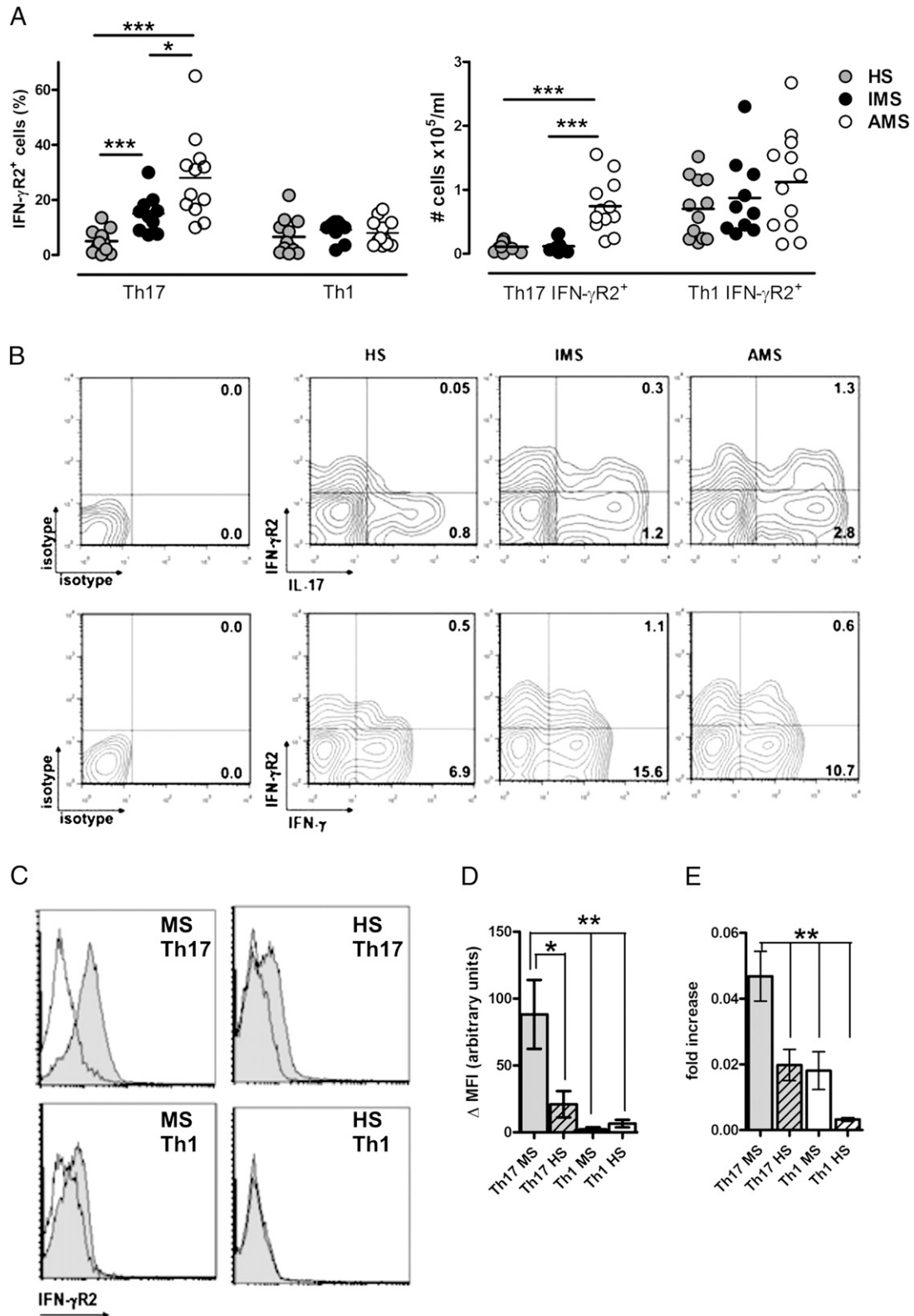


FIGURE 1. Th17 lymphocytes from AMS patients express IFN- γ R2 on their surface. *A* and *B*, PBMCs from HS, AMS, and IMS patients were stimulated for 5 h with PMA, ionomycin and BFA, and surface IFN- γ R2 and intracellular IL-17 and IFN- γ expression was detected by FACS analysis on CD4⁺ cells. *A*, The *left panel* shows the percentage of Th17 and Th1 cells expressing IFN- γ R2 in each subject, calculated using the formula: Absolute number of upper right quadrant cells/(Absolute number of lower right quadrant cells + Absolute number of upper right quadrant cells) \times 100. The *right panel* shows the absolute number of circulating Th17 IFN- γ R2⁺ and Th1 IFN- γ R2⁺ cells. *B*, Contour plots show representative isotype negative controls or IL-17/IFN- γ R2 or IFN- γ /IFN- γ R2 staining; numbers indicate the percentage of cells. *C* and *D*, FACS analysis of IFN- γ R2 expression in Th1 and Th17 clones generated from AMS and HS. *C*, Expression of IFN- γ R2 (gray histograms) and the background of mouse IgG1 negative control (open histograms) are shown for four representative clones. *D*, The graph shows the mean \pm SEM of IFN- γ R2 specific cell-associated mean fluorescence (Δ MFI) from Th1 and Th17 clones generated from three AMS and three HS PBMC. *E*, IFN- γ R2 mRNA expression was evaluated by quantitative RT-PCR in the same Th1 and Th17 clones. Graphs show mean \pm SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, Pearson *t* test.

have previously demonstrated that the sensitivity of T cells to IFN- γ is regulated by their surface expression of the IFN- γ R2 signaling chain (4). We have now compared the expression of IFN- γ R2 in peripheral Th17 cells from MS patients. A similar percentage of peripheral CD4⁺ IFN- γ producing cells (Th1) was observed in HS ($n = 12$) and patients with IMS ($n = 10$) and AMS ($n = 12$). By contrast, the percentage of peripheral CD4⁺ IL-17-producing (Th17) cells in AMS patients increased ~ 3 -fold compared with HS and IMS (Supplemental Fig. 1). The percentage of CD4⁺ cells that produce both IL-17 