[Frontiers in Bioscience 9, 1547-1555, May 1, 2004]

ABNORMAL LEVELS OF INTERFERON-GAMMA RECEPTORS IN ACTIVE MULTIPLE SCLEROSIS ARE NORMALIZED BY IFN-β THERAPY: IMPLICATIONS FOR CONTROL OF APOPTOSIS

James Ahn, Xuan Feng, Nilesh Patel, Neetu Dhawan, and AT Reder

Department of Neurology, The University of Chicago, Chicago, IL

TABLE OF CONTENTS

1. ABSTRACT

Interferon-γ is produced by immune cells before MS exacerbations, and exogenous IFN-γ treatment causes MS attacks. IFN-β production, conversely, rises after exacerbations. IFN-β therapy ameliorates MS, possibly by lowering IFN-γ secretion and inhibiting responses to IFN-γ. IFN-γ effects are regulated by IFN-γ receptor (IFNGR) expression. IFN-γ is pro-inflammatory at low IFNGR levels, but induces apoptosis in cells with high IFNGR levels. We studied effects of IFN-β1a therapy on IFNGR expression on PMA/ionomycin-stimulated PBMNC's in 29 patients with active and stable MS. Surface IFNGR-alpha (the binding chain) and IFNGR-beta (signaling chain), as well as intracellular IFN-γ and IL-10, were measured with flow cytometry. Before IFN-β therapy, intracellular IL-10 was depressed and the IFN-γ/IL-10 ratio was elevated in MS, particularly during clinical activity. With IFN-β therapy IL-10 levels increased, suggesting that a Th2 deficit was reversed. The IFNGR-alpha chain was significantly elevated on lymphocytes in stable and active MS patients not receiving IFN-β therapy. Expression of the IFNGR-beta chain was low during active untreated disease. After IFN-β therapy, the IFNGR-beta/alpha ratio increased at 3 months and fell at 12 months. Increased susceptibility to apoptosis with high IFNGR-beta chain expression at 3 months is likely to remove activated T cells during IFN-β therapy.

2. INTRODUCTION

IFN-γ is a pro-inflammatory Th1 cytokine that amplifies cell-mediated immune responses and regulates immunity in multiple sclerosis. A therapeutic trial of IFN-γ in MS was halted because it increased exacerbation rates 4-fold (1). Before MS exacerbations, IFN-γ is elevated in mononuclear cells (MNCs)(2) and in serum (3). During clinical activity, immune cells are also more sensitive to IFN-γ's ability to induce intracellular calcium (4). In contrast to these proinflammatory effects, however, IFN-γ limits expansion of activated T cells by causing their apoptosis (5). IFN-γ-induced T cell death limits EAE (6) and apoptosis of dysregulated T cells is a potential mechanism of action of IFN-β therapy for MS (7).

The interferon-γ receptor (IFNGR) is expressed on all cells of the body except RBCs. It is composed of a ligand-binding chain, IFNGR-alpha, and a signal transducing chain, IFNGR-beta. IFN-γ binding causes phosphorylation of latent signal transducer and activator of transcription-1 (STAT1) (8, 9), P-STAT1 then regulates antiviral responses as well as cytokine secretion, cell proliferation, and cell death.

IFN-γ receptors control apoptosis in normal human lymphocytes. The IFNGR-alpha chain is required, because blocking the receptor prevents activation-induced apoptosis (10). However, fine control of apoptosis resides in the beta chain. With high IFNGR-beta expression, IFN-γ triggers a strong activation of STAT1 and elevates levels of

interferon regulatory factor-1 (IRF-1), which prompts apoptosis. When IFNGR-beta expression is low, IFN-γ weakly activates STAT1 and causes moderate elevation of IRF-1, which induces proliferation (11, 12). Thus, IFNGR levels influence the intensity of activation of STAT1 (11), creating an intricate balance between IFNGR expression and cytokine levels, cell proliferation, and apoptosis.

IFN-β and IL-10 regulate disease activity in MS. Whole blood production of IFN-β rises as attacks resolve, in contrast to IFN-γ production, which rises before attacks (3). IFN-β-1a therapy ameliorates clinical and MRI markers of MS disease activity (13). IFN-β also both inhibits responses to IFN-γ and lowers IFN-γ secretion (14). IL-10 is a Th2 cytokine that inhibits macrophage and Th1 function and reduces secretion of proinflammatory cytokines (*e.g.*, IFN-γ) (15). IL-10 protein levels in MS immune cells and serum are significantly lower than in normal controls (16, 17). The ratio of Th1/Th2 cells, reflected by the IFN-γ/IL-10 ratio, is higher in active MS than stable MS; and higher in both forms of MS than in NL controls (18, 19). IFN-β therapy corrects these abnormalities – it increases IL-10 in PBMCs and elevates IL-10-producing T cells (14, 20, 21). Moreover, increased CSF IL-10 correlates with a positive therapeutic response to weekly IFN-β injections (22).

Normal T cells vary in IFNGR expression. The amount of IFNGR-alpha is greater on human Th2 than on Th1 clones (10). IFNGR-beta is present at low levels on both human Th1(23) and Th2 clones,(10) and increases with activation of these cells (10). The IFNGR-beta chain becomes undetectable on Th1 cells when they secrete IFN-γ, which down-regulates IFNGR in long-term cultures (10, 11, 24). With more acute exposure, IFN-γ hampers Th2 proliferation and causes apoptosis of Th1 cells with high IFNGR expression (10).

Since IFN-γ regulates and reflects MS disease activity, we hypothesized that IFN-γ receptors would vary during clinical activity. Four-color flow cytometry was used to evaluate lymphocyte subsets, IFN-γ and IL-10 content, and expression of IFNGR-alpha and IFNGR-beta on peripheral blood MNC from patients with clinically stable and active MS, before and after IFN-β-1a therapy.

