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ORIGINAL ARTICLE

Elevated frequencies of leukemic myeloid and plasmacytoid dendritic cells in acute myeloid leukemia with the *FLT3* internal tandem duplication

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Abstract Some 30% of acute myeloid leukemia (AML) patients display an internal tandem duplication (ITD) mutation in the FMS-like tyrosine kinase 3 (*FLT3*) gene. *FLT3*-ITDs are known to drive hematopoietic stem cells towards *FLT3* ligand independent growth, but the effects on dendritic cell (DC) differentiation during leukemogenesis are not clear. We compared the frequency of cells with immunophenotype of myeloid DC (mDC: Lin^-, $HLA\text{-}DR^+$, $CD11c^+$, $CD86^+$) and plasmacytoid DC (pDC: Lin^-, $HLA\text{-}DR^+$, $CD123^+$, $CD86^+$) in diagnostic samples of 47 *FLT3*-ITD^- and 40 *FLT3*-ITD^+ AML patients. The majority of ITD^+ AML samples showed high frequencies of mDCs or pDCs, with significantly decreased HLA-DR expression compared with DCs detectable in ITD^- AML samples. Interestingly, mDCs and pDCs sorted out from ITD^+ AML samples contained the ITD insert revealing their leukemic origin and, upon ex vivo culture with cytokines, they acquired DC morphology. Notably, mDC/pDCs were detectable concurrently with single lineage mDCs and pDCs in all ITD^+ AML (n=11) and ITD^- AML (n=12) samples analyzed for mixed lineage DCs (Lin^-, $HLA\text{-}DR^+$, $CD11c^+$, $CD123^+$). ITD^+ AML mDCs/pDCs could be only partially activated with CD40L and CpG for production of IFN-α, TNF-α, and IL-1α, which may affect the anti-leukemia immune surveillance in the course of disease progression.

Keywords Acute myeloid leukemia · Dendritic cells · Flt3 ITD (FMS like tyrosine kinase internal tandem duplication)

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Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) derived from bone marrow precursors. DCs internalize and process antigens for presentation to T and B cells in the lymphatic tissue for induction of adaptive cellular and humoral responses. There are two major subpopulations of DCs in the human peripheral blood, $CD11c^+CD123^-$ (mDC, also known as myeloid DC or DC1) and $CD11c^-CD123^+$ DC (known as plasmacytoid DC, pDC, or also DC2). DCs occur in very low frequency in the peripheral blood of both humans and mice (<1%) [1], and the homeostatic mechanism regulating DC frequency has not been fully elucidated. It is established that DC progenitors require factors such as granulocyte–macrophage colony-stimulating factor (GM-CSF: mDC) and FMS-like tyrosine kinase 3 ligand (FL: mDC, pDC) to expand and differentiate [2, 3]. FL is proposed to be a key regulator of the DC compartment as it is expressed on early hematopoietic stem and progenitor cells, particularly DC precursors [4, 5]. Therefore, the exposure of myeloid and lymphoid committed precursors to soluble FL can dramatically increase mDC and pDC frequencies in mice and humans [4, 6].

FLT3, the cognate FL receptor, is a frequent target for mutations in leukemia. An internal tandem duplication (ITD) mutation within *FLT3* is found in approximately 30% of acute myeloid leukemia (AML) cases with normal

cytogenetics. The leukemic population is commonly monoclonal and its occurrence is linked with a particularly poor prognosis and increased incidence of relapse [2, 7–10]. A hallmark of *FLT3*-ITD$^+$ AML blasts is a constitutive activation of the tyrosine kinase, leading to prolonged survival and promoted proliferation of hematopoietic stem/progenitor cells (independent of FL), as well as a partially blocked differentiation along the granulocytic/monocytic lineages [2, 5, 7, 11]. *FLT3*-ITD seems to be preferentially associated with myelomonocytic and monocytic leukemia (M4, M5) [12]. Although some DC markers have been detected in *FLT3*-ITD$^+$ AML cells (HLA-DR, CD123, CD4) [9], the association of *FLT3*-ITD$^+$ with abnormal frequencies of mDCs or pDCs has not been described previously in humans. Attempts to generate AML-DCs using in vitro culture systems with cytokines have previously indicated that *FLT3*-ITD would hamper the differentiation of AML blasts towards dendritic cells [13], but these studies did not specifically address the presence of ITD$^+$ AML-DCs in the original diagnostic samples. Incidentally, results obtained from murine bone marrow transplantation assays demonstrated that a knock-in of an ITD into murine *FLT3* conferred myeloproliferative disease with increment in the frequency of circulating and spleen mDCs (CD11c$^+$, CD86$^+$) [14].

