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ORIGINAL ARTICLE

Plasmacytoid Dendritic Cells in Multiple Sclerosis: Intracerebral Recruitment and Impaired Maturation in Response to Interferon-β

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Abstract

The roles of plasmacytoid dendritic cells (pDCs) and their response to interferon (IFN)-β therapy in multiple sclerosis (MS) patients are poorly understood. We identified pDC accumulation in white matter lesions and leptomeninges of MS brains and abundant expression of the Type I IFN-induced protein MxA, mainly in perivascular $CD3^+$ lymphocytes in lesions, indicating Type I IFN production by activated pDCs. The pDC chemoattractant chemerin was detected in intralesional cerebrovascular endothelial cells, and the chemerin receptor was expressed on infiltrating leukocytes, including pDCs. The effect of IFN-β on pDC phenotype and function was evaluated in MS patients before and during IFN-β treatment. Although IFN-β did not modify the frequency and immature phenotype of circulating pDC, they showed lower expression of major histocompatibility complex Class II and blood-dendritic cell antigen 2 molecules and upregulation of CD38 and B7H1 costimulatory molecules. On exposure to CpG (a site where cytosine [C] lies next to guanine [G] in the DNA sequence [the p indicates that C and G are connected by a phosphodiester bond]) oligodeoxynucleotides in vitro, pDCs from IFN-β-treated MS patients showed reduced expression of the pDC maturation markers CD83 and CD86 molecules; in vitro IFN-β treatment of pDCs from healthy donors resulted in lower secretion of proinflammatory cytokines, including IFN-α, and a decreased ability to stimulate allogeneic T cells in response to maturative stimuli. These data indicate that IFN-β modulates the immunologic functions of pDC, thus identifying pDCs as a novel target of IFN-β therapy in MS patients.

Key Words: IFN-β therapy, Multiple sclerosis, Plasmacytoid dendritic cells

INTRODUCTION

Plasmacytoid dendritic cells (pDCs) represent the main cellular source of Type I interferons (IFNs) in the immune system. They constitute a minor population of DCs that lacks myeloid cell markers, displays a plasmacytoid morphology, and matures in vitro after exposure to various inflammatory stimuli such as cytokines, CD154 (CD40 ligand), viruses, and bacterial DNA (1, 2). Blood dendritic cell antigen (BDCA) 2^+/$CD123^+$/$CD11c^-$ pDCs are normally present at a low frequency in the blood and in secondary lymphoid organs (3). Their maturation process involves increased cell surface expression of major histocompatibility complex (MHC) and costimulatory molecules and the production of increased amounts of Type I IFN. Plasmacytoid dendritic cells are the only cell type in the peripheral blood that efficiently and rapidly produces Type I IFN in response to most viruses (1). Recent studies performed in inflammatory skin diseases, rheumatoid arthritis, and allergic diseases have shown that pDCs accumulate in the target tissues and contribute to the local inflammatory processes (4–8).

Plasmacytoid dendritic cells have been detected in the cerebrospinal fluid of patients with various neuroinflammatory diseases, including multiple sclerosis (MS), the most common inflammatory disease of the central nervous system (CNS) (9). Stasiolek et al (10) have recently described dysregulated pDC functions that may contribute to impairment of immunoregulatory mechanisms in MS patients. Recently, we detected pDCs in demyelinated lesions and leptomeninges of highly inflamed MS brains; abnormal accumulations of B cells and plasma cells infected with Epstein-Barr virus (EBV) and evidence of $CD8^+$ T-cell activation and cytotoxicity toward infected cells were also found (11). Interferon β is frequently used to treat patients with relapsing-remitting (RR) MS, and it has an impact on disease activity (12, 13). The precise mechanisms through which this compound exerts its therapeutic effect, however, remain uncertain. Several studies indicate that IFN-β may influence the inflammatory response in MS through different

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mechanisms (14). For example, IFN-β decreases expression of MHC molecules on antigen-presenting cells, T-cell activation, and release of inflammatory cytokines, and enhances T-cell suppressor function and the release of neurotrophic factors by astrocytes and brain endothelial cells (14). Moreover, IFN-β treatment was reported to reduce expression of different metalloproteinases in peripheral blood leukocytes from patients with RRMS (15) and to stabilize the blood-brain barrier (16). Taken together, these findings indicate that IFN-β acts by attenuating the inflammatory process in MS.

Investigating the immune phenotype of pDCs in MS and how their immunologic functions are affected by IFN-β may provide relevant information on the immune mechanisms implicated in MS pathogenesis and contribute to understanding the disease-modifying effects of IFN-β. In this study, we have addressed these issues from several perspectives. We first analyzed postmortem brain tissue from MS cases in search of molecular signatures of pDC activation, including the expression of MxA induced by Type I IFN, and of chemoattractants that regulate homing of pDCs in inflamed tissues. We then investigated whether treatment of MS patients with IFN-β had any effect on the frequency and immunophenotype of blood pDC. We then used an in vitro system to evaluate the effects of IFN-β on pDC maturation induced by different activating stimuli. Our findings indicate that pDCs are actively recruited and become activated in the CNS, and that IFN-β impairs pDC maturation, thereby identifying pDCs as a potential cellular target of IFN-β therapy in MS patients.

MATERIALS AND METHODS

Patients and Specimens

Blood samples were obtained from 11 patients (3 men and 8 women) with definite MS according to the revised McDonald criteria (17). The mean age of the patients was 35.7 ± 13.9 years (range, 20–62 years; 2 patients were 62 and 53 years old; the remaining patients were <45 years old). Nine patients had RRMS, and 2 had secondary progressive MS (SPMS). Mean Expanded Disability Status Scale was 1.5 (range, 0–6). The patients had not taken steroids during the 3 months preceding enrollment, nor had they ever received IFN-β.

We analyzed blood samples of MS patients at baseline, 24 hours after the first IFN-β administration (Avonex; Biogen, Inc., Cambridge, MA; 6 million IU i.m., once a week s.c.) and 3 months after the beginning of treatment during a stable clinical phase of the disease. All MS patients analyzed were clinically stable by magnetic resonance imaging and had at least 9 T2-weighted lesions. Seven sex- and age-matched healthy donors were also studied. The study was approved by the Ethics Committee of S. Andrea Hospital, and all of the subjects in the study gave written informed consent.

Isolation and Stimulation of pDCs

Plasmacytoid dendritic cells from peripheral blood of healthy donors were purified from freshly collected buffy coats (Blood Bank of the University "La Sapienza", Rome, Italy). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lympholyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada), followed by positive sorting using anti-BDCA-4-conjugated magnetic microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). The purity of the recovered cells were greater than 85% to 95%. Plasmacytoid dendritic cells (0.5×10^6/ml) were cultured in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 2 mmol/L of L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin (Gibco, Grand Island, NY), and 15% fetal calf serum (BioWhittaker Europe). Plasmacytoid dendritic cells were stimulated with 1 ng/ml (used as survival factor) or 10 ng/ml (together with tumor necrosis factor [TNF] or CD40L) of interleukin (IL) 3 (R&D Systems, Minneapolis, MN), 3 μg/ml of phosphorothioate CpG 2395 oligodeoxynucleotide (18) (Genset Oligos, Proligo, Boulder, CO), 50 ng/ml of TNF (PeproTech EC Ltd, London, England), and 100 or 2,000 U/ml of IFN-β (Avonex). Human-soluble CD40L (Alexis Corporation, San Diego, CA) was used at a concentration of 200 ng/ml together with 1 μg/ml of enhancer (Alexis Corporation). Where indicated, a pretreatment of 4 hours with IFN-β (2,000 U/ml) was performed.

Flow Cytometry Analysis

The following monoclonal antibodies (mAbs) used for flow cytometry analysis. Monoclonal antibodies to CD83, CD86, human leukocyte antigen (HLA)-DR, B7-H1, CD123, immunoglobulin (Ig)G1, IgG2a, and IgG2b (BD Pharmingen, San Diego, CA) were used. Goat $F(ab')_2$ anti-mouse IgG fluorescein isothiocyanate (FITC) was used as secondary Ab where necessary (Southern Biotech, Birmingham, AL). Phycoerythrin (PE)-conjugated anti-BDCA-2 was obtained from Miltenyi Biotec, whereas Serotec (Oxford, UK) provided PE-conjugated anti-CD38. The expression of chemerin receptor (ChemR23) was analyzed by flow cytometry analysis using a mAb, namely, clone 4C7 (19).

Cells (5×10^4–10^5) were washed once in phosphate-buffered solution (PBS) containing 2% fetal calf serum and then incubated with purified mAbs at 4°C for 30 minutes. After washing, the cells were fixed with 2% paraformaldehyde before analysis on a FACScanto using FACSDiva software (BD Pharmingen). Apoptosis was detected by staining with AnnexinV-FITC/propidium iodide (PI) (ApoAlert Annexin V-FITC apoptosis kit; BD Pharmingen) according to the manufacturer's protocol.

