Immune functions in untreated and treated multiple sclerosis patients
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Citation for published version (APA):
Chapter 6

Interferon-β treatment enhances CD95 and interleukin-10 expression but reduces interferon-γ producing T cells in MS patients


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Journal of Neuroimmunology, in press
Chapter 1

Interleukin-2 treatment enhances CD8+ and interleukin-10 expression but reduces interleukin-2-producing T cells in MS patients.
Interferon (IFN)-β has been shown to favorably alter the disease course of relapsing-remitting multiple sclerosis (RRMS) patients. Although its mode of action is still unclear, there is ample evidence from in vitro studies that IFN-β directly modulates the function of immune cells. We here analyzed the effects of IFN-β treatment on immune functions in vivo in a group of 25 RRMS patients that received IFN-β (8 MIU) on alternate days. At baseline and at 1, 3 and 6 months from the start of the treatment, parameters for differentiation and activation states of both monocytes and T lymphocytes were assessed. A transient increase was seen in plasma (p) interleukin (IL)-10 level whereas pIL-12 (p40) was not affected. A similar change was found in the ability of monocytes to secrete these cytokines in vitro. Notably, patients that in vitro readily respond to IFN-β with enhanced IL-10 production had highest pIL-10 levels. Concerning T-cell differentiation, flow cytometric analysis of cytokine production showed that treatment with IFN-β moderately decreased mean percentages of CD8^pos^ T cells producing IL-2 and IFN-γ and CD8^neg^ T cells producing IL-4 (p<.05 for all cytokines), whereas a more significant decline was seen in mean percentage of CD8^neg^ T cells producing IFN-γ (p<.01). This resulted in a significant lower ratio Thelper(1) vs. Thelper(2) type cells in the CD8^pos^ T-cell subset (p<.05), but not in the CD8^neg^ T-cell subset. Finally, IFN-β treatment resulted in an initial rise in mean percentage of CD95^pos^ T cells and in a gradual increase in mean level of soluble CD95 (sCD95) in plasma (p<.01). Additional in vitro studies showed that IFN-β indeed rapidly (within 24 hours) upregulates CD95 expression on both primed and unprimed T cells and augments the release of sCD95 in culture supernatants.

Thus, we confirm here that IFN-β treatment leads to similar changes in cytokine production of T cells and monocytes as previously described in vitro. Enhanced IL-10 secretion may downmodulate cytokine secretion by activated T cells and in this way dampen newly-induced and/or ongoing immune responses. In addition, we identified a novel effect of IFN-β treatment, i.e. induction of CD95 expression. The augmentation of CD95 expression may directly interfere with T-cell selection, notably of autoaggressive T cells. Future studies are needed to show whether this increased CD95 expression indeed leads to increased apoptosis of immune cells.

Introduction

Based upon the concept that immunological processes play a major role in the pathogenesis of multiple sclerosis (MS) an array of immunomodulating drugs have been tested in clinical trials over the past years. Although most of these were only marginally successful, a few years ago it was published that recombinant IFN-β (rIFN-β) has a beneficial effect on the disease course of RRMS patients. Patients receiving rIFN-β had significantly fewer relapses, a lower Magnetic Resonance Imaging (MRI)-detected burden of disease, and a marked decrease in gadolinium-enhanced lesions, as compared with placebo-treated patients. These results were confirmed to a large extent after five years of treatment. Similar results were obtained with rIFN-β1a and, in addition, patients in this study showed a significant decrease in the time to sustained disability progression.

Because viral infections were presumed to be involved in the etiology and/or pathogenesis of MS, the rationale for the application of IFN-β was initially based upon the
anti-viral capacities of this cytokine. However, IFN-β, together with IFN-α classified as a type 1 interferon, also has immunomodulatory effects (for a review see ). It has been demonstrated in vitro that IFN-β significantly enhances secretion of the immunomodulatory cytokine IL-10 by human monocytes as well as by T cells and also induction of IL-10 mRNA by IFN-β has been documented. Importantly, shortly after injection of IFN-β in patients as well as in healthy controls elevated levels of IL-10 can be found in plasma. Although in man IL-10 is produced by a variety of cells including TH1 and TH2 type T-cells, a major function of this cytokine appears to be the downregulation of helper-1 type cytokines. The enhanced secretion of IL-10 by IFN-β is of particular interest in view of the hypothesis that activation of TH1 type cells and/or an insufficient counterbalance by TH2 type cells plays a disease promoting role in the pathogenesis of MS and many other autoimmune diseases.