3. MATERIALS AND METHODS

3.1. Patients

Twenty-nine patients (24 women and 5 men) had clinically definite MS with a mean age of 41.0±2.1 years. 26 patients had relapsing/remitting MS (15 stable, 11 active); 3 had active relapsing/progressive MS. "Active MS" is defined as a severe exacerbation within 2 weeks, or progression over the preceding 6 months with a drop in the EDSS of at least 1 point (25). "Stable MS" patients had no exacerbations and no clinical progression during the prior 6 months. Eight normal controls (3 women and 5 men), 37.4±3.8 years old, had no history of neurological or immune disorders.

Patients were examined before and after treatment with weekly IM IFNβ-1a (Avonex, Biogen; Cambridge, MA), 30 ug (6 million units). Consecutive blood samples were obtained before start of treatment (baseline), and after 3 months and 12 months of treatment. No subjects in the study had been treated with steroids or other immunomodulatory drugs for at least 4 months, none had ever been treated with chemotherapy, and none had ongoing infections.

3.2. Cell Preparation and Activation

Venous blood was collected in heparinized tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and processed within 2 h. Peripheral blood-derived mononuclear cells (PBMC) were isolated with Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation, and cryopreserved on liquid N_2. Sampling from patients treated with IFN-β took place between 12 h and 5 days after the last injection. Serial samples were run together after thawing. Freshly thawed cells were stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin calcium (Sigma; St. Louis, MO), each at 10 ng/ml, in AIM-V medium (Invitrogen; Carlsbad, California), and incubated at 37° C for 2 h. Monensin (Sigma) at 1μM/μl was then added to the culture for 4 h. After a total of 6 h, cells were centrifuged and washed with Hank's Balanced Salt Solution (Invitrogen). Six hours of PMA/ionomycin and 4 h of monensin exposure had optimal effects on maximizing T cell cytokine induction while minimizing cell death. Other consequences of PMA/ionomycin stimulation for 6-hours included the disappearance of almost all monocytes, and a slight increase in size and auto-fluorescence of lymphocytes, but no change in IFNGR expression.

3.3. Flow Cytometry

Four-color fluorescence analysis (FloJo software, Tree Star, Inc.; San Carlos, CA) was performed with an LSR II (Becton Dickinson; Sunnyvale, CA) equipped with three lasers (325nm UV, 488nm, and 688nm HeNe). Activated and media control cells were blocked with 4% normal mouse serum and then stained with surface and intracellular antibodies (Caltag; Burlingame, CA). 5-10 μL of each mAb were added to 100 μL of the MNC suspension. Cell surface stains included mouse anti-human antibodies for CD3 (T cells), CD4 (helper T cells), CD8 (cytotoxic/suppressor T cells), CD14 (monocytes), CD20 (B cells), CD56 (NK cells), CD119a (IFNGR-alpha chain), and CD119b (IFNGR-beta chain). After 30 minutes at 4° C with the primary antibody, the cells were washed and then stained with strepavidin-allophycocyanin (SA-APC) (Caltag) for 30 minutes at 4°C. Cells were washed, fixed, and permeabilized (Fix and Perm, Caltag), and then stained with intracellular antibodies for IFN-γ and IL-10. (Permiflow [Invirion; Frankfort, MI] proved less effective than Fix and Perm.) The cells were then washed and fixed with 4% paraformaldehyde in HBSS.

3.4. Standardization of Assays

Short-term lymphocyte activation modified CD4 protein, but not IFNGR-expression. CD4 proteins were progressively modulated off the cell surface during PMA/ionomycin stimulation (Figure **1**); this loss prevented accurate quantification of CD4 cells. CD8 and CD3

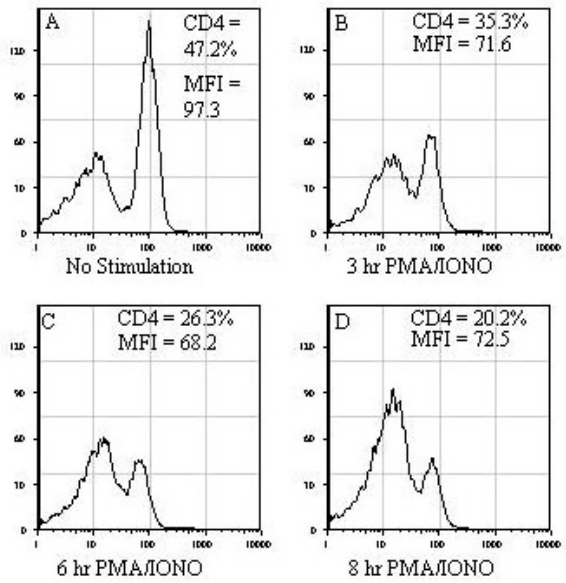

Figure 1. PMA/ionomycin activation removes CD4 from the cell surface, and interferes with accurate quantitation of CD4+ lymphocytes. CD4 expression on MNC (a) before, and (b) 2h, (c) 4h, and (d) 6h after activation with PMA/ionomycin. Note large reduction in the percentage of CD4+ cells and in the amount of CD4 expressed per cell (MFI). MFI = CD4 median fluorescence intensity.

proteins were not removed with activation. The percentage of CD3+ and CD3+CD8- cells did not change with disease activity or IFN-β therapy. There was a small decrease in the percentage of CD8+ cells during active MS, as previously demonstrated (26) (Active MS = 68% of NL, $p<0.02$; and 76% of stable MS, NS).

Intracellular and extracellular IFNGR-beta chain levels were compared because the beta chain is recycled between surface and intracellular pools (27). Intracellular IFNGR-beta was expressed at consistent, high levels in 70% of cells, paralleling (27). Intracellular IFNGR-beta expression was independent of disease state and IFN therapy and time after IFN-β injection (data not shown). IFN-γ (100 ng/ml) and IFN-β (160 U/ml) did not have any effect on IFNGR-alpha and -beta chain expression after 6 h of *in vitro* stimulation. This, and the presence of monensin to prevent secretion of IFNs and other cytokines, obviates an effect of endogenous IFN secretion on IFNGR-alpha or -beta chain expression. There were no significant sex differences in IFNGR expression. Finally, there was no difference in IFNGR expression when cells were prepared in AIM-V medium *vs*. 10% fetal calf serum.