Cytokine Measurement by ELISA or by Cytometric Bead Array

Supernatants from control, IFN-β-, CpG-, IFN-β, and CpG-treated pDC cultures were harvested and stored at −80°C. Interleukin 6, IL-10, CXCL-8, and TNF were measured with the human inflammation cytometric bead array (BD Pharmingen). An ELISA kit for IFN-α was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). Supernatants from polarized T cells were harvested 6 days after coculture with pDCs and analyzed using T_H1/T_H2 cytometric bead array (BD Pharmingen).

Mixed Lymphocyte Reaction

Cord blood $CD4^+$ T cells were purified by indirect magnetic sorting with a $CD4^+$ T-cell isolation kit (Miltenyi Biotech). The proliferative response was assessed at various T cells/pDC ratios using a fixed number of T cells (3×10^4) and evaluated after 6 days by measuring thymidine incorporation (0.5 μCi per well of [^{3}H] thymidine; Amersham, Little Chalfont, UK).

Immunohistochemistry

Immunohistochemical studies were performed on snap-frozen, postmortem brain tissue obtained from 4 MS cases with SPMS who had not been treated with IFN-β, 2 control subjects without signs of neurologic complications, and 1 patient with Alzheimer disease. Brain tissues were provided by the UK MS Tissue Bank at Imperial College London directed by Prof. R. Reynolds and by the Institute of

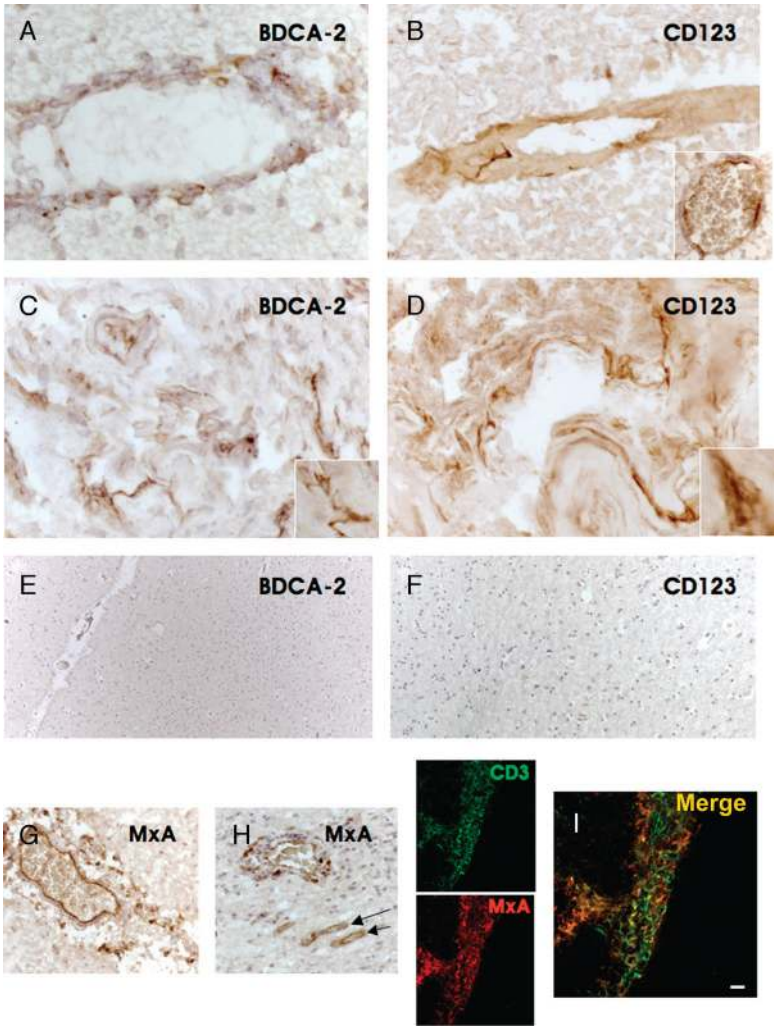

FIGURE 1. Immunohistochemical detection of plasmacytoid dendritic cells (pDCs) in the MS brain. Immunohistochemistry for blood dendritic cell antigen (BDCA)-2 **(A)** and CD123 **(B)** reveals immunostaining of both pDC markers in the brains of 2 cases of secondary progressive multiple sclerosis (SPMS). Blood dendritic cell antigen 2-positive and $CD123^+$ cells surround heavily **(A)** and minimally **(B)** inflamed blood vessels in an active lesion **(A)** and a chronic active lesion **(B)**; the stained cells seem to be in close contact with endothelial cells (**B**; inset). Blood dendritic cell antigen 2-positive and $CD123^+$ cells are also present in the cerebral meninges of the MS case shown in **(A**; **C**, **D)**. Blood dendritic cell antigen 2-positive and $CD123^+$ cells have elongated shapes and long cytoplasmic processes (**B–D**; insets). No immunoreactivity for BDCA-2 **(E)** or CD123 **(F)** was observed in control brain tissue samples. Panels from an SPMS case show expression of MxA in the inflamed meninges and in the intralesional perivascular cuffs, respectively **(G**, **H)**. In the meninges, MxA immunoreactivity is localized on round cells and on some vascular endothelial cells **(G)**. MxA immunoreactivity is also found on endothelial cells lining some mildly inflamed blood vessels in an active lesion (**H**; arrows). Double immunofluorescence for MxA (red) and CD3 (green) shows that MxA is expressed in $CD3^+$ T cells accumulating in the leptomeninges **(I)**. Original magnifications: **(E**, **F)** 250×; **(A–D**, **G**, **H)** 500×. **(I)** Bar, 20 μm.

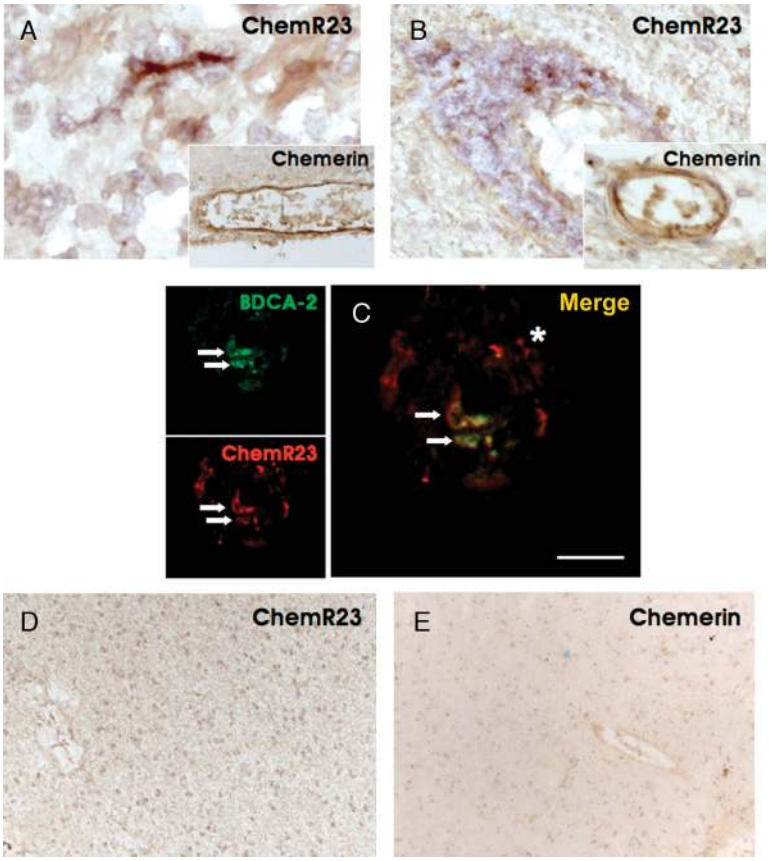

FIGURE 2. Chemerin and chemerin receptor (ChemR23) expression in the multiple sclerosis (MS) brain. Immunostaining with anti-ChemR23 monoclonal antibodies reveals ChemR23 immunoreactivity on scattered cells in the inflamed leptomeninges **(A)** and in perivascular inflammatory infiltrates in a chronic active lesion **(B)**. Vascular endothelial cells in the meninges and chronic active lesions of 2 secondary progressive MS cases express chemerin (**A**, **B**; insets). Chemerin receptor-positive cells have elongated shapes similar to those of blood dendritic cell antigen (BDCA) 2-positive/CD123-positive cells (compare **A** with insets in Figs. 1C and D). Double-immunofluorescence staining for ChemR23 (red) and BDCA-2 (green) reveals 2 BDCA-2$^+$ cells expressing membrane ChemR23 (**C**; arrows) and several ChemR23$^+$/BDCA-2$^-$ cells in an inflammatory cuff in a chronic active lesion (**C**; asterisks). No immunoreactivity for ChemR23 **(D)** or chemerin **(E)** is detected in normal brain samples. Original magnifications: **(D**, **E)** 250×; (**B** and insets in **A**, **B**) 500×; 1,000×. **(A)** Bar in **(C)**, 20 μm.

