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T helper type 1 and 17 cells Determine Efficacy of IFN- β in Multiple Sclerosis and Experimental Encephalomyelitis

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Abstract

Interferon- β is the major treatment for multiple sclerosis (MS). However, this treatment is not always effective. Here we see congruence in outcome between responses to IFN- β in experimental autoimmune encephalomyelitis (EAE) and relapsing-remitting MS (RRMS). IFN- β is effective in reducing EAE induced by T_H1 cells, but exacerbated disease induced by T_H17. Effective treatment in T_H1 EAE correlated with increased IL-10 in the spleen. In T_H17 disease, the amount of IL-10 was unaltered by treatment, though unexpectedly IFN- β still reduced IL-17 without benefit. Both inhibition of IL-17 and induction of IL-10 depended on IFN- γ . In the absence of IFN- γ signaling, IFN- β therapy was ineffective in EAE. In RRMS, IFN- β non-responders had higher IL-17F in serum compared to responders. Non-responders had worse disease with more steroid usage and more relapses than responders. Hence, IFN- β is pro-inflammatory in T_H17 induced EAE. Moreover, high IL-17F in the serum of RRMS patients is associated with non-responsiveness to therapy with IFN- β .

IFN- β is widely prescribed for the treatment of relapsing-remitting MS (RRMS). However, only about two thirds of relapsing RRMS patients respond to treatment¹. Furthermore, IFN- β can exacerbate symptoms in some individuals². Therefore, we analyzed how cytokine networks, particularly the T_H1 and T_H17 pathways influence IFN- β therapy in RRMS and in EAE.

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Author contributions

R.C.A., L.S. and C.R. discussed, designed and wrote this report; L.S. and C.R. contributed equally as senior authors and R.C.A. conducted and/or supervised all experiments. C.H.P., J.K. and L.F.V. characterized RRMS clinical data and collected serum. B.A.J., M.H. and I.K. assisted in analyzing multiplex data from RRMS. P.D.S., R.N., J.G.C., I.K., and R.M. assisted in EAE experiments. B.A.J. and L.K. performed histology on EAE spinal cords. A.C. and F.Z. performed the STAT1 activation assays. R.B., J.G.C. and A.C. assisted with mouse T-cell experiments. R.W.M. and K.B. performed the human T-cell experiments.

There are many reported effects of IFN- β ^{3–8}. IFN- β reduces T_H1 pathologies by blocking the pro-inflammatory properties of IFN- γ and IL-12^{6–7}. IFN- β also inhibits differentiation of T_H17 cells^{5,8,9}. Besides inhibition of inflammation, IFN- β leads to increased regulatory cytokine production for IL-10, IL-27 and IL-4^{4,8,10}. Despite inhibition of T_H1 and T_H17 and enhancement of regulatory cytokines, a full understanding of why IFN- β therapy works in some with RRMS and not in others, is still unknown.

Results

IFN- β blocks mouse T_H17 differentiation through STAT1

First, we analyzed the effect of IFN- β on T_H17 differentiation of mouse CD4 T-cells stimulated with IL-6, TGF- β and antigen-presenting cells (APCs)¹¹. IFN- β reduced T_H17 differentiation (Fig. 1a). This decrease in T_H17 differentiation is not due to inhibition of T-cell proliferation (data not shown).

In mice, IL-23 is important in driving effector/memory T_H17 cell function^{11–14}. However, naïve cells respond weakly to IL-23 and require IL-6 and TGF- β to produce IL-17^{11,12}. We found that IFN- β decreased IL-17 production in naïve CD4 T-cells stimulated with IL-6 and TGF- β in the presence of APC's (Fig. 1b). Similarly, IFN- β attenuated IL-17 in effector/memory cells stimulated with either IL-6 and TGF- β or IL-23 alone (Fig. 1b). This demonstrates that IFN- β decreases early and late stage T_H17 differentiation.

The major pathway activated by IFN- β is a complex containing STAT1, STAT2 and IRF9, ISGF3¹⁵. We found that IFN- β failed to suppress T_H17 differentiation of *STAT1*^{-/-} CD4 T-cells implying that suppression of TH17 is mediated by ISGF3 signaling (Fig. 1c).

