Immune functions in untreated and treated multiple sclerosis patients
Rep, M.H.G.

Citation for published version (APA):
Chapter 5

Recombinant interferon-β blocks proliferation but enhances interleukin-10 secretion by activated human T cells

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\textit{Journal of Neuroimmunology} 67 (1996) 111-118
Recombinant interferon-β blocks proliferation but enhances interleukin-10 secretion by activated human T-cells

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Received 27 September 1995; revised 11 March 1996: accepted 12 March 1996

Abstract

Results from recent clinical trials have indicated that recombinant interferon-β (rIFN-β) is a promising drug for the treatment of Multiple Sclerosis (MS), a disease of supposed autoimmune etiology. To gain insight into the immunoregulatory properties of this cytokine, we analyzed effects of interferon-β (IFN-β) on T-cell functions in vitro. Interferon-β inhibited T-cell proliferation, as well as T-cell-dependent immunoglobulin secretion, in a dose-dependent manner. IFN-β did not inhibit upregulation of CD40L on activated T-cells, but blocked induction of CD25 on stimulated T- and B-lymphocytes. Secretion of interferon-gamma (IFN-γ), tumour necrosis alpha (TNF-α) and IL-13 was inhibited by the addition of IFN-β, whereas IL-4 secretion was unaffected. Interestingly, IFN-β enhanced secretion of IL-2 about two-fold and secretion of IL-10 nearly four-fold. In summary, these findings suggest that IFN-β may exert direct effects on T- and B-cell function in vivo. In addition, enhanced secretion of IL-10 by activated T-cells may interfere with newly initiated and ongoing inflammatory immune reactions.

Keywords: Recombinant interferon-β (rIFN-β); Multiple sclerosis; T-cell activation; B-cell differentiation; Interleukin-10

1. Introduction

Although the etiology of the disease has not yet been elucidated, evidence for immunoregulatory disturbances in Multiple Sclerosis (MS) has accumulated (Selmaj et al., 1991; Hirsch, 1986; Antel et al., 1986; Rose et al., 1985; Selmaj et al., 1986). For this reason, several immunomodulating drugs have been tested in clinical trials, but so far none has proved to be very successful (Hughes, 1994). Recently, results of clinical trials with relapsing-remitting MS patients (RRMS) have indicated that recombinant interferon-β (rIFN-β) is a promising drug for the treatment of the disease (Paty and Li, 1993; The IFNB Multiple Sclerosis Study Group, 1993; Jacobs et al., 1994). Patients were reported to have fewer clinical exacerbations and a substantial reduction in disease activity on magnetic resonance imaging (MRI) scans after treatment.

Interferon-β (IFN-β), a type I interferon, is known for its anti-viral activity (Balkwill, 1989), but immunomodulatory effects of type I interferons have also been described. IFN-β reduces secretion of IFN-γ, TNF-α and lymphotoxin (TNF-β) by activated PBMC (Noronha et al., 1993; Abu Khabar et al., 1992). Moreover, IFN-β inhibits dose-dependently proliferation of circulating T-cells in vitro in response to concanavalin A (ConA) or CD3 mAb, irrespective of whether cells are obtained from MS patients or from healthy controls (Noronha et al., 1993; Rudick et al., 1993).

To obtain more insight into the immunoregulatory properties of rIFN-β, we investigated the influence of rIFN-β on T- and B-cells in vitro. Our findings indicate that mitogenic responses of T-cells may be directly influenced...
by IFN-β, and perhaps more importantly, that IFN-β strongly modulates the cytokine secretion pattern of activated T-cells.

