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An Imbalance of Two Functionally and Phenotypically Different Subsets of Plasmacytoid Dendritic Cells Characterizes the Dysfunctional Immune Regulation in Multiple Sclerosis

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Plasmacytoid dendritic cells (pDCs) are instrumental in peripheral T cell tolerance and innate immunity. How pDCs control peripheral immunotolerance and local parenchymal immune response and contribute to the altered immunoregulation in autoimmune disorders in humans is poorly understood. Based on their surface markers, cytokine production, and ability to prime naïve allogenic T cells, we found that purified BDCA-2BDCA-4pDCs consist of at least two separate populations, which differed in their response to oligodeoxynucleotides and IFNs (IFN-β), and differently induced IL-17- or IL-10–producing T cells. To evaluate the potential immunoregulatory role of these two types of pDCs in multiple sclerosis (MS) and other human autoimmune disorders (myasthenia gravis), we studied the phenotype and regulatory function of pDCs isolated from clinically stable, untreated patients with MS (n = 16). Patients with MS showed a reversed ratio of pDC1/pDC2 in peripheral blood (4.4:1 in healthy controls, 0.69:1 in MS), a phenomenon not observed in the other autoimmune disorders. As a consequence, MS pDCs had an overall propensity to prime IL-17–secreting cells over IL-10–secreting CD4+ T cells. Immunomodulatory therapy with IFN-β induced an increase of the pDC1 population in vivo (n = 5). Our data offer a plausible explanation for the disturbed immune tolerance in MS patients and provide evidence that immunomodulatory therapy acts at the level of reconstituting homeostasis of pDC, thus reconstituting the disturbed balance. The Journal of Immunology, 2010, 184: 000–000.

Plasmacytoid dendritic cells (pDCs) are considered to be safeguards of peripheral T cell tolerance and key players in innate immunity (1, 2). During immunoregulation, pDCs have both stimulatory (3, 4) and regulatory (5–7) effects on T cells. However, little is known regarding the contribution of pDCs to the balance of T cell activation during autoimmunity in the periphery or in the local control of immune reactions in inflamed tissue.

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS and is thought to be autoimmune in nature (8–10). The combination of genetic and environmental factors predisposes to disease susceptibility, severity, and course. Because of their tolerogenic and immunogenic functions, pDCs are assumed to play an important role in the immunoregulatory network in MS. A first hint to functional abnormalities of pDCs isolated from the peripheral blood of MS patients has been described previously (11).

pDCs are rarely detected in the CNS under nonpathologic conditions, but they are elevated under neuroinflammatory conditions (including MS) in the cerebrospinal fluid (12). Active recruitment of pDCs into the CNS and their accumulation in white matter lesions and leptomeninges of MS brains has also been demonstrated (13). In experimental autoimmune encephalomyelitis, an animal model of human MS, a critical tolerogenic effect of pDCs was demonstrated (14). Unlike myeloid DCs, pDCs had only a minor role in T cell activation and epitope spreading, but negatively regulated pathogenic CNS CD4+ T cell responses, suggesting a regulatory role during experimental neuroinflammation (14). However, it has to be noted critically that the exact mechanism of pDC accumulation and their role during CNS inflammation are not well understood.

In this study, we provide a detailed characterization of two phenotypically and functionally different subsets of pDCs in human peripheral blood using specific markers for pDCs (15). These subsets (pDC1 and pDC2) clearly differ in their properties to induce proinflammatory versus regulatory T cell responses. We noted that MS is characterized by a different distribution of those pDC subsets in the periphery, which results in a high propensity of MS–derived pDCs to generate IL-17 over IL-10–producing CD4+ T cells in vitro. Immunomodulatory therapy with IFN-β (an established standard treatment of MS) was capable to reverse the altered pDC distribution in vivo. Our data therefore suggest that the alterations in pDC subsets might be responsible for the failure of immunotolerance in MS patients.
Materials and Methods

Patients

Fresh blood samples were obtained from 16 patients referred to the Department of Neurology (University of Wuerzburg). Diagnoses of MS were made according to the criteria of McDonald et al. (16). Patients included in the study were clinically stable and had not yet received immunomodulatory treatment except for corticosteroids, with the last dose at least 6 mo prior to study. In addition, we included five patients who received de novo IFN-β and were followed for 12 mo. All patients gave informed consent in accordance with the Declaration of Helsinki and a protocol approved by the Ethics Committee of the University of Wuerzburg Medical School.

