Supporting Information

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SI Materials and Methods

Patients, Healthy Controls, and IFN-β Stimulation. Eleven patients with relapsing-remitting multiple sclerosis (RRMS; median age, 49.0 y; range, 28–59 y; median Expanded Disability Status Scale score, 1.5; range, 0–3.5) who received IFN-β1a therapy for various amounts of time (median time, 5.0 y; range, 0–15 y), consented to give whole blood (according to an institutional review board-approved protocol at the Cleveland Clinic) before injection with IFN-β1a, and nine of the 11 patients with RRMS also donated blood at different times after injection with IFN-β1a (Avonex; 30 μg/0.5 mL prefilled syringe, 12×10^6 IU/mL; Biogen Idec). Four of the 11 patients returned after approximately 3 y to donate blood before and 18 h after IFN-β1a injection, to enable the determination of ex vivo and in vitro induction of TRAIL stimulated by IFN-β. To obtain whole blood from patients with RRMS for in vitro stimulation with IFN-β1a, venipuncture always preceded the weekly injection with IFN-β1a. Concerning neutralizing antibodies (NAb) to IFN-β1a, information was available for only one of the 11 patients with RRMS. Patient 7, who was previously determined to have developed NAbs, did not show any activation of STAT1/3/5 in response to injection of IFN-β1a (Fig. S3). Because the other 10 patients did show activation of STATs in response to IFN-β1a, we assume they did not develop any NAb. Ten healthy controls (median age, 25.5 y; range, 21–48 y) also consented to give blood according to an institutional review board-approved protocol at the Cleveland Clinic. One of the healthy donors returned after 1 wk and donated blood again. For in vitro stimulation of whole blood from patients with RRMS and healthy subjects, undiluted whole blood was stimulated with IFN-β1a (varying amounts as indicated) in 6- or 10-cm culture dishes or 50 mL Falcon tubes (BD Biosciences) within 15 min after venipuncture. All in vitro stimulations were performed in a Thermo Electron Steri-Cycle HEPA class 100 CO_2 incubator at 37 °C (Thermo Fisher Scientific). After ex vivo or in vitro stimulation, whole blood was fixed immediately and erythrocytes were lysed for intracellular detection of tyrosine-phosphorylated STATs, phosphorylated kinases, or serine-phosphorylated NF-κB p65 in leukocyte subsets, by using flow cytometry. For each staining, 130 μL of whole blood was used.

Intracellular Detection of Activated STAT, Akt, p38, ERK, and NF-κB by Flow Cytometry. We used the exact same protocol that we used previously to study induction of intracellular phosphoproteins in human leukocytes present in whole blood (1). After fixation, lysis of RBCs, and methanol incubation, the following antibodies were used additionally: PT(180)/PY(182)-p38 MAPK, PS(529)-NF-κB p65, and PT(202)/PY(204)-ERK (clones 36/p38, K10-895.12.50, G263-7 respectively; BD Biosciences) or PS(473)-Akt-fluorescein (R&D Systems), and were added in amounts as advised by the manufacturer and incubated at room temperature in the dark for 1 h. Flow data were analyzed with WinList (Verity Software). An example of the analysis can be seen in our previously published study (1). The method described earlier results in phosphoflow data with excellent repeatability because duplicates show little variance.

