Interferon-α: A Key Factor in Autoimmune Disease?

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**Purpose.** Interferon (IFN)-α is an effective drug for treatment of uveitis in Behcet’s disease. This study was undertaken to investigate the mechanism of action of IFN-α in the treatment of various types of noninfectious sight-threatening uveitis.

**Methods.** Eleven patients with refractory uveitis, and 13 healthy individuals were enrolled. The number of circulating plasmacytoid dendritic cells (pDCs) and their capacity to produce IFN-α in culture on stimulation with synthetic oligodeoxynucleotides containing the CpG-motif were studied. Peripheral blood CD4⁺ T-cell phenotype and activation status were evaluated by flow cytometry at 0, 2, and 8 weeks after treatment for expression of CD69, CD62L, chemokine receptors (CCR4, CXCR3, and CCR5), and intracellular cytokines (TNF-α, IFN-γ, and IL-10).

**Results.** All patients experienced a positive clinical response to IFN-α treatment. There was no significant difference between patients and control subjects in the number of circulating pDCs, but there was a significant decrease in the capability of patients’ pDCs to produce IFN-α in response to CpG (P < 0.001). Peripheral blood CD4⁺ T cells expressed reduced levels of surface CD62L (P < 0.005) as a measure of activation and higher levels of chemokine receptors CXCR3, CCR4, and CCR5 (P < 0.005, P < 0.05, and P < 0.05, respectively); in addition, intracellular T-cell IL-10 levels were increased once the treatment was initiated (P < 0.01).

**Conclusions.** The data suggest that IFN-α may control uveitis by promoting induction of IL-10-producing T-cells, possibly T-regulatory cells. Dysregulation of the T-cell population in patients with uveitis may be associated with a defect in the pDCs’ ability to produce IFN-α, which can be circumvented with administration of exogenous IFN-α. (Invest Ophthalmol Vis Sci. 2006;47:3946–3950) DOI:10.1167/iovs.06-0058

Noninfectious posterior segment intraocular inflammation (uveitis) is a sight-threatening CD4⁺ T-cell-mediated, organ-specific autoimmune disease requiring treatment with systemic immunosuppressants or biological agents such as monoclonal antibodies. Type 1 interferon (IFN) has been used anecdotally in Behcet’s disease for almost 20 years, and only recently, a 95% success rate in controlling uveitis in Behcet’s disease with IFN-α2a was reported.

IFN-α is a member of the type I IFN family and was the first cytokine discovered as well as the first used in human clinical trials and the first approved by regulatory authorities for human use. The biological effects of receptor–ligand engagement are to mediate antiviral, antiproliferative, and immunostimulatory responses in cells. Whereas some of the effects of IFN-α are well recognized, such as antiproliferative effects, the apoptosis enhancement, the cytostatic–cytotoxic effects in the treatment of tumors, and the antiviral effect in viral infections, much remains to be discovered regarding its immunomodulatory effects.

IFN-α is a naturally occurring glycoprotein secreted in response to viral infections by cells, and the principal IFN-α-secreting cell in the blood is the plasmacytoid dendritic cell (pDCs). Synthetic oligodeoxynucleotides (ODNs), which contain specific unmethylated CG dinucleotides in particular sequence contexts (CpG motifs), can mimic pathogen-associated molecular patterns recognized by the immune system, especially by pDCs. Among different CpG-containing ODNs, particular sequences have been identified that induce maturation of pDCs and stimulate the production of high amounts of IFN-α (e.g., ODN 2216; up to 5 pg/single pDC). Of note, pDCs are the only cell type within peripheral blood mononuclear cells (PBMCs) to produce IFN-α on stimulation with ODN 2216.

Because pDCs are the major producers of type I interferons and do so both constitutively and in increased amounts after induction, it is possible that defects in pDC function may be associated with failure to evoke an interferon response when challenged. Of interest, it has been shown that HIV⁺ patients display a persistent decrease in the number and function of blood pDCs, despite a good response to antiretroviral therapy. Similarly, patients with chronic active hepatitis have defects in the number and function of pDCs. In addition, abnormalities in pDC function have been observed in some patients with autoimmune disease such as systemic lupus erythematosus (SLE) and dermatomyositis.

We hypothesized therefore that if patients with sight-threatening intraocular inflammation respond well to IFN-α therapy, they may have an underlying defect in the number or function of pDCs, which may lead to a defect in the control of autoimmune responses through a failure of T-cell regulation.