Since the characterization of DC frequencies in clinical ITD$^+$ AML samples were not described before, we examined whether the presence of the *FLT3*-ITD mutation in diagnostic peripheral blood samples obtained from AML patients would affect the occurrence of mDCs and pDCs. Samples obtained from healthy donors and ITD$^-$ AML patients were used as a comparative parameter. Here, we demonstrate that at leukemia presentation, *FLT3*-ITD$^+$ AML patients and *FLT3*-ITD$^-$ AML patients have a conspicuously high frequency of circulating mDCs, pDCs, and also mixed lineage mDCs/pDCs. Mixed lineage mDCs/pDCs detected in *FLT3*-ITD$^+$ AML samples could only be partially activated in vitro to produce inflammatory cytokines.

Materials and methods

Patient samples

The collection of peripheral blood samples from healthy volunteers (n=10) and AML patients was approved by the local ethics committee of the Hannover Medical School (MHH) and were obtained after an informed consent. A total of 40 *FLT3*-ITD$^+$ and 47 *FLT3*-ITD$^-$ AML patients were included in the study (Table 1). Due to limitations on the number of viable cells present in diagnostic leukemia samples required for more complex flow cytometry analyses (detection of mixed lineage DCs and analyses of cytokine production), an additional second cohort was included in the study, corresponding to 11 *FLT3*-ITD$^+$ patients and 12 *FLT3*-ITD$^-$ patients (Table 1).

Cytogenetics and *FLT3*-ITD analyses

Cytogenetic and molecular genetic studies were performed by the German–Austrian Acute Myeloid Leukemia Study Group at Hannover Medical School or at the University of Ulm. Blood diagnostic samples were analyzed for the presence of ITD mutations in the *FLT3* gene by polymerase chain reaction as described previously [15]. For *FLT3*-ITD molecular analyses, DNA was extracted from approximately 5×10^6 cells using Qiagen Blood Mini Columns according to the protocol of the manufacturer. Polymerase chain reaction (PCR) was performed with genomic DNA using primer molecules *FLT3*-14f-6F 5′-GCA ATT TAG GTA TGA AAG CCA GC-3′ and *FLT3*-E15R 5′-CTT TCA GCA TTT TGA CGG CAA CC. A 5-ng DNA was amplified in a total volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.001% (*w*/*v*) gelatine, 200 μM dNTPs, primer oligonucleotides (*FLT3*-E14-6F [5-prime labeled with 6-FAM] and *FLT3*-E5R; 0.5 μM each), and 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CO, USA). The PCR consisted of an initial incubation step at 95°C for 11 min followed by 27 cycles at 94°C for 30 s, 57°C for 60 s, and 72°C for 120 s, and a final elongation step at 94°C for 30 s, and 60°C for 45 min in ABI 9700 PCR machines. The PCR products were resolved on a 2% agarose gels stained with ethidium bromide. DNA of fluorescence-activated cell sorting (FACS)-sorted cells with DC immunophenotype was amplified by PCR and sequencing was performed on an Applied Biosystems 3130 sequencer and analysed with GeneMapper 4.0 (Applied Biosystems, Delaware/US).