Table I. Demographic and baseline characteristics of patients.

<table>
<thead>
<tr>
<th>variable</th>
<th>value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sex (female/male)</td>
<td>21/4</td>
</tr>
<tr>
<td>age in years</td>
<td>37.4 ± 7.6</td>
</tr>
<tr>
<td>disease duration in years</td>
<td>6.7 ± 5.1</td>
</tr>
<tr>
<td>number of clinical relapses previous 2 years</td>
<td>3 (2-6)</td>
</tr>
<tr>
<td>EDSS at inclusion</td>
<td>4.0 (1.5-6.0)</td>
</tr>
<tr>
<td>last methylprednisolone before inclusion in months</td>
<td>9.7 (2.4-40.9)</td>
</tr>
</tbody>
</table>

1 mean ± SD
2 median (range)
3 EDSS: Expanded Disability Status Scale

In this study we set out to document the in vivo effects of IFN-β treatment on markers for differentiation and activation of immune cells. We show that IFN-β induces a transient increase in pIL-10 and a decrease in cytokine secreting T cells, most notably of IFN-γ secreting cells. Unexpectedly, IFN-β induced enhanced expression of both membrane and soluble CD95. The potential relevance of these findings for the mode of action of IFN-β will be discussed.

Methods
Study design
An open trial was carried out with 25 MS patients at the Department of Neurology, Free
University Hospital, Amsterdam, The Netherlands. All patients had to fulfill the following inclusion criteria: RR disease type, more than 2 exacerbations in the 2 years before the start of the treatment, mild to moderate handicap (EDSS (Expanded Disability Status Scale) 6 or lower at the start of the treatment), above 17 years of age. Demographic and baseline characteristics are given in Table I.

Treatment consisted of a self-administered subcutaneous injection of 8 million international units (8 MIU) recombinant interferon-ß1b (Betaferon, Schering AG, Berlin, Germany) on alternate days for 6 months. Blood was taken at fixed time-points: before treatment (baseline), and after 1, 3 and 6 months. At all of these visits an EDSS score was obtained. At month 1 and at month 3 data from 24 patients were obtained. At month 6 data from 21 patients were obtained. Ten patients were treated 1 till 4 times for relapses with methylprednisolone during the study period. Because these patients did not show major differences in immune parameters compared to patients not treated with methylprednisolone, no further attention has been given to this subject.

Reagents
Monoclonal antibodies (mAb) directed against CD2 (CLB-T11.1/1, CLB-T11.1/2, and Hik27) and CD28 (CLB-CD28/1) were generated at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. PerCP-labelled CD3 mAb, CD4 mAb and CD8 mAb, phycoerythrin (PE)-labeled CD69 mAb, IgG1 control mAb, interferon (IFN)-γ, interleukin (IL)-4, and interleukin (IL)-2 mAb were purchased from Becton Dickinson (Immunocytometry Systems, San Jose, CA). PE-labeled CD45RA mAb (2H4-RD1) was obtained from Coulter Clone (Hialeah, FL). FITC-labeled CD95 (Fas) mAb was purchased from Immunotech S.A. (Marseille, France). PMA (CMC Cancer Research, Katonah, NY) and ionomycine (Calbiochem, La Jolla, CA) were prepared as stock solutions in DMSO, stored at -20 °C and diluted properly before use.

Blood samples and cell separation
At each visit venous blood was collected in evacuated blood collection tubes (Vacutainer, Becton Dickinson, Meylan, France) containing sodium heparin (143 USP Units). The samples were kept at room temperature and processed within 24 hr. Plasma was frozen at -20 °C.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Isopaque density gradient centrifugation, and cryopreserved immediately. To exclude interassay variability, samples from all timepoints from each individual patient were analyzed in one experiment. Viability of the cells was over 95 %, as indicated by trypan-blue exclusion.