Thirty to 50 ml of blood was collected by venous puncture and tourniquet. In parallel, 21 sex- and age-matched healthy donors (HDs), and 15 autoimmune disease controls (other disorders [OD]) were included in the study (15 patients with myasthenia gravis [MG]).

Isolation and stimulation of pDCs

pDCs were purified from freshly collected peripheral blood samples (<24 h processing time). PBMCs were isolated by density gradient centrifugation using lymphocyte separation medium (PAA Laboratories, Linz, Austria), followed by positive sorting using anti–BDCA-4-conjugated magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity of the recovered cells was determined by flow cytometry and was >90%. Where indicated, BDCA-4–isolated pDC were stained with anti-CD123/anti-CD58 and sorted by flow cytometry based on CD123HIGH/CD58LOW (named pDC1) expression and CD123LOW/CD58HIGH (named pDC2) using a fluorescence-activated cell sorter (MoFlo; Cytomation, Freiburg, Germany).

Ex vivo isolated or cultured pDCs were washed with FACS-buff (PBS supplemented with 1% BSA and 0.1% sodiumazide) and stained with fluorescence-labeled mAbs together with blocking mouse IgG (Sigma-Aldrich, Munich, Germany) at a concentration of 2 × 10^4 per well in RPMI 1640 (PAA, Linz, Austria) supplemented with 2 mmol/l l-glutamine, 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), and 10% human serum (AB serum; PAA). The cells were stimulated with type A (CpG_2A) or type B (CpG_2B) unmethylated CpG oligodeoxynucleotides (ODN) (2 μM, CpG 2216 and CpG 2016, respectively; TIB MOLBIOL, Berlin, Germany), unless specified otherwise in results. IFN-γ concentration was 1000 U/ml.

Flow cytometry

For intracellular staining, cells were fixed and permeabilized (BD Biosciences, Heidelberg, Germany) and FlowJo (Tree Star, Ashland, OR) software. The following mAbs were used for counterstaining of BDCA-4–isolated pDCs: anti–HLA-DR (M30, BD Biosciences), anti–HLA-ABC (HLA-A24, BD Pharmingen), anti-IL-12 (HP-F1), anti–ILT3 (ZM 3.8), anti–ILT4 (42D1, all Beckman Coulter, Krefeld, Germany), and anti-CD86 (2331, BD Pharmingen), anti-CD58 (HP-F1), anti-ILT2 (LT27:295; Miltenyi Biotec), anti–TNF-α (cA2; Miltenyi Biotec), anti–CD80, CD86, MHCII, TLRs, ICOS-L), this population was not also be identified (Fig. 1A, right panel). Since then, these markers have been used widely to identify and purify pDCs from human blood. We found that pDCs purified by BDCA-4* contain at least two distinct subpopulations in the peripheral blood of HDs that can be identified based on the cell size and granularity (Fig. 1A, left panel). Using mAb to CD123 (IL-3Rα) we could distinguish between CD123HIGH (pDC1 subset) and CD123LOW cells (pDC2 subset). A subpopulation that was negative for CD123 expression could also be identified (Fig. 1A, left panel). Because CD123 negative cells were also negative for other important cell surface proteins (e.g., CD80, CD86, MHCII, TLRs, ICOS-L), this population was not studied in detail here.

Further phenotypic analysis revealed a lower expression of MHC class II and other markers associated with DC maturation (e.g., CD86, TLR2, and CD58) by pDC1 in comparison with pDC2 (Fig. 1A, right panel). In addition to MHCII, CD123, and CD86, pDC1 and pDC2 differed in the expression of CD58, ILT2, ILT3, ILT4, CD1c, CD303, and TLR2 (Fig. 1A, right panel). No differences between pDC1 and pDC2 could be observed in the expression of TLR1, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 (data not shown). To rule out the autofluorescence-nonspecific Ab binding common for matured DCs, isotype stains were performed differentially on pDC1 and pDC2 (Supplemental Fig. 1). The stains revealed a weak increase in the fluorochromes FITC, PE, and APC and some increase on pDC2 in the PE-Cy5-channel.

pDCs represent a population of DCs with relatively low frequency (~0.4%) in human peripheral blood (1). Recently, two additional markers (BDCA-2 and BDCA-4) were identified with a restriction to human pDCs in peripheral blood and bone marrow (15). Since then, these markers have been used widely to identify and purify pDCs from human blood.