IFN- β requires IFN- γ to inhibit mouse T_H17 differentiation

Several studies demonstrated cross-talk between IFN- β and IFN- γ signaling pathways^{16–18}, however IFN- γ -dependent IFN- β -signaling has not been elucidated in CD4 T-cells. IFN- β or IFN- γ alone induced STAT1 activation in CD4 T-cells. This STAT1 activity was increased and prolonged when both IFN- β and IFN- γ were used together compared to either cytokine alone (Supplementary Fig. 1a). Additionally, the intensity and duration of STAT1 activation was reduced in *IFN γ R*^{-/-} CD4 T-cells stimulated with IFN- β as compared with WT T-cells (Supplementary Fig. 1b).

Next we tested whether IFN- β requires IFN- γ signaling to inhibit T_H17 differentiation. We examined the effect of IFN- β on CD4 T-cells when stimulated with IL-6, TGF- β and APC's and in the presence or absence of neutralizing IFN- γ antibody. Without IFN- γ neutralization, IFN- β decreased the frequency of IL-17 expressing T-cells by 60% (Fig. 1d). However, when IFN- γ was neutralized, the ability of IFN- β to inhibit IL-17 was reduced to 24%. Neutralizing IFN- γ in the absence of IFN- β increased the frequency of IL-17⁺ CD4 cells. This is likely due to the inhibition of T_H17 by IFN- γ . Two other cytokines have been implicated in the inhibition of T_H17 differentiation, IL-10 and IL-27^{10,19}. Neutralization of IL-10 or IL-27 did not affect the ability of IFN- β to inhibit IL-17 expression (Fig. 1d).

IFN- β and IFN- γ may suppress IL-17 production in CD4 T-cells by acting either on T-cells or on APCs. We found that IFN- β inhibited IL-17 in WT CD4 cells co-cultured with WT APCs or *IFN γ R*^{-/-} APC (Fig. 1e). In contrast IL-17 was not inhibited in *IFN γ R*^{-/-} CD4 T-cells when co-cultured with either WT or *IFN γ R*^{-/-} APCs (Fig. 1e). Additionally, we found IFN- β alone did not inhibit IL-17 production in purified naïve CD4 cells (Supplementary Fig. 2a). T_H17 inhibition likely requires the synergistic effects of IFN- β and IFN- γ . Indeed, we found that IFN- β or IFN- γ alone could not attenuate IL-17 production, but, IFN- β and IFN- γ together reduced IL-17 significantly (Fig. 1f).

Effects of IFN- β on differentiation mouse T_H1 and Tregs

IFN- β induces T_H1 differentiation^{20,21}. In non-polarizing conditions, IFN- β induces IFN- γ expression in CD4 cells in the presence (Fig. 2a) or absence of APCs (Supplementary Fig. 2b). However, IFN- β had no significant effect on IFN- γ production when T-cells were cultured in T_H1 or T_H17 conditions. We next examined the cooperative effects between IFN- β and IFN- γ in T_H1 differentiation. In non-polarizing conditions, IFN- β induced IFN- γ production equally in WT and *IFN- γ R*^{-/-} CD4 cells cultured with WT APCs (Fig. 2b), but, IFN- γ production was reduced when CD4 cells were cultured with *IFN γ R*^{-/-} APCs (Fig. 2b). During T_H1 polarization, IFN- γ production was not affected by IFN- β or IFN- γ signaling (Fig. 2c).

The development of T_H17 cells is closely linked to regulatory T-cells^{11,12,22}. Therefore, we explored the possibility that while IFN- β inhibits T_H17 differentiation, via Foxp3⁺ Treg development. However, we found that IFN- β does not induce the development Foxp3⁺ Treg cells in T_H17 differentiation and had no effect on Treg development in cultures with TGF- β in the absence of IL-6 (Supplementary Fig. 3).

IFN- β increases IL-10 in mouse CD4 T-cells

MS patients who respond to IFN- β have increased IL-10 after treatment^{23,24}. Therefore, we investigated whether IFN- β regulates IL-10 during mouse T-helper cell differentiation. We found that IFN- β increased IL-10 in non-polarizing, T_H1 and T_H17 culture conditions (Fig. 2d). However, upon incubation with IFN- β both the T_H1 and non-polarizing culture conditions showed significantly higher amounts of IL-10 as compared to the T_H17 condition (Fig. 2d).

We next explored whether IFN- γ works synergistically with IFN- β to up-regulate IL-10. When IFN- γ was neutralized in non-polarizing (Fig. 2e), T_H1 (Supplementary Fig. 4a), and T_H17 (Supplementary Fig. 4b) cultures, up-regulation of IL-10 by IFN- β was impaired. Furthermore, IL-10 production was impaired when IFN- γ signaling was disrupted in either CD4 T-cells or APCs, demonstrating that IL-10 induction by IFN- β requires IFN- γ signaling in CD4 T-cells and APCs (Fig. 2f). Up-regulation of IL-10 may promote an autocrine loop, further increasing the expression of IL-10. We found that neutralizing IL-10 had no effect on expression of IL-10 (Fig. 2e and Supplementary Fig. 4).