Cytokine levels in vivo
Interleukin (IL)-10 levels in plasma were measured by ELISA (CLB, Amsterdam). IL-12 p40 was determined in an enzyme-linked immunosorbent assay (ELISA) as previously described. IL-12 p70 was measured in an ELISA as described, identical to the IL-12 p40 ELISA, but now using p70-specific mAb 20C2 as a coating antibody (kindly provided by Dr M. Gately, Hofmann La Roche, Nutley, NJ).
Monokine secretion \textit{in vitro}

Whole blood cultures were performed as described before\cite{23}. In short, blood was diluted 1:10 in IMDM, supplemented with 0.1 \% FCS, antibiotics, and 50 IU/ml sodium heparin. Monocytes were stimulated either with or without 100 pg/ml lipo-oligosaccharide (LOS) or with 0.1 \% Staphylococcus Aureus (Cowan strain) (SAC). Supernatants were harvested after 24 hours and frozen at -20\textdegree C. IL-12 p40, IL-12 p70 and IL-10 secretion was measured in specific ELISA as described above.

\textit{In vitro} cytokine secretion by PBMC

PBMC (25x10^4 cells/ml) were cultured in IMDM, supplemented with 10 \% fetal calf serum (FCS), penicillin, streptomycin and \(\beta\)-mercaptoethanol, in a final volume of 200 \(\mu\)l, and stimulated in triplicate cultures with a triplet of CD2 mAb (CLB-T11.1/1, CLB-T11.2/1 and Hik27; all 5 \(\mu\)g/ml) in the presence of CD28 mAb CLB-CD28/1 (5 \(\mu\)g/ml)\cite{26} in flat-bottom microtiter plates (Greiner, Langenthal, Switzerland). Cells cultured without stimuli served as negative controls. To measure sensitivity of patients to IFN-\(\beta\) before initiation of treatment, these cultures were also performed in the presence of IFN-\(\beta\) (1-1,000 U/ml)\cite{15}.

Cytokine secretion by PBMC was measured in culture supernatants in the presence or absence of IFN-\(\beta\) (1-1,000 U/ml), IL-4, IFN-\(\gamma\) and IL-10 were measured after 72 hours of culture in specific ELISA (CLB, Amsterdam).

From at total of 21 patients data were obtained on pre-treatment \textit{in vitro} IL-10 secretion. To test whether pIL10 levels are correlated with the ability of PBMC to respond to IFN-\(\beta\) \textit{in vitro}, patients were subdivided into a low responder group (n=11) and a high responder group (n=10) based on their IL-10 secretion in standard cultures in the presence of IFN-\(\beta\). High responders are arbitrary defined as individuals who produce 50\% of the maximally-induced IL-10 (1000 U/ml IFN-\(\beta\)) at an IFN-\(\beta\) dose below 10U/ml; low responders are arbitrary defined as individuals who produce 50\% of the maximally-induced IL-10 (1000 U/ml IFN-\(\beta\)) at an IFN-\(\beta\) dose above 10U/ml.

Flow cytometric measurement of intracellular cytokine production

Measurement of intracellular cytokine production was performed as previously described\cite{11,29}. Briefly, 0.5x10^6 cells/ml were stimulated for 4 h with PMA (1 ng/ml) and ionomycine (1 \(\mu\)M) in the presence of the protein-secretion inhibitor monensin (1 \(\mu\)M). All subsequent steps were performed at 4\textdegree C. After cell surface staining with CD8-PerCP and CD95(Fas)-FITC, cells were washed twice with PBS and fixated with PBS/4\% paraformaldehyde (5 min). Fixation was followed by permeabilization with PBS/0.1\% saponin (Sigma)/10\% human pooled serum (10 min). For all subsequent washing and incubation steps PBS/0.1\% saponin/0.5\% BSA was used. Cytoplasm was stained with PE-labeled cytokine mAb (IFN-\(\gamma\), IL-4, IL-2, all 5 \(\mu\)g/ml) (20 min). Analysis was performed as described for the measurement of membrane markers.

Soluble CD95 levels in plasma

Levels of soluble CD95 in plasma were measured by ELISA as described before\cite{4}. All steps were performed at room temperature. Microtiter plates (Nunc-Immuno plate Maxisorp surface, Nunc. Denmark) were coated with 100 \(\mu\)l/well CLB-CD95/2 (2 \(\mu\)g/ml) in 0.1 M
NaHCO₃/Na₂CO₃ buffer (pH=9.6) overnight. Coated plates were washed 5 times with 100 µl/well of phosphate buffered saline (PBS) containing 0.02 % Tween-20 (PBST). Samples and standards were diluted in high performance ELISA (HPE) buffer (CLB, Amsterdam, The Netherlands) and 100 µl of each sample dilution was added to the plate. To each sample dilution 10 µl of a 10 µg/ml solution of biotin-coupled CLB-CD95/6 was added and the plate was incubated for 2 hours. After 5 washes with 100 µl/well PBST 100 µl of streptavidine-polyHRP (CLB, Amsterdam, The Netherlands) diluted 1:10,000 in PBS containing 2 % whole milk was incubated for 30 minutes. The plates were washed 5 times with 100 µl/well PBST, and developed with 100 µl substrate solution (0.1 mg/ml 3,5,3',5'-tetramethylbenzidine, Merck, Darmstadt, Germany), containing 0.003 % H₂O₂ in 0.11 M NaAc (pH=5.5) for 10 minutes. The enzyme reaction was stopped with 100 µl 2 M H₂SO₄. Plates were read at 450 nm in a Titertek Multiskan reader (Labsystems Multiskan Multisoft, Helsinki, Finland).