Detection of Surface TRAIL Protein on Leukocytes by Flow Cytometry. Whole blood was drawn before and 18 h after IFN-β1a injection, and surface markers to distinguish leukocyte subsets in whole blood were stained with anti–human-CD4-PC5, -CD19-PC7, and -CD14-FITC (clones 13B8.2, J3-119, and RMO52, respectively; Beckman Coulter), anti–CD3-Pacific blue, -CD123-biotin, -CD25-APC-cy7, -CD94-APC (clones UCHT1, 9F5, M-A251, and HP-3D9, respectively; BD Biosciences), anti–CD14-AF700 (clone TüK4; Invitrogen), and anti–TRAIL-PE (clone 75402, mouse IgG1; R&D Systems) or mouse IgG1-PE isotype control (clone MOPC-21; BD Biosciences) antibodies, using amounts as indicated by the manufacturers and incubating for 30 min at 4 °C (150 μL whole blood was used per tube). After a wash with 3 mL wash buffer (tubes centrifuged at $300 \times g$ for 10 min), and bringing the volume back to 150 μL per tube by adding wash buffer, samples were incubated with 3 μL of 10×-diluted streptavidin–Pacific orange (Invitrogen) for 30 min at 4 °C. Whole blood samples were subsequently fixed by adding 102 μL prewarmed 10% formaldehyde (Polysciences) and incubating for 10 min at 37 °C. The erythrocytes were lysed by adding 1,275 μL of 0.12% Triton X-100 (Pierce) to each tube and incubation at room temperature for 30 min while shaking. After three washes with 3 mL wash buffer and centrifugation at $300 \times g$ for 10 min, the cells were resuspended in 300 μL wash buffer, and 50,000 cells were collected in the live gate with a LSRII flow cytometer (BD Biosciences). Flow data were analyzed with WinList (Verity Software). The percentage of IFN-β–induced TRAIL in leukocyte subsets was determined by subtracting the percentage of positive cells in unstimulated cells (before injection) from the percentage of positive cells in stimulated cells (after injection).

The induction of TRAIL on leukocytes after stimulation with 1,500 IU/mL IFN-β1a in vitro for 18 h was done by using essentially the same experimental approach described above, with the only exception that whole blood was diluted (1:2) with plain RPMI media for detection of TRAIL after IFN stimulation in vitro for 18 h. In addition, to study the dependency of TRAIL induction on activation of PI3K/Akt and p38 MAPK, whole blood was pretreated with 25 mM Ly294,002 or 20 mM SB203580, respectively (Sigma-Aldrich; both specific chemical inhibitors dissolved in DMSO) for 30 min and subsequently stimulated with 1,500 IU/mL IFN-β1a. The percentage of TRAIL-positive monocytes and granulocytes of the four patients with RRMS was set at 100% for DMSO pretreated (i.e., control) leukocyte subset, and the percentage change in TRAIL caused by pretreatment with the specific inhibitors was calculated.

Statistical Analysis. InStat 3 software (GraphPad Software) was used for statistical analyses. The nonparametric Friedman test was used to test whether the four (monocytes, B cells, and $CD8^+$ and $CD4^+$ T cells) or five (granulocytes, monocytes, B cells, and $CD8^+$ and $CD4^+$ T cells) leukocyte subsets differed with respect to activation of p38 MAPK, STAT1, STAT3, and STAT5, or induction of TRAIL protein. When the Friedman test showed a significant difference ($P < 0.05$), post-hoc analysis was subsequently performed by using a Dunn test to detect which blood subsets differed significantly from each other. When calculating the P values, the Dunn test takes into account the number of comparisons one is making (i.e., Bonferroni adjustment).

1. van Boxel-Dezaire AH, et al. (2010) Major differences in the responses of primary human leukocyte subsets to IFN-beta. *J Immunol* 185:5888–5899.