IFN- β indirectly induces IL-10 in mouse CD4 T-cells

Since IFN- β and IFN- γ directly inhibit IL-17 production in CD4 cells, we hypothesized that there is a similar effect on IL-10. We found that IFN- β alone had no effect on IL-10 production from purified CD4 cells with no APCs in all conditions (Supplementary Fig. 2c), this includes non-polarizing and T_H1 conditions that produce large amounts of IFN- γ (Supplementary Fig. 2b). Also, the addition of IFN- γ with IFN- β to purified CD4 cells did not significantly increase IL-10 (Fig. 2g).

IL-27, which is produced by APC's, induces IL-10 in CD4 T-cells^{10,25}. Furthermore, IFN- β induces IL-27. Therefore, we analyzed the effect IFN- β has on IL-27 production. We found that in both non-polarizing and T_H1 conditions, IL-27 was significantly up-regulated by IFN- β compared to T_H17 conditions (Supplementary Fig. 5a). Furthermore, we found that neutralizing IFN- γ inhibited IL-27 production (Supplementary Fig. 5b,c).

Effects of IFN- β on antigen-driven mouse T_H differentiation

Next we assessed the effects of IFN- β during T_H1 and T_H17 differentiation during MOG-peptide re-stimulation. We re-stimulated T-cells isolated from the draining lymph nodes of myelin oligodendrocyte glycoprotein (MOG)-immunized mice with MOG peptide and either

IL-12 or IL-23, to polarize cells to T_H1 and T_H17 respectively, and measured the concentration of cytokines and chemokines in culture supernatants by Luminex and ELISA (Supplementary Fig. 6). IFN- β significantly reduced IL-17 production in T_H17 cultures, greatly induced IL-10 production in T_H1 cells but not in T_H17, and had no significant effect on IFN- γ production (Supplementary Fig. 6a–c).

In addition, IFN- β did not up-regulate IL-4 and TGF- β , two cytokines that have been implicated in regulating autoimmunity (Supplementary Fig. 6d,i). Also of note, under T_H1 conditions, IFN- β induced TNF, IL-6, IL-1 α and IL-1 β (Supplementary Fig. 6e–h). Under T_H17-culture conditions IFN- β inhibited expression of the chemokines G-CSF and MCP-1 (Supplementary Fig. 6n,o).

IFN- β is effective in T_H1 EAE but exacerbates T_H17 EAE

Our *in vitro* experiments demonstrate that IFN- β has anti-inflammatory effects in T_H1 and T_H17 differentiation. This led us to examine whether IFN- β would be effective in treating EAE induced by T_H1 or T_H17 cells. We found considerable difference in the effect IFN- β treatment on EAE induced by adoptive transfer of T_H1 and T_H17 cells. In C57BL/6 mice, IFN- β treatment significantly attenuated the progression of EAE symptoms in T_H1-induced EAE (Fig. 3a), but in contrast, symptoms of T_H17-induced EAE were exacerbated by IFN- β treatment (Fig. 3b). This was not a strain-dependent phenomenon as a similar effect was observed in the SJL/PLP model (Supplementary Fig. 7a,b). In accordance with the clinical course of EAE, histological analysis demonstrated that IFN- β treatment blocked inflammation in the spinal cords of mice with T_H1-EAE but not in T_H17-EAE (Fig. 3c).

Next we assessed the amount of IFN- γ , IL-17, and IL-10 produced in the CNS 45 days after adoptive transfer of T-cells. The frequency of CD4 T-cells producing IL-17 and IFN- γ in the spinal cord was decreased after IFN- β treatment of T_H1-EAE, but in T_H17-EAE both T_H1 and T_H17 cells were elevated with IFN- β (Fig. 3d,e). IL-10, on the other hand, was decreased in the spinal cords of both the T_H1 and T_H17 disease after IFN- β treatment (Fig. 3f).