**In vitro effects of IFN-β on CD95 expression**

PBMC (5x10⁵ cells/ml) from two healthy donors were cultured in IMDM, supplemented with 10 % fetal calf serum (FCS), penicillin, streptomycin and β-mercaptoethanol, in a final volume of 1 ml. IFN-β was simultaneously added in a concentration of 1 U/ml. Cells cultured without stimuli served as negative controls. Cells were harvested after 16 hours and stained for CD4, CD45RA and CD95. Analysis was performed as described for the measurement of membrane markers.

**Statistical analysis**

Treatment effects after 1 month were evaluated by Student's T-test, or by Wilcoxon Signed-ranks test in case of non-normally distributed variables. P-values are depicted as p(3). Repeated measurements MANOVA with age as a covariate was used to evaluate long-term treatment effects. P-values indicating linear and parabolic trends are depicted as p(1) and p(2) respectively. Log transformation was used in case variables were non-normally distributed.

The use of in vitro assays for the prediction of a patient’s response to therapy was analyzed by repeated measurements MANOVA. Differences between groups at baseline was analyzed by Mann-Whitney U test. We considered p-values <.05 to be statistically significant.

**Results**

IFN-β induces a transient increase in IL-10 in vivo as well as in vitro but does not affect in vivo levels of IL-12 (p40).

We and others¹⁵,¹⁶,²⁰ have previously shown that IFN-β augments production of IL-10 in vitro. This is in agreement with in vivo findings, as treatment with IFN-β resulted in a rise in the mean pIL-10 level after one month (Fig. 1; p<.05, Wilcoxon). After the first month mean IL-10 levels returned to levels comparable to baseline values. In most plasma samples the p70 dimer of IL-12, the bioactive form of the Th1 associated cytokine, was not detectable, so this parameter could not be evaluated. In contrast, significant amounts of IL-12 (p40) were measured in plasma (Fig. 1), but no changes were found as a result of treatment.
Figure 1. Levels of IL-10 and IL-12 in plasma. Data are given as mean concentration ± SEM. No significant trend was seen in mean level of IL-12 (p40) in plasma during six months of treatment with IFN-ß. Mean level of pIL-10 increased from 7.1 ± 1.5 pg/ml at baseline to 16.7 ± 6.7 pg/ml at month 1 (p<.05). At month 6 mean level of pIL-10 had returned to 6.3 ± 2.8 pg/ml. *(3) p<.05 compared to baseline (Wilcoxon).

Figure 2. In vitro IL-10 secretion as a predictor of plasma IL-10 levels upon treatment with IFN-ß. Data are given as mean level of pIL-10 ± SEM. From a total of 21 patients data were obtained on in vitro IL-10 secretion. They were subdivided in a high-responder (n=10) and a low-responder group (n=11) as described in the results. At baseline a significant difference was seen in mean level of pIL-10 between the two groups (high- vs. low-responders 11.8 ± 2.8 pg/ml vs. 2.7 ± 1.6 pg/ml p<.01, Mann-Whitney U). After one month there was a significant increase in mean level of pIL-10 in the high responder group (33.5 ±18.6 pg/ml at month 1; p<.05), but not in the low responder group (6.1 ± 3.3 pg/ml at month 1). Mean level of pIL-10 in the high responder group decreased to 4.3 ± 3.3 pg/ml at month 6. *(2) p<.05 parabolic trend (MANOVA).