3.5. Statistics

Student's t-test, two tailed, was used to compare values between controls and different forms of MS. A paired two-sample t-test, two tailed, was used for multiple comparisons of longitudinal serial values of IFNGR-alpha and –beta chains, IFN-γ, and IL-10. p values < 0.05 were considered significant. There were no sex or age differences in receptor or cytokine expression.

4. RESULTS

4.1. IFNGR expression on lymphocytes reflects clinical disease activity in MS

IFNGR-alpha chain expression was significantly increased in both active and stable MS, in comparison to healthy normal lymphocytes (Figure 2A). IFNGR-alpha expression was not different between stable and active MS. IFNGR-beta expression during active MS, in contrast, was decreased compared to NL controls (Figure 2B). IFNGR-beta levels in stable MS patients were not appreciably different from NL or active MS.

The IFNGR-beta chain is the deciding factor in whether IFN-γ-stimulated cells will proliferate or die (28). The ratio of beta/alpha chains reflects signaling efficiency for cellular responses. The ratio of IFNGR-beta/alpha expression fell with disease activity (Figure **2C**). The ratio in both MS disease states was decreased in comparison to NL controls. This suggests that lymphocytes in clinically active MS would be less responsive to certain effects of IFN-γ.

Changes in lymphocyte subsets paralleled changes in the overall lymphocyte population. On CD3+ cells, IFGR-alpha chain expression was greatest in active disease ($p<0.01$ *vs*. NL) and intermediate in stable MS. IFNGR-beta chain levels were decreased during active MS *vs*. NL ($p<0.05$) and stable MS *vs*. NL ($p<0.003$). CD3- cells had a parallel rise in IFNGR-alpha in stable MS ($p<0.09$) and in active MS ($p<0.02$ vs. NL). However, there were no significant differences in IFNGR-beta chain expression in CD3- cells with disease activity. IFNGR levels were not substantially different between CD8+ and CD8- cells. These data suggest that the changes in IFNGR-beta chain expression during active disease are predominantly on CD3+ lymphocytes, and probably within the CD4+ subset.

4.2. IFN-β therapy modifies IFNGR expression on lymphocytes from MS patients – Longitudinal analysis at 0, 3, and 12 months of therapy

Since IFN-β ameliorates disease activity in MS through an undefined mechanism of action, we studied whether IFN-β therapy would normalize IFNGR expression in MS. IFNGR-alpha levels decreased from month 0 to month 3 of IFN-β therapy, and from month 0 to month 12 (Figure **3A**). The lower levels of IFNGR-alpha expression at both times remained significantly greater than NL controls.

Levels of the IFNGR-beta chain rose significantly from pre-treatment to month 3, and were elevated compared to normal controls at this time (Figure 3B). The beta/alpha chain ratio also rose after 3 months of IFN-β therapy (Figure 3C). Over-expression of the IFNGR-beta chain increases susceptibility to apoptosis (11, 28). Possibly as a consequence, there was a considerable decline of IFNGR-beta chain expression from months 3 to 12, back to NL control levels, and the IFNGR-beta/alpha chain ratio fell to below NL control levels (Figure 3B).

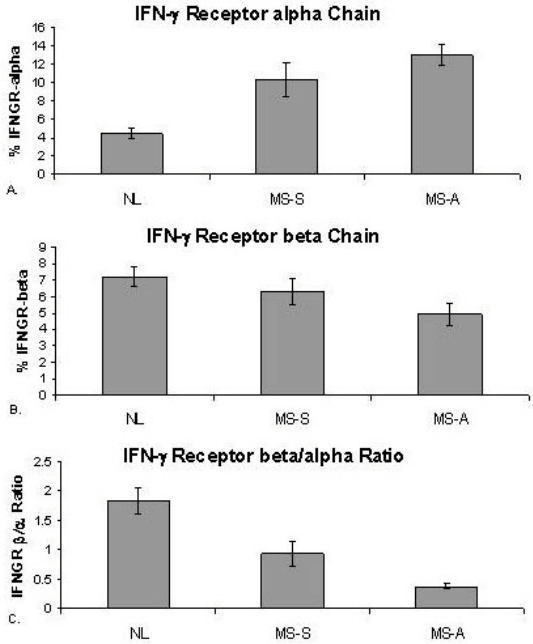

Figure 2. Percentage of IFNGR positive cells in lymphocyte population from untreated MS and NL subjects. (A) IFNGR-alpha chain expression is increased, compared to 10 normal controls, in both 10 active ($p<0.001$) and 13 stable MS patients ($p<0.00001$, student's t-test) [NL<stable MS#active MS]. IFNGR-alpha expression was not different between stable and active MS. (B) IFNGR-beta expression in active MS patients was decreased ($p<0.024$, student's t-test) compared to NL patients. Stable MS patients showed no appreciable change from NL or active MS. (C) The IFNGR-beta/alpha chain ratio fell with disease activity (stable MS *vs*. NL, $p<0.03$; and active MS *vs*. NL, $p<0.00003$; student's t-test) [NL>stable MS>active MS]. NL = normal, MS-s = stable MS, MS-a = active MS.