Immunophenotypic analyses and sorting of mDCs and pDCs

Peripheral blood mononuclear cells (PBMCs) obtained from patients and healthy volunteers were isolated by standard density gradient centrifugation using Ficoll (Biocoll separating solution, Greiner, Bio-One, Germany) separation and cryopreserved in 90% FBS and 10% DMSO. The mDCs and pDCs were identified using a commercially available kit ("peripheral blood dendritic cell detection by flow cytometry", Becton Dickinson BD, San Jose, CA, USA). The protocol is based on a four color staining. For detection of myeloid DCs, we used lineage cocktail 1 (FITC) containing monoclonal antibodies (mABs) against CD3, CD14, CD16, CD19, CD20, and CD56 as a negative selection, a mAB against CD11c (APC, clone S-HCL-3), a

Table 1 Patients' characteristics for the first cohort (mDCs or pDCs analyses) and second cohort (mDCs or pDCs or mDC/pDC analyses) ($p \leq 0.05$)

	First cohort *FLT3* ITD positive (N=33)	First cohort *FLT3* ITD negative (N=42)	p value	Second cohort *FLT3* ITD positive (n=11)[a]	Second cohort *FLT3* ITD negative (n=12)[b]
Age, median (range) years	62 (32–84)	56.5 (18–82)	0.17	60.38 (44–83)	56.8 (35–79)
Sex (no./%)					
Male	18 (55)	25 (60)	0.67	6 (54)	6 (60)
Female	15 (45)	17 (40)		5 (46)	4 (40)
WBC median ($\times 10^9$/L)	95.19	65.69	0.1	67.7	63.52
FAB (no./%)	N=18	N=21	0.29	N=10	N=8
M0	2 (11)	2 (10)		1 (9)	0 (0)
M1	1 (6)	5 (24)		2 (18)	2 (16)
M2	4 (22)	3 (14)		1 (9)	0 (10)
M3	3 (17)	0 (0)		0 (0)	0 (0)
M4	3 (17)	5 (24)		3 (27)	3 (25)
M5	5 (27)	6 (28)		5 (45)	3 (25)
Not FAB classified	(15)	(21)		(1)	(4)
Cytogenetics (no./%)					
Favorable	3 (9)	3 (7)	0.13	0 (0)	0 (0)
Intermediate	26 (79)	28 (67)		7 (63)	7 (58)
Adverse	2 (6)	10 (24)		2 (18)	4 (33)
Missing	2 (6)	1 (2)		2 (15)	1 (10)
Probability of relapse (%)	15	24	0.51	N.A.	N.A.
Allo-transplantation (%)	21	31	0.33	36	33
Complete remission to date (CR) (no./%)	15 (45)	22 (54)	0.48	4 (36)	9 (75)

FAB French–American–British classification, *FLT3* FMS-like tyrosine kinase 3, *ITD* internal tandem duplication, *WBC* white blood cells, *N.A.* not applicable

[a] Four of the patients in this cohort were previously included in *FLT3*-ITD + cohort 1

[b] Seven of the patients in this cohort were previously included in *FLT3*-ITD- cohort 1

mAB against HLA-DR (PerCp, clone L243), and a mAB against CD86 (clone FUN-1) or CD83 (clone HB15e) (both PE). Detection of plasmacytoid DCs was similar, except that instead CD11c, CD123 detection (PE, clone 9F5) was performed. Stained cells were analyzed on a FACSCalibur cytometer using CellQuest software (BD, San Jose, CA, USA). Fifty thousand viable cells gated on the FSC/SSC scatter were negatively selected using the lineage markers. The resulting lin^- population was analyzed for HLA-DR/CD11c- (mDCs) or HLA-DR/CD123- (pDCs) expressing cells. The activation/maturation status of DCs was evaluated by analyses of CD86 and CD83 expression.

Eleven additional ITD^+ AML samples for which we could obtain higher viable cell numbers were further characterized as mixed lineage mDCs and pDCs upon analyses of $lin^-/HLA\text{-}DR^+/CD11c^+/CD123^+$ cells. Four of the 11 patients were analyzed with an analysis based on $lin^-/HLA\text{-}DR^+/CD4^+/CD11c^+/CD123^+$. The samples were incubated for 2 h with Golgi Plug (BD, Heidelberg) to inhibit cytokine secretion. Cells were prepared following a BD cytofix/cytoperm protocol for intracellular staining. The cells were harvested on ice, washed, and stained for the expression of cell surface lineage markers (FITC), HLA-DR (V450, BD), CD11c (APC, BD), CD123 (PerCp Cy-5.5, BD), and CD4 (Alexa700, BD). The cells were subsequently stained intracellularly for tumor necrosis factor (TNF)-α (PE-Cy 7) and INF-α (PE-Green A, BD) or IL-1α (PE-Green A, BD), respectively. After staining, cells were acquired at a minimum of 100,000 events. Five color flow cytometry analysis was performed using an LSR II apparatus. Analyses were made with BD FACS DIVA.