We also observed profound differences in the cytokine secretion by both pDC subsets (Fig. 1B). pDCs were analyzed either directly (no stimulus) or after stimulation using CpG_2A (ODN 2216), which was shown to induce high amounts of IFN-α/β in pDCs, or CpG_2B (ODN 2006), which strongly promotes pDC maturation and activation but induces only small amounts of IFN-α/β (17–19). Unlike CpG_2A, pDC stimulation with CpG_2B altered the pDC1/pDC2 ratio observed under noninflammatory conditions. CpG_2B treatment stimulated a profound shift of total pDCs into pDC2, as indicated by lower expression of CD123 (Fig. 1B) and high expression of various maturation markers (not shown). In our assays, pDC1 was the main source of IFN-α under both steady state and proinflammatory stimulus delivered by CpGs, whereas pDC2 was a poor cytokine producer at a steady state and produced TNF-α upon CpG treatment (Fig. 1C, left and middle rows). TNF-α could be differentially induced in pDC1 in response to CpG_2A and in pDC2 by CpG_2B (Fig. 1C, middle and lower rows). pDC2 also produced more IL-6 than did pDC1 (data not shown). This behavior further corroborates our finding that pDCs in human blood contain at least two phenotypically different subsets.

We next studied whether the two identified pDC populations would change phenotype and/or function in response to CpGs. As already indicated in Fig. 1, pDC1 displayed a more immature
phenotype with low expression of MHCII, CD86, and CD58 molecules. As a result, this cell population was more prone to mature upon CpG stimulation. CpG A and CpG B treatments both resulted in an increase of CD86 and MHCII expression by pDC1 with a percentage of double-positive cells increasing from 36% (unstimulated) to 58% (upon CpG A) and 83% (upon CpG B) (data not shown). pDC2 consistently revealed CD86 and MHCII expression before or after CpG treatments (data not shown). Therefore, we suggest that CpG A activated total pDCs, but did not induce their maturation, whereas CpG B appeared to result in maturation of pDC1 into cells with pDC2 phenotype. Thus, blood-derived pDCs could be converted by CpG B into pDC2, whereas the immunomodulatory agent IFN-β, had an opposite effect on pDCs, inducing a pDC1 phenotype (Fig. 2A). After demonstrating the behaviors of pDC1 (CD123<sup>high</sup>CD58<sup>low</sup>) and pDC2 (CD123<sup>low</sup>CD58<sup>high</sup>) in response to different stimuli, and their relative changes, we were interested in the effects these cells could have under conditions of allogeneic T cell priming. Thus, we sorted pDC1 (CD123<sup>high</sup>CD58<sup>low</sup>) and pDC2 (CD123<sup>low</sup>CD58<sup>high</sup>) by flow cytometry from human blood-derived pDCs and directly cocultured them with allogeneic naive T cells. Interestingly, pDC1 induced 3.8-fold more IL-10–producing T cells (Tr1) (Fig. 2B) than did pDC2, whereas pDC2 induced 4.8-fold more IL-17–secreting IL-17–producing T cells (Th17) than did pDC1 (Fig. 2B). Because matured DCs are generally prone to reduced viability, we included 7-AAD staining to control the ability of pDC subsets to survive over time and under different experimental conditions. Although both pDC1 and pDC2 were stable and viable in culture under all conditions, CpG A treatment resulted in reduced viability of mainly pDC2 (Supplemental Fig. 2 and data not shown).
pDC1/pDC2 distribution influences the generation of IL-10–producing T cells versus Th17

We next investigated the influence of prestimulated total pDCs (containing pDC1 and pDC2 subsets) on T cell priming in vitro to corroborate our previous observations. Total pDCs were isolated and treated with CpG_A or CpG_B for 48 h prior to coculture with allogeneic naïve T cells. After 7 d, the cells were collected and analyzed for the expression of T cell-secreted cytokines (Fig. 2C). After showing that CpG_A does not alter the composition of pDCs (Fig. 2A), we compared how in vitro-shifted pDCs differed in the T cell priming assay. CpG_B-treated pDCs (therefore mainly consisting of pDC2; Fig. 2C, left panel) induced no Th1 (Fig. 2C, right panel), and IFN-β–driven pDCs (therefore mainly consisting of pDC1) induced Th1.