Pathological Anatomy, A. Gemelli Hospital, Rome, Italy. A snap-frozen tonsil from a child undergoing tonsillectomy was used as positive control.

Eight tissue blocks from subcortical and periventricular areas of the cerebral hemispheres from 4 cases with SPMS, 1 block from each control subject and 1 block from a patient with Alzheimer disease were analyzed. Multiple sclerosis lesions were characterized and classified by histopathologic methods, including hematoxylin and eosin staining, Oil Red O, and the combined Luxol fast blue–periodic acid–Schiff reaction to estimate myelin breakdown and the presence of myelin-loaded macrophages, as previously described (20).

To evaluate the degree of inflammation and lesion activity, we performed immunohistochemical stainings with anti-CD3 (clone PS1) and anti-CD20 (clone L26) mAbs (Immunotech, Marseille, France) to identify T and B cells, respectively; anti-CD138 (clone B-B4) mAb (Serotec) to identify plasma cells; anti-DC-SIGN (clone DCN 46) mAb (BD Pharmingen) to identify myeloid DCs; anti-CD68 mAb (clone KP1, Dako, Carpinteria, CA) to identify macrophages; and an anti-MHC Class II molecule mAb (clone CR3/43, Dako). Lesions were classified according to Lassmann et al (21) and De Groot et al (22). The MS tissue blocks contained active (n = 2) and chronic active (n = 9) subcortical lesions, 1 largely inactive periventricular lesion, and leptomeninges. Major histocompatibility complex Class II$^+$ activated microglia were present throughout active lesions and at the edges of chronic active lesions where myelin phagocytosis was also detected. Prominent inflammatory cell infiltrates comprising T cells, B cells, DCs, and macrophages were observed in the meninges of 3 MS cases analyzed.

For immunohistochemical staining, 10-μm-thick serial sections were cut in a cryostat, mounted on SuperFrost Plus slides, dried overnight at room temperature, and fixed in cold acetone for 10 minutes. After blocking endogenous peroxidase activity with 0.1% H_2O_2 in PBS for 20 minutes, sections were incubated with 10% normal rabbit serum for 1 hour and then overnight at 4°C with anti-BDCA-2 (Miltenyi Biotech) and anti-CD123 (IL-3R; BD Pharmingen) mAbs diluted 1:15 and 1:100 in PBS, respectively. Anti-Chemerin

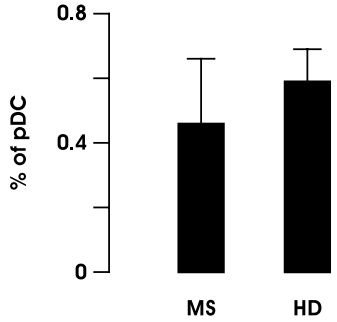

FIGURE 3. Percentages of circulating plasmacytoid dendritic cells (pDCs) in multiple sclerosis (MS) patients versus healthy donors (HD). The percentages of pDCs were determined in whole blood samples from healthy donors (n = 7) and MS patients (n = 11) as described in Materials and Methods section. Results of pDC enumeration are presented as the mean ± standard error of the percentage of blood dendritic cell antigen 2-positive cells. $p > 0.05$ by analysis of variance.

(clone 4G10) and anti-ChemR (ChemR 23, clone 4C7) mAbs were diluted 1:30 and 1:15 in PBS + 1% bovine serum albumin, respectively (19). For MxA immunostaining, sections were incubated for 1 hour at room temperature with anti-MxA mAb (clone M143; courtesy of Dr. O. Haller, Institute für Medizinische Mikrobiologie und Hygiene, Universitat Freiburg, Freiburg, Germany) diluted 1:400 in PBS containing 2% bovine serum albumin. The binding of biotinylated secondary Abs (rabbit anti-mouse IgG from Jackson Laboratory, Bar Harbor, ME) was visualized with the avidin-biotin-horseradish peroxidase complex technique (ABC Vectastain Elite kit; Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) as substrate. Sections were counterstained with hematoxylin and viewed with a Zeiss Axiophot photomicroscope (Zeiss, Oberkochen, Germany). Negative controls included the use of IgG isotype controls and omission of the primary Abs. For double-immunofluorescence stainings and confocal analysis of ChemR23 and BDCA-2, after an initial blockade with 10% normal sera in PBS, sections were incubated overnight at 4°C with unconjugated primary mAb specific for ChemR23, 1 hour with biotin-conjugated rabbit anti-mouse second Ab, followed by tetrarhodamine isothiocyanate-conjugated streptavidin. Finally, sections were incubated with mAb anti-BDCA-2 FITC-conjugated for 2 hours, washed, and sealed. For MxA and CD3 double immunofluorescence, sections were incubated overnight with a mixture of the primary antibodies (anti-CD3 goat polyclonal Ab and anti-MxA mAb) and then for 1 hour with a mixture of Cy2-conjugated donkey anti-goat and biotinylated rabbit anti-mouse

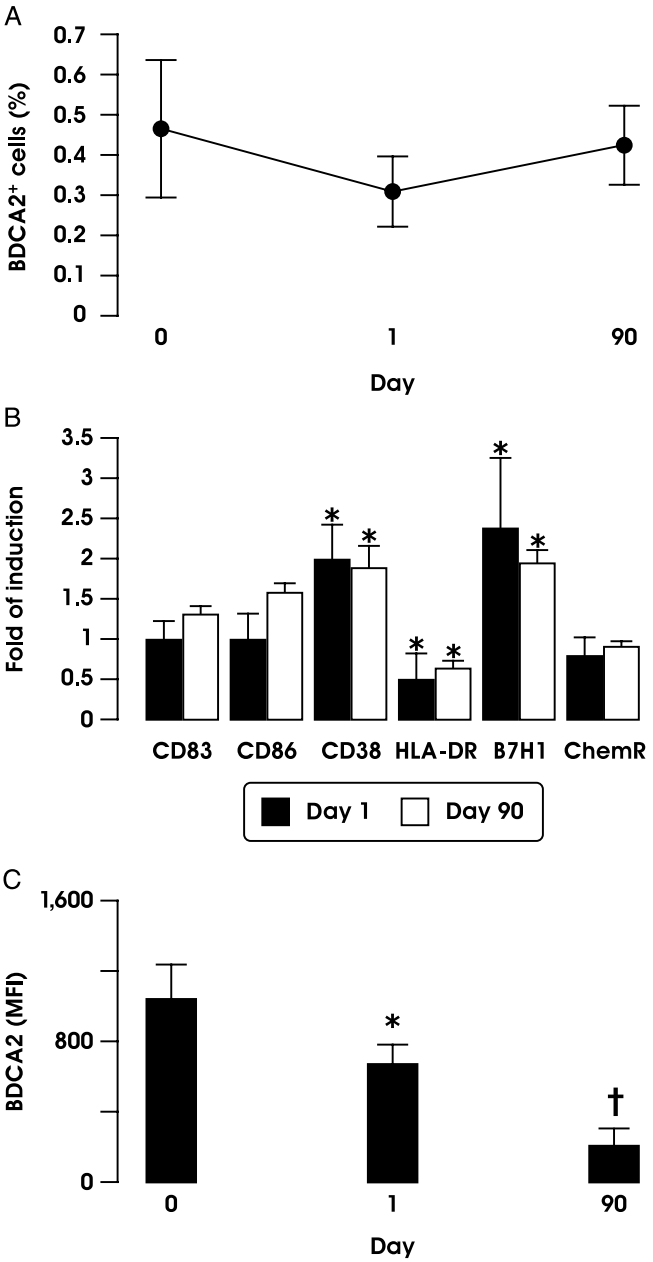

FIGURE 4. Influence of interferon (IFN)-β therapy on the percentages and phenotype of circulating plasmacytoid dendritic cells (pDCs) in multiple sclerosis (MS) patients. Blood samples from 11 MS patients were collected at different time points (Days 1 and 90) after IFN-β administration, and pDC percentages were evaluated as in Figure 3. The frequency **(A)** and the mean fluorescence intensity (MFI) **(C)** of blood dendritic cell antigen (BDCA) 2-positive cells are shown (mean ± standard error); *, $p = 0.035$ Day 0 versus Day 1; †, $p = 0.012$ Day 0 versus Day 90 by Student *t*-test. **(B)** Peripheral blood mononuclear cells were obtained from MS patients (n = 11) before, at Day 1, and Day 90 after IFN-β administration. The maturation of pDCs was studied through the analysis of CD83, CD86, CD38, human leukocyte antigen-DR, B7H1, and ChemR in the BDCA-2^+/CD123^+ population. Results are expressed as mean fold induction ± standard error of the expression of each molecule calculated by dividing the MFI values of pDCs collected from MS patients at Days 1 and 90 after treatment with IFN-β by the MFI values of pDCs obtained from the same patients at baseline. *, $p < 0.05$ Student *t*-test.