To test whether the effects on cytokine levels in vivo were associated with the capacity of PBMC to secrete these particular cytokines in vitro, we first stimulated monocytes for 24 hours with either LOS or SAC in a whole blood culture system, and measured monokines in culture supernatant. IL-10 secretion in vitro showed approximately a similar pattern as was seen for levels of this cytokine in vivo. Upon stimulation with LOS, a significant increase in mean IL-10 secretion was seen initially (Table II, p<.05, MANOVA), which was followed by...
IFN-ß treatment enhances CD95 and interleukin-10 expression in MS

**Table II. In vitro monokine secretion (whole blood culture).**

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>month 1</th>
<th>month 3</th>
<th>month 6</th>
<th>p(1)</th>
<th>p(2)</th>
<th>p(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10¹</td>
<td>58.0 ± 8.5³</td>
<td>84.8 ± 10.9</td>
<td>86.0 ± 14.2</td>
<td>48.8 ± 8.4</td>
<td>n.s.</td>
<td>&lt;.05</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>IL-12(p40)¹</td>
<td>437.9 ± 66.7</td>
<td>582.7 ± 68.8</td>
<td>635.1 ± 81.3</td>
<td>566.1 ± 63.3</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-12(p70)²</td>
<td>4.9 ± 1.2</td>
<td>5.3 ± 1.6</td>
<td>3.6 ± 0.7</td>
<td>7.0 ± 2.0</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

¹ upon stimulation with LOS (100 pg/ml)
² upon stimulation with SAC (0.1 %)
³ values are given as mean pg/ml ± SEM

A decrease after 6 months of treatment. In contrast, no effects of treatment with IFN-ß were found on levels of IL-12 (p40) upon stimulation with LOS. Low levels of IL-12 (p70) could be detected in culture supernatant upon stimulation with SAC, but also no effects were seen as a result of treatment with IFN-ß. No treatment-induced changes were seen in the ability of T cells to secrete IL-10 after polyclonal stimulation in vitro (data not shown).

There was a clear difference between the high-responder group and the low-responder group (as described in the methods) in the pattern of IL-10 levels in plasma (Fig. 2). Although there is substantial interindividual variation, mean level of IL-10 in plasma in the high-responder group was increased after one month as compared with the low-responder group (p<.05, MANOVA).

**Percentages of IFN-γ, IL-4 and IL-2 producing T cells are diminished upon IFN-ß treatment**

Since the balance between TH1 and TH2 type T cells might play a role in the disease process in MS, we measured percentages of CD8neg (i.e. CD4pos) and CD8pos cells positive for IFN-γ (type 1), IL-4 (type 2) or IL-2 (both type 1 and 2). Within the CD8neg subset a clear decline was observed in the percentage of T cells producing the TH1 type cytokine IFN-γ over the whole treatment period (Table IIIa, p<.01, MANOVA). Remarkably, although less apparent, the same trend was seen for CD8neg T cells producing the TH2 type cytokine IL-4 (p<.05). As a result, the ratio TH1 vs. TH2 type CD8neg T cells was not significantly affected (Fig. 3).

Within the CD8pos T-cell subset similar results were found for IFN-γ (Table IIIb, p<.05), but since the mean percentage of IL-4 producing cells did not change, this led to a moderate decrease in the TH1/TH2 ratio over the whole treatment period (Fig. 3, p<.05). Furthermore, while T cells producing IL-2 were not significantly affected in the CD8neg subset (Table IIIa), in the CD8pos subset a decline was seen in mean percentage T cells producing this cytokine (Table IIIb; p<.05), which was most prominent in the first phase of the treatment.
### Table IIIa. Changes in percentages of cytokine producing cells within the CD8\(^{\text{neg}}\) subset.

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>month 1</th>
<th>month 3</th>
<th>month 6</th>
<th>p(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-(\gamma)</td>
<td>13.8 ± 1.3(^1)</td>
<td>11.9 ± 1.0</td>
<td>13.1 ± 1.1</td>
<td>10.6 ± 0.9</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.7 ± 0.3</td>
<td>2.6 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>IL-2</td>
<td>30.5 ± 2.0</td>
<td>28.1 ± 1.9</td>
<td>30.4 ± 1.8</td>
<td>27.1 ± 2.4</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