Morphological analyses of thawed/sorted and ex vivo cultured samples

In order to analyze the morphology of cells with mDC or pDC immunophenotypes, diagnostic samples from six

ITD$^+$ patients were thawed and sorted for lin$^-$CD11-c$^+$HLA-DR$^+$ (mDCs) and lin$^-$CD123$^+$HLA-DR$^+$ (pDCs) on a FACSAria (BD). Cells were analyzed directly after sorting by cytospin, Giemsa staining, and microscopy (Olympus CKX 41) or were further cultivated under DC differentiation/ maturation conditions. ITD$^+$ mDC-sorted cells were cultured in 12-well culture plates in an X-vivo medium (Lonza, Basel, Switzerland) in the presence of recombinant human (rh) GM-CSF (20 ng/μl, Cell Genix GmbH, Freiburg, Germany) and recombinant human (rh) interleukin (IL)-4 (20 ng/μl, Cell Genix GmbH, Freiburg, Germany). ITD$^+$ pDC-sorted cells were cultured in the presence of rhIL-3 (10 ng/ml, R&D systems, Cologne, Germany). After 5 to 7 days in culture, mDCs and pDCs were stimulated with rhCD40-ligand (CD40L) (50 ng/ml, R&D Systems, Cologne, Germany).

Results and discussion

ITD negative and positive AML samples show elevated frequencies of mDCs and pDCs

We used a commercially available analytical procedure for quantification of DC subsets in whole blood PBMC. CD3$^-$, CD14$^-$, CD16$^-$, CD19$^-$, CD20$^-$, and CD56$^-$ cells were analyzed for expression of HLA-DR/CD11c (mDCs) or HLA-DR/CD123 (pDCs) (Fig. 1a). Activated and matured DCs were further characterized through analyses of CD86 and CD83 expression, respectively [1]. In order to validate the methodology for DC frequency analyses in a healthy control group, we initially analyzed the frequency of mDCs and pDCs in fresh and cryopreserved samples obtained from ten healthy volunteers (Fig. 1b). The fresh samples were analyzed on the day of blood draw, whereas the cryopreserved samples were maintained frozen for at least 1 week prior to analysis. Although the numbers of detectable mDCs (lin$^-$/CD11c$^+$/HLA-DR$^+$) and pDCs (lin$^-$/CD123$^+$/HLA-DR$^+$) in cryopreserved samples were lower than fresh samples, the difference was found not to be statistically significant (Fig. 1b). Cryopreserved mDCs of healthy volunteers (0.82±0.23% of PBMC) contained mainly activated (CD86$^+$) and mature (CD83$^+$) cells. In contrast, in pDCs (0.44±0.19 of PBMC), only approximately half of the cells were activated and mature (Fig. 1c). In contrast to the homogeneous frequencies of the mDCs (in the range between 0.4% and 1.2%) and pDCs (0.3–0.8%) in the PBMC obtained from healthy volunteers, extremely variable frequencies of mDCs (0.3–60%) and pDCs (0.2–70%) were observed in AML