and IFN- γ was also increased in AMS (Supplemental Fig. 1), even if this expansion was limited compared with that of CD4⁺ IL-17 producing cells in accordance with what we have previously reported (1). In PBMCs from AMS, IMS and HS IFN- γ R2 expression was evaluated on IFN- γ and IL-17-producing CD4⁺ cells by FACS. Cell surface IFN- γ R2 was expressed in a similar low percentage of Th1 cells in all three groups (HS, 6.5 ± 1.9 ; AMS, 8.0 ± 1.4 ; IMS, 9.1 ± 1.1 ; Fig. 1A, 1B). By contrast, it was expressed on a low percentage of Th17 cells in HS (5.0 ± 1.2), whereas this percentage was increased in IMS (14.9 ± 2.1), and to a larger extent in AMS (28.1 ± 4.4 ; Fig. 1A, left panel; 1B). The increase of IFN- γ R2 in Th17 but not in Th1 cells of AMS was confirmed when the absolute number of circulating CD4⁺ cells was also evaluated (Fig. 1A, right panel). The IFN- γ R2 expression was also increased in the small fraction of CD4⁺ IL-17 and IFN- γ -producing cells in AMS (Supplemental Fig. 2). To confirm this observation, Th1 and Th17 clones were generated from AMS and HS (Supplemental Table I). Both mRNA and surface expression of IFN- γ R2 were higher in Th17 than in Th1 clones, regardless of the disease status. IFN- γ R2 was significantly higher in Th17 clones from AMS compared with those from HS (Fig. 1C–E).

To evaluate the sensitivity of AMS Th cells to IFN- γ , PBMCs from five AMS patients were stimulated for 72 h with anti-CD3 mAb with or without IFN- γ (100 and 1000 U/ml), and the percentage of IL-17 or IFN- γ -producing CD4⁺ cells was measured. IFN- γ induced a significant decrease of the percentage of Th17 cells, but did not affect that of Th1 cells (Fig. 2A). The effect of IFN- γ in suppression of the proliferation of T cells was evaluated on Th1 and Th17 clones from AMS cultured with anti-CD3 mAb for 2 d in the absence or presence of IFN- γ (1000 U/ml). IFN- γ did not affect the proliferation of Th1 clones, whereas it reduced that of Th17 clones (Fig. 2B). These data confirm that high IFN- γ R2 expression in Th17 cells sensitizes them to the antiproliferative effects of IFN- γ .