\(^1\) values are given as mean percentage of CD8\(^{\text{neg}}\) cells ± SEM

### Table IIIb. Changes in percentages of cytokine producing cells within the CD8\(^{\text{pos}}\) subset.

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>month 1</th>
<th>month 3</th>
<th>month 6</th>
<th>p(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-(\gamma)</td>
<td>28.2 ± 3.1(^1)</td>
<td>24.8 ± 2.6</td>
<td>25.6 ± 2.3</td>
<td>22.9 ± 2.5</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.6 ± 0.5</td>
<td>2.9 ± 0.7</td>
<td>3.0 ± 0.6</td>
<td>2.5 ± 0.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-2</td>
<td>15.1 ± 1.7</td>
<td>15.1 ± 2.6</td>
<td>14.5 ± 1.6</td>
<td>12.9 ± 1.7</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

\(^1\) values are given as mean percentage of CD8\(^{\text{pos}}\) cells ± SEM

**IFN-\(\beta\) induces a decrease in CD69\(^{\text{pos}}\) peripheral blood T cells but an increase in CD95\(^{\text{pos}}\) peripheral blood T cells and in levels of soluble CD95 in plasma**

We investigated whether IFN-\(\beta\) influences, next to T-cell differentiation, also the state of activation of peripheral blood T cells. We first measured expression of the very early activation marker CD69. Fig. 4A shows that the percentage of CD69\(^{\text{pos}}\) cells within the CD3\(^{\text{pos}}\) T-cell subset is reduced upon treatment with IFN-\(\beta\) (p<.05, MANOVA). Secondly, we analyzed 'primed' versus 'unprimed' T cells by measuring expression of CD95, which is rapidly upregulated upon T-cell activation\(^{28}\). Double-colour immunofluorescence analysis revealed that patients showed both in the CD8\(^{\text{pos}}\) (Fig. 4A, p<.05) and in the CD8\(^{\text{neg}}\) T-cell subset higher percentages of CD95\(^{\text{pos}}\) T cells upon treatment with IFN-\(\beta\), although in the latter subset this effect was transient (p<.05). Interestingly, the increase in the percentage of CD95\(^{\text{pos}}\) T cells was accompanied by a significant gradual increase in levels of soluble CD95 (Fig. 4B, p<.01). This was most prominent in the last five months of the treatment period. The
increase in percentage of CD95 expressing T cells did not show a statistical significant correlation with sCD95 in plasma.

The increase in the percentage of CD95$^{\text{pos}}$ T cells could either be due to an alteration in the redistribution of "unprimed" versus "primed" from the secondary lymphoid tissues and solid organs to the peripheral blood or to a direct effect of IFN-β on CD95 expression. To address this latter possibility, T cells from healthy donors were stimulated in vitro with graded amounts of IFN-β in the absence or presence of polyclonal activators (CD2 mAb). As can be seen in figure 5, already low dose IFN-β (1 IU/ml) induced a marked upregulation of CD95 on "unprimed" (CD45RA$^{\text{pos}}$) T cells, regardless of whether these cells had been stimulated in vitro or not. Although the majority (>95%) of the "primed" (CD45RA$^{\text{neg}}$) T-cell subset is already positive for CD95, still an increase was seen in mean expression of CD95 upon addition of IFN-β. Concomitant with increased membrane expression, IFN-β increased the release of sCD95 in culture supernatants of stimulated cells (data not shown).

Discussion

Treatment with IFN-β has been shown to have beneficial effects on the disease course of relapsing-remitting MS patients$^{12,27,30}$. For several reasons it is important to study the
underlying mechanisms of action of this treatment. First, it would provide insight into the pathophysiological processes underlying RRMS. Second, although IFN-β is a promising drug that delays disease progression, it is no cure for the disease and more refined therapies need to be designed which may expand on the positive effects of IFN-β. Thus far in only a few studies in vivo effects of IFN-β have been investigated. Here we choose an open label design to analyze the effects of IFN-β on the immune system of patients undergoing treatment with this drug.

Cytokine levels in plasma of IFN-β treated patients were measured to analyze whether treatment with IFN-β resulted in changes in parameters of immune activation in vivo. In our study no evidence was found that IFN-β might have an effect on the in vivo secretion of either the p40 chain or the p70 heterodimer of IL-12. For the latter no definite conclusions can be made, because p70 was not detected in plasma, and was also secreted in very low amounts in...
IFN-ß treatment enhances CD95 and interleukin-10 expression in MS