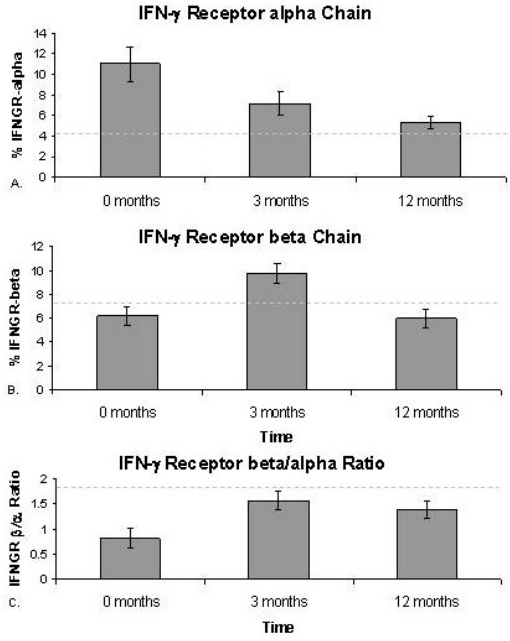

Figure 3. Percentage of IFNGR positive cells in lymphocyte population during IFN-β therapy in MS. (A) IFNGR-alpha expression decreased from month 0 to month 3 of therapy ($p<0.05$, paired t-test), and from month 0 to month 12 ($p<0.004$, N=21). There was a non-significant trend for a further drop in expression from month 3 to month 12 ($p=0.11$). Both points were still significantly greater than NL ($p<0.003$, student's t-test). Dashed line indicates IFNGR-alpha expression in NL subjects, as seen in Figure **2**. (B) The IFNGR-beta chain rose from pre-treatment to month 3 ($p<0.003$, paired t-test) and was higher than in normal controls at 3 months ($p<0.002$). IFNGR-beta chain expression declined from month 3 to 12 ($p<0.003$, student's t-test), back to NL levels (dashed line). (C) The IFNGR-beta/alpha chain ratio rose after 3 months of IFN-β therapy ($p<0.05$), but it tended to fall at 12 months ($p<0.06$). By 12 months, the ratio was below control levels (dashed line; $p<0.01$). NB, with paired t-tests, mean and SEM in figure are used for orientation but not in the statistical analysis. (Abbreviations as in Figure 2.)

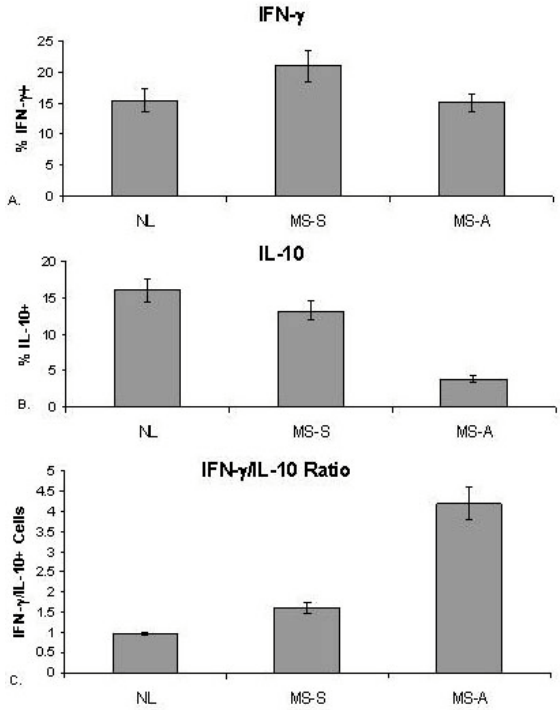

Figure 4. Percentage of IFN-γ and IL-10 positive cells in the lymphocyte population of normal controls and untreated MS patients. (A) IFN-γ+ cells were equivalent between NL controls and the stable and active MS groups (student's t-test). (B) IL-10+ cells decreased in patients with clinically active MS compared to normal controls ($p<0.0001$, student's t-test), and the percentage in active MS was also less than in stable MS ($p<0.00001$). The downward trend in IL-10+ cells in stable MS compared to NL was not statistically significant. (C) The IFN-γ/IL-10 cell ratio was higher in stable and active MS than in normal subjects (both $p<0.0001$, student's t-test). The ratio was also higher in active MS compared to stable MS patients ($p<0.0001$). (Abbreviations as in Figure **2**.)

Changes in CD3- lymphocytes during treatment paralleled the changes in the entire lymphocyte population. The IFGR-alpha chain levels fell after 3 months of therapy ($p<0.05$ *vs*. baseline), and fell further at 12 months ($p<0.04$ *vs*. baseline). IFNGR-beta chain expression rose from 0 to 3 months ($p<0.004$), but fell at 12 months ($p<0.005$ *vs*. 3 months), back to baseline values. There was no change in IFNGR on CD3+, CD8+, and CD8- lymphocytes. The variation in the IFNGR-alpha and beta chains with therapy was statistically significant only in CD3- cells. NK and B cells, which are CD3-, have higher baseline expression of IFNGR (28). In the present data, IFNGR-beta chain expression was greater on CD3- than on CD3+ cells before and after IFN-β therapy ($p<0.007$ to 0.0001). This suggests that IFN-β therapy predominantly modifies IFNGR expression on CD3- cells, whereas pretreatment fluctuations caused by disease activity are mainly within CD3+ cells.

4.3. The number of Th1 and Th2 lymphocytes varies with disease activity in MS.

Th1 cells are IFN-γ-positive and inhibit Th2 cytokine production. Th2 cells are IL-10-positive and inhibit Th1 cells and may inhibit inflammation in MS. Flow cytometry measures a T cell's potential to secrete these cytokines, but does not measure cytokine secretion.

The percentage of IFN-γ+ cells was similar in NL and in stable and active MS groups (Figure 4A). However, patients with active MS had significantly fewer IL-10+ cells than NL controls, and also had fewer IL-10+ cells than stable MS patients (Figure 4B). The IFN-γ/IL-10 ratio in lymphocytes was significantly higher in stable and active MS than in NL subjects (Figure 4C). This indicator of the Th1/Th2 balance was also higher in active MS than in stable MS patients.

IFNGR expression on Th1 and Th2 cells was evaluated. IL-10-positive T cells (Th2), as well as IFN-γ-positive cells (Th1), in NL controls and in stable and active MS had equivalent expression of the IFNGR-alpha chain. There was high expression of the IFNGR-beta chain on IL-10+ normal CD3+ T cells. However, in all forms of MS, before and after therapy, there was minimal IFNGR-beta expression on IL-10+ and IFN-γ+ T cells. Potential causes of low IFNGR-beta chain in MS include downregulation of the IFNGR-beta chain by serum IFN-γ, or apoptotic death of IFNGR-beta^{hi} cells.