**Methods**

**Patients and Study Design**

Eleven patients with severe refractory, sight-threatening, noninfectious posterior uveitis were recruited to a prospective open-label study between April 2002 and May 2004. The clinical details are shown in Table 1. Informed consent was obtained from all patients. The research adhered to the tenets of the Declaration of Helsinki.

**Protocol for Administration of IFN-α**

Before the initiation of the treatment with IFN-α2b (Intron A), other immunosuppressants apart from steroids were discontinued 24 hours before treatment, because of probable antagonistic effects. Oral steroid therapy, if in use, was reduced to a maximum dose of 15 mg per day, in that ethical concerns mitigated against complete cessation. IFN-α was injected subcutaneously daily, initially at a dose of 3 MU/d for 3 days, increased to 6 MU/d for several weeks, and tapered down gradually, depending on the activity of the disease. The minimum...
duration of IFN-α-2b administration was 1 month, the maximum, 29 months (Table 1).

### Isolation of Peripheral Blood Dendritic Cells

PBMCs were isolated from whole blood of IFN-α-treated patients and age-matched, healthy control subjects into cell preparation tubes (Vacutainer; BD Biosciences, Erembodegem, Belgium) in one step. DCs were then isolated from PBMCs (Blood Dendritic Cell Isolation Kit II; Miltenyi Biotec, Surrey, UK) by cell-sorting bead separation (MACS; Miltenyi). The isolation was performed in two steps: first, B cells and monocytes were magnetically labeled and depleted by using a cocktail of CD19 and CD14 beads (MicroBeads; Miltenyi). Subsequently, pre-enriched DCs in a nonmagnetic flow-through fraction were magnetically labeled and enriched with a cocktail of antibodies against DC markers: BDCA-4 (pDCs), BDCA-3 (mDC2), and BDCA-1 (mDC1). Purified DC samples were separated by positive-bead separation (MACS; Miltenyi), counted, and dispensed into 96-well, flat-bottomed tissue culture plates.

### Culture of DCs

Serum-free medium (X-VIVO 20; BioWhittaker, Cambrex Bio Science Wokingham, UK) supplemented with 0.4% bovine serum albumin (BSA) was used throughout these experiments. The sorted DCs were cultured in 96-well, flat-bottomed tissue culture plates at $4 \times 10^4$ cells in 200 μL of medium per well. To each well, recombinant human cytokine IL-3 (10 ng/mL; PeproTech, London, UK) was added. To half of the wells, CpG ODN 2216 (6 μg/mL; Autogen Bioclear, Mile Elm, UK) was added.

### IFN-α ELISA

DC culture supernatants were harvested after 30 hours and assayed by ELISA at 10hare dilution if cultured with IL-3 and CpG or neat if cultured with IL-3 alone, to determine IFN-α levels (Human Interferon Alpha ELISA kit; PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturer’s protocol (high-sensitivity protocol: 10–500 pg/mL).

### Flow Cytometry Analysis of Cultured Cell Populations

Cells from each well after 30 hours of incubation were washed with phosphate-buffered saline (PBS) containing 0.4% fetal calf serum (wash buffer) and incubated for 30 minutes with a cocktail of antibodies: CD14FITC, CD11cPE, CD3PerCP, BDCA-4bio, and CD19APC-Cy7 (7.5 μL of each; BD Biosciences) and washed twice. Cells were incubated in secondary antibody streptavidin-APC (30 minutes; 50 μL per tube, dilution 1:200 in PBS), washed twice, and analyzed by flow cytometry (LSR flow cytometer; with Cellquest Pro software; BD Biosciences).

### Blood Dendritic Cell Enumeration Assay

Blood dendritic cells (pDCs and two types of myeloid DCs: mDC1 and mDC2) were enumerated in whole blood (human Blood Dendritic Cell Enumeration kit; Miltenyi). The kit includes all reagents needed for a complete analysis: premixed antibody cocktails for DC detection (BDCA-1 for mDC1, BDCA-2 for pDCs, and BDCA-3 for mDC2) and for isotype control, a dead-cell discrimination dye and reagents for erythocyte lysis and cell fixation. Appropriate antibody controls for detection and exclusion of B cells, granulocytes, and monocytes are included in the kit.