Moreover, we challenged the capability of general pDCs as well as pDC1 versus pDC2 differentially to induce FoxP3 regulatory T cells, as previously suggested (20). As a positive control, we used TGFβ3, which has been reported to induce FoxP3-expressing cells under in vitro conditions (21). In our experimental settings, we did not observe the induction of FoxP3-expressing cells by pDC (data not shown).

The ratio of pDC1 versus pDC2 is inverted in peripheral blood-derived pDCs of patients with MS

To investigate whether MS might be associated with a disturbance of the pDC1/pDC2 ratio, we next evaluated phenotype and distribution of pDC1/pDC2 in peripheral blood of patients with MS. pDCs isolated from human peripheral blood of stable, untreated, relapsing-remitting MS cases were compared with HDs and another autoimmune disease (OD), MG. In HDs, the ratio of pDC1 to pDC2 was rather stable at an average number of 4.4:1 to 1 (pDC1 to pDC2: n = 21, range, 0.6:1–18.2:1; Fig. 3A). Interestingly, pDCs of patients with MS showed a reversed ratio of pDC1 to pDC2 (n = 16; average, 0.69:1; range, 0.1:1–18.1:1), which was not observed in OD. Patients with MG (n = 15), a prototypic Ab-mediated neurologic disorder, had a slightly altered pDC1/pDC2 balance in the same direction as MS (average ratio, 0.9:1), but this ratio did not reach the level of significance and the pDC1/pDC2 distribution was not as apparent as in MS blood samples (Fig. 3A). In addition, we investigated several patients with rheumatoid arthritis, where the pDC1/pDC2 ratio was not altered (data not shown). We did not observe significant differences in cell surface markers expression for both pDC1 and pDC2 when compared between HD and MS (Supplemental Fig. 3).

pDC derived from patients with MS induce more Th17 in comparison with HD controls

To address whether the inverted pDC1-to-pDC2 ratio in the total pDCs from patients with MS might have consequences for the balance of primed immune responses—similar to our in vitro experiments (Fig. 2)—we compared the properties of HD- and MS-derived pDCs in coculture assays with naïve allogeneic T cells. MS-derived pDCs, predominantly containing the pDC2 subset (Fig. 3A), induced a higher amount of IL-17–secreting Th17-type cells in individual experiments in comparison with HD controls (Fig. 3B). In addition, T cells that were double positive for IL-17 and IFN-γ secretion were also detected in cocultures with MS-derived pDCs. Interestingly, these cells were not found in assays with HD-derived pDCs (Fig. 3B). Using a Transwell membrane separating pDCs and T cells, we could not detect any induction of IL-17 and IFN-γ production by T cells in the absence of pDCs, suggesting that a direct contact, possibly in a form of costimulation, could be required for the instruction of lineage (data not shown).

Immunomodulatory treatment with IFN-β reconstitutes the pDC1/pDC2 imbalance in MS

Because an inverted ratio of pDC1 to pDC2 toward pDC2 could be shown for patients with MS (Fig. 3A) and IFN-β treatment could induce a pDC1 phenotype in vitro (Fig. 2A), we finally questioned whether immunomodulatory therapy with IFN-β, a well-established basic therapy in clinical practice, would exert its effects by influencing the pDC phenotype. We investigated samples from MS cases that were serially acquired before and at different times during clinical treatment. Blood-derived pDCs were analyzed in the samples before IFN-β therapy and at 6 and 12 mo after treatment. Analogous to our observations in vitro, systemic IFN-β therapy resulted in
A recovery of the underrepresented pDC1 population in MS blood (Fig. 4). In an extreme case, before therapy, there was no detectable pDC1 population (Fig. 4A). The pDC1 population reached 39% after 6 mo and 52% after 12 mo of treatment. We examined five patients in whom this shift could be observed in differing frequencies (Fig. 4B, 4C). The first sign of a recovery of the pDC1-to-pDC2 ratio could be observed as early as after 2 mo of therapy (data not shown). Similar to earlier observations (11), this finding rules out that the subset imbalance is a fixed abnormality not amenable to therapeutic modification.

