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Plasmacytoid Dendritic Cells in Multiple Sclerosis: Chemokine and Chemokine Receptor Modulation by Interferon-beta

Latt Latt Aung, MD¹, Patricia Fitzgerald-Bocarsly, PhD², Suhayl Dhib-Jalbut, MD¹, and Konstantin Balashov, MD, PhD^{1,*}

¹Department of Neurology, UMDNJ-RWJMS, New Brunswick, NJ

²Department of Pathology and Laboratory Medicine, UMDNJ-NJMS, Newark, NJ

Abstract

Plasmacytoid dendritic cells (pDCs) are present in peripheral blood, leptomeninges and demyelinating lesions in patients with multiple sclerosis (MS). The ability of pDCs to produce chemokines and express the chemokine receptor CCR7 in MS is not known. We studied pDCs in MS patients and healthy subjects. The ability of pDCs to up-regulate CCR7 was significantly increased in untreated MS patients as compared to healthy subjects. IFN-beta treatment significantly inhibited TLR9 agonist-specific secretion of chemokines, which are ligands for CCR5-positive Th1 cells (CCL3, CCL4, and CCL5), and impaired TLR9 agonist-induced up-regulation of CCR7 and IFN-alpha in MS patients. This finding represents a new immunomodulatory effect of IFN-beta in patients with multiple sclerosis.

Introduction

Multiple sclerosis (MS) is considered to be an immune-mediated disorder of the central nervous system (CNS) (McFarland and Martin, 2007, Sospedra and Martin, 2005). Although the immunopathogenesis of the disease is not completely understood, both polygenic and environmental factors contribute to disease onset and/or clinical exacerbation (Hafler et al., 2007, Noseworthy et al., 2000, Compston and Coles, 2002). Viral pathogens have been implicated in the etiology and pathogenesis of MS (reviewed in (Cook et al., 1996). Among those, strong data implicates Epstein-Barr Virus (EBV) a human DNA virus (Haahr and Hollsberg, 2006, Serafini et al., 2007, Giovannoni et al., 2006, Levin et al., 2003, Ascherio and Munger, 2008). The association of HHV-6 with MS has been suggested and is being investigated (Swanborg et al., 2003). For established MS patients, the risk of disease exacerbation was found to be increased at the time or shortly after clinical viral infections (Edwards et al., 1998, Sibley et al., 1985, Panitch, 1994) (and reviewed by Rutschmann et al. (Rutschmann et al., 2002)). In addition to infection, the antiviral protein interferon (IFN)-gamma, a T helper type 1 (Th1)-type cytokine produced mainly by NK and T cells, is able to trigger multiple sclerosis exacerbation (Panitch et al., 1987b, Panitch et al., 1987a).

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***Corresponding Author:** Konstantin E. Balashov MD, PhD, Department of Neurology, UMDNJ-Robert Wood Johnson Medical School, 97 Paterson Street, Rm 208, New Brunswick, NJ 08901, Phone: (732) 235-7732 (office), Fax: (732) 906-5705, kbalashov@yahoo.com.

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Plasmacytoid dendritic cells (pDCs), characterized as CD11c(−)Lin(−)CD123(++)DR(++)BDCA2(+)BDCA4(+) cells, have been intensively investigated due to their important role in both innate and adaptive immunity (Colonna et al., 2004, Gilliet et al., 2008, Fitzgerald-Bocarsly et al., 2008). Compared to other peripheral blood mononuclear cells, pDCs express a high level of Toll-like receptor 9 (TLR9) (Liu, 2005) which recognizes viral DNA within the early endosomes at the initial phase of viral infection. Activated via TLR9, pDCs secrete multiple immunoregulatory cytokines/chemokines such as IFN-Type I cytokines, TNF-alpha, IL-6, CCL3, CCL4, CCL5, CXCL10 and IL-8 (Decalf et al., 2007) and up-regulate expression of chemokine receptor CCR7 directing them to secondary lymph organs to prime naïve T cells (Sozzani et al., 1998). Among cytokines produced by pDCs are IFN-Type I cytokines promoting Th1 cell differentiation via STAT4 transcriptional factor pathway (reviewed in (Korman et al., 2008)), IL-6 which promotes myelin antigen-specific Th17- and Th1-responses in experimental autoimmune encephalomyelitis (EAE) (Serada et al., 2008), and TNF-alpha which induces oligodendrocyte apoptosis (Akassoglou et al., 1998) and mediates neuronal injury (Iliev et al., 2004). In addition, pDCs produce chemokines CCL3, CCL4 and CCL5 which are ligands for CCR5 positive T cells secreting high level of IFN-gamma in MS patients (Balashov et al., 1999). PDCs are found in the CSF of MS patients (Pashenkov et al., 2001, Pashenkov et al., 2002, Lopez et al., 2006, Stasiolek et al., 2006) and accumulate in leptomeninges and MS lesions (Serafini et al., 2007, Lande et al., 2008). Both pDCs and TLR9 appear to be important in the pathogenesis of EAE, the most widely used animal model of MS. The generation of Th17 cells is decreased in pDC-depleted mice and is associated with less severe clinical and histopathological signs of EAE (Isaksson et al., 2009). Activation of antigen-presenting cells through TLR9 can overcome tolerance and precipitate EAE (Segal et al., 2000, Ichikawa et al., 2002, Waldner et al., 2004). Thus, pDCs may serve as a strong link between viral infection and MS exacerbation.