Analysis of JAK2 expression on Th17 cells from MS patients

In addition to their tyrosine kinase activity, JAK proteins have been shown to regulate the stability of their cognate receptors at the cell surface (13–17). As IFN- γ R2 is associated with JAK2 (18), and as IFN- γ signaling in cells partially defective for IFN- γ R2 was restored by overexpression of JAK2 (10), the expression of JAK2 was evaluated in Th17 and Th1 clones from HS and AMS patients. In a large screening done by microarray analysis, JAK2 was found to be significantly higher in Th17 clones obtained from AMS patients than from HS; therefore, we analyzed the JAK2 mRNA by quantitative PCR in Th17 clones. The mRNA expression of JAK2 was significantly higher in the Th17 clones generated from AMS patients than in those from HS (Fig. 3A). FACS analysis of JAK2 confirmed its higher expression in Th17 clones from AMS compared with those from HS, whereas JAK2 expression was low in Th1 clones from both AMS and HS (Fig. 3B). Moreover, FACS analysis of peripheral PBMCs showed that JAK2 was more expressed in CD4⁺ cells from AMS than from HS and IMS, while it was similarly expressed in CD4⁺ cells from AMS, IMS, and

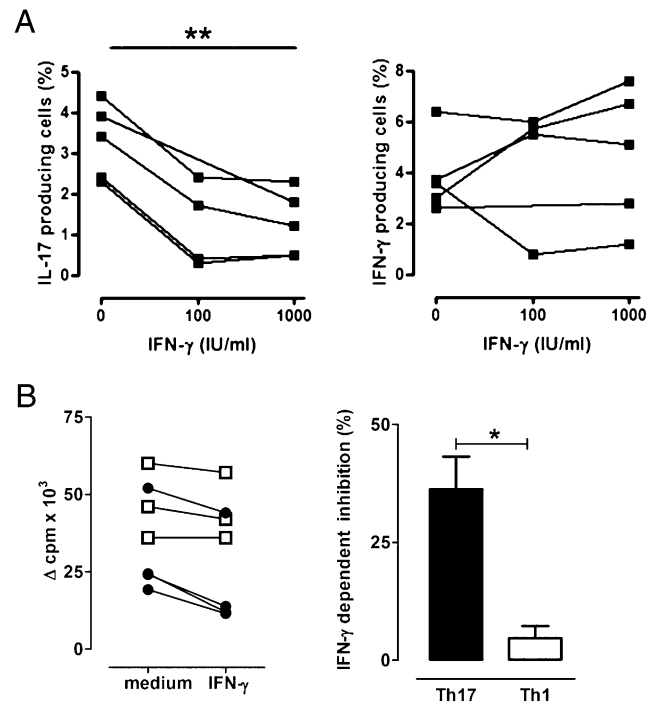


FIGURE 2. Th17 lymphocytes from AMS patients are sensitive to IFN- γ inhibitory effects. **A**, FACS analysis of IL-17 or IFN- γ production from CD4⁺ lymphocytes from AMS patients stimulated for 72 h with anti-CD3 mAb and treated with increasing doses of IFN- γ . Graphs show the percentage of Th17 or Th1 cells in five patients. $**p < 0.01$, $F = 8.444$; one-way ANOVA for repeated measures. **B**, [³H]TdR uptake of Th1 (white) and Th17 (black) clones generated from three AMS patients cultured with or without 1000 U/ml IFN- γ for 48 h. The results are expressed as the Δ cpm measured for each clone (left graph) and as the mean \pm SEM of IFN- γ dependent inhibition of [³H]TdR uptake in Th1 and Th17 clones (right graph). $*p < 0.05$, $**p < 0.01$, Pearson t test.

HS (Fig. 3C). In particular, the percentage of Th17 cells that expressed JAK2 was significantly higher in AMS (46.8 ± 4.1) than HS (6.6 ± 0.9) and IMS (13.3 ± 3.6 ; Fig. 3D, 3E).

IFN- γ R2 and JAK2 increase in Th17 cells from HS depends on activation stimuli

The selective increased expression of IFN- γ R2 in Th17 cells from AMS patients suggested that in vivo chronically stimulated Th17 cells may respond to the antiproliferative effect of IFN- γ . Because the expression of IFN- γ R2 and JAK2 in Th17 cells was low in both HS and IMS, we examined whether their increase is dependent on the activation state of Th17 cells.

IFN- γ R2 surface expression was evaluated on peripheral blood CD4⁺ T cells from five HS stimulated with anti-CD3 plus anti-CD28 mAbs and cultured for 4 d in the absence (nonpolarized) or presence of either IL-12 or IL-23, cytokines polarizing to Th1 or Th17 cells respectively (Supplemental Fig. 3A). IFN- γ R2 was expressed at low levels on the surface of T cells activated in nonpolarizing or Th1 conditions. In nonpolarizing conditions, expression of IFN- γ R2 slightly increased 2 d after activation and then decreased again, whereas in Th1 cells, expression of IFN- γ R2 completely disappeared in keeping with our previous data (4). On Th17 cells, IFN- γ R2 increased from d 1 to d 2, peaked on d 3, and then disappeared (Fig. 4A). By contrast, IFN- γ R1 was constantly expressed at high levels (90–95% IFN- γ R1⁺ cells) in all conditions (Supplemental Fig. 3B). Because IFN- γ R2 is constitutively endocytosed in T lymphocytes (19), we measured protein expression and mRNA for IFN- γ R2 in Th1 and Th17 cells.