2. Materials and methods

2.1. Reagents

Monoclonal antibodies (mAb) directed against CD2 (CLB-T11.1/1, CLB-T11.1/2 and Hik27), CD3 (CLB-T3/3), CD28 (CLB-CD28/1), CD16 (CLB-gran/11), CD14 (CLB-mon/1), CD40 (CLB-CD40), IgM (CLB-MH15), IgG (CLB-MH16), CD19 (FITC-labeled, CLB-CD19F), CD4 (FITC-labeled, clone 10A12), CD25 (biotinylated, clone 4E10), biotinylated IgG1 control, and FITC-conjugated goat anti-mouse immunoglobulin (GAM–FITC) were generated at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. Streptavidin–phycoerythrin (SA–PE), phycoerythrin (PE)-labeled CD69 mAb and CD4 mAb were purchased from Becton Dickinson (Immunocytometry Systems, San Jose, CA). Anti-IgM (PE)-labeled antibodies were purchased from Southern Biotechnology Associates (Birmingham, USA).

CD40-hIg was kindly provided by Peter Lane (Basel Institute of Immunology). PMA (CMC Cancer Research, Katonah, NY) and ionomycin (Calbiochem, La Jolla, CA) were prepared as stock solutions in DMSO, stored at −20°C and diluted properly before use. Recombinant human interferon-β (IFN-β1; Betaseron®) was kindly provided by Dr. Pablo Valenzuela (Chiron, Emeryville, CA).

2.2. Cell separation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll–Isopaque density gradient centrifugation. Peripheral blood lymphocytes (PBL) were depleted for monocytes by adherence to plastic (2 h, 37°C). The final population contained < 5% CD14+ cells, as determined by flow cytometry. Viability of the cells was over 95%, as indicated by trypan-blue exclusion.

Tonsils taken from children at tonsillectomy were finely minced, and cells were suspended in Earles medium, supplemented with 3% FCS, 10% TNC and antibiotics, centrifuged for 10 min at 1000 rpm. After two washes with IMDM, supplemented with 10% FCS, the resulting cell suspension was incubated with saturating amounts of CD3 (CLB-T3/4/1), CD2 (CLB-T11.1/1), CD14 mAb (CLB-mon/1) and CD16 mAb (CLB-gran/11) for 30 min at 4°C in Earles medium, supplemented with 2% FCS. After two washes, the cells were incubated for 45 min at 4°C with sheep anti-mouse Ig-coated magnetic beads (Dynabeads M450, Dynal A.S., Oslo, Norway). Bead-coated cells were removed with a Dynal magnetic particle concentrator. The final cell suspensions contained > 98% CD19+ cells, as determined by flow cytometry.

2.3. T-cell stimulation

PBMC (5 × 106) were cultured in a final volume of 200 μl IMDM, supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin and β-mercaptoethanol, and stimulated with a triplet of CD2 mAb (Van Kemenade et al., 1994) (final dilution ascites 1:1000), in the presence of CD28 mAb (final dilution ascites 1:400). Alternatively, cells were stimulated with immobilized CD3 mAb (CLB-T3/3, 5 μg/ml) (van Lier et al., 1989), or with a combination of PMA (1 ng/ml) and ionomycin (10 μM). Cells were cultured in the presence or absence of IFN-β (1–10,000 U/ml) for four days. 0.4 μCi/well of [3H]thymidine (200 mCi/nmol, Amersham, Buckinghamshire, UK) was added during the last 24 h of culture and the median cpm [3H]thymidine of triplicate cultures was measured. Results are given as percentage inhibition:

\[
\% \text{ inhibition} = \left( \frac{\text{cpm control} - \text{cpm with IFN-β}}{\text{cpm control}} \right) \times 100\%
\]

2.4. Cytokine secretion

Cytokine secretion by PBL was measured in culture supernatants upon stimulation with a combination of CD2 mAb and CD28 mAb, in the presence or absence of IFN-β (1–10,000 U/ml), as described above. IL-2 secretion was measured after 24 h of culture using the IL-2-dependent CTLL-2 line as described by Gillis et al. (1978). Other cytokines were measured after 72 h of culture. IL-4 (Van der Pouw-Kraan et al., 1992), IFN-γ (Van der Meide et al., 1985), IL-10 (IL-10 ELISA was developed at DNAX Research Institute, Palo Alto, CA), TNF-α (TNF-α ELISA was developed at the CLB, Amsterdam) and IL-13 (van der Pouw-Kraan et al., 1996) secretion was measured in specific ELISA. Results are given as fold increase in comparison with control values:

relative increase = \left( \frac{\text{concentration with IFN-β} - \text{concentration control}}{\text{concentration control}} \right) \times 100\%