We hypothesized that pDCs may trigger MS exacerbation in response to viral pathogens but are inhibited by disease-modifying therapy such as IFN-beta, consequently decreasing the frequency of MS attacks. Here we describe a new immunodulatory effect of IFN-beta which impairs the ability of pDCs to up-regulate CCR7 expression and to produce CCL3, CCL4 and CCL5 chemokines.

Materials and Methods

1. Patients and Controls

Patients and healthy donors, 18–60 years old, were enrolled in the study. Patients were diagnosed with clinically definite relapsing-remitting MS (RR MS) or clinically isolated syndrome (CIS) as described (Jacobs et al., 2000), and were not receiving any immunomodulatory drugs other than IFN-beta based treatment (Avonex, Rebif, or Betaseron). Patients were not taking any immunosuppressive treatment for at least three months prior to the study (e.g., Mitoxantrone). The patients with infection-like symptoms or history of chronic inflammatory diseases were excluded. Patients with secondary progressive MS and primary progressive MS, patients with EDSS score 6 or higher, or patients who received IV steroids or any other non-IFN-beta immunomodulatory drugs less than 2 months prior to blood drawing were excluded. The patients were treated with IFN-beta 1a (Avonex, Rebif) or IFN-beta 1b (Betaseron) in doses approved by the FDA and recommended by the drug manufacturers. The participation of patients and healthy subjects in the study was approved by institutional review boards. Informed consent was obtained from all subjects. The MS patients and healthy subjects presented in Figures 1, 2 and 3 are described in Tables 1, 2 and 3 respectively. The same patients were analyzed for chemokine production before and after treatment with IFN-beta (Table 2 and Figure 2). Not all patients included in Figure 1 and 3 were tested at two time points (before and after IFN-beta treatment).

2. Cell separation

Peripheral blood mononuclear cells (PBMC) were isolated from 80–100 ml of heparinized blood samples within 4 hours after venipuncture as described (Balashov et al., 1999). Fresh pDCs were separated from PBMC by positive immunomagnetic sorting and BDCA-4 cell isolation kit (Miltenyi Biotec) with 2 steps of pDCs enrichment on magnetic columns. Such procedure typically yields 200,000–250,000 pDCs with more than 90% purity based on flow cytometry analysis of CD11c⁺CD123⁺DR⁺⁺ cells. Separated pDCs had less than 2% of CD14(+) cells (monocytes) and less than 1% of CD3(+), CD16(+) and CD20(+) cells (T cells, NK cells and B cells, respectively). Based on gene expression analysis by RT-qPCR, separated pDCs have approximately 100 fold higher level of TLR9 as compared to PBMC.