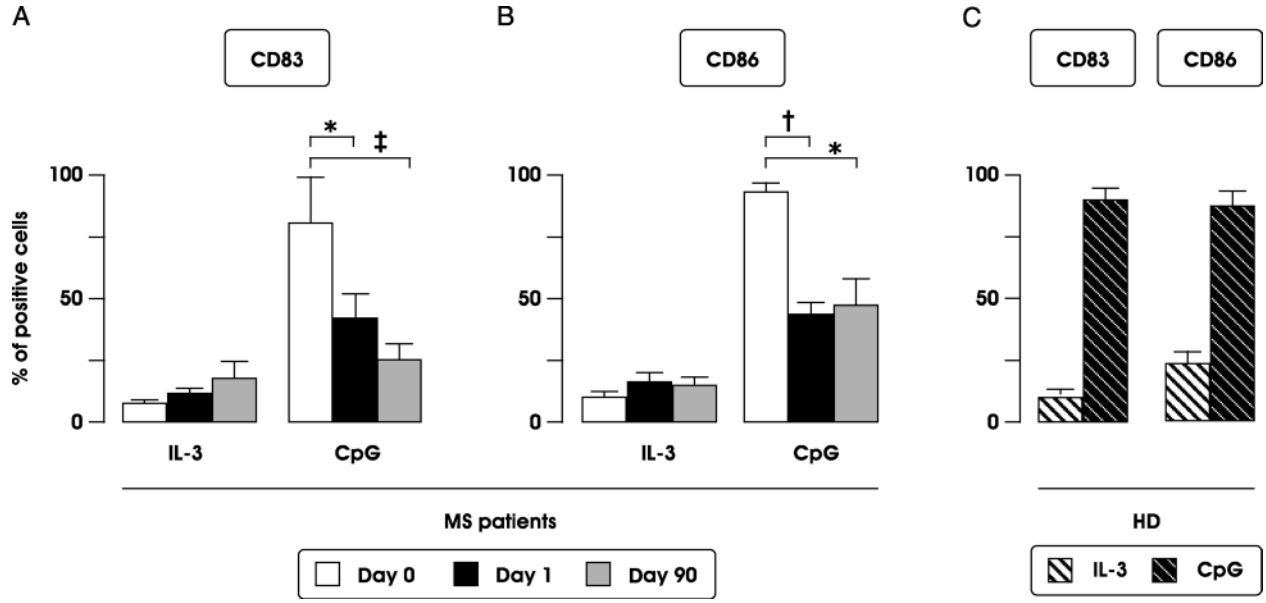

FIGURE 5. Influence of interferon (IFN)-β therapy on the in vitro maturation of plasmacytoid dendritic cells from multiple sclerosis (MS) patients. Peripheral blood mononuclear cell (PBMC) collected from MS patients at baseline (Day 0), at Day 1, and Day 90 of IFN-β treatment and PBMC from healthy donors (HD) were cultured in vitro with CpG (3 μg/ml) or with interleukin 3 (10 ng/ml). After 24 hours, blood dendritic cell antigen 2/CD123 double-positive cells were stained with monoclonal antibodies for CD83 (**A**, **C**; left side) and CD86 (**B**, **C**; right side). The results are expressed as the mean ± standard error of mean fluorescence intensity in 5 MS patients and 3 HD. *, p = 0.046; †, p = 0.013; ‡, p = 0.004 by Student *t*-test.

secondary Abs, followed by incubation with tetrarhodamine isothiocyanate-conjugated streptavidin. For negative controls, primary Abs were replaced with preimmune serum and IgG isotype controls. Images were analyzed and acquired with a laser scanning confocal microscope (LSM 5 Pascal; Carl Zeiss).

Cell Enumeration

Flow cytometric analysis was used to enumerate different hematopoietic lineages in whole blood by use of a blood dendritic cell enumeration kit (Miltenyi Biotech). Briefly, scatter properties of the cells and staining with CD19 PE-Cy5 and CD14 PE-Cy5 mAbs enabled enumeration of B cells and monocytes, respectively. Simultaneous labeling with anti-BDCA-1 PE and anti-BDCA-2 FITC mAbs allowed identification of $CD19^-$ cells and enumeration of the BDCA-1^+ myeloid DC and the BDCA-2^+ pDC subsets. Absolute numbers of the respective cell populations were normalized to the total number of blood cells to obtain the relative percentage of each cell type according to the manufacturer's protocol. Blood samples from 11 MS patients and 7 healthy donors (HD) were analyzed.

Characterization and Stimulation of PBMCs From MS Patients

Peripheral blood mononuclear cells were isolated from MS patients at baseline and after IFN-β (Avonex) administration. Heparinized venous blood was separated by density gradient centrifugation using Lympholyte-H (Cedarlane Laboratories).

To evaluate the immunophenotype of circulating pDC, PBMCs were isolated from MS patients (n = 11), and the expression of CD86, CD83, HLA-DR, CD38, B7H1, and ChemR was performed on CD123 and BDCA-2 double-positive cells.

To evaluate the effects of IFN-β treatment on pDC maturation, PBMCs from MS patients (n = 5) were washed twice in PBS and cultured in RPMI 1640 supplemented with 2 mmol/L of L-glutamine, 10% fetal calf serum (BioWhittaker), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco) at 1.5×10^6 cells/ml for 24 hours. Peripheral blood mononuclear cells were cultured in the presence of IL-3 (10 ng/ml) or CpG 2395 (3 μg/ml). The maturation of pDCs was studied through the analysis of CD83 and CD86 surface markers on BDCA-2^+/$CD123^+$ population.

Statistical Analysis

Statistical significance of differences was determined by the Student *t*-test for paired data or with ANOVA with Bonferroni-Dunn posttest ($p < 0.05$ was considered significant).

RESULTS

Detection of pDCs in the MS Brain

We first analyzed the presence and distribution of pDCs in postmortem brain tissue from 4 cases of SPMS using immunohistochemistry for the pDC markers BDCA-2 and CD123. We previously showed that in cryosections of human tonsil, these markers stain pDCs, which are mainly localized in T-cell areas (7). Blood dendritic cell antigen 2–positive and $CD123^+$ cells with irregular cell bodies and

long cytoplasmic processes were intermingled among inflammatory cells accumulating around blood vessels in acute and chronic active MS lesions (Figs. 1A, B). The perivascular cuffs mainly contained $CD3^+$ T cells and $CD68^+$ macrophages, fewer DC-SIGN$^+$ DCs, and variable numbers of $CD20^+$ B cells and $CD138^+$ plasma cells (data not shown). No staining for BDCA-2 or CD123 was observed in the demyelinated parenchyma (data not shown). Numerous dendritiform BDCA-2^+ and $CD123^+$ cells were also detected in the inflamed leptomeninges, often near blood vessels (Figs. 1C, D). As previously shown (11), accumulation of BDCA-2^+ pDCs was also noted inside ectopic B-cell follicles in the meninges (data not shown). No immunoreactivity for BDCA-2 or CD123 was detected in brain tissues of 2 nonneurologic control cases or in 1 case of Alzheimer disease (Figs. 1E, F and data not shown, respectively).

Because pDC activation results in increased Type I IFN production, we analyzed the expression of MxA, a Type I IFN-induced protein (23). No immunoreactivity for MxA was detected in control brains (not shown), whereas numerous MxA-expressing cells were present in the inflamed meninges and in the inflammatory cell infiltrates surrounding blood vessels in periventricular and subcortical white matter lesions (Figs. 1G, H). Immunoreactivity for MxA was detected in perivascular and intrameningeal cells with round or irregular cell bodies and in some cerebrovascular endothelial cells (Fig. 1H). Double-immunofluorescence staining for MxA and CD3 indicated that MxA was expressed predominantly in $CD3^+$ T lymphocytes (Fig. 1I) but also in $CD3^-$ leukocytes within the inflammatory infiltrates. This suggests that several cell types respond to Type I IFN stimulation. No MxA immunoreactivity was detected within the parenchyma (data not shown).