We then assessed the effects IFN- β treatment on splenic T-cells isolated 45 days after adoptive transfer of T-cells. In both T_H1 and T_H17 disease, IFN- γ production remains unchanged with IFN- β treatment (Fig. 3g). However, IFN- β had differential effects on IL-17 and IL-10 in these disease models. In T_H1 EAE, levels of IL-17 produced by spleens were low and treatment with IFN- β did not affect the production of this cytokine (Fig. 3h). However, in T_H17-EAE, splenic T cells produced higher amounts of IL-17 compared to T_H1-EAE, and surprisingly, IL-17 was significantly reduced in the T_H17 mice treated with IFN- β (Fig. 3h). In contrast, IL-10 production in spleens from T_H1 diseases was significantly elevated by IFN- β , but in T_H17-EAE, IL-10 remained very low after treatment (Fig. 3i).

IFN- β treatment requires IFN- γ to suppress EAE

Since IFN- β is highly effective in T_H1-EAE and we find that IFN- β requires IFN- γ to suppress IL-17 and induce IL-10 in cultures; we speculated that IFN- β treatment requires IFN- γ to effectively treat active EAE. To explore this hypothesis, we tested the efficacy of IFN- β treatment of MOG_{35–55}-induced EAE in C57BL/6 mice and IFN γ R^{-/-} mice. Daily injections of Rebif, IFN- β 1a, a popular form of IFN- β treatment, beginning seven days after EAE induction, significantly delayed the onset and reduced the severity of EAE symptoms in the C57BL/6 mice (Fig. 4a). This protective effect of IFN- β treatment was transient, since the mice developed severe symptoms after the withdrawal of treatment, 18 days after induction of the disease. In contrast to the C57BL/6 mice, IFN- β treatment had no effect on

the development of disease in *IFN γ R*^{-/-} mice (Fig. 4b). Similar to Rebif treatment, recombinant mouse IFN- β attenuated EAE in C57BL/6 mice (Supplementary Fig. 7c) but not in *IFN γ R*^{-/-} mice (Supplementary Fig. 7d).

Mice were treated daily with Rebif from day 0 to day 6 post-induction of EAE to model disease prevention. IFN- β treatment was modestly protective in C57BL/6 mice (Fig. 4c), while *IFN γ R*^{-/-} mice had a trend towards more severe symptoms when treated with IFN- β (Fig. 4d). This is similar to the effect of IFN- β following adoptive transfer of T_H17 cells (Fig. 3b). Next we tested whether IFN- β treatment can reverse the progression of EAE after the onset of symptoms. We treated C57BL/6 and *IFN γ R*^{-/-} mice with recombinant murine IFN- β (rmIFN- β) when they attained a clinical score of 2 or 3. We found that rmIFN- β treatment attenuated the progression of symptoms in C57BL/6 mice scores compared to PBS treated mice (Supplementary Fig. 7e). In accordance with the other regimens, IFN- β did not attenuate the disease in the *IFN γ R*^{-/-} mice (Supplementary Fig. 7f).

Our *in vitro* data demonstrated that cooperative signaling of IFN- β and IFN- γ indirectly induces IL-10 in CD4 T-cells via APCs. To test whether this occurs in disease, we induced EAE in *IFN γ R*^{-/-} recipient mice by transferring WT encephalitogenic T_H1 cells and treated with rmIFN- β or PBS every second day from day 0 to 10. We found that the recipient mice treated with IFN- β exhibited severe acute clinical symptoms which were significantly increased compared to the PBS treated mice (Fig. 4e).

In the CNS, C57BL/6 mice treated with IFN- β had reduced frequencies of T_H1 and T_H17 cytokine positive CD4 T cells, as well as reduced frequencies of the population of CD4 cells co-expressing IL-17 and IFN- γ in spinal cords (Supplementary Fig. 8a) and brainstem and cerebellum (Supplementary Fig. 8b) 12 days post induction of disease. In contrast, IFN- β treatment increased the frequencies of these T-helper populations in the CNS of *IFN γ R*^{-/-} mice (Supplementary Fig. 8a,b).

Effect of IFN- β on human T_H differentiation

To confirm the observed effects of IFN- β on human T_H differentiation, we stimulated naïve CD4 T-cells from 4 donors for 5 and 11 days in non-polarizing, T_H1-polarizing and T_H17-polarizing conditions in the presence or absence of IFN- β . IFN- β had no effect on IFN- γ production in the all polarizing conditions after 5 or 11 days of culture (Fig. 5a,d). IL-17 production was also not modulated by IFN- β after 5 days of culture (Fig. 5b). However, by day 11, IFN- β inhibited IL-17 production during T_H17 differentiation of 3 donors (578 ng/ml to 85 ng/ml, 696 ng/ml to 304 ng/ml, 284 ng/ml to 90ng/ml), while in one donor IFN- β increased IL-17 production (385 pg/ml to 998 pg/ml) (Fig. 5e). During T_H1 polarization, IFN- β significantly increased IL-10 production by day 5 (Fig. 5c) in all donors and the induction of IL-10 was greater in T_H1 compared to the non-polarizing and T_H17 polarizing conditions on both day 5 and day 11 (Fig. 5c,f).