### Peripheral Blood CD4+ T-Cell Analysis

Patients underwent peripheral blood CD4+ T-cell analysis from whole blood before and 2 and 8 weeks after receiving IFN-α treatment, as described elsewhere. In brief, expression of the activation markers CD69 and CD10, chemokine receptors CCR4, and CCR5, and the intracellular cytokines TNF-α, IFN-γ, and IL-10 was determined with flow cytometry after a 4-hour incubation in either basal medium (unstimulated culture) for CD69 and chemokine receptors, or PMA (phorbol-12-myristate-13-acetate) with ionomycin and the Golgi inhibitor monensin (stimulated culture) for intracellular cytokines. Monensin was used because it interrupts intracellular transport, leading to accumulation of cytokines in the Golgi apparatus. Using monensin increases the signal-to-noise ratio and allows detection of intracellular cytokines that would otherwise be only weakly fluorescent.

### Statistical Analysis

Statistical analysis was performed using the Wilcoxon matched-pair test for paired data and the Mann-Whitney test for unpaired data (Prism, ver. 4.01; GraphPad, San Diego, CA). Significance was attributed when $P < 0.05$. 

### RESULTS

#### Analysis of Blood Dendritic Cell Populations

Patients with active sight-threatening uveitis who were refractory to broad-spectrum immunosuppressants were taken off...
treatment with the exception of oral prednisolone (maximum dose, 15 mg; as mentioned, for ethical reasons it was not possible to cease prednisolone therapy completely) and treated with IFN-α-2b according to the regimen described in the Methods section. All patients gained substantial clinical improvement on IFN-α (Table 1). Blood samples were analyzed for dendritic cell populations by using recently developed DC markers\textsuperscript{18} while the patient was taking IFN-α. The data show that the total number of circulating DCs was not different between uveitis patients and control subjects, despite the use of steroids (Table 2). Circulating pDCs were not significantly lower in the patient group (0.18% ± 0.13%, n = 7 vs. 0.30% ± 0.145%, n = 8, P > 0.05; Fig. 1A, Table 2). In contrast, the number of mDC2 but not mDC1 cells was markedly reduced in the patients (P < 0.005; Table 2).

**Production of IFN-α by Dendritic Cells on Stimulation with CpG**

pDC-enriched leukocyte populations from whole blood samples of patients with uveitis who were on IFN-α treatment and from healthy control subjects were cultured in serum-free medium with recombinant human IL-3 (30 hours), and their supernatants were analyzed for IFN-α by ELISA. pDC-rich cultures incubated with IL-3 alone produced minimal levels of IFN-α, and in some individuals the cytokine was undetectable, even when analyzed by high-sensitivity methodology. In pDC cultures stimulated with CpG, IFN-α secretion was greatly increased. However, this response was significantly greater in pDC cultures from healthy individuals than from patients with uveitis (P < 0.001; Fig. 1B) in cultures using an equal number of cells per well (4 × 10\textsuperscript{4} cells) from patients and control subjects under identical conditions. This suggests that there was a functional defect in pDCs from patients with uveitis. Flow cytometry of the DC cultures after incubation with CpG showed that there were approximately 30% pDCs, 25% mDCs, and 40% to 50% T-cells after 30 hours of cell culture (Table 3). Although it is possible that non-pDCs produce IFN-α,\textsuperscript{19,20} as indicated earlier,\textsuperscript{8} CpG ODN 2216 is a selective stimulator of IFN-α from pDCs alone, and therefore we consider that any IFN-α detected in our culture supernatants is derived exclusively from the pDC population.

**Analysis of Circulating T-Lymphocytes in Patients with Uveitis in Response to IFN-α Treatment**

Whole-blood samples from patients with uveitis and healthy control subjects were analyzed for T-cell phenotype before and after treatment with IFN-α. Flow cytometry of the CD\textsubscript{5}\textsuperscript{+}/CD\textsuperscript{4}\textsuperscript{+} T-cell population showed no significant difference in CD69 expression between patients and healthy control subjects, in contrast to previous reports.\textsuperscript{21,22} There was also no change in CD69 expression in uveitis patients after treatment with IFN-α (Table 4). In contrast, patients with uveitis showed consistently reduced expression of CD62L on CD\textsubscript{4}\textsuperscript{+} T-cells compared with healthy control subjects, which became statistically significant 2 weeks after IFN-α treatment commenced (Table 4).