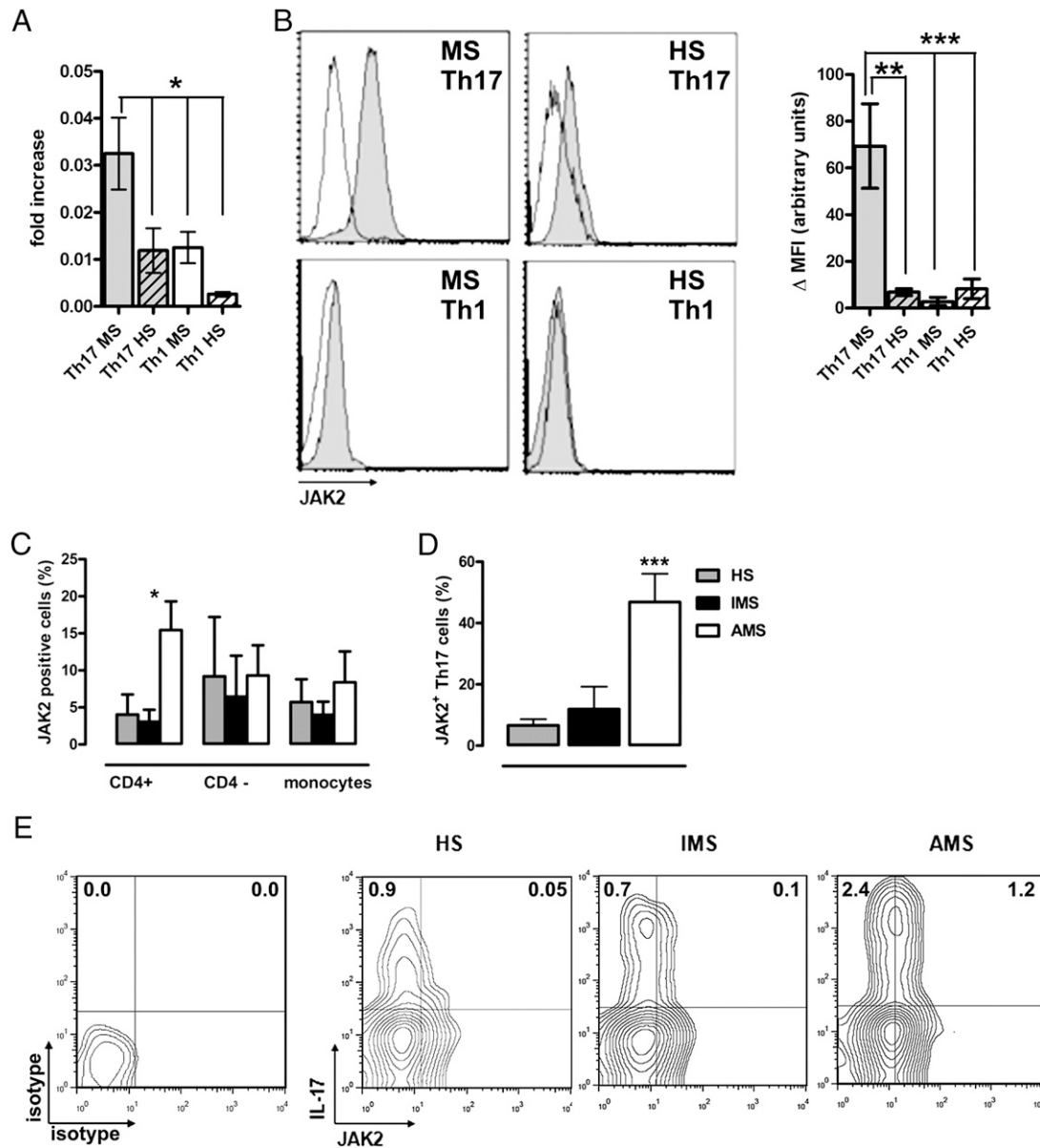


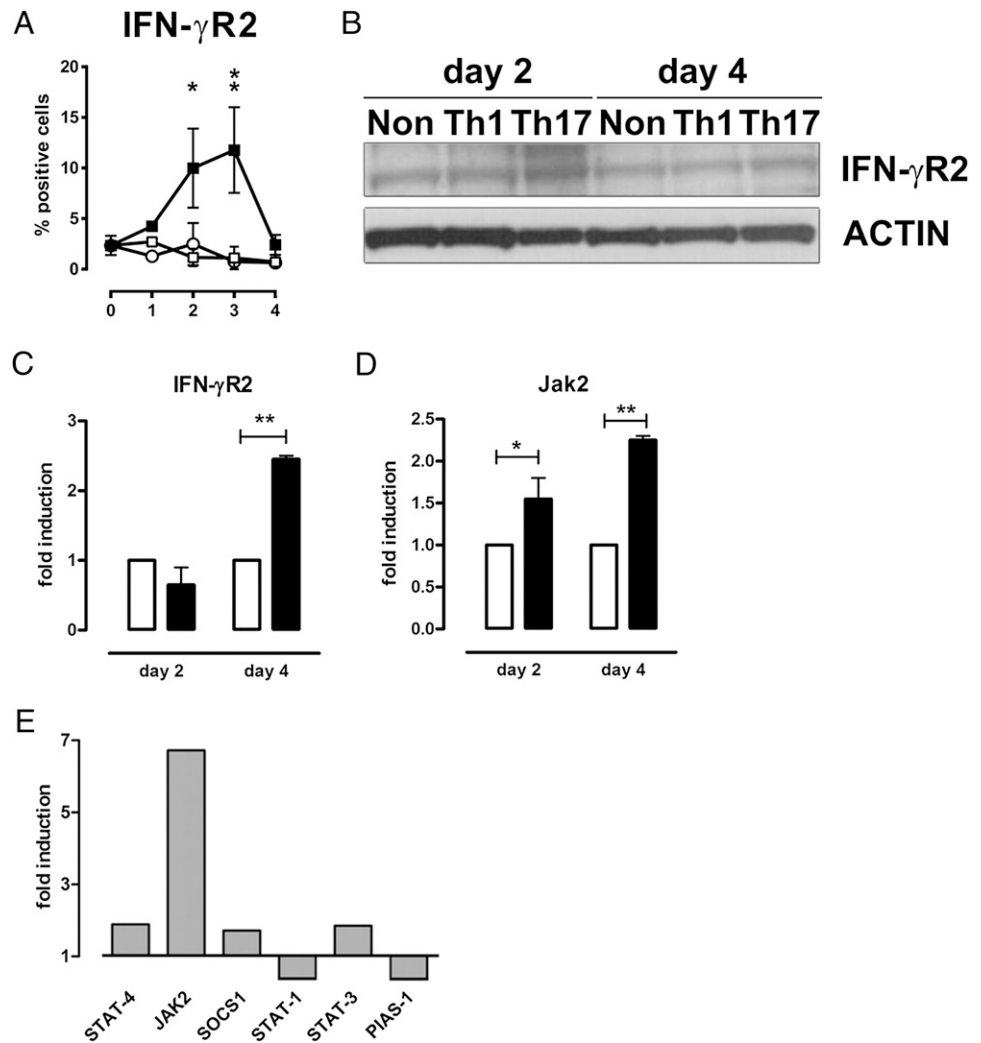
FIGURE 3. Th17 lymphocytes from AMS patients express higher levels of JAK2 compared with Th17 from IMS and HS. *A*, JAK2 mRNA expression was evaluated by quantitative RT-PCR in Th1 and Th17 clones generated from three AMS and three HS. Graphs show mean \pm SEM. *B*, Expression of JAK2 (gray histograms) and the background of mouse IgG1 negative control (open histograms) are shown for four representative Th1 and Th17 clones generated from AMS and HS. The graph shows the mean \pm SEM of JAK2 Δ MFI from the same Th1 and Th17 clones. *C–E*, PBMCs from HS ($n = 5$), AMS ($n = 5$) and IMS patients ($n = 5$) were stimulated for 5 h with PMA, ionomycin and BFA, and JAK2 expression was detected by FACS analysis. *C*, The graph shows the mean \pm SEM of the percentage of JAK2 expression on CD4⁺, CD4⁻, and monocytes. *D*, The graph shows the mean \pm SEM of the percentage of Th17 cells expressing JAK2 in HS, AMS, and IMS, calculated using the formula: % Upper right quadrant cells/(% Upper left quadrant cells + % Upper right quadrant cells) \times 100. *E*, The contour plots show a representative isotype negative control or IL-17/JAK2 staining in HS, AMS, and IMS; numbers indicate the percentage of cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Pearson t test.