3. Cell culture and cytokine measurement

Freshly isolated pDCs (100,000/ml) were cultured with or without 5 µg/ml (0.735mM) CpGA (ODN#2336, Coley Pharmaceutical) for 16 hours and supernatants were collected and stored at –80°C until analyzed. In preliminary experiments, control oligo (ODN#2243, Coley Pharmaceutical) was tested at concentration similar to TLR9 agonist CpG type A oligo (ODN#2336) but was not able to induce detectable IFN-α production by pDCs. Detection of selected chemokines (CCL3, CCL4, CCL5 and CXCL10) was done by the BioMarker Services (Millipore Corporation, St. Charles, MO) using MILLIPLEX Multi-Analyte Profiling with Luminex xMAP Multiplexing Technology (http://www.millipore.com/drugdiscovery/dd3/map_portfolio) with standard curve ranges from 3.2 to 10,000 pg/ml and analyzed using Milliplex Analyst data reduction software (Millipore). If any sample had a undetectable level of chemokine, the chemokine concentration was assigned a value 3.2 pg/ml,

4. Flow cytometry analysis

Intracellular flow cytometry for IFN-α with surface phenotyping was performed as described earlier (Dai et al., 2004) with modifications. In brief, PBMC (1×10⁶/ml) were cultured with or without 5 µg/ml CpGA (ODN#2336, Coley Pharmaceutical) for a total of 4 hours and BFA (5 µg/ml) (Sigma) was added for the last two hours of the culture. Cultured cells were washed with PBS/0.1% BSA buffer and stained with APC-labeled anti-BDCA2 mAb (Miltenyi Biotec) and PE-labeled anti-CD123 mAb (Biolegend). Cells were fixed with 1% paraformaldehyde. To detect IFN-α producing pDCs, cells were permeabilized with 0.1% Triton (Sigma). Biotin labeling of mouse monoclonal anti-human IFN-α Ab (PBL) with Zenon labeling kit (Molecular Probes) was done according to the manufacturer's instructions. Streptavidin-PE-Cy7 (Biolegend) was used as secondary reagent to detect IFN-α producing pDCs. Three-color flow cytometry analysis was done on FC500 flow cytometer (Beckman Coulter). IFN-α producing pDCs were defined as IFN-α-positive pDCs (IFN-α+CD123+BDCA2+). Results were analyzed with CXP software (Beckman Coulter).

To detect CCR7-positive pDCs (CCR7+CD123+BDCA2+), PBMC activated with TLR9 agonist were stained with PE-Cy7-labeled anti-human CCR7 mAb (BD Biosciences), APC-labeled anti-BDCA2 mAb (Miltenyi Biotec) and PE-labeled anti-CD123 mAb (Biolegend) followed by a three-color flow cytometry analysis as described above.

CCR7 and IFN-α expression was also evaluated by Mean fluorescence intensity (MFI) ratio, calculated as the ratio of CCR7 (of IFN-α) mAb mean/negative control mean.

5. Statistical analysis

Within patient change from baseline, laboratory measures were tested by a paired *t*-test. The difference between groups of patients and healthy donors was tested by unpaired *t*-test.

Results

1. IFN-beta treatment decreases IFN-alpha production

pDCs are the major source of the Th1-promoting cytokine IFN-alpha. We compared IFN-alpha production by pDCs activated with TLR9 agonist in healthy donors (HD), untreated and treated RR MS patients. As shown in Figure 1, the proportion of IFN-alpha producing pDCs in IFN-beta treated MS patients (Mean $\pm$ SEM = 25.99 ± 2.25 , n=8) was significantly decreased as compared to untreated MS patients (Mean $\pm$ SEM = $47.34 \pm 3.324\%$, n=8), $p < 0.0001$. IFN-beta MFI ratio for treated patients was 14.21 ± 1.22 , $p < 0.0001$ as compared to untreated MS patients (35.01 ± 3.61). The frequency of IFN-alpha producing pDCs had a non-significant trend to be elevated in untreated RR MS patients as compared to healthy subjects (Mean $\pm$ SEM = $37.00 \pm 4.965\%$, n=8), $p > 0.05$. There was no statistically significant difference in the frequency of IFN-alpha producing pDCs between healthy subjects and IFN-beta treated MS patients, $p > 0.05$. The frequency of pDCs producing IFN-alpha without stimulation with TLR9 agonist was less than 1.7%.