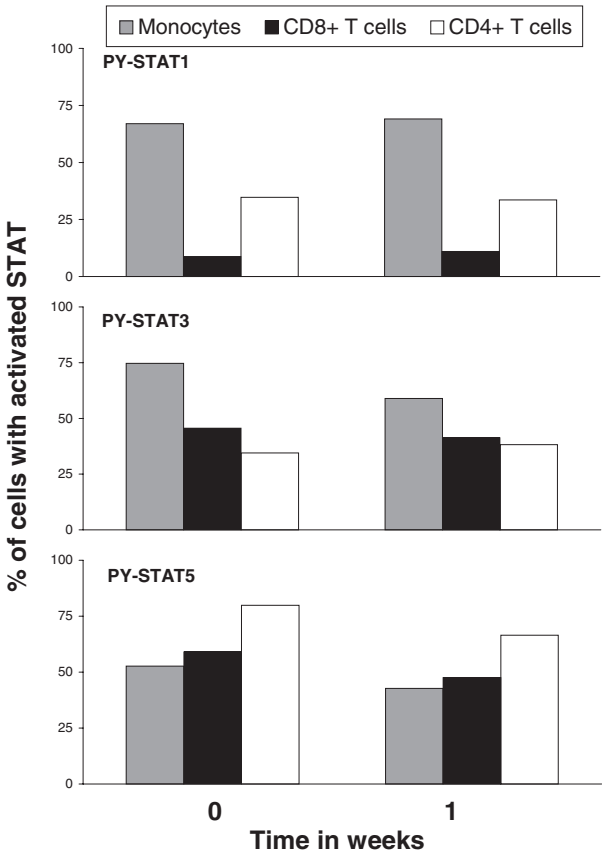

Fig. S1. Whole blood of a single donor repeatedly stimulated with IFN-β in vitro shows a similar STAT activation pattern over time. Whole blood of a healthy donor was stimulated in vitro with 1,000 IU/mL IFN-β1a (Avonex) for 25 min. Exactly 1 wk later, blood was drawn from the same donor and stimulated in the same manner, revealing a similar STAT activation pattern in leukocyte subsets (monocytes and $CD4^+$ and $CD8^+$ T cells) as the week before.

