Methods

Cell culture reagents and antibodies

Cells were cultured in RPMI 1640 media supplemented with 2 nM L-glutamine, 5 mM HEPES, and 100 U/μg/ml penicillin/streptomycin (Biowhittaker, Walkersville, MD), 0.5 mM sodium pyruvate, 0.05 mM nonessential amino acids (Life Technologies, Rockville, MD), and 5% human AB serum (Gemini Bio-Products, Woodland, CA). The antibodies used for stimulation were anti-human CD3 (clone UCHT1) and anti-human CD28 (clone 28.2) (BD Biosciences, San Jose, CA) at 1μg/ml. Treg Inspector Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used in suppression assays. IL-12, IL-23 and IL-27 (R&D Systems, Minneapolis, MN) were used at 10 ng/ml, 20 ng/ml and 50 ng/ml, respectively. IL-2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) and was used at 25 U/ml.

Study subjects

Peripheral blood was drawn from healthy individuals and 25 MS subjects after informed consent and approval by the Institutional Review Board at the Brigham and Women's Hospital. The patients were of ages of 47.1±10.5 years, and all had relapsing-remitting disease with Kurtzke Expanded Disability Status Scale scores between 0 and 2.5. "Untreated" patients (n=13) were not treated with any immunomodulatory drugs. "Treated" patients (n=12) had been receiving IFNβ for at least 12 months .Healthy donors were between were age and sex paired with no history of autoimmune diseases.

Cell isolation and FACS sorting of T cell populations

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors after informed consent was obtained in accordance with the Declaration of Helsinki by Ficoll Hypaque gradient centrifugation. Total CD4+ T cells were isolated by negative selection using CD4+ T cell isolation kit II (Miltenyi Biotec) and stained for fluorescence-activated cell sorting (FACS) with the following antibodies: anti-CD62L (clone Dreg56), CD45RA (clone HI100), CD25 (clone M-A251) (all from BD Biosciences) and CD127 (clone eBioRDR5) from eBioscience (San Diego, CA). The Treg ($CD4^+CD45RA^-CD25^{hi}CD127^{low/neg}$), Tmem ($CD4^+CD45RA^-CD25^{low/neg}CD62L^+$)

and Tnaïve ($CD4^+CD45RA^+CD25^{low/neg}CD62L^+$) populations were sorted on a FACS Aria (BD Biosciences).

Cell activation and intracellular staining

Cell populations were stimulated with 1 µg/ml plate-bound anti-CD3, 1 µg/ml soluble anti-CD28 and 25 U/ml IL-2 in the presence or absence of IL-12. At day 4, cells were stimulated with 30 nM phorbol-12-myristate-13-acetate (PMA) and 200 nM ionomycin for 4 hours in the presence of GolgiStop (BD Biosciences) and intracellular staining of cytokines (IFNγ, IL-10, IL-17, IL-4), Foxp3 and T-bet was performed with Foxp3 staining buffers (eBioscience) per manufacturer´s recommendations and the following antibodies: IFNγ (clone 4S.B3), IL-17 (clone BL168) and T-bet (clone 4B10) from Biolegend (San Diego, CA), IL-10 (clone JES3-19F1) and IL-4 (clone 3010.211), from BD Biosciences and Foxp3 (clone PCH101) from eBioscience.

Foxp3 locus methylation analysis

FACS sorted Tregs from healthy controls were stimulated for 4 days in the presence and absence of IL-12 and *ex vivo* FACS sorted Tregs from healthy controls and RRMS patients were stimulated for four hours with PMA and ionomycin. Samples were intracellularly stained for Foxp3 and IFNγ. Fixed $IFN\gamma^+Foxp3^+$ and $IFN\gamma^-Foxp3^+$ Tregs were resorted and subjected to genomic DNA isolation as previously described {Hansmann #111}. Sodium bisulfate treatment was performed of purified genomic DNA using the EpiTect Bisulfite kit (QIAGEN), following manufacturer´s recommendations. PCR and purification of gel bands were performed as previously described {Floess, 2007 #112} and sequenced directly. Trace files were interpreted using 4Peaks software (Mekentosj).

Enzyme-linked immunosorbent assay

ELISA measurement of IFNγ and IL-10 from stimulated Treg clone supernatants was performed according to manufacturer's recommendations (BD Biosciences). Antibodies used were human IFNγ MAb (clone 2G1) and human IFNγ MAb biotin-labeled (Thermo Scientific, Rockford, IL) and rat anti-human and viral IL-10 and biotin anti-human and viral IL-10 (BD Biosciences).

Quantification of mRNA expression levels by RT-PCR

RNA was isolated using QIAGEN RNeasy Micro Kit (QIAGEN, Valencia, CA), following manufacturer´s guidelines and converted to cDNA by reverse transcription (RT) with random hexamers and Multiscribe RT (TQMN, Reverse Transcription Reagents; Applied Biosystems, Foster City, CA). For mRNA gene expression assays, probes were purchased from Applied Biosystems (Table 1) and the reactions were set up following manufacturer´s guidelines and run on a 7500 Fast Real-Time PCR System (Applied Biosystems). Values are represented as the difference in Ct values normalized to β2-microglobulin for each sample as per the following formula: Relative RNA expression = (2^{-dCt}) x 1000.

Single-cell Treg clones

Tmem and Treg cells were sorted at one cell per well in XVIVO15 medium containing 5% human serum and stimulated with soluble CD3 and CD28 (both at 1 µg/mL), irradiated APCs (10^5/well) and 50 U/ml IL-2. Half of the medium was replaced with fresh medium containing IL-2 (50 U/m) starting at day 10 and every 3 to 4 days thereafter. After 4 weeks of expansion, each clone was tested for Foxp3 expression, IL-17, IFNγ, IL-10 and IL-4 production by intracellular staining.

Suppression assays

Previously IL-12-treated or untreated Tregs were co-cultured with CFSE-labelled responder CD4+CD25- T cells at a 1:2 Treg:Tresp ratio (1250 Tregs and 2500 Tresp). The stimuli used were plate-bound anti-human CD3 at 0.3µg/ml and $15x10^3$ irradiated CD2-depleted APC or Treg Inspector Beads (Miltenyi) at manufacturer´s recommended concentration. At day 3, co-cultures were stained for viability with LIVE/DEAD stain kit (Invitrogen, Carlsbag, CA) and fixed using Foxp3 staining buffer (eBioscience), and proliferation of viable responder T cells was analyzed on a LSRII flow cytometer (BD Biosciences). For suppression assays with *ex vivo* FACS sorted Tregs and Treg clones, Treg Inspector Beads were used.

Statistics

A standard two-tailed *t test* was used for statistical analysis with p values of 0.05 or less considered significant.