2. Production of chemokines CCL3, CCL4 and CCL5 specific for chemokine receptor CCR5 are reduced in IFN-beta treated MS patients

pDCs produce detectable amounts of CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CXCL10 (IP10) chemokines within 8 to 12 hours of viral infection (Megjugorac et al., 2004, Piqueras et al., 2006). CCL3, CCL4, and CCL5 chemokines are all ligands for chemokine receptor CCR5. CXCL10 interacts with chemokine receptor CXCR3. CCR5 is expressed on T cells, macrophages and dendritic cells while CXCR3 is mostly expressed on T cells and NK cells. Both CCR5 and CXCR3 are preferentially expressed on Th1 cells as compared to Th2 cells in healthy subjects (Sallusto et al., 1998, Loetscher et al., 1998). CCR5 positive T cells are also major producers of IFN-gamma in MS patients (Balashov et al., 1999). On the functional level, CCL3, CCL4, and CCL5 induce a dose-dependent chemotactic transmigration of Th1 cells, while Th2 cells were not attracted by these chemokines (Siveke and Hamann, 1998). Thus, modulation of CCL3, CCL4 and CCL5 secretion in patients may affect migration of Th1 cells towards activated APCs and, therefore, the induction of Th1 responses in MS.

We evaluated chemokine production by pDCs activated with TLR9 agonist in fourteen MS patients before and after a 3 month course of IFN-beta treatment (Figure 2 and Supplementary Table 1). TLR9 agonist-specific production of CCL3, CCL4, and CCL5 by pDCs was significantly decreased (194 ± 36.09 pg/ml, $p = 0.0004$; 287.7 ± 41.18 pg/ml, $p = 0.0001$; 23.3 ± 5.5 pg/ml, $p = 0.027$) after 3 months of IFN- β treatment compared to the pre-treatment levels (409.9 ± 59.73 pg/ml; 722.9 ± 110.5 pg/ml; 53.2 ± 12.7 pg/ml), respectively. However, CXCL10 chemokine production remained unchanged (1982 ± 535.6 pg/ml before treatment and 1909 ± 379.6 pg/ml after treatment). The levels of CCL3, CCL4, CCL5 and CXCL10 chemokines produced by non-activated pDCs (baseline) before treatment were 10.82 ± 2.69 pg/ml, 10.71 ± 2.60 pg/ml, 103.1 ± 30.18 pg/ml, and non-detectable (less than 3.2 pg/ml), respectively. The levels of CCL3, CCL4, CCL5 and CXCL10 chemokines produced by non-activated pDCs (baseline) after IFN-beta treatment were 38.58 ± 14.44 pg/ml, 44.48 ± 18.7 pg/ml, 201.0 ± 69.31 pg/ml and 5.57 ± 2.11 pg/ml, respectively. The difference between baseline chemokine production (non-activated pDCs) before and after IFN-beta treatment was significant ($p = 0.0465$) only for CCL3 (Supplementary Table 1). As a reference, chemokine production by pDCs separated from five healthy donors (Age: 31 ± 4.0 ; Sex: 5 Females) was studied and was not found to be statistically different from MS patients before treatment (Supplementary Table 1).

3. CCR7 up-regulation is decreased in IFN-beta treated MS patients

Immature pDCs express many chemokine receptors including CCR1, CCR2, CCR5, CCR6, CXCR1, and CXCR2. However, upon stimulation, pDCs selectively increase expression of CCR7 which directs activated pDCs towards CCR7 ligands, chemokines CCL19 and CCL21, expressed abundantly by stromal cells in the T cell-rich lymph node areas where pDCs play an important role in the initiation of the immune response (Sozzani et al., 1998, Penna et al., 2001, Penna et al., 2002b, Penna et al., 2002a). Recently, a variety of reports have indicated that, apart from chemotaxis, CCR7 regulates the cytoarchitecture, the rate of endocytosis, the survival, the migratory speed, the maturation of the DCs and the expression of MHC and costimulatory molecules which are required in the presentation of antigen to naïve T cells (Sanchez-Sanchez et al., 2006, Randolph et al., 2005).

We investigated CCR7 expression in stimulated pDCs in MS patients. The level of TLR9 ligand-induced CCR7 expression in untreated MS patients ($28.36 \pm 3.08\%$, $n=9$) was significantly increased compared to healthy subjects ($18.06 \pm 3.55\%$, $n=7$), $p = 0.0455$. The ability of TLR9 ligand to up-regulate CCR7 expression in IFN- β treated MS patients ($14.78 \pm 1.95\%$, $n=9$) was significantly decreased compared to untreated MS patients, $p=0.0018$ (Figure 3). CCR7 MFI ratio for IFN-beta treated patients was 1.41 ± 0.09 , $p = 0.0355$ as compared to untreated MS patients (1.97 ± 0.22). Overall, IFN-beta treated MS patients had levels of CCR7 expression comparable to untreated healthy subjects.