in vitro in whole blood culture. Results from our study and that of others\textsuperscript{3,16} show that production of the immunosuppressive cytokine IL-10 is enhanced by IFN-ß treatment. However, we do not know which cells are the major source. Although we here show that monocytes ex vivo are affected by IFN-ß, we can not exclude the possibility that also T cells produce more IL-10 in treated patients, because in contrast to the experiments with monocytes, in assays where cytokine secretion was measured after T-cell stimulation, IFN-ß was washed out from the cultures.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure5.png}
\caption{IFN-ß in vitro enhances expression of CD95 on both CD45RA\textsuperscript{pos} and CD45RO\textsuperscript{pos} CD4\textsuperscript{pos} T cells. Data are shown of one representative experiment with PBMC of a healthy donor. Without stimulation the percentage of CD95\textsuperscript{pos} CD45RA\textsuperscript{pos} T cells increased from 6 (A) to 74 (B) in the presence of 1 U/ml IFN-ß (in the same experimental conditions mean fluorescence intensity of CD95 on CD45RA\textsuperscript{pos} T cells increased from 43 to 59). When cells were stimulated with CD2 mAb the percentage of CD95\textsuperscript{pos} T cells within the CD45RA\textsuperscript{pos} subset increased from 61 (C) to 95 (D) (mean fluorescence intensity of CD95 on CD45RA\textsuperscript{pos} T cells increased from 69 to 80).}
\end{figure}

In general, effects of IFN-ß on IL-10 secretion seem to have a transient character. Two distinct phenomena might be at play here. First, in earlier studies it was found that IFN-ß as well as IL-10 levels in vivo in treated patients rapidly decrease (within days and even within several hours) after injection of IFN-ß\textsuperscript{14,16}. Since blood was drawn at varying timepoints after IFN-ß injection, this may indeed explain interindividual changes in IL-10 levels in plasma observed in our study. In addition, this transient effect might point towards a direct effect of IFN-ß on IL-10 transcription rather than to a change in cellular differentiation. The second phenomenon we observe is that IL-10 plasma levels and enhanced secretion by monocytes in
vitro decline after one month. Interestingly, although we find a down-modulation of TH1 responses, also this effect is transient in a similar fashion. It could be that the system becomes 'adapted' to the addition of IFN-β possibly through the induction of counteracting mechanisms. An important counteracting mechanism that occurs in a considerable number of patients treated with IFN-β, and which might diminish therapeutic effects, is the formation of neutralizing antibodies\(^7\). However, this can not fully explain our findings, because in most cases antibodies only develop after 6 months of treatment\(^19\). Nevertheless, whatever the nature of the counteracting mechanism may be, it could be an interesting option be to consider building in a drug-free period to optimize efficacy of the treatment.

Our observation that treatment with IFN-β results in enhanced expression of CD95 in vivo, both at the membrane level and in soluble form, leads to some points of discussion. Upregulation at the membrane level is most likely caused by an effect of IFN-β on CD95 mRNA expression\(^22\). Consequently, an increase in deletion of activated immune cells would possibly occur\(^1,8\). Our data on diminished expression of CD69 on T cells indeed would lend support for this hypothesis. Furthermore, also other, non-immune cells may show increased sensitivity to CD95L mediated apoptosis. This may partly explain the fact that no correlation was found between levels of sCD95 and percentages of circulating CD95\(^508\) T cells, because CD95 may also be produced by other cells after treatment with IFN-β. Another explanation, which does not exclude the former, could be that interindividual variation in production and clearance of CD95 may exist.

It is conceivable that upregulation of IL-10 and downmodulation of T-cell cytokine production in vivo are causally related. Indeed, in vitro experiments have shown that IL-10 is at least partially involved in downregulation of IFN-γ production by IFN-β\(^2\). The same may hold for the diminished expression of CD69. On the other hand, upregulation of IL-10 and CD95 expression may be independent phenomena both associated with direct or indirect effects of IFN-β on transcription cq stabilization of the respective messengers. Comparison of clinical scores, MRI data and immunological parameters in future studies will show which laboratory parameters correlate best with clinical efficacy of IFN-β. It will be important to know whether this will confirm our findings on the correlation between in vitro responses to IFN-β with in vivo reactions (pIL-10), and whether consequently laboratory protocols may be designed to select individuals that will benefit most from this type of treatment.

**Acknowledgements**

The authors would like to thank Paul Baars and people working in the lab of Marijke Roos (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) for technical assistance. This study was supported by grant 94-192 from the
IFN-β treatment enhances CD95 and interleukin-10 expression in MS

Dutch Society for Support of Research on Multiple Sclerosis
References


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45:1277.