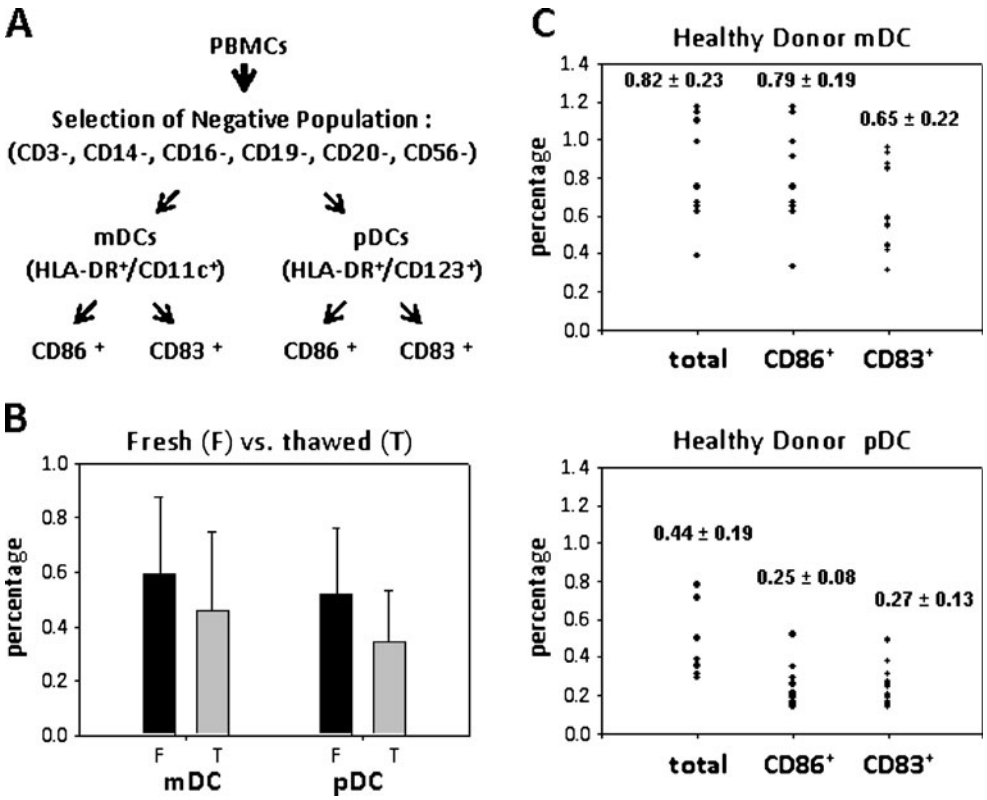

Fig. 1 Experimental design and validation of methods. **a** Schema of the flow cytometry analyses for detection of mDCs and pDCs. **b** Frequencies of DCs detectable in fresh (*black*) versus thawed (*grey*) PBMCs obtained from healthy donors (n=10) do not differ significantly (p values of mDCs, 0.15; pDCs, 0.44.). **c** Frequencies of cells with mDC and pDC immunophenotypes as total cells or with additional expression of the activation marker CD86 and maturation marker CD83 (numbers indicate the average and standard deviation for each analyses; n=10)