Discussion

Human pDCs comprising only 0.2–0.8% of PBMC are being intensively investigated due to their important role in both innate and adaptive immunity (Colonna et al., 2004). The investigation of pDCs represents a significant challenge as sufficient numbers of cells can only be obtained from buffy coats. However, the application of sensitive techniques such as intracellular staining followed by three-color flow cytometry and detection of multiple cytokines in the same sample by multi-analyte cytokine profiling allowed us to study those cells ex-vivo.

In this manuscript we addressed the effect of IFN-beta in MS on three important functions of activated pDCs, namely intracellular IFN-alpha production, chemokine secretion and CCR7 receptor up-regulation. Several studies examined the effect of IFN-beta on pDC function. IFN-beta treatment did not affect the concentration of pDCs in peripheral blood from MS patients (Lopez et al., 2006, Lande et al., 2008).

Based on our results, the frequency of IFN-alpha producing pDCs in untreated MS patients was comparable to healthy subjects. However, IFN-beta treatment was associated with significantly reduced frequency of pDCs producing IFN-alpha (Figure 1). Type I Interferons produced in high amounts by activated pDCs were shown to promote Th1 cell differentiation via the STAT4 transcriptional factor pathway (reviewed in (Korman et al., 2008)). Lande et al. observed that IFN-beta inhibited IFN-alpha production by pDCs separated from healthy subjects; however, the authors did not address this in MS patients (Lande et al., 2008). Stasiolek et al. reported that TLR9 ligand-induced IFN-alpha production was decreased in MS patients (Stasiolek et al., 2006). In contrast to Stasiolek et al. findings (Stasiolek et al., 2006) who used ELISA to measure IFN-alpha secretion by unfractionated peripheral blood mononuclear cells, we applied flow cytometry to link IFN-alpha production specifically to pDCs (Figure 1) which, in part, could explain the different outcomes between the two studies. Recently, Bayas et al. (Bayas et al., 2009) studied TLR9-agonist activated IFN-alpha secretion by pDCs in MS by using methods of pDC separation, culture condition and IFN-alpha detection different from our protocol. The difference between MS patients and healthy subjects was not statistically significant for strong IFN-

alpha inducer (CpG Type A oligo which is similar to TLR9 agonist used in our study) but was significant for weak IFN-alpha inducer (CpG Type B oligo which was not used in our study) if data from 24 hour-and 72 hour-long cultures were combined together (Bayas et al., 2009).

The ability of pDCs activated with viral pathogens to produce multiple chemokines (Fonteneau et al., 2003, Piqueras et al., 2006, Decalf et al., 2007) and to express the chemokine receptor CCR7 directing pDCs to secondary lymph organs to prime naïve T cells (Sozzani et al., 1998) is well-documented in healthy subjects. However, this pathway has not been previously examined in MS patients. The chemokines CCL3, CCL4, and CCL5 have been implicated in the pathogenesis of MS and they are highly expressed in brain tissue of MS patients (Boven et al., 2000). CCR5 positive T cells were increased in peripheral blood and CSF of MS patients (Balashov et al., 1999, Sorensen et al., 1999) and CCR5 positive lymphocytes, macrophages, and microglia were detected in active MS lesions (Sorensen et al., 1999, Trebst et al., 2001, Mahad et al., 2004). CCR5 positive T cells expressed a Th1 phenotype in MS patients as they secreted high levels of IFN-gamma (Balashov et al., 1999). Note that CCL3, one of several CCR5 ligands, was able to enhance IFN- γ production by activated T cells directly (Karpus et al., 1997). The link between CCR5 expression and clinical severity of MS was analyzed in a population of MS patients expressing CCR5 delta32, a truncated allele of the gene encoding a non-functional CCR5 receptor. The onset of MS was delayed by approximately 3 years (Barcellos et al., 2000) and a risk of recurrent clinical disease activity was decreased (Sellebjerg et al., 2000) as compared to MS patients expressing functional CCR5 chemokine receptor.