Recent studies have identified chemerin as a novel chemoattractant for DCs, including pDCs and macrophages, through its receptor ChemR23 (19). Chemerin receptor expressed on pDCs is thought to regulate pDC traffic in secondary lymphoid organs and inflamed tissues (24, 25). Immunohistochemical analysis revealed that ChemR23 was expressed in inflammatory cells accumulating in the leptomeninges and in perivascular cuffs of MS lesions (Figs. 2A, B), whereas chemerin immunoreactivity seemed confined to endothelial cells of some inflamed blood vessels in the meninges and white matter lesions (insets in Figs. 2A and B). Many ChemR23$^+$ cells had elongated, ramified shapes similar to those of BDCA-2^+/$CD123^+$ pDCs (compare Fig. 2A with Fig. 1B and insets in Figs. 1C and D and Fig. 2B with Fig. 1A). By double-immunofluorescence staining, some of the ChemR23$^+$ cells detected in perivascular cuffs were identified as BDCA-2^+ pDCs (Fig. 2C). No specific immunoreactivity for ChemR23 and chemerin was detected in control brains (Figs. 2D, E). Together, these data suggest that pDCs accumulating in the MS brain become activated to produce Type I IFN, and that the chemerin/ChemR axis may be implicated in the regulation of the intracerebral homing of pDCs.

Frequency of Circulating pDCs in MS Patients Versus Healthy Donors

The frequencies of pDCs in whole blood samples of 7 HD and 11 MS patients who had not received any pharmacologic treatment were determined. After normalizing pDC numbers to total numbers of blood cells, no differences in the relative percentages of pDCs were found between MS patients and HD (Fig. 3) or in 9 patients with RRMS versus 2 with SPMS (data not shown). These results indicate that no alterations in pDC numbers are evident in MS patients, irrespective of the clinical course. Moreover, the immature phenotype of circulating pDCs, that is, a moderate expression of MHC Class II molecules and absence of maturation markers such as CD83 and CD86, did not differ between MS and healthy donor samples (data not shown).

Effect of IFN-β Therapy on the Number, Phenotype, and In Vitro Maturation of Circulating pDCs From MS Patients

To determine whether IFN-β influences the numbers and phenotype of circulating pDCs, we compared the frequency of pDCs in whole blood of MS patients before and after treatment with IFN-β. Whole blood from 11 MS patients at baseline (Day 0), 24 hours after the first IFN-β administration (Day 1), and after 3 months of therapy (Day 90; 24 hours after the IFN-β injection) was analyzed. Compared with baseline levels, the frequency of pDCs

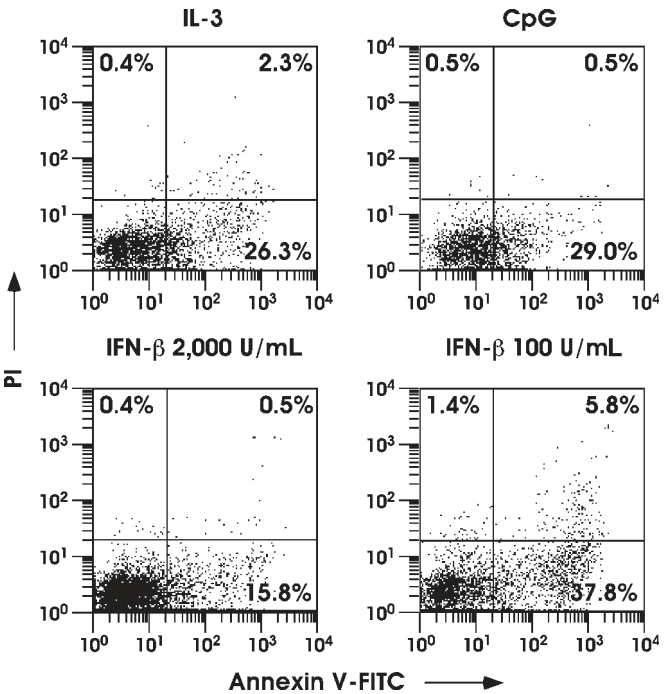

FIGURE 6. Viability of cultured plasmacytoid dendritic cells. Plasmacytoid dendritic cells were purified from healthy donors and stimulated with interleukin 3 (1 ng/ml), IFN-β (2,000 or 100 U/ml), or CpG (3 μg/ml). Cell viability after 24 hours was assessed by double staining with Annexin V-fluorescein isothiocyanate (V-FITC) propidium iodide (PI). Flow cytometry analysis of 1 representative experiment of at least 3 performed is shown. Percentages of cells positive for Annexin V, PI, or both are indicated in each quadrant. IFN, interferon; IL, interleukin.

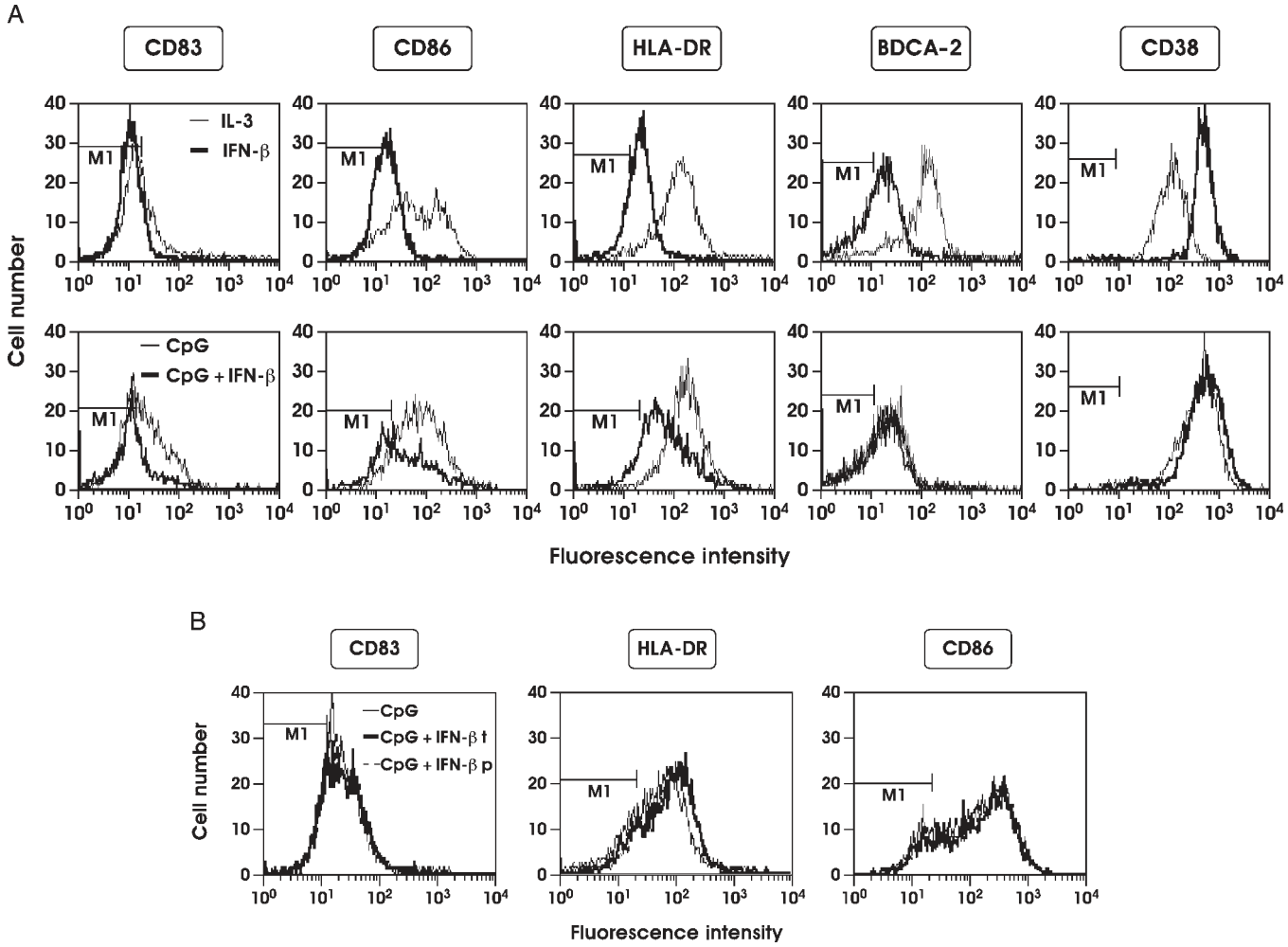

FIGURE 7. Effect of interferon (IFN)-β on the immunophenotype of in vitro CpG-stimulated plasmacytoid dendritic cell (pDC). Plasmacytoid dendritic cells (5×10^4) isolated from peripheral blood mononuclear cells of healthy donors were stimulated with interleukin (IL)-3 (1 ng/ml), IFN-β (2,000 U/ml), or CpG (3μg/ml), or pretreated for 4 hours with IFN-β before the addition of CpG. After 24 hours, cells were stained and analyzed by flow cytometry as described in Materials and Methods section. **(A)** Top panel, pDCs stimulated with IFN-β (bold line) or IL-3 (thin line). Bottom panel, pDCs cultured with CpG alone (thin line) or pretreated with IFN-β (bold line). **(B)** Plasmacytoid dendritic cells were cultured with CpG alone (thin line), CpG for 4 hours before the addition of IFN-β (CpG + IFN-β p; hatched line), or CpG and IFN-β added simultaneously (CpG + IFN-β t; bold line). Isotype-matched controls were contained in the M1 bar. Histogram plots of flow cytometry analysis of 1 representative experiment of at least 6 performed are shown. BCDA, blood dendritic cell antigen; p, posttreatment; t, together.

was slightly, but not significantly, reduced at Day 1 only (Fig. 4A).