Discussion

Our study addressed the questions of how human pDCs contribute to the immune regulatory networks and how phenotype and function of blood-derived pDCs might differ in a common autoimmune disorder of the CNS (i.e., MS) from healthy volunteers, thereby providing a further hint to the immunopathogenesis of MS.

We could show that human peripheral pDCs contain at least two major subpopulations, which we propose to be called pDC1 and pDC2 subsets. Under basal conditions, these subpopulations exist in a certain ratio and have different effects on the generation of proinflammatory versus anti-inflammatory or regulatory T cell responses. Although previous reports have recognized differences in the overall pDC population in human blood (15, 22) and MS (11, 23), distinct populations of pDCs as identified by specific markers have not yet been analyzed in detail. Based on the size and activation marker expression of pDCs under basal conditions (24, 25), our data suggest that pDC1 (CD123highCD86 MHCIIlow) resemble rather immature DC, whereas pDC2 behave similar to DC showing a mature phenotype.

Accumulating evidence suggests that pDCs exert both immunogenic and tolerogenic properties during various immune responses.

Thus, pDCs can induce Tr1 (26) and FoxP3+ (20) regulatory T cells in direct coculture assays. Although we could not induce FoxP3 expression in naive T cells by pDC (data not shown), we observed a generation of Tr1-type T cells through direct, cell-to-cell contact dependent pDC interaction. Specifically, our results proved to be in accordance with other work (26) showing that the pDC1 subset could induce Tr1.

It is generally accepted that the functions of pDCs are not fixed, but can be modulated by a variety of environmental factors (e.g., innate stimuli). Thus, pDC act as immune regulators and contain a high percentage of the pDC1 subset under steady-state conditions. However, under pathogenic conditions (e.g., with viral or other inflammatory stimuli resulting in pDC maturation) pDCs effectively switch into pDC2-containing immunogenic cell population to presumably fight pathogens during infection (27).

IFN-β, an approved immunomodulatory drug for MS (28), had a long-lasting effect on the pDC1 to pDC2 distribution, shifting the ratio of the two subsets to a pDC1 phenotype in MS blood samples. IFN-β–modulated pDCs with enhanced CD123 expression and IL-10 production were previously described as a subset of myeloid DCs (29–31). Interestingly, these cells resemble the pDC1 subset described in this study and suggest that both pDC1 and pDC2 are a subset of plasmacytoid DCs and not, as hypothesized earlier (22), a possible subtype of myeloid DCs.

An impaired maturation profile and a lower cytokine secretion in response to innate stimulation of pDCs derived from patients with MS have been demonstrated recently (11, 23). However, the factors responsible for this impairment remained elusive, as was the link with the disordered immunoregulation in MS. We found a remarkable imbalance in the distribution of pDC1 versus pDC2 subsets in MS. Although the overall frequency of pDCs in peripheral blood did not differ between MS and HDs, and the cell
surface markers on both pDC1 and pDC2 between MS and HDs proved to be similar, the functional consequences of pDC–T cell interactions were significantly different. Thus, the number of $T_{H17}$ after pDC interaction was clearly higher in the presence of MS-derived pDCs. In addition, unlike pDCs from HD controls, MS-derived pDCs induced double-positive IFN-γ+IL-17+ T cells in vitro, which are believed to represent a highly proinflammatory and, in the proper antigenic setting, encephalitogenic subpopulation of CD4+ T cells. The generation of these cells has recently been reported (32), suggesting a synergetic action of two cytokines. As a side note, our results differ in one data point from those of Stasiokel et al. (11); whereas they found CD86 to be expressed less in MS pDCs ex vivo, we observed that pDC2 is CD86+ and patients with MS have a much higher percentage of pDC2. Other discrepancies between our studies can be attributed to cell preparation (PBMCs versus leukapheresis) and general differences in scientific questioning (cocultures versus monocolonies).