In our study, IFN-beta treatment significantly inhibited TLR9 agonist-specific secretion of chemokines, which are ligands for CCR5-positive Th1 cells (CCL3, CCL4, and CCL5), when tested in the same MS patients before and after treatment (Figure 2). pDCs separated from MS patients before treatment had a nonsignificant trend for decreased chemokine production as compared to healthy subjects (Supplementary Table 1). Thus, IFN-beta may potentially impair the chemotaxis of CCR5 positive Th1 cells and monocytes to the MS lesions and ameliorate the severity of the disease as pDCs are highly expressed in MS lesions (Serafini et al., 2007, Lande et al., 2008). On another hand, IFN-beta treatment was associated with the tendency to induce baseline chemokine production by non-activated pDCs. This effect was statistically significant for CCL3. It was shown earlier that IFN-beta inhibited CCL3 and CCL5 gene expression in T cells (Zang et al., 2001) but induced CCL3, CCL4 and CCL5 chemokines in microglia cells (Kitai et al., 2000).

According to our results, pDCs separated from untreated MS patients and activated with TLR9 agonist had CCR7 up-regulation at levels significantly increased compared to healthy subjects. However, IFN-beta treatment was associated with significantly reduced ability of pDCs to up-regulate CCR7 expression as compared to untreated patients (Figure 3). Up-regulation of CCR7 chemokine receptor on activated pDCs is a critical event directing pDCs to secondary lymph organs to prime naïve T cells (Sozzani et al., 1998). It was also reported that CCL19, the ligand for CCR7, was elevated in MS lesions (Krumbholz et al., 2007). In addition, CCR7 positive dendritic cells were found in the CSF and MS lesions (Kivisakk et al., 2004). Both CCL19, CCL21 (another CCR7 ligand) and CCR7 transcripts were expressed in the CNS of mice with EAE and CCL21 protein was confined to the endothelium of inflamed blood vessels (Columba-Cabezas et al., 2003). We hypothesize that expression of CCL19 and CCL21 in the CNS may be a very early step in perivascular demyelinating lesion formation. This will facilitate migration of CCR7 positive leukocytes such as activated dendritic cells from the peripheral blood. Once in the CNS, activated pDCs will contribute to the demyelinating process by secreting chemokines attracting CCR5-positive Th1 cells. They will also promote generation of Th17 and Th1 cells via production

of IL-6 and IFN- α , respectively. We report that patients with MS have increased ability to up-regulate CCR7 expression if activated via TLR9 pathway, a pathway specific for viral and bacterial DNA pathogens. In contrast, IFN- β treatment associated with decreased up-regulation of CCR7 may potentially lead to the impaired trafficking of activated pDCs to the CNS which, in turn, would diminish formation of new demyelinating lesions.

The exact mechanism of how IFN- β affects TLR9-mediated pDC responses remains to be understood. The direct effect on intracellular transport of TLR9 ligands, the level of TLR9 expression or TLR9-mediated intracellular signaling pathways cannot be excluded. It was reported that the full-length TLR9 has to be cleaved from the N-terminal to generate a functional (processed) TLR9 C-terminal (Ewald et al., 2008, Park et al., 2008). Subsequently, we found that TLR9 processing was significantly impaired in pDCs separated from IFN- β treated MS patients while TLR9 gene and protein expression were not decreased (Balashov et al., in preparation). The effect of IFN- β on other phases of TLR9-mediated activation of pDCs has not been studied.

If our findings are confirmed by others and are shown to be linked to clinical disease activity, then modulation of TLR9-mediated pathway may prove to be an important target for a new generation of MS immunomodulatory drugs such as TLR antagonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Intracellular secretion of IFN- α in pDCs