4.4. IFN-β therapy modifies intracellular IFN-γ and IL-10 content in lymphocytes from MS patients – Longitudinal analysis at 0, 3, 12 months of therapy

The number of IFN-γ-positive cells dropped significantly at 3 months compared to pretreatment levels, and remained low through 12 months of IFN-β therapy (Figure 5A). The lower IFN-γ content in treated patients did not differ from healthy NL controls after 3 to 12 months of IFN-β therapy.

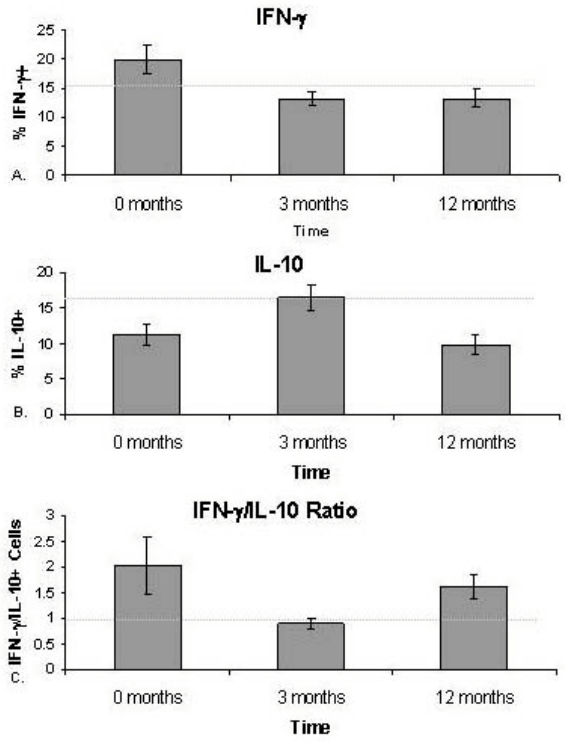

Figure 5. Percentage of IFN-γ and IL-10 positive cells in the lymphocyte population during IFN-β therapy in MS. (A) IFN-γ+ cells dropped at 3 months ($p<0.004$ compared to pre-treatment, two-tailed paired t-test), and remained low through 12 month of IFN-β therapy. The reduced IFN-γ content during IFN-β therapy did not differ from healthy controls. Dashed line indicates IFN-γ expression in NL subjects, as seen in Figure 4. (B) IL-10+ cells rose from 0 to 3 months ($p<0.05$), up to normal levels, suggesting a Th2 deficit is reversed with IFN-β therapy. The number of IL-10+ cells then dropped at 12 months compared to 3 months ($p<0.00004$) and compared to NL controls (dashed line; $p<0.01$). (C) The IFN-γ/IL-10 cell ratio dropped significantly from 0 to 3 months of IFN-β therapy ($p<0.0003$), and was equivalent to control levels (dashed line). However, at 12 months, the ratio rebounded back to baseline levels. At 12 months, the IFN-γ/IL-10 ratio had increased up to levels seen in stable MS, and was greater than the ratio in NL controls ($p<0.001$, student's t-test) but not up to levels in untreated active MS ($p<0.000005$ *vs*. treated at 12 mo). (Abbreviations as in Figure 2.)

IL-10-positive cells rose significantly from 0 to 3 months (Figure 5B, up to normal control levels, suggesting a Th2 deficit is reversed with IFN-β therapy. The number of IL-10+ cells then dropped at 12 months, equivalent to or below pre-treatment levels.

The IFN-γ/IL-10 ratio fell significantly from 0 to 3 months of IFN-β therapy, and was then equivalent to NL control levels (Figure 5C). This implies there was an early shift from a Th1 towards a Th2 bias with therapy. However, at 12 months, the IFN-γ/IL-10 ratio rebounded back to baseline levels, and had increased up to levels exhibited in untreated, stable MS patients. At 12 months, the ratio was greater than in NL controls, but did not rise up to levels in untreated, active MS (12 month treated *vs*. active untreated, $p<0.0000004$, student's t-test).

5. DISCUSSION

The Th1/Th2 balance and IFN-γ receptor expression change with fluctuations in clinical MS activity and after IFN-β therapy. The IFN-γ/IL-10 ratio is elevated in stable MS and rises even more during active MS (Figure **4**). The IFN-γ/IL-10 ratio falls markedly after 3 months of IFN-β treatment, but is followed by a partial rebound after 12 months of treatment (Figure **5**). These data extend previous studies (18, 20, 21), and serve as an internal control for changes in IFNGR expression in MS. IFN-β has continued clinical efficacy over years (25). However, there is some tachyphylaxis to IFN-β with long-term therapy, including reduced IFN-β induction of IFN-stimulated genes (29), MxA protein (7), and monocyte IL-10 production (30, 31), inhibition of T cell penetration of the BBB (32), and less susceptibility to apoptosis in MNC(7) and monocytes (33).

The IFNGR wields a two-edged sword — IFN-γ is pro-inflammatory with low IFNGR expression, but it causes apoptosis with high IFNGR levels. When IFNGR-alpha levels are adequate to permit binding, the less prevalent IFNGR-beta chain controls signaling intensity (11). A change in IFNGR-beta/alpha ratio is thus likely to regulate immune responses relevant in MS.

IFNGR expression increases with T cell activation (10, 34). This may explain the high levels of IFNGR-alpha chain expression in MS (Figure **2**), where peripheral immune cells have a Th1, memory phenotype (Figure 4, and (35)), and where cells are sensitive to IFN-γ induction of intracellular calcium fluxes (4). After IFN-β therapy, IFNGR-alpha chain expression decreased toward NL levels, perhaps a consequence of less T cell activation, or fewer activated T cells--removed through apoptosis (Figure 3). Other explanations are unlikely, including a persistent increase in IFN-γ (the elevated IFN-γ secretion falls after IFN-β therapy (36)), or a rise in Th1/Th2 cells (IL-10-secreting Th2 cells rise in the periphery with therapy (21)).