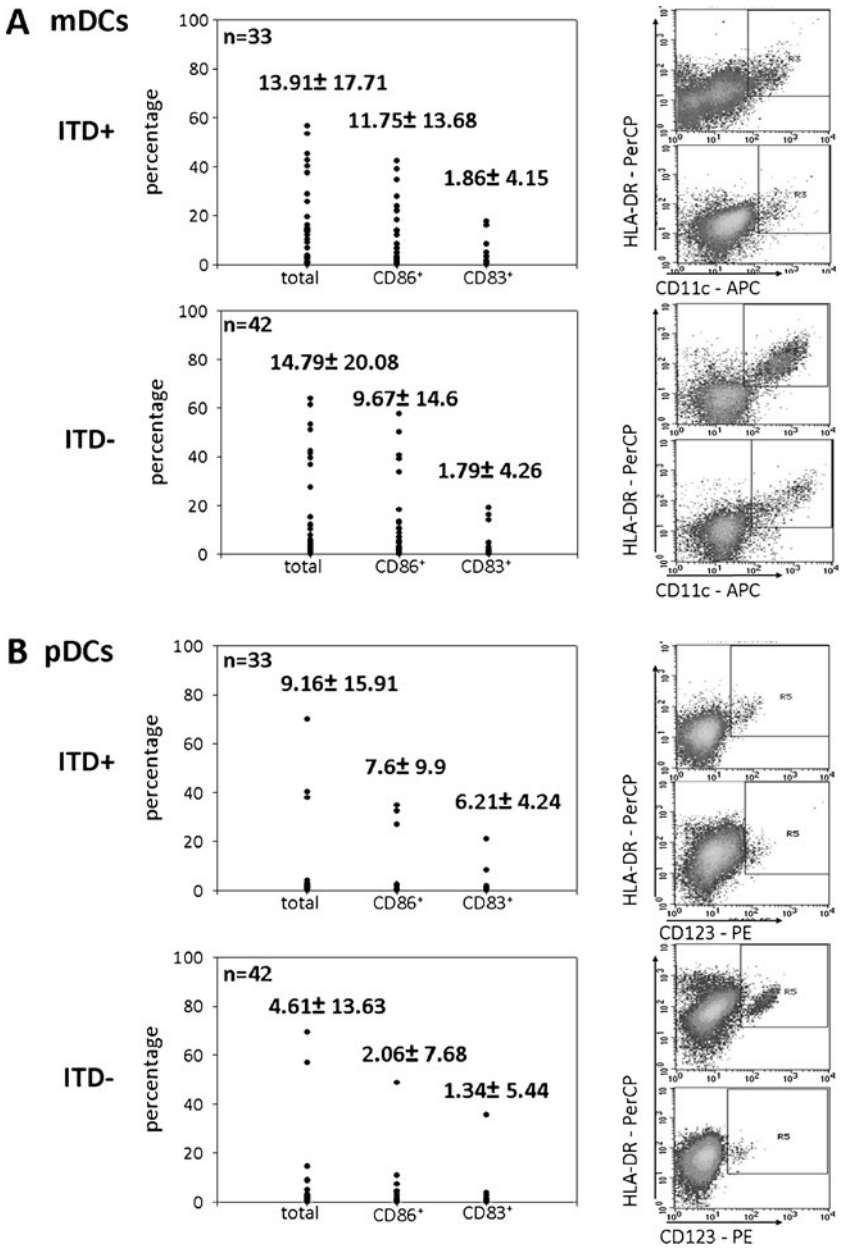

Fig. 2 Flow cytometry analyses of cells with DC immunophenotype in AML diagnostic samples. **a** Frequencies of mDCs, and **b** pDCs detected in *FLT3*-ITD$^+$ (*upper graphs*) and *FLT3*-ITD$^-$ AML samples (*lower graphs*). All analyses indicated significantly higher frequencies of mDCs and pDCs in ITD$^+$ and ITD$^-$ AML samples in comparison with DCs analyses in healthy subjects ($p<0.05$). The *panels* on the right side show representative FACS analyses samples with the gating strategy

diagnostic samples of both ITD$^+$ and ITD$^-$ patients (Fig. 2a, b).

Due to the high variability in the frequencies of cells with mDC immunophenotype, we considered an "aberrant" mDC frequency for those samples showing mDC frequencies equal or greater than 10% of the total viable cells included in the analyses (thus, roughly corresponding to tenfold higher than the normal mDC frequency observed in healthy controls). A remarkable proportion of AML samples had an "aberrant" mDC frequency (42% for ITD$^+$ and 38% for ITD$^-$). We did not observe significant differences between the distributions of activated and matured mDCs in ITD$^+$ and ITD$^-$ samples (Fig. 2a). Nevertheless, what was noticeable in these AML samples in comparison to the samples of healthy volunteers, was that a much smaller fraction of the AML-mDCs co-expressed CD86 or CD83, indicating a lower activation and maturation status, respectively.

The average frequency of cells with pDC immunophenotype was also dramatically increased in AML diagnostic samples compared to healthy controls (Fig. 2b). To facilitate the analyses of the highly variable pDC frequen-

cies, we considered "aberrant" the samples with equal or greater than 2% frequency of pDCs (thus, more than fivefold higher than healthy controls). Approximately, 40% of the samples analyzed in the ITD$^+$ group and 26% in the ITD$^-$ group were classified as "aberrant", indicating an overall trend for high accumulation of pDCs.

Mohty et al. previously reported the aberrant frequencies of DCs in cryopreserved AML samples [16]. In their work, however, samples were not stratified according to the presence of *FLT3*-ITD and they used immunoglobulin-like transcript 3 instead of HLA-DR as co-expressed DC marker. In view of our current results, ITD$^+$ AML samples seem to demonstrate an even more pronounced pattern of aberrant DC frequencies than ITD$^-$ AML samples.