PBMC (1×10^6 /ml) were separated from healthy subjects (HD), non-treated MS patients (MS: No Rx) and MS patients treated with IFN-beta (MS: IFN- β) as described in Table 1. Cells were stimulated for 4 hours with TLR9 agonist CpGA. Cell surface staining with anti-CD123 and anti-BDCA-2 mAbs followed by intracellular staining with anti-IFN- α mAb were performed as described in Materials and Methods. **Figure 1a:** The population of pDCs was defined as double-positive BDCA-2⁺CD123⁺ cells and was gated (small square in the right upper quadrant of exemplary Dot Plot **A** (unstimulated cells) or **C** (cells stimulated with TLR9 agonist)) by flow cytometry analysis. The gated population of pDCs was analyzed for frequency (in %) of IFN- α producing pDCs (the number (%) in right upper quadrant of exemplary Dot Plot **B** (unstimulated pDCs) or **D** (stimulated cells)). The frequency of pDCs producing IFN-alpha without stimulation with TLR9 agonist was always less than 1.7%. **Figure 1b:** The final results for TLR9-ligand induced IFN-alpha secretion by pDCs (% of IFN- α producing pDCs) for healthy donors (HD), untreated MS patients (MS: No Rx) and IFN-beta treated MS patients (MS: IFN- β) are shown. MFI ratio for HD was 22.53 ± 4.59 . MFI ratio for IFN-beta treated patients was 14.21 ± 1.22 , $p < 0.0001$ as compared to untreated MS patients (35.01 ± 3.61). Statistical analysis was done with unpaired t-test.

Figure 2. Chemokine production by activated pDCs

pDCs were isolated from peripheral blood of patients described in Table 2 before and 3 month after treatment with IFN-beta. Cells were stimulated with or without TLR9 agonist CpGA for 16 hours and concentration of CCL3 (A), CCL4 (B), CCL5(C), CXCL10 (D) in cell supernatants was measured as described in Materials and Methods. The figure depicts the difference between the level of chemokines produced by pDCs activated with TLR9 agonist and the level of chemokines produced by non-activated pDCs (baseline). Statistical analysis was done with paired t-test.

TLR9 ligand-induced CCR7 expression

Figure 3. Up-regulation of CCR7 in activated pDCs

PBMC were separated from healthy subjects (HD), non-treated MS patients (MS: No Rx) and MS patients treated with IFN-beta (MS: IFN- β) as described in Table 3. Cells were stimulated for 4 hours with or without TLR9 agonist CpG-A. The population of pDCs was selected based on gating of BDCA-2 and CD123 double-positive cells. The frequency of pDCs expressing surface CCR7 was measured by three-color flow cytometry as described in Materials and Methods. The level of CCR7 up-regulation in stimulated pDCs was obtained by subtracting the baseline level of CCR7 expression for each individual. MFI ratio for HD was 1.65 ± 0.21 . MFI ratio for IFN-beta treated patients was 1.41 ± 0.09 which was significantly decreased compared to untreated MS patients (1.97 ± 0.22), $p = 0.0355$. Statistical analysis was done with unpaired t-test.

Table 1

Patients presented in Figure 1

Subjects:	Type of MS (number of subjects)	Age (mean $\pm$ SEM)	Female/Male	Disease Modifying Drugs (number of patients treated)
Healthy donors	N/A (8)	35.88 $\pm$ 3.512	6/2	N/A
MS: No Rx	RR MS (5), CIS (3)	36.88 $\pm$ 4.168	7/1	N/A
MS: IFN- β	RR MS (7), CIS (1)	37.38 $\pm$ 4.157	6/2	Rebif (1) Betasteron (2) Avonex (5)

Table 2

Patients presented in Figure 2

Subjects:	Type of MS (number of subjects)	Age (mean $\pm$ SEM)	Female/Male	Disease Modifying Drugs (number of patients treated)
MS: No Rx	RR MS (10), CIS (4)	36.29 $\pm$ 2.833	9/5	N/A
MS: IFN- β	RR MS (10), CIS (4)	36.29 $\pm$ 2.833	9/5	Betasteron (8) Avonex (6)

Table 3

Patients presented in Figure 3

Subjects:	Type of MS (number of subjects)	Age (mean $\pm$ SEM)	Female/Male	Disease Modifying Drugs (number of patients treated)
Healthy donors	N/A (7)	32.57 $\pm$ 3.845	6/1	N/A
MS: No Rx	RR MS (8), CIS (1)	31.67 $\pm$ 2.967	7/2	N/A
MS: IFN- β	RR MS (7), CIS (2)	40.67 $\pm$ 4.871	7/2	Betaseron (5) Avonex (4)