Western blot analysis showed similar levels of IFN- γ R2 expression in nonpolarized Th1 and Th17 cells after 2 and 4 d of activation (Fig. 4*B*), suggesting that, as the whole pool of IFN- γ R2 remained constant in these cells, its accumulation on Th17 cells was the outcome of stabilization of IFN- γ R2 on their surface. Quantitative RT-PCR showed that mRNA for IFN- γ R2 was equally expressed in Th1 and Th17 cells recovered and purified after 2 d of activation, and 2-fold increased on d 4 in Th17 cells only (Fig. 4*C*). In Th17 cells, IFN- γ -dependent STAT1 phosphorylation (Supplemental Fig. 3*C*) and inhibition of IL-17 production (Supplemental Fig. 3*D*) peaked at d 2 and then decreased to almost zero on d 4, in parallel with the IFN- γ R2 surface ex-

pression. Inhibition of Th17 cells was mediated by the IFN- γ anti-proliferative effect because its neutralization resulted in a strong enhancement of Th17 cell proliferation, whereas it did not affect the proliferation of nonpolarized or Th1 polarized cells (Supplemental Fig. 3*E*).

Compared to Th1 cells at day 2 and 4 after anti-CD3 and anti-CD28 mAb stimulation, the expression of JAK2 mRNA on Th17 cells was increased (Fig. 4*D*). To confirm that JAK2 levels in Th17 cells are dependent on their activation, induction of mRNA for JAK2 was evaluated on resting and polyclonally activated CD4⁺ Th17 clones from HS by real-time PCR. Among mRNA of genes involved in the promotion or inhibition of cytokine signaling, the

FIGURE 4. Modulation of IFN- γ R2 and JAK2 expression in Th17 cell induction. Nonpolarized Th1 or Th17 cells were generated from HS PBMCs. **A**, FACS analysis of the kinetics of IFN- γ R2 positive cells (mean \pm SD) in nonpolarized (\circ), Th1 (\square) and Th17 (\blacksquare) cells from five experiments performed on different HS. * p < 0.05, ** p < 0.01, Mann-Whitney U test. **B**, IFN- γ R2 total protein expression was analyzed in nonpolarized, Th1, or Th17 cells from HS PBMCs on days 2 and 4 after activation. **C** and **D**, Quantitative RT-PCR analysis of IFN- γ R2 and JAK2 mRNA expression in Th1 (white bars) and Th17 (black bars) cells harvested and purified on days 2 and 4. Graphs show the means \pm SEM from three experiments performed on different donors. * p < 0.05, ** p < 0.01, Pearson t test. **E**, mRNA expression of STAT-4, JAK2, SOCS1, STAT-1, STAT-3, and PIAS-1 was analyzed in resting and polyclonally activated CD4⁺ Th17 clones from three HS by real-time PCR analysis. Data are reported as mean \pm SEM of the fold induction of mRNA expression in polyclonally activated compared with resting Th17 clones.



highest variation was observed in JAK2, which increased \sim 7-fold following activation (Fig. 4E).

These data indicate that the increase of surface IFN- γ R2 and JAK2 expression on Th17 cells from HS depends on activation stimuli, and suggest that the high levels of IFN- γ R2 on Th17 cells from AMS depend on their activation.

Re-expression of JAK2 stabilized cell surface expression of IFN- γ R2 in JAK2-deficient cells

Overexpression of JAK2 in activated Th17 cells suggests that JAK2 stabilizes IFN- γ R2 at the cell surface. To address this point, IFN- γ R2 expression and stability were analyzed in JAK2-deficient γ 2a or JAK2-expressing γ 2aJ2 cells (10). FACS analysis revealed that IFN- γ R2 was more expressed at the cell surface on γ 2aJ2 than on γ 2a cells (Fig. 5A). Similarly, following cell surface immunoprecipitation with anti-IFN- γ R2 mAb, Western blot analysis showed more IFN- γ R2 on the surface of γ 2AJ2. The amount of IFN- γ R2 present in the total lysates of both γ 2AJ2 and γ 2A was the same. In γ 2AJ2 cells, the IFN- γ R2 immunoprecipitated from the cell surface was associated with JAK2 (Fig. 5B). The stability of IFN- γ R2 in γ 2A and γ 2AJ2 cells was evaluated. Cells were treated with cycloheximide for 30 min, and the cell surface expression of IFN- γ R2 was assessed by flow cytometry at different times. At $T = 0$, IFN- γ R2 expression was faint, but still detectable on γ 2A cells. After 10 min, it had almost disappeared, after 15 and 30 min it was no longer detectable (Fig. 5C). By contrast, γ 2AJ2

showed a higher cell surface expression of IFN- γ R2 at $T = 0$, and a stable expression was still detectable after 30 min (Fig. 5C). IFN- γ R2 internalization in γ 2A and γ 2AJ2 was assessed by FACS analysis. Cells were labeled with anti-IFN- γ R2 mAb at 4°C and then incubated for the indicated times at 37°C. The ratio between IS and IZ IFN- γ R2 decreased rapidly in γ 2A cells, whereas it was almost stable in γ 2AJ2 (Fig. 5D).