The description of two phenotypically and functionally different pDC populations in conjunction with their alteration in MS raises several important questions, such as: 1) is the imbalance between pDC1/pDC2 a phenomenon associated with autoimmune conditions in general or is it specifically associated with MS, and 2) could the reversion of the pDC1/pDC2 imbalance be a strategy of reconstituting parts of the disordered immunoregulatory networks in MS?

First, the significant imbalance in pDC1 to pDC2 subset distribution could be observed only in MS and not in MG, which we used as a neurologic autoimmune disease control. This finding suggests that the detected imbalance of pDC1/pDC2 is characteristic of MS. Therefore, it is tempting to speculate about specific pathogenic conditions that might induce this phenomenon in MS. For example, through its stimulation, a viral infection present in patients with MS could drive the pDC maturation toward the pDC2 phenotype. Indeed, the contribution of viruses (e.g., herpesviruses) to the development of MS (33, 34) and modulation of pDC subpopulations in blood and tissue under human CMV (HCMV) infection (35) have been described. Thus, the expression of cell surface markers on pDCs was shown to be differently regulated in response to HCMV infection (35). These results imply that, similar to HCMV infection, a yet unidentified low-virus infection persistency in patients with MS could selectively target pDC population in the blood, changing their phenotype and function. However, from the observations in the current study, it is not possible to determine whether pDC maturation in the blood of patients with MS is occurring before the disease develops as a phenotypic variant or during the course of disease. This question is possibly addressed by assessing very early MS (e.g., pDCs from juvenile cases of MS), in which the number of environmental triggers influencing the immunoregulatory networks is lower than in cases of adult MS.

The fact that systemic therapy with IFN-β had an effect on the pDC1-to-pDC2 shift in MS blood samples not only corroborates our findings on pDC1/pDC2 shifting in vitro, but also strengthens the notion of its pathogenic relevance. Despite the fact that IFN-β therapy acts at various levels within the putative pathogenesis of MS (36), the examples how IFN-β therapy reconstitutes tolerogenic factors of the immune systems are of specific importance (e.g., recovery of FoxP3 Treg function) (37) and upregulation of tolerogenic molecules on APC (e.g., B7H1 on monocytes) (38). It has been shown that pDCs show an impaired maturation in response to IFN-β (13), which among other markers is reflected by a lower MHC class II expression. This finding fits well with our observation that IFN-β induces the pDC1 phenotype (pDC1 are MHCIIlow).

As a note of caution, we did not purify and test the remaining pDC population with negative CD123 expression. It cannot be excluded that this subset has also some regulatory functions in the disordered immune network of MS.

Our work identifies and characterizes that human blood-derived pDCs contain at least two different subpopulations. These subpopulations differ in cell surface markers, cytokine expression, and their ability to prime naive T cells toward tolerogenic or immunogenic/inflammatory directions. The phenotype of these pDC subpopulations can be modulated in vitro. There is a clear underrepresentation of immature pDC1 in conjunction with functional evidence in MS, and immunomodulatory therapy IFN-β recovers the pDC1 population, regenerating a pDC1/pDC2 ratio as observed in healthy controls. Our work provides an additional mechanism of action for IFN-β in vivo and shows how immunomodulatory therapy can act at the cellular level in recovering immunoregulatory dysfunctions in patients with MS.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure Legends:

**Supplementary Figure 1**: Isotype control stainings of magnetically isolated pDC gated on pDC1 (blue) or pDC2 (red) and stained with fluorescence-labelled antibody isotypes (mouse, IgG1, fluorochromes: FITC, PE, PE-Cy5, APC).

**Supplementary Figure 2**: 7ADD immunostaining on cultured pDC in vitro. pDC were magnetically separated and cultured for 24h either alone or with CpGA, CpGB, and IFNβ. The cells were then stained for CD58 to allow differentiation between pDC1 and pDC2 and co-stained with 7-AAD to detect cell death. Graphs show one experiment out of two performed.

**Supplementary Figure 3**: Surface phenotype of pDC1 and pDC2 subsets derived from HD and MS patients. pDC of HD and MS patients were magnetically isolated and then stained for several surface markers (CD123, CD86, ILT-2, ILT-3, ILT-4, and MHC2 (HLA-DR)). Shown is one representative example out of eight experiments performed for a HD (grey line) and an MS patient (black line) gated for pDC1 (left column) and pDC2 (right column) (n=8).