Cytokine profile in RRMS patients before IFN- β treatment

We analyzed the pre-treatment serum levels of 28 cytokines and chemokines in RRMS patients. Twelve patients were classified as responders and fourteen as non-responders to IFN- β treatment. Non-responders have exacerbated disease course in terms of both their relapse rate and steroid usage. Steroids are used in the clinic when there is acute neurologic deterioration. The median relapse rate in the two years following initiation of IFN- β treatment in the non-responders was two, while it was zero in the responder population. Likewise, the median number of steroid interventions was two in the non-responders and zero in the responders (Supplementary Table 1).

Cluster analysis of the cytokine profiles grouped 6 non-responders together (Fig. 6a). This group of non-responders had significantly elevated serum concentrations of both IL-17F and IFN- β compared to the responders (Fig. 6b,c). Furthermore, there was a significant correlation with IL-17F and IFN- β levels in the serum of responders, non-responders and healthy individuals (Supplementary Fig. 9a. This was not found when comparing IL-17F or IFN- β to MIP1 β (Supplementary Fig.9b,c) or other analytes (data not shown).

Discussion

IFN- β is one of the most widely used treatments for MS. However, a major limitation with IFN- β is that a 30 to 50% of MS patients do not respond to IFN- β therapy. Therefore, it is desirable to identify responders and non-responders prior to the initiation of treatment. In a series of experiments in mice, we identified that mice with T_H1-EAE benefit from IFN- β treatment with reduction in levels of disability, while mice with T_H17-EAE do not respond and disease worsens.

In T_H1-EAE, induction of anti-inflammatory cytokine, IL-10, was increased with beneficial IFN- β treatment. Since IFN- β treatment inhibited T_H1-EAE, we speculated that effective IFN- β treatment depended on high IFN- γ levels during EAE. In fact, IFN- β was effective in treating active EAE C57BL/6 mice, but worsened EAE in *IFN γ R*^{-/-} mice. Furthermore, when EAE was induced via passive transfer of C57BL/6 T_H1 cells into *IFN γ R*^{-/-} recipients, IFN- γ influences a broad range of cells in its complex interactions with IFN- β . Cell culture experiments, concordant with *in vivo* studies in EAE, revealed that the induction of IL-10 by IFN- β in T-cells required APCs and also required cooperative IFN- γ signaling, indicating that the APC's were the target of this activity of IFN- β . Popular speculation on the mechanism of IFN- β treatment is that it attenuates disease by inhibiting the differentiation of T_H17 cells^{5,8,26}. We find that IFN- β inhibits IL-17 production *in vitro* and in EAE, yet IFN- β is ineffective in treating T_H17-induced EAE and increases symptoms in these mice.

In RRMS a subset of non-responders had high serum levels of the T_H17 cytokine, IL-17F before IFN- β therapy was initiated. IL-17F is produced by T_H17 cells in EAE²⁷ suggesting that this group of MS patients is skewed towards a T_H17 version of disease. Furthermore, these patients also had high levels of endogenous IFN- β compared to responders. This correlation between high IL-17F and IFN- β concentration in the serum suggests a tight biological association between these two cytokines. Two hypotheses, which may not be mutually exclusive, could explain this phenomenon.

One hypothesis is that these non-responders have aggressive T_H17 disease. To counteract inflammation, their immune systems up-regulates IFN- β . Since endogenous IFN- β levels are already high, IFN- β treatment is ineffective. A second hypothesis is that IFN- β is pro-inflammatory during T_H17 disease. Not only would IFN- β treatment be ineffective, it could worsen symptoms. This is supported by observations in EAE, where symptoms worsened with IFN- β treatment in T_H17-EAE, a finding concordant with the data in RRMS, where patients with high IL-17F and IFN- β have exacerbated disease. Observations in neuromyelitis optica (NMO), another demyelinating disease, provide evidence for this hypothesis. NMO lesions are granulocytic^{28,29} and T_H17 responses attract granulocytes to sites of inflammation^{30–32}. Furthermore, NMO patients have high levels of IL-17 in the CSF³³. Finally, IFN- β treatment of NMO induces relapses^{2,34,35}. Therefore, the disease processes of NMO and the IL-17F^{hi} non-responders could be very similar.