2.5. Immunoglobulin secretion

To measure T-cell-dependent immunoglobulin secretion, PBMC were cultured in flat-bottomed microritester plates (Nunc, Roskilde, Denmark) coated with CD3 mAb (CLB-T3/3, 5 μg/ml) (van Lier et al., 1989). Cells were stimulated in the presence or absence of IFN-β (1–10,000 U/ml). IgM and IgG secretion was measured in super-

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IFN-ß blocks proliferation but enhances IL-10 secretion by human T cells (Rümké et al., 1982). Results are given as a percentage of the control value, i.e. Ig secretion in the absence of IFN-ß.

2.6. Flow cytometry

PBMC (1 × 10^6 cells/well) were cultured in 24-well culture plates for 24 h in the presence of a combination of CD2 mAb and CD28 mAb, as described above, with or without 1,000 U/ml IFN-ß. Purified tonsillar B cells were treated similarly, except that they were stimulated with a combination of IgM mAb (final dilution ascites 1:1000) and CD40 mAb (final dilution ascites 1:1000). After isolation, cells were washed twice with medium consisting of phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA). Immunofluorescence staining was performed by incubation of PBMC with saturating amounts of combinations of FITC- and either PE-labeled mAb or biotinylated mAb in PBS/BSA. Stained cells were washed twice and in a second step streptavidin-PE was added which binds to biotinylated mAb. 10^4 viable lymphocytes were analyzed using a fluorescence-activated cell sorter (FACS, Becton Dickinson, Sunnyvale, CA).

2.7. Statistical analysis

Effects of IFN-ß were analyzed using the Wilcoxon signed Rank-test for paired comparisons. Correlations between effects were measured using Spearman’s Rank-correlation test.

3. Results

3.1. In vitro T-cell proliferation and differentiation is inhibited by IFN-ß

T-cell proliferation was measured after optimal stimulation of peripheral blood lymphocytes with either immobilized CD3 mAb or a triplet of CD2 mAb in combination with CD28 mAb. IFN-ß significantly inhibited T-cell proliferation at a concentration of 10,000 U/ml. Mean percentage of inhibition ± S.E.M. was 39 ± 12% after stimulation with immobilized CD3 mAb, and 45 ± 13% after stimulation with a triplet of CD2 mAb in combination with CD28 mAb (P = 0.008 in both conditions; Fig. 1A). A similar effect could be seen when surface receptors were bypassed through direct activation of PKC and release of Ca^{2+} by a combination of PMA and ionomycin (mean percentage inhibition ± S.E.M. was 42 ± 20%; P = 0.011). Interestingly, although this dose-dependent decrease in

Fig. 1. In vitro proliferation of peripheral blood T-cells. (A) Effect of IFN-ß (10,000 U/ml) on T-cell proliferation using different stimuli. Results are given as mean percentage inhibition ± S.E.M. (n = 9). Mean proliferation ± S.E.M. in the absence of IFN-ß after stimulation with immobilized CD3 mAb was 35090 ± 17351 cpm, after stimulation with a combination of a triplet of CD2 mAb and CD28 mAb 56337 ± 17708 cpm, and after stimulation with PMA and ionomycin 11429 ± 2212 cpm. With medium alone, either in the presence or absence of IFN-ß, values were always < 200 cpm. (B) Interindividual difference in dose–response curves of T-cell proliferation after activation with a combination of a triplet of CD2 mAb and CD28 mAb. Results are given as percentage of the control value, i.e. proliferation in the absence of IFN-ß. Dotted line represents mean value at 10,000 U/ml IFN-ß.
proliferative response was a general finding, there appeared to be a rather large interindividual difference in the amount of inhibition (Fig. 1B).