Discussion

In this study, we show that during in vitro-induced Th17 cell expansion, the upregulation of their IFN- γ R2 chain makes them sensitive to IFN- γ /STAT1 inhibitory signals. More importantly, the surface expression of IFN- γ R2 in peripheral Th17 cells was higher in AMS patients than in IMS patients and HS. This difference probably reflects Th17 cell activation during the phases of disease activity. By contrast, in vitro-induced Th17 cells displayed an early IFN- γ R2 surface accumulation that decreased after 4 d of culture, suggesting that the increase in IFN- γ R2 and the acquisition of IFN- γ /STAT1 sensitivity are dependent on TCR-mediated activation.

Expansion of peripheral Th17 cells is associated with AMS (3). IFN- γ signaling plays a role in Th17 cell differentiation and function. Indeed our data indicate that Th17 cells from AMS express high surface levels of IFN- γ R2 and are sensitive to the antiproliferative effect of IFN- γ . This observation is in keeping with the recent finding indicating that the inhibition of the Th17

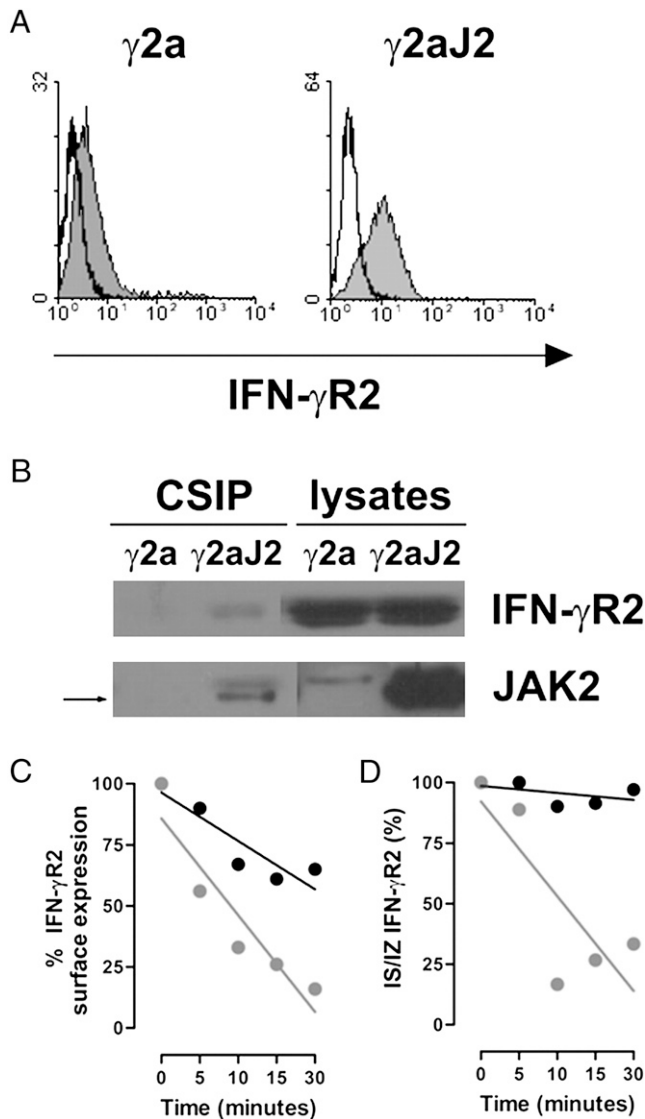


FIGURE 5. JAK2 stabilizes IFN- γ R2 cell surface expression. *A*, FACS analysis of IFN- γ R2 surface expression on JAK2-deficient $\gamma 2a$ and JAK2-expressing $\gamma 2aJ2$ cells. Gray histograms represent IFN- γ R2 expression, and open histograms represent the background of isotype-matched negative control. *B*, Western blot analysis of IFN- γ R2 and JAK2 expression in the CSIP or lysates from $\gamma 2a$ and $\gamma 2aJ2$ cells. CSIP was obtained by immunoprecipitating cell surface proteins with an anti-IFN- γ R2 mAb. *C*, IFN- γ R2 stability on $\gamma 2a$ and $\gamma 2aJ2$ cell membrane, evaluated by treating cells with cycloheximide (50 μ g/ml) for 30 min at 37°C and analyzing their IFN- γ R2 surface expression by FACS at different time points. IFN- γ R2 stability was calculated by dividing the MFI of the untreated cells by the MFI of cycloheximide-treated cells. *D*, IFN- γ R2 internalization in $\gamma 2a$ and $\gamma 2aJ2$ cells. Cells were labeled with anti-IFN- γ R2 mAb at 4°C and then incubated for the indicated times at 37°C. The ratio of surface (IS)/internalized (IZ) IFN- γ R2 was calculated as described in *Materials and Methods*. Each panel shows one representative of three independent experiments.