We next evaluated the effect of IFN-β therapy on the immunophenotype of circulating pDCs by studying the cell surface expression of maturation, antigen-uptake, and adhesion molecules (Figs. 4B, C). After IFN-β treatment, pDCs displayed increased expression of 2 IFN-inducible markers, CD38 and B7H1, indicating that IFN-β had activated its target genes (Fig. 4B). Interferon-β treatment also significantly and progressively reduced expression of BDCA-2, which has been implicated in antigen uptake, on the surface of pDCs at 1 day and 3 months after treatment (Fig. 4C). At both points analyzed, there was a significant reduction of MHC Class II (HLA-DR) molecule expression on pDC, whereas expression of the costimulatory molecules CD83 and CD86 and of ChemR23 was not altered (Fig. 4B). The downregulation of molecules involved in antigen (Ag) uptake and presentation (BDCA-2 and MHC Class II, respectively; Figs. 4B, C) and the concomitant induction of B7H1, a molecule with an inhibitory effect on T-cell activation (26, 27), suggest that IFN-β can profoundly affect the Ag-presenting and T-cell stimulatory function of circulating and possibly CNS-infiltrating pDCs in MS patients. Conversely, the absence of an effect on ChemR23 expression indicates that the ability of pDCs to migrate in response to chemerin may not be affected by IFN-β treatment.

To understand whether IFN-β therapy might have an influence on the maturation process of pDC, an in vitro

TABLE. Effect of IFN-β on the Maturation of pDCs Stimulated In Vitro With TNF or CD40L*

Stimulus	CD83 (% positive cells)	CD86 (% positive cells)	BDCA-2 (MFI)	HLA-DR (MFI)	CD38 (MFI)
IL-3	7.5 ± 1.5	15.7 ± 8	452 ± 108	458 ± 150	531 ± 145
IFN-β	3.3 ± 1	6 ± 1.6	215 ± 42	170 ± 31.5	1,987 ± 997
IL-3 + TNF	39 ± 6.2	60 ± 9	245 ± 70	1,730 ± 848	739 ± 264
IFN-β + IL-3 + TNF	12.4 ± 1.8†	25 ± 3.5†	201 ± 52	337 ± 19§	1,390 ± 280
IL-3 + CD40L	19 ± 5.4	35 ± 15	380 ± 95	549 ± 115	387.5 ± 56
IFN-β + IL-3 + CD40L	10 ± 2.6	18 ± 8¶	242.5 ± 45	367 ± 104¶	1,600 ± 200

*Plasmacytoid dendritic cells (5×10^4) isolated from PBMCs of HDs were stimulated as indicated. Pretreatment with IFN-β started 4 hours before the addition of IL-3 + TNF or IL-3 + CD40L (for the doses, see Materials and Methods section). After 24 hours, cells were stained and analyzed by flow cytometry as described in Materials and Methods section. The results shown are the mean ± SE values obtained in 6 experiments.

†, $p < 0.05$; §, $p < 0.02$.

†, $p < 0.005$ for IFN-β + IL-3 + TNF versus IL-3 + TNF.

¶, $p < 0.05$ for IFN-β + IL-3 + CD40L versus IL-3 + CD40L.

BCDA, blood dendritic cell antigen; HD, healthy donors; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; pDCs, plasmacytoid dendritic cells; SE, standard error; TNF, tumor necrosis factor.

experiment was performed in which PBMCs from MS patients were collected at baseline (Day 0), 24 hours after the first IFN-β administration (Day 1), and after 3 months of therapy (Day 90; 24 hours after the last IFN-β injection). Peripheral blood mononuclear cells were maintained in vitro for 24 hours in the presence of IL-3 (10 ng/ml) or CpG, a strong maturation stimulus for pDCs that acts through activation of Toll-like receptor (TLR) 9 (18). The maturation of pDCs was studied by analyzing the expression of CD83 and CD86 surface markers on the blood-derived BDCA-2^+/$CD123^+$ population in MS patients and healthy donors. CpG treatment induced CD83 and CD86 expression to the same extent in MS patients and in healthy donors (Fig. 5). Compared with pDCs analyzed before IFN-β treatment, however, pDCs of the MS patients analyzed after IFN-β administration (Days 1 and 90) showed reduced expression of CD83 and CD86 after in vitro stimulation with CpG (Figs. 5A, B). This finding indicates that IFN-β can inhibit the response of pDCs to TLR-9 stimulation.

Effect of IFN-β Treatment on the Viability and Maturation of pDCs In Vitro

To investigate the effects of IFN-β on the activation and functional properties of pDCs further, BDCA-4^+ cells from HD were isolated by positive sorting. This procedure allowed us to obtain a sufficient number of purified pDCs to analyze the effects of IFN-β in vitro. Because isolated pDCs undergo spontaneous apoptosis when cultivated in vitro in the absence of appropriate factors, different doses of IFN-β were used to test the survival of pDC. The ability of IFN-β to influence the viability of cultured pDCs was measured by evaluating annexin$^+$ and PI^+ cells and was compared with the survival-promoting activity of a low dose of IL-3 (1 ng/ml) or

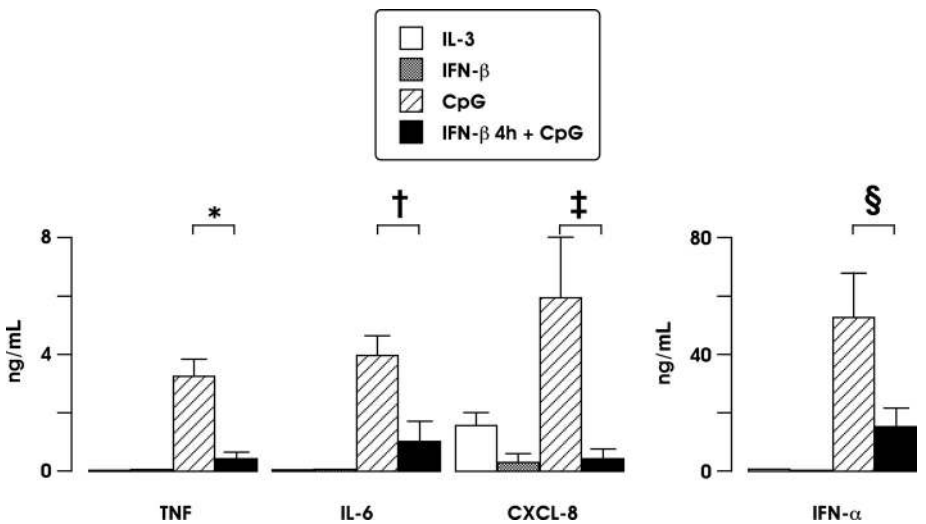

FIGURE 8. Cytokine production by interferon (IFN)-β-pretreated plasmacytoid dendritic cells. Plasmacytoid dendritic cells (5×10^4) from healthy donors were stimulated as in Figure 6A, and the amounts of tumor necrosis factor (TNF), interleukin (IL)-6, IL-8, and IFN-α were measured in the supernatants by cytometric bead array or ELISA. The results are expressed as the mean ± standard error of cytokine levels obtained in 6 experiments. *, $p = 0.012$; †, $p = 0.032$; ‡, $p = 0.045$; §, $p = 0.037$ by Student *t*-test.