The data presented in this study demonstrates a phenomenon often seen with cytokines. IFN- β has polar effects in different contexts, leading to benefit in T_H1 conditions, but harm in T_H17 conditions. This duality of functions for cytokines is often referred to as a Janus-

like, quality after the two headed God from Roman mythology, with opposite activities attributed to the very same molecule³⁷. This very feature of IFN- β provides an opportunity to explore some logical biomarkers that might have predictive value in assessing the response to a popular therapy for MS.

Materials and Methods

EAE induction and treatment

Active EAE was induced in C57BL/6 and *IFN γ R*^{-/-} (described supplementary methods) who were treated daily with 1 μ g/ml of IFN β -1A (Rebif®, Serono) or PBS from 0 to 6, or 7 to 17 days after the induction of EAE. In some experiments, mice were also treated daily with 5000 units of rmIFN- β (PBL) from day 10 to 17. We monitored mice daily for clinical signs.

For adoptive transfer of EAE in C57BL/6 or SJL mice, on day 10, we re-stimulated splenic and axillary lymph node cells with MOGp35–55 or PLPp139–151 and 10ng ml⁻¹ of IL-12 (T_H1) or IL-23 (T_H17) for 3d and transferred 30x10⁶ cells into healthy recipients. The frequency of donor CD4 T-cells producing IL-17 and IFN- γ was assessed by fluorescence-activated cell sorting (FACS) prior to transfer (data not shown). Mice received 1000U/dose of rmIFN- β or PBS every second day from day 0–10 post transfer and clinical signs were monitored. Spinal cords were fixed and sectioned for histology (LFB and H&E).

Infiltrating cells were isolated from spinal cords or brainstem/cerebellum from 3–4 perfused animals. CNS homogenates were incubated with collagenase and DNase for 1h at 37°C and cells were purified by a Percoll gradient.

Mouse T_H differentiation

We depleted spleen cells of CD8 T-cells by magnetic sorting (Miltenyi) and stimulated the cells for 3d with 1 μ g ml⁻¹ of anti-CD3 (Ebioscience) in non-polarizing, T_H1 (10ng ml⁻¹ IL-12), T_H17 (1ng ml⁻¹ rhTGF- β and 20 ng ml⁻¹ rmIL-6), or Treg (TGF- β 1ng ml⁻¹) conditions in the presence or absence of rmIFN- β (100U ml⁻¹). Where indicated, 10 μ g ml⁻¹ of antibodies to IFN- γ (Ebioscience), IL-10 (Ebioscience) or IL-27p28 (R&D) were added to cultures.

We obtained naïve and activated/memory CD4 T-cells by magnetic selection (Miltenyi) and stimulated the cells with anti-CD3 and APCs (T-cell depleted splenocytes) at a 1:5 ratio in non-polarizing, T_H1 or T_H17 conditions or IL-23 (10ng/ml) alone. Purified CD4⁺ T-cells cultured without APCs were stimulated by plate-bound anti-CD3 (5 μ g ml⁻¹) and anti-CD28 (0.5 μ g ml⁻¹).

For antigen specific differentiation, we re-stimulated LN cells from MOGp35–55 immunized mice with MOGp35–55 for 3d in IL-12 or IL-23 (10ng ml⁻¹) with or without IFN- β .

Analysis of mouse cytokines

For FACS, cells were stimulated with of PMA and ionomycin (Sigma-Aldrich) and monensin for 4 h, stained for CD4 (BDBiosciences), fixed and permeabilized with Cytofix/Cytoperm (BDBiosciences), then stained for IFN- γ , IL-17, IL-10 or FoxP3 (BD Biosciences) before analysis by FACS. ELISAs for IL-17 (R&D Systems), IL-10 (BD Biosciences), IFN- γ (BDBiosciences), and IL-27p28 (R&D) were performed on culture supernatants. Cytokines from antigen specific differentiation were analyzed by multiplex-bead-analysis (Panomics).

T-Cell Proliferation

5×10^5 splenic cells, depleted of CD8 T-cells, were cultured in triplicate with $1 \mu\text{g/ml}$ anti-CD3 in the presence of the indicated concentration of rmIFN- β . After 48 hrs, cultures were pulsed with [^3H]thymidine for an additional 18 hrs, and counts per minute (c.p.m.) of incorporated [^3H]-thymidine were read using a beta counter.

In vitro STAT1 activation

We stimulated splenocytes from C57BL/6 and *IFN γ R $^{-/-}$* for 15m with 100 U ml^{-1} of rmIFN- β . Cells were fixed with 1.6% paraformaldehyde and permeabilized with 100% methanol before staining for CD4 (Ebioscience) and pSTAT1 (BDBiosciences).