T-cell-dependent immunoglobulin secretion was measured after stimulation of PBMC with an optimal concentration of immobilized CD3 mAb, which is an efficient way to induce T-cell-dependent Ig secretion (van Lier et al., 1989). Secretion of IgM and IgG was dose-dependently inhibited upon addition of IFN-β (Fig. 2). In the presence of 1,000 U/ml IFN-β, mean IgM and IgG secretion ± S.E.M. was resp. 36 ± 8% and 33 ± 8% of the value measured in the absence of IFN-β. This inhibition was nearly maximal, because values observed at a concentration of 10,000 U/ml IFN-β were resp. 31 ± 10% and 24 ± 7%.

3.2. Secretion of Th1- and Th2-type cytokines by T-lymphocytes is differentially affected by IFN-β

Effects of IFN-β on cytokine secretion by circulating T-cells were measured after optimal stimulation with a triplet of CD2 in combination with CD28 mAb (Fig. 3). Previous studies in our laboratory have demonstrated that this mode of activation is superior to immobilized CD3 mAb in inducing (paracrine) cytokine production (De Jong et al., 1991). At the highest concentration (10,000 U/ml) used, IFN-β reduced secretion of the cytokines IFN-γ and TNF-α to a similar extent (up to 50% of the initial value; P = 0.008 for both cytokines), while IL-2 secretion was almost doubled (P = 0.038). In contrast, IFN-β did not have any effect on the secretion of the Th2 cytokine IL-4. Interestingly, IL-10 secretion was dramatically increased, to about 3.6 times the initial value (P = 0.012), while secretion of IL-13 was reduced to about 50% of the initial value (P = 0.048), upon addition of IFN-β.

To determine the relationship between sensitivity for effects of IFN-β on T-cell proliferation and sensitivity for effects on lymphokine secretion, correlations were calculated between T-cell proliferation and resp. IL-2, IFN-γ, TNF-α and IL-10 secretion upon stimulation with a triplet of CD2 mAb in the presence of CD28 mAb. While effects of IFN-β on IL-2 secretion showed no relationship with effects on T-cell proliferation, the inhibitory effects of IFN-β on secretion of IFN-γ (R = 0.74; P = 0.01) and TNF-α (R = 0.81; P = 0.01) correlate well with the reduction of T-cell proliferation (Table 1). Interestingly, enhancement of IL-10 secretion was most pronounced in

Fig. 2. Immunoglobulin secretion in a T-cell-dependent activation system, measured 14 days after stimulation of 5×10⁴ PBMC with an optimal concentration of immobilized CD3 mAb. IFN-β was used at the indicated concentrations. (A) IgM secretion: mean IgM secretion ± S.E.M. in the absence of IFN-β was 29.2 ± 19.7 μg/ml. (B) IgG secretion: mean IgG secretion ± S.E.M. in the absence of IFN-β was 12.3 ± 3.9 μg/ml. Results are given as percentage of the control value, i.e. Ig secretion in the absence of IFN-β. In unstimulated cultures, mean IgM and IgG secretion ± S.E.M. was 2.0 ± 1.6 μg/ml and 1.5 ± 0.7 μg/ml, respectively. Mean percentage of the control value ± S.E.M. in the presence of 10,000 U/ml IFN-β was 11 ± 1 and 28 ± 7 for IgM and IgG, respectively. Different lines represent 5 different donors.
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![Graph](image)

**Fig. 3.** Effect of IFN-β on cytokine secretion by peripheral blood lymphocytes stimulated with a combination of a triplet of CD2 mAb and CD28 mAb. Mean secretion ± S.E.M. of cytokines in the absence of IFN-β was, respectively: IL-2: \(171 ± 168 \text{ U/mL}\), IFN-γ: \(27.6 ± 18.4 \text{ pg/mL}\), TNF-α: \(8.8 ± 4.8 \text{ pg/mL}\), IL-4: \(228 ± 292 \text{ pg/mL}\), IL-10: \(335 ± 245 \text{ pg/mL}\), IL-13: \(291 ± 164 \text{ pg/mL}\). With medium alone, either in the presence or in the absence of IFN-β, no cytokine secretion could be measured.