The signal-transducing IFNGR-beta chain determines the function and death of T cells. Expression of the beta chain declined with disease activity (NL > stable MS > active MS) (Figure 2B). This hierarchy matches 125I-IFN-γ binding to the IFNGR-beta/alpha heterodimer on non-adherent cells (NL > stable MS > active progressive/relapsing MS) (37). The IFNGR-beta chain could be downregulated by high levels of serum IFN-γ during active MS (2, 3). Death of immune cells with high expression of IFNGR-beta is also likely (see below), as immune cells are activated during MS exacerbations (38, 39). However, in later, progressive stages of MS the majority of lymphocytes are resistant to induction of apoptosis (35). Low expression of the IFNGR-beta chain during active MS also suggests that MS cells will be less sensitive to IFN-γ-induced apoptosis.

IFN-β treatment caused a rise in IFNGR-beta expression and increased the IFNGR-beta/IFNGR-alpha ratio at 3 months of therapy (Figure 3). Cells with high IFNGR-beta levels are more responsive to IFN-γ, creating more intense STAT1 phosphorylation and higher likelihood of apoptosis (11, 28). A rise in the IFNGR-beta chain from direct induction by IFN-β therapy is not likely, as no change is seen in vitro (Methods). However, the rise in IFNGR-beta expression may derive from a prolonged therapy-induced fall in serum IFN-γ, allowing more IFNGR expression.

After 12 months of IFN-β therapy, IFNGR-beta chain expression and the IFNGR-beta/alpha ratio fell (Figure **3**). Importantly, lymphocytes expressing high levels of IFNGR-beta may have been killed. Lymphocytes are removed through apoptosis during the first months of IFN-β therapy (7, 40). Similarly, IFN-γ terminates inflammation in EAE by causing apoptosis of T cells (6).

IFN-β therapy may enhance apoptosis by increasing signaling through the IFNGR. Apoptosis is induced by IFN-γ, and serum IFN-γ levels fluctuate during disease activity. There are high numbers of IFN-γ-secreting cells in untreated MS patients--a "cytokine storm"(2, 41) that could cause apoptosis of immune cells expressing high levels of IFNGR (5). IFN-β therapy increases STAT1 protein levels (42), activates STAT1,(39) and increases IFNGR-beta levels, all leading to IFN-γ-induced apoptosis. During the first several months of IFN-β therapy when IFNGR-beta chain expression is highest, there is a transient early rise of IFN-γ-secreting cells in some patients (43, 44), and then an eventual decrease in IFN-γ-secreting cells and in serum IFN-γ levels (30, 36). Even though average levels decline with IFN-β therapy, IFN-γ is present in serum and is elevated in inflammatory microenvironments. In concert, IFN-β reduces anti-apoptotic proteins (45) and synergizes with apoptosis-inducing, high-dose glucocorticoids (46, 47).

The proposed mechanism: 1) IFN-β-1a normalizes lymphocyte function in MS by initially raising IFNGR-beta expression. This intensifies the IFN-γ-induced activation of STAT1, causing apoptosis of Th1 cells (7, 11). 2) With more prolonged IFN-β therapy, cells become insensitive to IFN-γ—manifested by lowered IFNGR-beta chains at 12 months of treatment.

7. ACKNOWLEDGEMENTS

Thanks to Gretchen Hendrie for typing, and to Biogen for financial support.

8. REFERENCES

1. Panitch, H. S., Hirsch, R. L., Schindler, J. & Johnson, K. P.: Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. *Neurology* 37, 1097-1102 (1987)

2. Beck, J., Rondot, P., Catinot, L., Falcoff, E., Kirchner, H. & Wietzerbin, J.: Increased production of interferon gamma and tumor necrosis factor precedes clinical manifestation in multiple sclerosis: Do cytokines trigger off exacerbations? *Acta Neurol Scand* 78, 318-323 (1988)

3. Dettke, M., Scheidt, P., Prange, H. & Kirchner, H.: Correlation between interferon production and clinical disease activity in patients with multiple sclerosis. *J Clin Immunol* 17, 293-299 (1997)

4. Grimaldi, L. M. E., Martino, G., Filippi, M., Martinelli, V., Brambilla, E., Gobbi, C. & Comi, G.: Interferon (IFN)-γ-induced intracellular calcium increase in T lymphocytes from patients with multiple sclerosis precedes clinical exacerbation and detection of active lesions on MRI. *Mult Scler* 3, 316 (1997)

5. Refaeli, Y., Van Parijs, L., Alexander, S. I. & Abbas, A. K.: Interferon γ is requireed for activation-induced death of T lymphocytes. *J Exp Med* 196, 999-1005 (2002)

6. Furlan, R., Brambilla, E., Ruffini, F., Poliani, P. L., Bergami, A., Marconi, P. C., Franciotta, D. M., Penna, G., Comi, G., Adorini, L. & Martino, G.: Intrathecal delivery of IFN-γ protects C57 BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. *J Immunol* 167, 1821-1829 (2001)

7. Gniadek, P., Aktas, O., Wandinger, K.-P., Bellmann-Strobl, J., Wengert, O., Weber, A., von Wussow, P., Obert, H.-J. & Zipp, F.: Systemic IFN-β treatment induces apoptosis of peripheral immune cells in MS patients. *J Neuroimmunol* 137, 187-196 (2003)

8. Pestka, S., Kotenko, S. V., Muthukumaran, G., Izotova, L. S., Cook, J. R. & Garotta, G.: The interferon gamma (IFN-γ) receptor: a paradigm for the multichain cytokine receptor. *Cytokine & Growth Factor Reviews* 8, 189-206 (1997)