Human T_H differentiation

We cultured naïve T cells from healthy donors for 5 d at a density of 5×10^5 cells per well in Yssel's media containing 1% human AB serum (Gemini Bio-Products) along with beads coated with antibodies to CD2, CD3 and CD28 (1 bead per 10 cells; Miltenyi) in non-polarizing conditions (no cytokines), T_H1-polarizing conditions (hIL-12 (5 ng ml^{-1} ; R&D)) or T_H17-polarizing conditions (hIL-23 (50 ng ml^{-1} ; DNAX)) in the presence or absence of hIFN- β (100 U/ml). Cells were split and cultured for 6 additional days with the various cytokines and IL-2 (100 U/ml ; R&D). For day 5 cultures, cytokines were directly assayed from cell supernatants. For Day 11 cultures, $1 \times 10^6 \text{ cells ml}^{-1}$ were stimulated with T cell-activation beads with IL-2 and after 48 h cytokines were assayed.

MS Patients Clinical Classification and Serum Collection

Twenty-six RRMS patients receiving IFN- β treatment for at least 12 months were identified as responders or non-responders to IFN- β therapy. The patients were classified based on Expanded Disability Status Scale (EDSS) progression, the number of relapses and steroid interventions in the two years before initiation of treatment compared to the two years after starting treatment (Supplementary Table 1). Two neurologists, blinded to the laboratory data, classified the selected patients as responder or non-responder. Serum samples were obtained the day before starting IFN- β therapy. The study received approval from the Medical Ethics Board of the VU University Medical Center, Amsterdam, Netherlands.

ELISA and multiplex analysis of human cytokines

Cytokines from human cultures supernatants were assayed by ELISA (Ebioscience). Cytokine analysis of sera from MS patients and healthy controls was performed by multiplex-bead-analysis. Multiplex results were analyzed using Gene Cluster software and samples were ordered using hierarchical clustering and results were presented as a heat map using TreeView³⁶.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Effect of IFN-β on mouse TH17 differentiation. a) IFN-β attenuates TH17 differentiation. Spleen cells, depleted of CD8 T-cells, were cultured with and without IFN-β in non-polarizing, TH1 and TH17 conditions. CD4 T-cells were analyzed for IL-17 production by flow cytometry. b) IFN-β stimulation of naïve CD4 T-cells (CD62⁺) cultured in TGF-β and IL-6 with APCs, effector/memory CD4 T-cell (CD62⁻) cultured in TGF-β and IL-6 with APCs and effector/memory CD4 T-cell (CD62⁻) cultured in IL-23 and APCs. All CD4 T-cells were cultured with APCs at a ratio of 1:5. Cytokine secretion was analyzed by ELISA. Results are the mean ± SD of triplicates. Results are representative 3 experiments. **P*<0.05. c) *STAT1*^{-/-} spleen cells, depleted of CD8 T-cells, were cultured with and without IFN-β in TH17 conditions. IL-17 production from CD4 T-cells was analyzed by flow cytometry. d) CD8-depleted spleen cells from C57BL/6 mice were cultured in TH17 conditions with or without IFN-β in the presence and absence of neutralizing antibodies to IFN-γ, IL-10 or IL-27p28. e) Purified WT or *IFNγR*^{-/-} CD4 T-cells were polarized in TH17 conditions with WT or *IFNγR*^{-/-} in the presence or absence of IFN-β and IL-17 production was assessed by ELISA. **P*<0.01. f) Purified CD4 T-cells were stimulated with plate-bound anti-CD3 and anti-CD28 in TH17 conditions in the presence or absence of IFN-β, IFN-γ or both; IL-17 production was assessed by ELISA. **P*<0.01. Results are the mean ± SD of triplicates. Results represent 1 of 3 similar experiments.

Fig. 2.