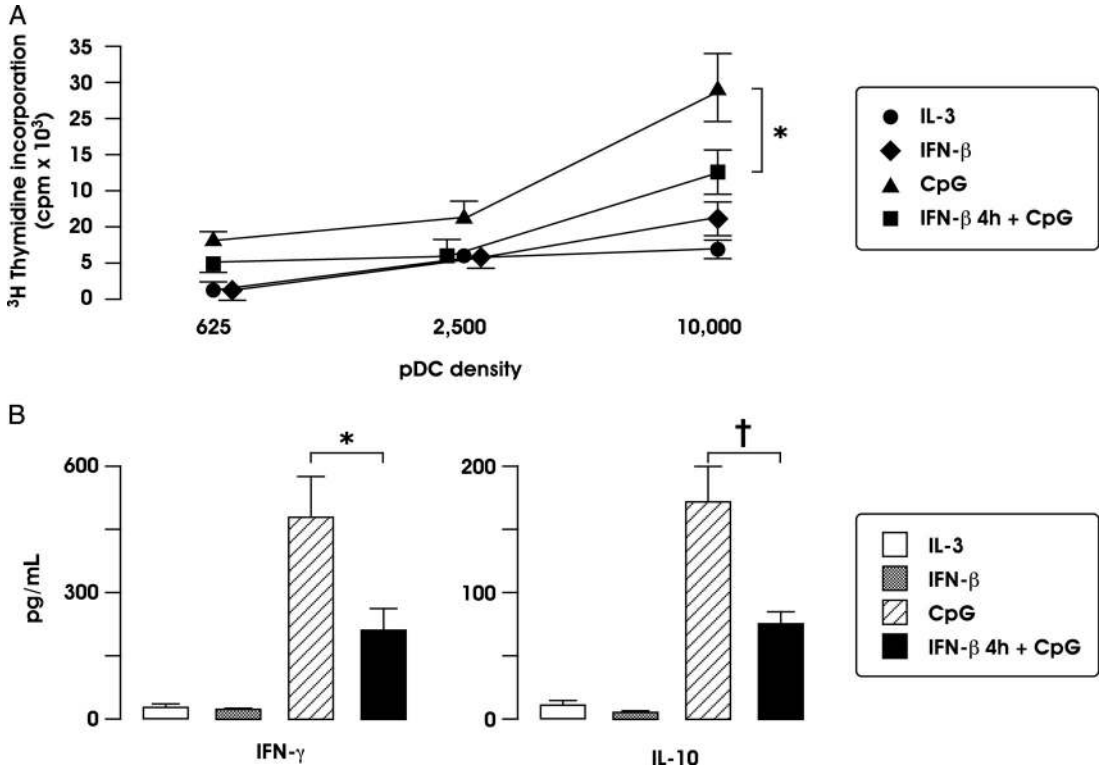

FIGURE 9. Immunoregulatory effect of interferon (IFN)-β-pretreated plasmacytoid dendritic cells (pDCs) in allogeneic cocultures. Plasmacytoid dendritic cells from healthy donors were stimulated for 24 hours as indicated. Cells were extensively washed and cultured at the indicated cell density with allogeneic cord blood naive T cells (3×10^4 per well) for 6 days. T-cell proliferation was measured by [^{3}H]thymidine incorporation. Results are reported as mean counts per minute ± standard error values of 4 independent experiments. *, p = 0.048 by Student *t*-test. **(B)** Plasmacytoid dendritic cells stimulated as in **(A)** were extensively washed and cultured at a 1:5 ratio (stimulator/effector cell) with allogeneic naive T cells (3×10^4 per well). After 6 days, supernatants were collected to measure IFN-γ and interleukin (IL)-10. *, p = 0.045; †, p = 0.048 by Student *t*-test.

of the maturative stimulus CpG (3 μg/ml). At the dose of 2,000 U/ml, IFN-β acted as a potent pDC survival factor (>80% annexin$^-$ and PI$^-$ cells after 24 hours of in vitro stimulation), whereas a lower dose (100 U/ml) was less effective in preventing the spontaneous apoptosis of isolated pDCs (Fig. 6). These data are consistent with previous results showing that IFN-α enhances the survival of sorted CD11$^-$ DC (28). As expected, IL-3 and CpG treatments supported the viability of pDCs (70%–75% annexin$^-$ and PI$^-$ cells after 24 hours of in vitro stimulation). Based on this result, the dose of 2,000 U/ml of IFN-β was used to treat pDCs in subsequent experiments, although this concentration is higher than the levels (50–100 U/ml) encountered by circulating pDCs in the peripheral blood of IFN-β-treated patients.

To determine whether IFN-β influenced pDC maturation in vitro, the effects of a 24-hour treatment with IFN-β (2,000 U/ml) were compared with those induced by a low dose of IL-3 (1 ng/ml). Interferon-β-treated pDCs displayed an immature phenotype, that is, absence of CD83 and CD86 expression, but a reduced expression of BDCA-2 and MHC Class II molecules compared with ex vivo pDCs (Fig. 7A; upper panels). As expected, strong upregulation of CD38, a surface molecule known to be induced by Type I IFN (29), was observed in pDCs treated in vitro with IFN-β (Fig. 7A; upper panels). In contrast, pDCs treated with IL-3 (1 ng/ml) showed a partially activated state indicated by upregulation of CD86 and HLA-DR but no changes in CD83 and BDCA-2 expression (Fig. 7A; upper panels).

The effect of IFN-β treatment was also evaluated on pDC maturation induced in vitro by CpG. Purified pDCs were grown for 4 hours in the presence or absence of IFN-β and then stimulated with CpG for 24 hours (Fig. 7A; lower panels). No differences were observed in the expression of CD38 and BDCA-2 (Fig. 7A; lower panels) or in the viability of pDC cultures (70%–75% of the cells were annexin$^-$ and PI$^-$ in both conditions; data not shown). Clear inhibition of pDC maturation induced by CpG was observed in IFN-β-pretreated cells versus untreated pDCs, as indicated by reduced expression of CD83, CD86, and HLA-DR molecules (compare Fig. 7A upper panels with lower panels). Conversely, no effect on the expression of these molecules was observed when pDCs were treated simultaneously with IFN-β and CpG or were exposed to IFN-β 4 hours after CpG stimulation (Fig. 7B).

We then asked whether IFN-β was also capable of blocking the maturation of pDCs induced by TLR-independent stimuli such as TNF or CD40 ligation. Interestingly, pretreatment of pDCs with IFN-β inhibited the induction of CD83, CD86, and HLA-DR in pDCs stimulated with TNF and IL-3 or with CD40L and IL-3 (Table). Pretreatment with IFN-β

further decreased the expression of BDCA-2 occurring in mature pDC, whereas a clear induction of CD38 was observed when IFN-β was added to pDC cultures, irrespective of the maturative stimulus. These results indicate that IFN-β can modulate the maturation process of pDCs triggered by TLR- and cytokine-dependent stimuli and by CD40 activation.

Effects of IFN-β Treatment on Cytokine Release From In Vitro-Activated pDC

To investigate the effects of IFN-β on pDC maturation further, we stimulated pDCs with CpG, either in the presence or absence of IFN-β, or with IL-3 and analyzed by cytometric bead array the secretion of inflammatory cytokines such as TNF and IL-6 and the chemokine CXCL8 in the culture supernatants. Interferon β alone failed to induce release of any of the previously discussed cytokines, whereas IL-3-treated pDC production of CXCL8 was upregulated (Fig. 8). Interestingly, CpG-induced release of TNF, IL-6, and CXCL8 by pDCs was significantly reduced upon pretreatment with IFN-β (Fig. 8). Because pDCs are known to release high amounts of Type I IFN after virus or CpG stimulation, IFN-α production was also analyzed by ELISA. Although IFN β or a low dose of IL-3 did not stimulate IFN-α release, a robust production of IFN-α occurred in CpG-treated pDC, which was significantly inhibited when the cells were pretreated with IFN-β (Fig. 8).

Effects of IFN-β on T-Cell Stimulation Induced by In Vitro Maturing pDCs

Finally, we analyzed the ability of CpG-matured pDCs to activate alloreactive T cells in the presence or absence of IFN-β. Figure 9.A shows that CpG-treated pDCs were more effective than pDCs stimulated with IFN-β (2,000 U/ml) or with a low dose of IL-3 (1 ng/ml) in inducing T-cell proliferation. In contrast, pretreatment of pDCs with IFN-β before stimulation with CpG decreased the proliferative response of T cells. We also evaluated the effects of IFN-β on the polarizing ability of CpG-stimulated pDCs. T cells stimulated with CpG-matured pDCs released IFN-γ and IL-10 (Figure 9B) Production of both cytokines was significantly reduced when T cells were stimulated with CpG-induced pDCs that had been pretreated with IFN-β (Fig. 9B). There was no induction of IL-4 observed when T cells were cocultured with CpG-induced pDCs in the presence or absence of IFN-β (data not shown).

DISCUSSION

This study provides novel insights on the role of pDCs in MS pathogenesis by showing that pDCs are actively recruited and become activated in MS lesions, and that pDC maturation is affected by IFN-β treatment both in vivo in MS patients and in vitro.