**Table 1.** Correlation between sensitivity for anti-proliferative effects and effects on cytokine secretion

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>(R)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>-0.38</td>
<td>0.32</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.74</td>
<td>0.01</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.81</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.67</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Spearman's Rho was calculated using percentages of the control value (i.e. stimulation in the absence of IFN-β), measured in the presence of 10,000 U/ml IFN-β.

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Donors that showed a modest IFN-β-induced decrease in T-cell proliferation \((R = 0.67; P = 0.01)\).

**3.3. Effects of IFN-β on the expression of early activation antigens on CD4+ T-cells and tonsillar B-cells**

To investigate whether IFN-β exerts its effects already during the early stages of activation of CD4+ T-cells in vitro, PBMC were stimulated for 24 h with a triplet of CD2 mAb in combination with CD28 mAb, in the absence or in the presence of 1,000 U/ml IFN-β. Analysis of CD4+ T-lymphocytes indicated that IFN-β did not affect the upregulation of CD40L. However, expression of the very early activation marker CD69 was enhanced (Table 2). In contrast, the upregulation of CD25 (IL-2R) was markedly inhibited.

Tonsillar B-cells were cultured for 24 h in the presence of IgM mAb in combination with CD40 mAb, to study whether IFN-β affected the expression of activation antigens on B-cells. Addition of 1,000 U/ml IFN-β resulted in a higher expression of CD69. However, similar to what could be seen in T-cells, CD25 upregulation after 24 h was diminished when IFN-β was added to these cultures.

**Table 2.** Phenotypes of activated CD4+ peripheral blood T-lymphocytes and activated purified tonsillar B-lymphocytes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>-IFN-β</th>
<th>+IFN-β</th>
<th>-IFN-β</th>
<th>+IFN-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1 control</td>
<td>11</td>
<td>8</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>CD69</td>
<td>77</td>
<td>127</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td>CD25</td>
<td>215</td>
<td>24</td>
<td>39</td>
<td>14</td>
</tr>
<tr>
<td>CD40L</td>
<td>44</td>
<td>56</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

PBMC and tonsillar B-cells were activated for 24 h as described in Section 2. Results from one representative experiment are given as mean fluorescence (MFI) 24 h after activation.

a) 1,000 U/ml IFN-β.

b) n.a. = not applicable.