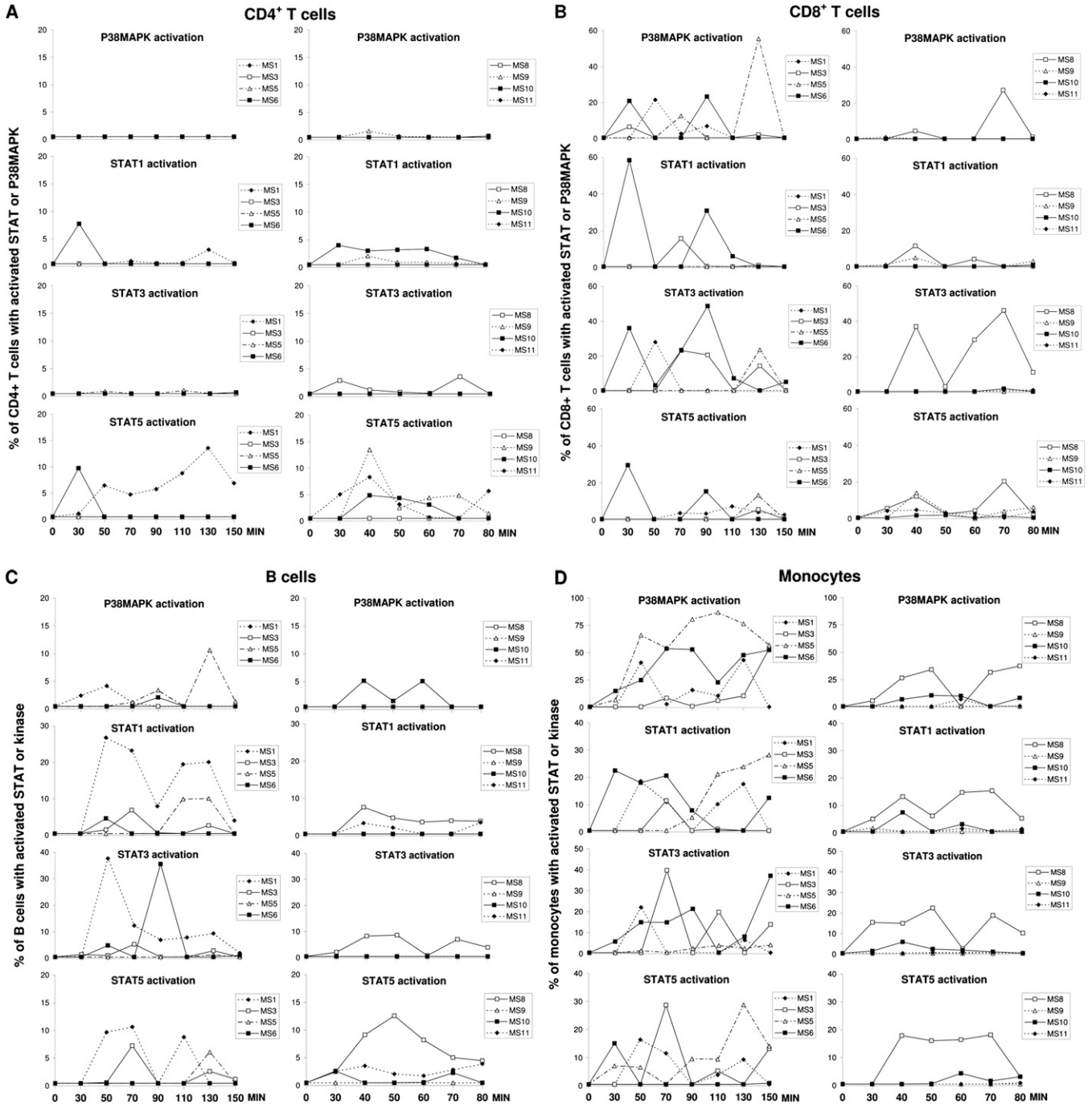

Fig. S2. Analysis of STAT and p38 activation after injection of IFN-β1a into patients with RRMS. Eight patients with RRMS (patients 1, 3, 5, 6, and 8–11) were injected i.m. with IFN-β1a and blood was sampled before injection and at 20-min intervals between 30 and 150 min ($n = 4$; *Left*) or at 10-min intervals between 30 and 80 min ($n = 4$; *Right*) after injection. Whole blood was immediately fixed and further processed as described in *Materials and Methods*. Flow cytometric analysis of $CD4^+$ T cells (*A*), $CD8^+$ T cells (*B*), B cells (*C*), and monocytes (*D*) revealed an oscillating pattern of STAT1, STAT3, STAT5, and p38 activation over time, with initial peak responses between 30 and 70 min after injection.