Using BDCA-2 and CD123 as pDC markers, we confirm and extend our previous findings that pDCs accumulate in the inflamed meninges and white matter lesions of MS patients (11), whereas no pDCs were detected in brain tissues from nonneurologic control cases and from a case with Alzheimer disease. Although it is not known whether intracerebral pDC recruitment can be considered as a negative or a positive prognostic factor in MS, the presence of pDCs in the MS brain is consistent with previous observations, indicating that these cells are involved in other chronic inflammatory diseases (4, 8). Plasmacytoid dendritic cells homing to cutaneous lesions in systemic lupus erythematosus are thought to play a key role in the pathogenesis of this autoimmune disease because disease progression is associated with high systemic levels of IFN-α (5, 6, 30). Similarly, the presence of pDCs in the inflamed synovium of rheumatoid arthritis patients (7) and in the inflamed skin (4) indicates that pDCs contribute to the local inflammatory processes. From our immunohistochemical data on the expression of MxA, which is frequently used as a surrogate marker for local Type I IFN production (7, 31), we infer that pDCs infiltrating the MS brain are activated and produce significant amounts of Type I IFN. Because the brain specimens analyzed were obtained from MS patients who had not received IFN-β therapy, MxA is likely to be induced in situ in an autocrine and/or paracrine fashion by Type I IFN released from activated pDCs. Most importantly, our recent finding of an abnormal accumulation of latently EBV-infected B cells and plasma cells in the brains of MS patients (11), together with the observation that EBV can undergo reactivation in CNS-infiltrating plasma cells, suggests that this herpesvirus might act as the main maturative stimulus for brain-infiltrating pDC. Accordingly, pDCs were found to accumulate not only in MS lesions but also in meningeal ectopic B-cell follicles in which the highest frequency of latently and lytically EBV-infected B cells was observed (11). Therefore, it is conceivable that stimulation of pDCs by EBV leads to Type I IFN production, which in turn promotes a cellular immune response against EBV through activation of natural killer and T cells, as recently demonstrated by Lim et al (32) in posttransplant lymphoproliferative disease. The present findings therefore support a link between EBV infection and the presence of activated pDCs in the MS brain, where production of biologically relevant amounts of Type I IFN can be directly involved in the establishment of an antiviral state, amplification of a proinflammatory T-cell response, and consequent tissue damage (33).

Our data also suggest that the chemerin/ChemR axis, which has been recently implicated in pDC migration (24), may play a role in the recruitment of pDCs into the CNS in MS. Interestingly, chemerin was found to be expressed in vascular endothelial cells in the meninges and white matter lesions, whereas the ChemR was expressed on infiltrating leukocytes, including pDCs. These observations are consistent with those in systemic lupus erythematosus in which pDC accumulation in skin lesions was recently shown to be driven by expression of chemerin in inflamed dermal endothelial cells (24). Because other chemokines that are expressed in the inflamed CNS such as CXCL12 and CXCL10 (34–37) may affect pDC migration, it is likely that homing of pDCs to the MS brain results from the concerted action of multiple chemokines.

To obtain more insight into the role of pDCs in the pathogenesis of MS, we analyzed the frequency and immunophenotype of circulating pDCs in MS patients versus healthy donors. In agreement with previous studies (10, 38), we found no alterations in the percentage and immature phenotype of circulating pDCs in MS patients compared with healthy donor subjects. Taken together with evidence of pDC accumulation in the cerebrospinal fluid (9) and brain tissue of MS patients (Reference 11 and this study), this suggests that pDC alterations in MS are mainly compartmentalized in the CNS, where pDCs may be recruited and activated by inflammatory stimuli.

To understand whether pDCs might represent a target of IFN-β therapy, ex vivo analysis of circulating pDCs was carried out in PBMCs of MS patients before and during IFN-β treatment. Although previous studies have shown that IFN-β may induce expression of CD123 in cultured human monocytes and on circulating pDCs from MS patients (38), the effects of IFN-β on pDC phenotype and function were not characterized. Here, we found no significant changes in the frequency of pDCs before or during IFN-β treatment, although the level of some cell surface molecules was markedly affected. The IFN-β-induced profile of membrane molecules indicates that pDCs respond to this cytokine by acquiring the immunophenotype of reduced expression of MHC Class II and BDCA-2 and increase of 2 IFN-inducible markers, CD38 and B7H1. Modulation of the latter molecule is particularly interesting in light of recent studies showing that B7H1 exerts a strong inhibitory effect on $CD4^+$ and $CD8^+$ T-cell activation (26, 27) and is also induced by IFN-β in monocytes and semimatured myeloid DC (39). Likewise, the absence of CD83 and CD86 expression and the downregulation of molecules involved in antigen uptake and presentation (i.e. BDCA-2 and MHC Class II) are consistent with the concept that IFN-β affects the T-cell stimulatory function of circulating pDC. Blood dendritic cell antigen 2 has recently been identified as a novel Type II C-type lectin capable of targeting an uncharacterized ligand or ligands into antigen-processing and peptide-loading compartments for presentation to T cells (40). Conversely, the absence of an effect on ChemR23 expression suggests that IFN-β does not impair the ability of pDCs to migrate in response to chemerin. Moreover, our observation that IFN-β inhibits the induction of CD83 and CD86 in blood pDCs stimulated ex vivo with a TLR-9 agonist indicates that IFN-β treatment may act on pDCs by reducing their ability to respond to maturative stimuli.

Additional information on IFN-β effects on pDC function induced by maturative stimuli was obtained by analyzing the response of pDCs isolated from the buffy coat of HD and exposed in vitro to IFN-β before activation with a TLR-9 ligand (CpG), proinflammatory cytokines (TNF and IL-3), or CD40L. We used this in vitro system because large numbers of pDCs are required to analyze the effects of IFN-β on a pure population of pDCs and to perform parallel mixed-lymphocyte reaction experiments. Having established the in vitro conditions to study pDC activation/maturation, we analyzed the effects of IFN-β on the release of proinflammatory soluble factors such as IFN-α, IL-6, TNF, and CXCL8 in response to TLR-9 triggering. These in vitro experiments showed that IFN-β inhibited production of all the cytokines induced by the TLR-9 agonist CpG, thus strengthening the concept that IFN-β impairs multiple effector functions associated with pDC maturation.

Plasmacytoid dendritic cells can drive T_H1 polarization through production of Type I IFN, but under certain conditions or depending on their tissue localization, they are also capable of promoting the differentiation of IL-10-expressing regulatory T cells (2, 3, 41, 42). Using a mixed-lymphocyte reaction assay, we found that treatment of pDCs with IFN-β before CpG stimulation reduces their capacity to stimulate T-cell proliferation and release of IFN-γ and IL-10. These results indicate that no further expansion of IL-10– and IFN-γ-producing T cells occurs after cocultivation with pDCs matured by CpG in the presence of IFN-β, thus confirming the ability of IFN-β to inhibit the functional activity of maturing pDCs. Experiments are in progress to evaluate the mechanisms through which IFN-β, by acting on the immunoregulatory capacity of pDC, can tune the activation of autoreactive T cells and regulatory T cells. This is of particular interest in light of recent findings indicating that a defect in $CD4^+CD25^{high}$ regulatory T-cell function occurs in MS patients (43).

The results described here shed new light on the mechanisms of IFN-β therapy in MS patients. This involves the tuning of the response to maturative stimuli of pDCs, a specialized leukocytic population that regulates multiple aspects of the immune response mainly through the release of Type I IFN. Our results clearly show that IFN-β interferes with pDC maturation and with their ability to release Type I IFN and other inflammatory mediators, and to stimulate T cells. Based on these findings, we propose that IFN-β induces a hyporesponsive state of pDCs to maturative stimuli, leading to reduced antigen-presenting and T-cell stimulatory activity. In parallel, through induction of the inhibitory molecule B7H1, IFN-β can enhance the ability of pDCs to downregulate pathogenic T cells. It can be envisaged that pDCs homing to the inflamed CNS, after being exposed to IFN-β in the peripheral blood, respond poorly to the maturative stimuli encountered in the inflamed tissue. Because only mature pDCs are able to induce the activation of memory $CD4^+$ and $CD8^+$ T cells (44–46) and to promote the differentiation of B cells into Ab-producing plasma cells (47), the present results suggest that pDCs accumulating in the brain of MS patients treated with IFN-β might display a reduced ability to promote pathogenic humoral and cellular immune responses due to the lack of an appropriate mature phenotype. It is thus conceivable that IFN-β therapy may affect the response of pDCs to EBV stimulation, and this, in turn, may restrain $CD8^+$ T-cell–mediated immunopathology, which is the major determinant of tissue destruction in EBV-associated diseases (48). In conclusion, a better understanding of the proposed involvement of pDCs in MS immunopathology and of their response to IFN-β open new opportunities to elucidate disease mechanisms and increase the therapeutic efficacy of this treatment.

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