CD\textsubscript{5}\textsuperscript{+}/CD\textsubscript{4}\textsuperscript{+} chemokine receptor expression was increased in patients’ cells compared with control subjects before treatment commenced. Of interest, both CXCR3 and CCR5 increased further in expression after treatment with IFN-α, whereas CCR4 declined toward normal, particularly after 2 weeks of treatment (Table 4). When intracellular cytokines were compared between the groups, inconsistent data were obtained from unstimulated cultured PBMLs. However, in PMA-stimulated cells, the intracellular IL-10 levels gradually increased to a statistically significant level at 8 weeks (P < 0.01), whereas the slightly elevated intracellular TNF-α levels tended to decline toward those of the healthy control subjects, although this change did not reach statistical significance. Intracellular IFN-γ levels remained essentially constant throughout the study (Table 4).

**DISCUSSION**

IFN-α is the major cytokine produced by cells in response to viral infection and represents the central platform of the organism’s innate immune response to viral pathogens.\textsuperscript{23,24} In recent years, its role in the pathogenesis of autoimmune diseases such as SLE has been recognized.\textsuperscript{25} This concept has been further developed by the suggestion that autoimmune diseases occur in two broad pathogenetic groups: a “systemic” autoimmune disease group mediated by IFN-α and including diseases such as SLE, thyroiditis, and diabetes mellitus and an organ-specific autoimmune disease group, in which TNF-α is

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td><strong>PDC enumeration in full blood</strong></td>
<td><strong>IFN alpha production in supernatant</strong></td>
</tr>
<tr>
<td><strong>CONTROLS</strong></td>
<td><strong>PATIENTS</strong></td>
</tr>
<tr>
<td><strong>CONTROLS</strong></td>
<td><strong>PATIENTS</strong></td>
</tr>
<tr>
<td>0.00</td>
<td>0.05</td>
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<tr>
<td>0.00</td>
<td>2500</td>
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</tbody>
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**FIGURE 1.** PDC analysis: (A) PDC enumeration assay in whole blood of patients and healthy age-matched control subjects. (B) Production of IFN-α by PDCs (per microliter of medium) in cell culture on stimulation with CpG (**P < 0.001).
prominent as the pathogenetic cytokine and which includes diseases such as rheumatoid arthritis and multiple sclerosis.\textsuperscript{26} This notion has been further refined to suggest that there is cross-regulation between IFN-\(\alpha\) and TNF-\(\alpha\) in the healthy state and that imbalance of either would induce autoimmune disease, excess IFN-\(\alpha\) promoting systemic autoimmunity, and excess TNF-\(\alpha\) production promoting organ-specific disease.\textsuperscript{28}

The results of the present study can be interpreted within this disease paradigm. First, clinical responsiveness of posterior uveitis to IFN-\(\alpha\)-2b therapy, described in this study and elsewhere, categorizes it within the group of organ-specific disease, in which tissue damage is mediated by TNF-\(\alpha\). This finding concurs with the known responsiveness of posterior uveitis to anti-TNF-\(\alpha\) therapy\textsuperscript{22,27} and with the extensive experimental evidence from animal models of the disease clearly demonstrating the pathogenic role of TNF-\(\alpha\) (for review, see Ref. 28). In addition, IFN-\(\alpha\) therapy has been shown to be effective in controlling experimental autoimmune uveoretinitis.\textsuperscript{29}

The effectiveness of IFN-\(\alpha\) therapy and of anti-TNF-\(\alpha\) therapy in control of clinical and experimental autoimmune non-infectious uveitis implies a defect in regulation of autoreactive T-cells. TNF-\(\alpha\) is produced by T effector cells and macrophages, themselves activated by myeloid DCs. In contrast, IFN-\(\alpha\) is produced by pDCs, mostly in response to viral infection. This response is necessary to control spread of virus but may also have implications for autoimmunity. Regulation of autoreactive T-cells, which are present in all individuals at low frequency due to incomplete thymic deletion during development, is mediated through T-regulatory cells (T-regs) in the periphery in adulthood. pDCs are known to be inducers of T-regs, and thus activation of pDCs during viral infection may also be accompanied with increases in the number or function of T-regs, as a fail-safe mechanism to prevent organ-specific autoimmunity during the course of a viral infection through cross-reactivity of foreign and autoantigenic epitopes. Indeed, the induction of T-regs after viral infection is well documented, and the fact that T-regs function in an antigen nonspecific manner may be beneficial to the organism in controlling autoimmunity.

It is possible to consider therefore that autoimmune disease may be induced by a failure in this type of regulatory mecha-
nism. For instance, if the response to viral infection is inadequate or incomplete because of a deficiency in the number or function of pDCs, the virus infection may be cleared, but the induction of T-regs may be impaired with the consequent unrestrained expansion of autoreactive T-cells.