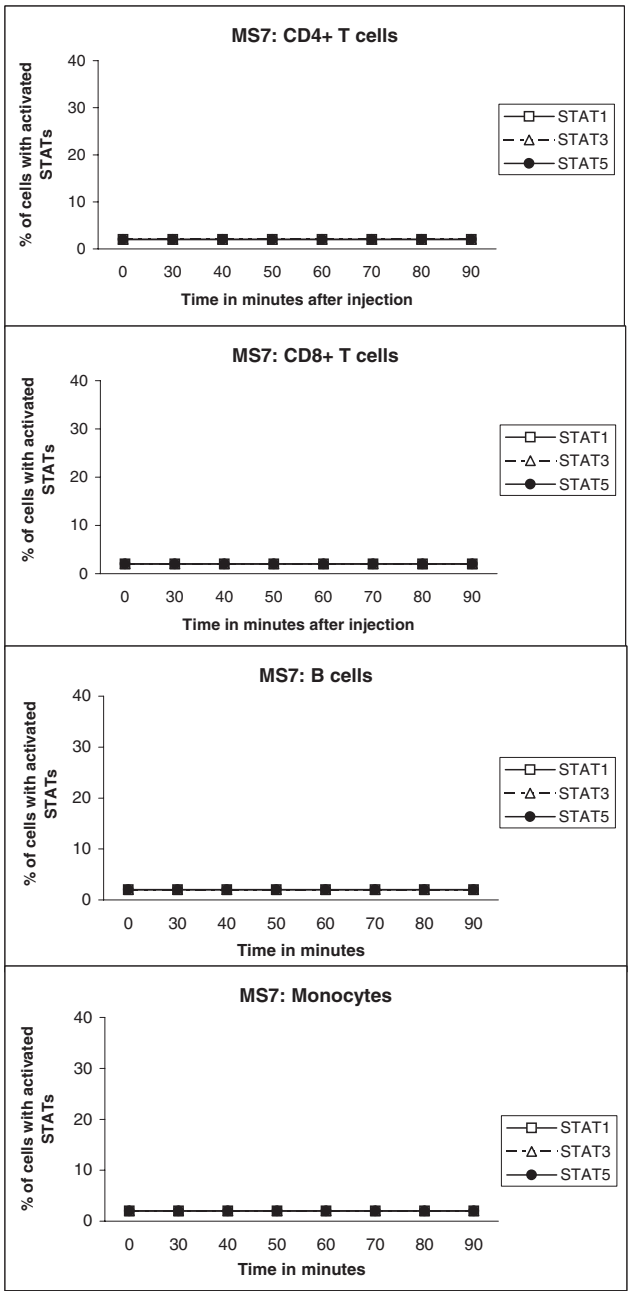

Fig. S3. In one patient with RRMS who developed NAbs against IFN-β1a, no activation of STATs could be detected after injection with IFN-β1a. RRMS patient 7 was injected i.m. with IFN-β1a and blood was sampled before injection and at 10-min intervals between 30 and 90 min after injection. Whole blood was immediately fixed and further processed as described in *Materials and Methods*. Flow cytometric analysis of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and monocytes revealed that no activation of STAT1, STAT3, or STAT5 occurred after injection.

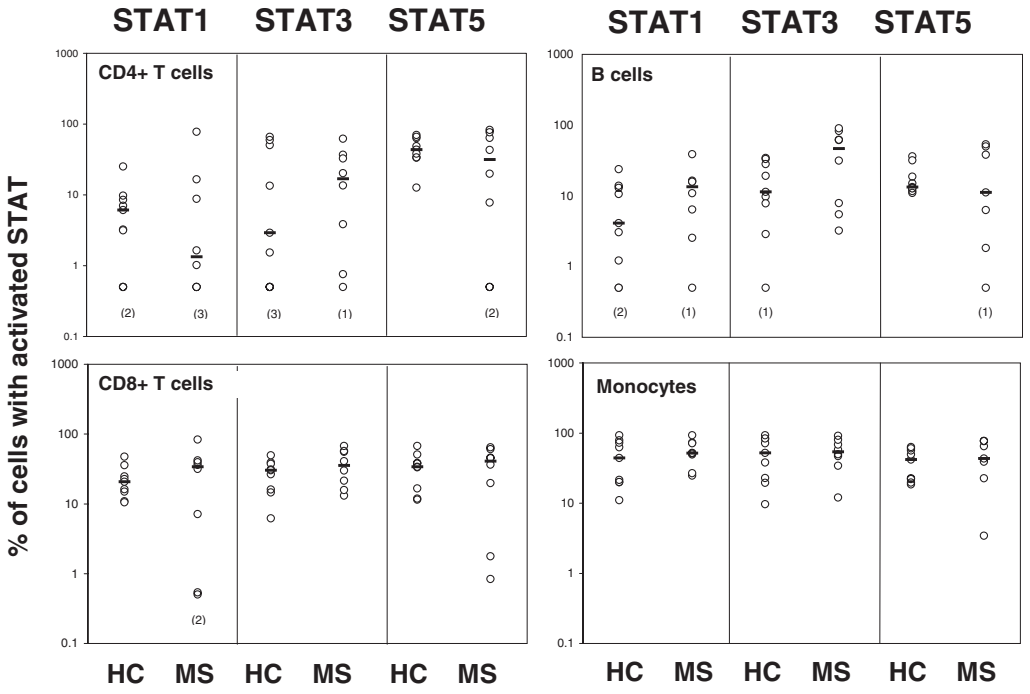

Fig. S4. Similar activation of STAT1, STAT3, and STAT5 in leukocyte subsets of healthy controls and patients with RRMS after stimulation with IFN-β1a in vitro. Whole blood from nine healthy donors and from eight patients with RRMS, whose blood was drawn before IFN-β1a injection, was stimulated with 500 IU/mL IFN-β1a for 25 min in vitro to determine activation of STAT1, STAT3, and STAT5. The median percentages of activated STATs in leukocytes are indicated with horizontal bars. The Mann–Whitney test indicated no significant differences between healthy controls or patients with RRMS with respect to the IFN-β–induced activation of STAT1, STAT3, and STAT5 in leukocytes in vitro. The numbers between brackets indicate the numbers of patients with RRMS (MS) or healthy controls (HC) who showed undetectable activation of STAT1, STAT3, or STAT5 within each leukocyte subset. Note that the RRMS data in this figure are the same as the in vitro data shown in Fig. 2, *Right*.