9. Decker, T. The molecular biology of type I interferons (IFN-α/β) (gene activation, promoters, proteins induced). *In* "Interferon Therapy in Multiple Sclerosis" (A. T. Reder, ed.), pp. 41-76. Marcel Dekker, Inc., New York. (1997)

10. Novelli, F., D'Elios, M. M., Bernabei, P., Ozmen, L., Rigamonti, L., Almerigogna, F., Forni, G. & Del Prete, G.: Expression and role in apoptosis of the α- and ß-chains of the IFN-γ receptor on human Th1 and Th2 clones. *J Immunol* 159, 206-213 (1997)

11. Bernabei, P., Allione, A., Rigamonti, L., Bosticardo, M., Losana, G., Borghi, I., Forni, G. & Novelli, F.: Regulation of interferon-γ receptor (IFN-γR) chains: a peculiar way to rule the life and death of human lymphocytes. *Eur Cytokine Netw* 12, 6-14 (2001)

12. Sanceau, J., Hiscott, J., Delattre, O. & Wietzerbin, J.: IFN-beta induces serine phosphorylation of Stat-1 in Ewing's sarcoma cells and mediates apoptosis via induction of IRF-1 and activation of caspase-7. *Oncogene* 19, 3372-3383 (2000)

13. Jacobs, L. D., Cookfair, D. L., Rudick, R. A., Herndon, R. M., Richert, J. R., Salazar, A. M., Fischer, J. S., Goodkin, D. E., Granger, C. V., Simon, J. H., Alam, J. J., Bartoszak, D. M., Bourdette, D. N., Braiman, J., Brownscheidle, C. M., Coats, M. E., Cohan, S. L., Dougherty, D. S., Kinkel, R. P., Mass, M. K., Munschauer, F. E., Priore, R. L., Pullicino, P. M., Scherokman, B. J., Weinstock-Guttman, B., Whitham, R. H. & Group, T. M. S. C. R.: Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. *Ann Neurol* 39, 285-294 (1996)

14. Rieckmann, P. The effects of IFN-β on cytokines and immune responses. *In* "Interferon Therapy of Multiple Sclerosis" (A. T. Reder, ed.), pp. 161-191. Marcel Dekker, Inc., New York. (1997)

15. Mosmann, T. R. & Moore, K. W.: The role of IL-10 in crossregulation of T_H1 and T_H2 responses. *Immunol Today* 12, A49-A53 (1991)

16. Inoges, S., Merino, J., Bandrés, E., de Castro, P., Subirá, M. L. & Sánchez-Ibarrola, A.: Cytokine flow cytometry differentiates the clinical status of multiple sclerosis (MS) patients. *Clin Exp Immunol* 115, 521-525 (1999)

17. Ozenci, V., Kouwenhoven, M., Huang, Y.-M., Kivisakk, P. & Link, H.: Multiple sclerosis is associated with an imbalance between tumour necrosis factor-alpha (TNF-α)-and IL-10-secreting blood cells that is corrected by interferon-beta (IFN-β) treatment. *Clin Exp Immunol* 120, 147-153 (2000)

18. Rieckmann, P., Albrecht, M., Kitze, B., Weber, T., Tumani, H., Broocks, A., Lüer, W. & Poser, S.: Cytokine mRNA levels in mononuclear blood cells from patients with multiple sclerosis. *Neurology* 44, 1523-1526 (1994)

19. Arnason, B. G. W. & Reder, A. T.: Interferons and multiple sclerosis. *Clin Neuropharm* 17, 495-547 (1994)

20. Byskosh, P. V. & Reder, A. T.: Interferon-β effects on cytokine mRNA in peripheral mononuclear cells in multiple sclerosis. *Mult Scler* 1, 262-269 (1996)

21. Rudick, R. A., Ransohoff, R. M., Peppler, R., VanderBrug Medendorp, S., Lehmann, P. & Alam, J.: Interferon beta induces interleukin-10 expression: Relevance to multiple sclerosis. *Ann Neurol* 40, 618-627 (1996)

22. Rudick, R. A., Ransohoff, R. M., Lee, J.-C., Peppler, R., Yu, M., Mathisen, P. M. & Tuohy, V. K.: In vivo effects of interferon beta-1a on immunosuppressive cytokines in multiple sclerosis. *Neurology* 50, 1294-1300 (1998)

23. Groux, H., Sornasse, T., Cottrez, F., de Vries, J. E., Coffman, R. L., Roncarolo, M.-G. & Yssel, H.: Induction of human T helper cell type 1 differentiation results in loss of IFN-γ receptor ß-chain expression. *J Immunol* 158, 5627-5631 (1997)

24. Bach, E. A., Szabo, S. J., Dighe, A. S., Ashkenazi, A., Aguet, M., Murphy, K. M. & Schreiber, R. D.: Ligand-induced autoregulation of IFN-γ receptor β chain expression in T helper cell subsets. *Science* 270, 1215-1218 (1995)

25. The IFN-Beta Multiple Sclerosis Study Group: Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. *Neurology* 43, 662-667 (1993)

26. Reder, A. T., Antel, J. P., Oger, J. J., McFarland, T. A., Rosenkoetter, M. & Arnason, B. G.: Low T8 antigen density on lymphocytes in active multiple sclerosis. *Ann Neurol* 16, 242-249 (1984)

27. Rigamonti, L., Ariotti, S., Losana, G., Gradini, R., Russo, M. A., Jouanguy, E., Casanova, J.-L., Forni, G. & Novelli, F.: Surface expression of the IFN-γR2 chain is regulated by intracellular trafficking in human T lymphocytes. *J Immunol* 164, 201-207 (2000)

28. Bernabei, P., Coccia, E. M., Rigamonti, L., Bosticardo, M., Forni, G., Pestka, S., Krause, C. D., Battistini, A. & Novelli, F.: Interferon-γ receptor 2 expression as the deciding factor in human T, B and myeloid cell proliferation or death. *J Leukoc Biol* 70, 950-960 (2001)