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**4. Discussion**

Type I interferons (IFN-α and IFN-β) have been tested in clinical trials with MS patients for several reasons. Jacobs et al. (1986) administered natural interferon-β intrathecally to MS patients, because of a suspected viral etiology of the disease. In this small trial a decrease in clinical exacerbation frequency was reported. Others have ascribed clinical effects of IFN-β to its immunomodulatory properties (Knobler et al., 1984; Camenga et al., 1986). In agreement with earlier findings (Noronha et al., 1993; Rudick et al., 1993), we found that IFN-β significantly inhibits T-cell proliferation in a dose-dependent manner. With respect to this it was remarkable that donors differ in their sensitivity to IFN-β. Therefore, it will be interesting to investigate whether this in vitro response might have a predictive value considering the therapeutic effects of IFN-β. Similarly, T-cell-dependent B-cell differentiation was inhibited by IFN-β. Diminished Ig secretion was not due to reduced CD40L expression, because IFN-β exerted no effect on the expression of this early activation molecule. Furthermore, secretion of cytokines needed for B-cell differentiation such as IL-2 and IL-4 (Zubler et al., 1984; Waldmann et al., 1984; Nakasheishi et al., 1984; Nakagawa et al., 1985) was not lowered by addition of IFN-β. As phenotypical analysis of activated B-cells showed that IFN-β inhibits the upregulation of CD25, a direct effect of IFN-β on B-cells can be envisaged. Indeed, IFN-β was found to inhibit proliferative responses of
eral blood T-cells was observed, whereas IL-4 secretion by Strikingly, a marked increase of Th2-like cytokines is also influenced by IFN-γ. The differentiation of CD4+ T-cells was downregulated by IFN-γ production (Del Prete et al., 1993), especially just prior to and during clinical attacks (Beck et al., 1988). IFN-γ activates accessory cells such as macrophages, astrocytes and microglial cells, resulting in enhanced MHC class II expression (Massa et al., 1986; Wong et al., 1984; McCarron et al., 1990) and augmented secretion of pro-inflammatory cytokines such as TNF-α (Collart et al., 1986). Importantly, TNF-α is thought to play a role in myelin breakdown and damage of oligodendrocytes in the CNS of MS patients (Selmaï and Raine, 1988).

Based on these findings, which suggest that MS might be a 'Th1-like' disease, it was of interest to determine whether IFN-β had any influence on the secretion of Th1-type cytokines by activated T-cells. IFN-β, when added to in vitro activated peripheral blood T-cells was found to inhibit IFN-γ as well as TNF-α secretion up to 50% at a concentration of 10,000 U/ml. Similar effects have been reported in previous studies (Noronha et al., 1993; Abu Khabir et al., 1992). Furthermore, we could confirm earlier findings (Noronha et al., 1993), that secretion of IL-2 by IFN-β-treated PBL is enhanced in comparison with untreated PBL. As also mentioned by these authors, this effect might be explained by a diminished contribution of IL-2, because we have demonstrated that IFN-β not only directly or indirectly regulates CD25 expression on activated T-cells, but also has an independent effect on IL-2 secretion. At this moment, no reports are known to us about the concentrations of IFN-β that are reached in vivo, in patients treated with this drug. Therefore, it remains difficult to determine whether the in vitro findings can be extrapolated to the in vivo situation. But, as we have already found a three-fold increase of IL-10 secretion in the presence of 1000 U/ml IFN-β (data not shown), it seems possible that modulation of IL-10 secretion might be relevant for the therapeutic effects of IFN-β.

It has been shown that serum IL-10 levels, especially in progressive MS patients is lower in comparison with controls (Salomaggi et al., 1994), and also that during the recovery phase of MS, IL-10 concentrations found in CSF are enhanced (Rieckmann et al., 1994). Studies in EAE mice have shown that IL-10, when given before the induction phase, prevents development of the disease (Rott et al., 1994). In mice it has been demonstrated that IL-10, which may also be produced by monocytes, selectively downregulates Th1-type responses (Fiorentino et al., 1989). In humans IL-10 is a strong inhibitor of monokines, especially IL-12. IL-12 is considered to be the most important cytokine for induction of Th1 responses, most likely by its IFN-γ-inducing effect on T-cells and NK cells (Trinchieri, 1994). This suggests that IL-10 might play an important role in the dampening of ongoing immune responses in man. Further in vitro studies, and also studies on patients treated with interferon-β, may provide an answer to the question whether the in vivo effects of this cytokine can be explained by this enhanced IL-10 secretion by T-cells, or that other mechanisms might play a role.

Acknowledgements

This work was supported by Grant 90-43 from the Dutch Society for Support and Research into Multiple Sclerosis. The authors wish to thank Drs. A.C. Lankester, F. Miedema, and T.C.T.M. van der Pouw Kraan for critically reviewing the manuscript, and Dr. T.C.T.M. van der Pouw-
Kraan for generously providing materials for IL-4, IL-13, IFN-γ and TNF-α ELISAs.

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