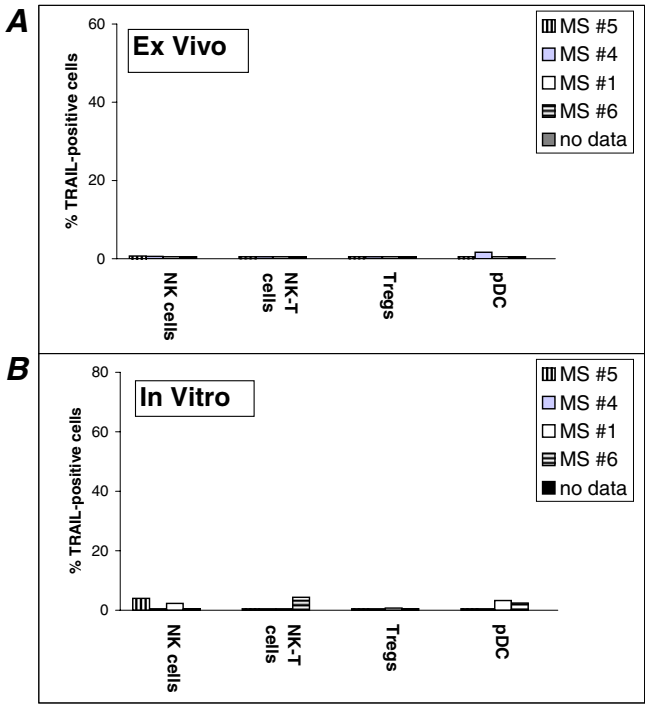

Fig. S5. No induction of TRAIL on certain leukocytes of patients with RRMS after stimulation with IFN-β1a in vitro and in vivo. The percentages of TRAIL-positive leukocytes were determined in four patients with RRMS (patients 1 and 4–6: same patients as in Fig. S2) 18 h after injection with IFN-β1a (Fig. S5*A*), or after stimulation of whole blood samples of four patients with RRMS with 1,500 IU/mL IFN-β1a in vitro for 18 h (Fig. S5*B*). For the latter, blood drawn before injection with IFN-β1a was treated in vitro. Surface TRAIL expression on NK cells ($CD94^+/CD3^-$), NK T cells ($CD94^+/CD3^+$), regulatory T cells (Tregs; $CD4^+/CD25high^+$), or pDCs ($CD123^+/CD14^-$) of patient 12 after IFN-β1a injection or stimulation with IFN-β1a in vitro was not determined.