(a–c) Effect of IFN- β on mouse T_H1 differentiation. a) Spleen cells, depleted of CD8 T-cells, were cultured with and without IFN- β in non-polarizing, T_H1 and T_H17 conditions. CD4 T-cells were analyzed for IFN- γ production by flow cytometry. b) Purified WT or *IFN γ R*^{-/-} CD4 T-cells were stimulated in non-polarizing conditions with APCs from WT or *IFN γ R*^{-/-} in the presence or absence of IFN- β . IFN- γ was assessed by ELISA. * $P < 0.05$. c) Purified WT or *IFN γ R*^{-/-} CD4 T-cells were stimulated in T_H1 conditions with APCs from WT or *IFN γ R*^{-/-} in the presence or absence of IFN- β . IFN- γ was assessed by ELISA. d) CD8 depleted spleen cells were stimulated with or without IFN- β in non-polarizing, T_H1 , and T_H17 conditions and IL-10 expression in CD4 T-cells was analyzed by flow cytometry. e) CD8 depleted spleen cells were stimulated with or without IFN- β in non-polarizing conditions in the presence or absence of antibodies to IFN- γ or IL-10. f) IFN- β requires IFN- γ signaling in mouse CD4 T-cells and APCs to induce IL-10. Purified WT or *IFN γ R*^{-/-} CD4 T-cells were cultured in non-polarizing conditions with WT or *IFN γ R*^{-/-} in the presence or absence of IFN- β and IL-10 was assessed by ELISA. * $P < 0.01$ and ** $P < 0.005$. g) Purified CD4 T-cells were stimulated with plate-bound anti-CD3 and anti-CD28 in non-polarizing conditions in the presence or absence of IFN- β .

Fig. 3.

IFN- β treatment blocks T_H1 induced EAE but exacerbates T_H17 induced EAE. (a and b) Clinical scores from mice with passive EAE induced by adoptive transfer of (a) T_H1 and (b) T_H17 cells that were treated with rmIFN- β or PBS every second day from day 0 to 10 post transfer ($n=9$ to 11 mice per group). * $P<0.05$. (c) Histology of spinal cord sections from T_H1 and T_H17 induced EAE treated with IFN- β or PBS. Sections of spinal cord were obtained 45 days after transfer and stained with H&E and Luxol fast blue. Scale bars, 50 μ m. (d–f) Frequency of CD4⁺ lymphocytes expressing IFN- γ (d), IL-17 (e) and IL-10 (f) in the spinal cords 45 day post transfer. The mean percentage $\pm$ standard deviation ($N=3$ experiments) of cytokine positive cells is given. Each experiment is a pool from 2–3 mice per group. (g–i) Concentration of IFN- γ (g), IL-17 (h) and IL-10 (i) from supernatants of MOGp35–55 stimulated spleens taken from mice 45 days post transfer. Data represent mean and standard deviation of 3–4 mice per group. * $P<0.05$.

Fig. 4.

IFN-β treatment requires IFN-γ signaling to suppress EAE symptoms.

(a and b) Clinical scores from active EAE in (a) C57BL/6 and (b) *IFNγR*^{-/-} mice that were treated with Rebif® or PBS daily from day 7 to day 17 post EAE induction (*n*=7 to 9 mice per group).

(c and d) Clinical scores from active EAE in (c) C57BL/6 and (d) *IFNγR*^{-/-} mice that were treated with Rebif® or PBS daily from day 0 to day 6 post EAE induction (*n*=4 to 5 mice per group).

e) Clinical scores from *IFNγR*^{-/-} mice with passive EAE induced by adoptive transfer of WT T_H1 and treated with IFN-β or PBS every second day from day 0 to 10 post transfer (*n*=6). Treatment doses indicated with arrows. **P*<0.05.

Fig. 5.

Effect of IFN- β on Human T_H differentiation. Naïve human CD4 T-cells were cultured in non-polarizing, T_H1-polarizing (IL-12) and T_H17-polarizing (IL-23) conditions for 5 days (a–c) or 11 days (d–f) and IFN γ , IL-17A and IL-10 were assessed by ELISA. Day 11 cultures were further reactivated in the presence of beads coated with antibodies to CD3, CD28 and CD2 Abs for 48 hrs prior to analysis. * P <0.01, ** P <0.02, and *** P <0.05.

Fig. 6.

Pre-Treatment cytokine profiles in serum of IFN-β responder and non-responder MS patients. a) Relative cytokine levels in responder and non-responder MS patients. Relative cytokine levels in serum from responder and non-responder MS patients are depicted as the difference in relation to healthy controls. Samples were analyzed by hierarchical clustering, and displayed as a heat map where red represents increased levels, black represents similar levels and green represents decreased levels of cytokine compared to healthy controls. b) Concentration of IL-17F in subsets of pre-treatment MS patients and healthy control. Cytokine concentrations were calculated from a standard linear regression of known quantities of IL-17F. c) Concentration of IFN-β in subsets of pre-treatment MS patients and healthy control. Cytokine concentrations were calculated from a standard linear regression of known quantities of IFN-β. * $P < 0.001$ and ** $P < 0.002$.