29. Koike, F., Satoh, J.-i., Miyake, S., Yamamoto, T., Kawai, M., Kikuchi, S., Nomura, K., Yokoyama, K., Ota, K., Kanda, T., Fukazawa, T. & Yamamura, T.: Microarray analysis identifies interferon β-regulated genes in multiple sclerosis. *J Neuroimmunol* 139, 109-118 (2003)

30. Rep, M. H. G., Schrijver, H. M., van Lopik, T., Hintzen, R. Q., Roos, M. T. L., Ader, H. J., Polman, C. H. & van Lier, R. A. W.: Interferon (IFN)-β treatment enhances CD95 and interleukin 10 expression but reduces interferon-γ producing T cells in MS patients. *J Neuroimmunol* 96, 92-100 (1999)

31. Byrnes, A. A., McArthur, J. C. & Karp, C. L.: Interferon-β therapy for multiple sclerosis induces reciprocal changes in interleukin-12 and interleukin-10 production. *Ann Neurol* 51, 165-174 (2002)

32. Prat, A., Al-Asmi, A., Duquette, P. & Antel, J. P.: Lymphocyte migration and multiple sclerosis: Relation with disease course and therapy. *Ann Neurol* 46, 253-256 (1999)

33. Van Weyenbergh, J., Wietzerbin, J., Rouillard, D., Barral-Netto, M. & Liblau, R.: Treatment of multiple sclerosis patients with interferon-beta primes monocyte-derived macrophages for apoptotic cell death. *J Leukoc Biol* 70, 745-748 (2001)

34. Allione, A., Bernabei, P., Bosticardo, M., Ariotti, S., Forni, G. & Novelli, F.: Nitric oxide suppresses human T lymphocyte proliferation through IFN-γ-independent induction of apoptosis. *J Immunol* 163, 4182-4191 (1999)

35. Correale, J., Gilmore, W., Lopez, J., Li, S. Q., McMillan, M. & Weiner, L. P.: Defective post-thymic tolerance mechanisms during the chronic progressive stage of multiple sclerosis. *Nat Med* 2, 1354-1360 (1996)
36. Revel, M., Chebath, J., Mangelus, M., Harroch, S. & Moviglia, G. A.: Antagonism of interferon beta on interferon gamma: inhibition of signal transduction in vitro and reduction of serum levels in multiple sclerosis patients. *Mult Scler* 1, S5-S11 (1995)

37. Bongioanni, P., Lombardo, F., Fioretti, C. & Meucci, G.: T-lymphocyte immunointerferon receptors in patients with multiple sclerosis. *J Neurol* 243, 605-610 (1996)

38. Genç, K., Dona, D. L. & Reder, A. T.: Increased $CD80^+$ B cells in active multiple sclerosis, and reversal by IFNβ-1b therapy. *J Clin Invest* 99, 2664-2671 (1997)

39. Feng, X., Petraglia, A. L., Chen, M., Byskosh, P. V., Boos, M. D. & Reder, A. T.: Decreased expression of interferon-stimulated genes in active multiple sclerosis is linked to subnormal phosphorylation of STAT1. *J Neuroimmunol* 129, 105-115 (2002)

40. Kaser, A., Deisenhammer, F., Berger, T. & Tilg, H.: Interferon-ß1b augments activation-induced T-cell death in multiple sclerosis patients. *The Lancet* 353, 1413-1414 (1999)
41. Link, H.: The cytokine storm in multiple sclerosis. *Mult Scler* 4, 12-15 (1998)

42. Dupont, S. A., Goelz, S., Goyal, J. & Green, M.: Mechanisms for regulation of cellular responsiveness to human IFN-β1a. *J Interferon Cytokine Res* 22, 491-501 (2002)

43. Dayal, A. S., Jensen, M. A., Lledo, A. & Arnason, B. G. W.: Interferon-gamma-secreting cells in multiple sclerosis patients treated with interferon beta-1b. *Neurology* 45, 2173-2177 (1995)

44. Petereit, H. F., Bamborschke, S., Esse, A. D. & Heiss, W. D.: Interferon gamma producing blood lymphocytes are decreased by interferon beta therapy in patients with multiple sclerosis. *Mult Scler* 3, 180-183 (1997)

45. Sharief, M. K., Noori, M. A. & Zoukos, Y.: Reduced expression of the inhibitor of apoptosis proteins in T cells from patients with multiple sclerosis following interferon-β therapy. *J Neuroimmunol* 129, 224-231 (2002)

46. Gasperini, C., Pozzilli, C., Bastianello, S., Koudriavtseva, T., Galgani, S., Millefiorini, E., Paolillo, A., Horsfield, M. A., Bozzao, L. & Fieschi, C.: Effect of steroids on Gd-enhancing lesions before and during recombinant beta interferon 1a treatment in relapsing remitting multiple sclerosis. *Neurology* 50, 403-406 (1998)

47. Leussink, V. I., Jung, S., Merschdorf, U., Toyka, K. V. & Gold, R.: High-dose methylprednisolone therapy in multiple sclerosis induces apoptosis in peripheral blood leukocytes. *Arch Neurol* 58, 91-97 (2001)

Abbreviations: IFN: Interferon, EAE: Experimental Allergic Encephalomyelitis, MS: multiple sclerosis, MS-a: Active, MS, MS-s: Stable MS, NK: Natural Killer cell, NL: Normal, PBMNC: Peripheral Blood Mononuclear Cells

Key Words: Nervous system, Brain, Cytokine, Disease, apoptosis, interferon-β, interferon-γ, interferon-γ receptor, multiple sclerosis

Send correspondence to: Anthony T Reder, MD, Department of Neurology, MC-2030, 5841 S. Maryland Avenue, The University of Chicago, Chicago, IL 60637, Tel: 773-702-6204, Fax: 773-702-9076, E-mail: areder@neurology.bsd.uchicago.edu