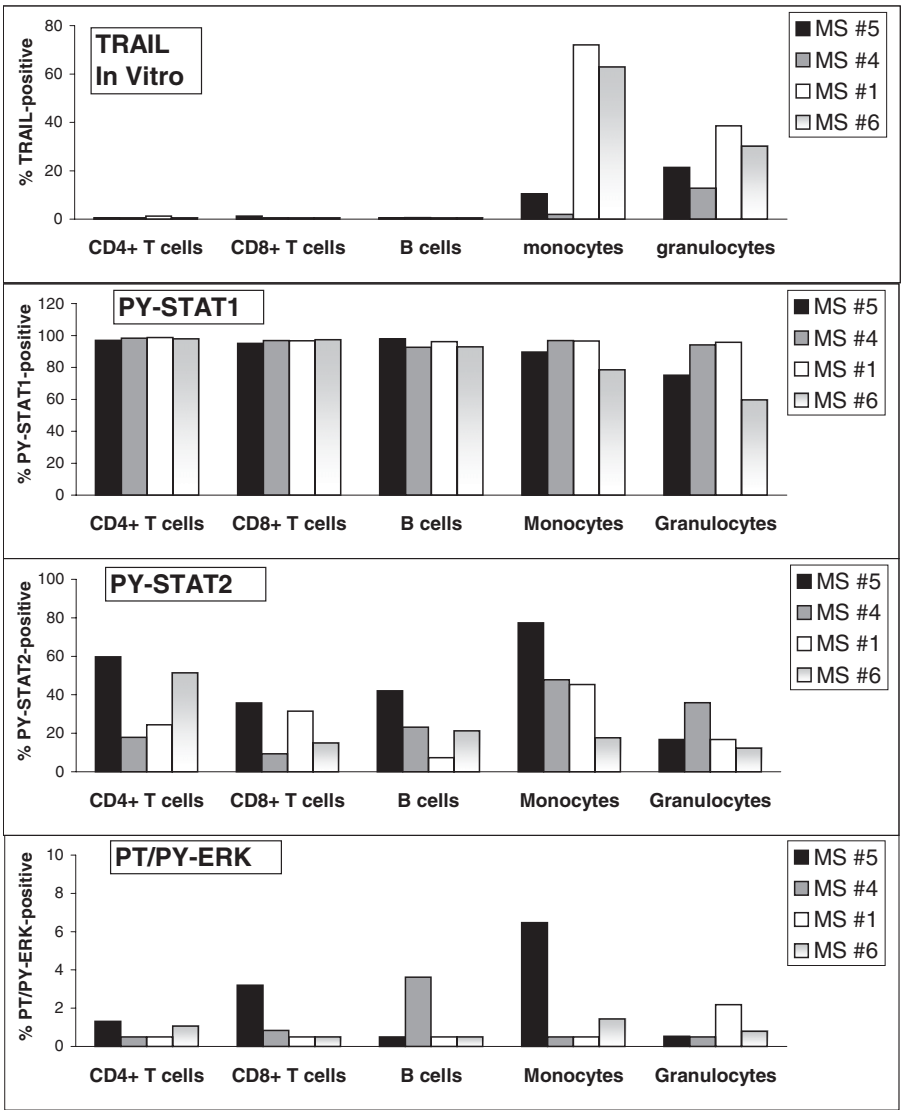

Fig. S6. Differential induction of TRAIL on leukocytes of patients with RRMS in response to IFN-β1a in vitro did not correlate with differential activation of STAT1, STAT2, and ERK. In whole blood of four patients with RRMS (patients 1 and 4–6: same patients as in Fig. S2), activation of STAT1, STAT2, and ERK, and induction of TRAIL was determined after short (15, 30, and 45 min) and long (18 h) stimulation with 1,500 IU/mL IFN-β1a in vitro. Shown for each individual patient are the highest percentages of activated STAT- or ERK-positive leukocytes observed within the 15- to 45-min timeframe.

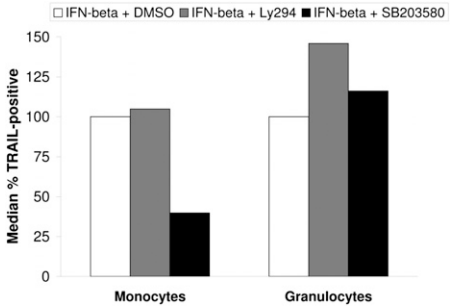

Fig. S7. An inhibitor of p38 activation inhibits the induction of TRAIL in response to IFN-β1a only in monocytes. Whole blood of four patients with RRMS (same as in Figs. 3 and 4) was pretreated with DMSO, 25 μM Ly294002 in DMSO (PI3K/Akt inhibitor), or 20 μM SB203580 in DMSO (p38 MAPK inhibitor) for 30 min, and subsequently stimulated with 1,500 IU/mL IFN-β1a in vitro for 18 h. The median percentages of TRAIL-positive monocytes and granulocytes of the four patients with RRMS are shown, and set at 100% for the DMSO-pretreated leukocyte subset.

Table S1. Differential activation of STATs and cell type-specific activation of p38 after IFN-β1a injection (*n* = 8)

Subset	PY-STAT1	PY-STAT3	PY-STAT5	PT/PY-P38
$CD4^+$ T cells, *n* (%)	2 (25)	1 (13)	5 (63)	0 (0)
$CD8^+$ T cells, *n* (%)	4 (50)	4 (50)	5 (63)	5 (63)
B cells, *n* (%)	5 (63)	4 (50)	5 (63)	2 (25)
Monocytes, *n* (%)	5 (63)	5 (63)	6 (75)	7 (88)

The data for eight patients with RRMS (patients 1, 3, 5, 6, and 8–11) are summarized. Only data for activation of STATs or p38 that was observed in more than 3% of the cells within each leukocyte subset between 30 and 70 min after injection has been included. Shown are also the percentages of patients in which the activation of STAT1, STAT3, STAT5, or p38 was observed (between brackets).