Data in the present report support the foregoing hypothe-
sis. Patients with uveitis had circulating pDCs that were not as responsive to CpG stimulation in IFN-\(\alpha\)-production as were pDCs from age-matched healthy control subjects. Although this effect may have been in part because the patients with uveitis were taking systemic steroids, the assays were performed on pDCs, which had been extensively washed and cultured in the absence of steroids for more than 24 hours. In addition, the maximum dose of steroids was 15 mg prednisolone daily, which is less than that reported to modify pDC behavior.\textsuperscript{30}

The failure of pDCs from patients with uveitis to secrete IFN-\(\alpha\) in vitro, as demonstrated in this study, may be due to an intrinsic defect in pDCs but may also be explained by other causes. For instance, it is possible that T-cells present in the culture system (see Table 3) had a negative effect on IFN-\(\alpha\)-production by the pDCs, but there is little direct evidence of this, although the possibility that increased production of adrennergic mediators\textsuperscript{31} or bystander release of nitric oxide by these cells may have affected IFN-\(\alpha\) secretion.\textsuperscript{32} Rather, the evidence supports a mechanism of T-regulatory function in the presence of IFN-\(\alpha\)-secreting DCs which would then be less effective in the absence of IFN-\(\alpha\), as suggested earlier.

In addition to the impaired function of pDCs in patients with uveitis, there were changes in T-cell populations that were of significance. For instance, IL-10 production by CD5\(^+\)/CD4\(^+\) T cells was reduced before treatment and was restored to normal after IFN-\(\alpha\) therapy, an observation consistent with an expansion of an IL-10-secreting T-reg population. There was also a decrease in CD62L expression and changes in chemo-
kine expression, which mirrored presumptive generalized Th1-cell function and activation, to complement the putative loss of T-reg cell function.

We thus suggest that, in organ-specific autoimmune uveoretinitis, there may be a defect in pDCs function, either intrinsic or extrinsic, with a concomitant reduction in the pDCs' responsiveness to antigen challenge and impaired IFN-\(\alpha\)-production, particularly to viral challenge (CpG). This leads to a

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**Table 3.** Cell Populations in the Cell Culture with IL-3 \(\pm\) CpG Defined by Flow Cytometry in Three Patients and 1 Control Subject

<table>
<thead>
<tr>
<th>Cells</th>
<th>pDC</th>
<th>mDC</th>
<th>T-Lymphocytes</th>
<th>B-Lymphocytes</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the well</td>
<td>IL-3 (\pm) CpG</td>
<td>IL-3 (\pm) CpG</td>
<td>IL-3 (\pm) CpG</td>
<td>IL-3 (\pm) CpG</td>
<td>IL-3 (\pm) CpG</td>
</tr>
<tr>
<td>Avg. % ± SD</td>
<td>28 ± 6.9</td>
<td>31 ± 9.2</td>
<td>24 ± 10.2</td>
<td>26 ± 10.2</td>
<td>40 ± 30.3</td>
</tr>
</tbody>
</table>

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**Table 4.** Flow Cytometry Analysis of CD3\(^+\)/CD4\(^+\) Cells after Treatment with IFN-\(\alpha\)

<table>
<thead>
<tr>
<th>Patients (n = 11)</th>
<th>Control Subjects (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td></td>
</tr>
<tr>
<td>CD69</td>
<td>39 ± 10.7</td>
</tr>
<tr>
<td>CD62L</td>
<td>67 ± 10.7</td>
</tr>
<tr>
<td>CXC3R3</td>
<td>27 ± 27.2</td>
</tr>
<tr>
<td>CCR4</td>
<td>42 ± 14.9***</td>
</tr>
<tr>
<td>CCR5</td>
<td>14 ± 19.8</td>
</tr>
<tr>
<td>Stimulated cells</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>2.1 ± 3.66</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>20 ± 18.1</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>78 ± 19.4</td>
</tr>
</tbody>
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secondary defect in T-reg cell activation, with unrestrained autoreactive Th1-cell activation, which if the cross-reactivity matches, leads to autoimmune inflammation. It also explains the responsiveness of patients to IFN-α and to anti-TNF-α therapy based on low endogenous levels of IFN-α in these patients and a cross-regulatory function of TNF-α and IFN-α, as described by Palucka et al.28

References