cell response by IFN- β is dependent on IFN- γ signaling (3). As MS is mediated by both autoreactive Th1 and Th17 cells, the higher expression of IFN- γ R2 on Th17 cells may favor the suppressive effect of IFN- γ produced by Th1 cells.

We also show that the higher surface expression of IFN- γ R2 on Th17 cells from AMS patients is associated with a higher expression of mRNA and protein for JAK2. Experiments in JAK2-

deficient $\gamma 2a$ cells demonstrated that they are related. Whether IFN- γ R2 surface accumulation and JAK2 upregulation in Th17 cells are a common feature of other chronic inflammatory diseases such as rheumatoid arthritis and Crohn disease is an intriguing issue that needs to be further evaluated.

During Th1 cell differentiation, the combination of TCR stimulation and the presence of IL-12 leads to a strong IFN- γ production that downregulates IFN- γ R2 transcription (20). We have demonstrated that IFN- γ R2 downregulation in T cells mainly results from its endocytosis that can be induced, independently from IFN- γ , by iron and IGF-1 (4, 12). Therefore, it is likely that the upregulation of IFN- γ R2 we observed in activated Th17 cells from MS patients results from the mobilization of IFN- γ R2 from intracellular stores to the cell surface (4). Upon in vitro activation, Th1 and Th17 cells displayed similar total protein levels of IFN- γ R2, even if Th17 cells expressed higher levels of mRNA at later times of culture. These findings suggest an increased stability of IFN- γ R2 mRNA in Th17-deflecting conditions. Accumulation of IFN- γ R2 on the surface of Th17 cells correlated with a higher level of JAK2 mRNA upon their polyclonal stimulation. This strongly indicates that the mobilization of IFN- γ R2 to the cell surface requires JAK2, which is overexpressed in Th17 cells upon TCR stimulation. Our results demonstrate that JAK2 stabilizes IFN- γ R2 at the cell surface, as proven by the reinstatement of its surface expression in JAK2-deficient $\gamma 2a$ cells upon JAK2 rescue. This evidence supports the role of JAK2 in stabilizing surface IFN- γ R2 expression and is in keeping with the well-established role of JAK2 in favoring Golgi processing and cell surface expression of EpoR (16, 21).

Th17 cells play a role in the pathogenesis of EAE and have been associated with MS exacerbations (2). Iron is increased in the brains of MS patients (22) and iron chelation ameliorates EAE (23). This suggests that strategies aimed to perturb IFN- γ R2 intracellular traffic, such as iron chelation or IGF-1 signaling blockade (4, 11), could enhance the predisposition to IFN- γ -induced apoptosis in autoreactive Th17 cells. In MS, this approach can be used to synergize with IFN- β treatment that selectively inhibits Th17 cells (1).

We have previously demonstrated that human Th17 cells acquire type I IFN- γ R2 expression and sensitivity to IFN- β /STAT1 inhibitory activity during their expansion (1). Once Th17 cells complete their IL-23 dependent expansion, they express high levels of IFNAR1 and become susceptible to the STAT1-dependent antiproliferative effect induced by type I IFN. This explains one of the IFN- β mechanisms of action in MS treatment (1).

Overall, the data reported in this and in our previous paper (1) suggest that STAT1-dependent antiproliferative signals are always inducible in Th17 cells by type I or II IFNs, depending on their receptor expression, which varies during their life. By contrast, type I and II IFN antiproliferative effects are always absent in Th1 cells, which downregulate both IFN- γ R2 and IFNAR1 during their differentiation (1). Thus, expansion and contraction of Th17 cells could be strictly controlled by Th1 and NK cells through IFN- γ production, and by activated dendritic cells through type I IFN production. Because IL-17 produced by Th17 cells inhibits Th1 cell differentiation (24), this study suggests that in a reciprocal manner IFN- γ produced by Th1 lymphocytes restrains the Th17 cell response, particularly in MS. This study provides a springboard for the assessment of new prognostic markers for the indication of IFN- β management in MS, and it opens the door to the evaluation of these mechanisms in other chronic inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

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