ITD negative and positive DCs detected in AML samples show downregulation of HLA-DR

Expression of HLA-DR (corresponding to the major histocompatibility complex II) in DCs detected in AML diagnostic samples was expressed at significantly lower levels than for DCs analyzed in the samples of healthy controls (Fig. 3), a finding that correlates with previous observations regarding the downregulation of HLA-DR in AML samples obtained from ITD$^+$ patients [17, 18]. Notably, within the AML groups, the expression of HLA-DR in DC populations detectable in ITD$^+$ AML was significantly lower than in DCs detectable in ITD$^-$ samples (Fig. 3). The profound downregulation of HLA-DR in ITD$^+$ DC subsets might be implicated in defective class II antigen presentation leading to dysregulation of these APCs. This could potentially affect presentation of leukemia antigens to CD4$^+$ T helper cells, hampering development of a stimulatory cytokine environment for anti-leukemia immune responses.

mDCs and pDCs detectable in ITD$^+$ AML samples are derived from leukemia and can be driven to further differentiate morphologically with cytokines in vitro

An important observation by Mohty et al. [16], who had previously reported high frequencies of mDC and pDC subsets in diagnostic AML samples, was that these cells were likely of leukemic origin since they carried the same translocations as the original leukemia. In order to extend this observation to ITD$^+$ leukemia, DCs obtained from three different ITD$^+$ AML samples were sorted and examined for the presence of the ITD by PCR. DCs sorted from the ITD$^+$ AML samples contained the ITD mutation, demonstrating that they originated from leukemic blasts (Fig. 4).

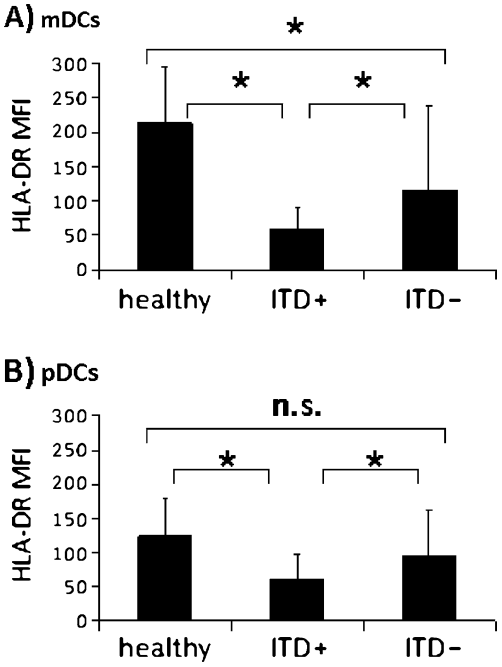

Fig. 3 Flow cytometry analyses of HLA-DR expression in AML cells. **a** Mean fluorescence intensity (M.F.I.) of HLA-DR expression on mDCs showing significant (*asterisk*) differences in the expression level comparing healthy (n=10), ITD$^-$ (n=42) and ITD$^+$ (n=33) PBMC. **b** M.F.I. of HLA-DR expression on pDCs

These ITD$^+$-sorted DCs were then used for cytospin/Giemsa preparations and morphological analyses (Fig. 5). Sorted mDCs (Lin$^-$, HLA-DR$^+$, CD11c$^+$) corresponded to cells with appearance of monocytic blasts with high nuclear-to-cytoplasmic ratio (Fig. 5a). Sorted pDCs demonstrated blast-like morphology with some cells resembling the normal pDC morphology originally described by Siegal et al. [19] and some presenting the "AML-cuplike" description of Kussick et al. [18], who previously described *FLT3*-ITD$^+$ blasts with prominent nuclear invagination and decreased HLA-DR expression (Fig. 5b). Sorted ITD$^+$ DCs that could be cultured ex vivo in the presence of cytokines commonly used for terminal differentiation of mDCs (GM-CSF, IL-4, CD40L) or pDCs (IL-3, CD40L) were analyzed. ITD$^+$ mDCs maintained in the presence of GM-CSF/IL-4 for 5 days resulted in a population of large cells with dendrites, and upon subsequent 24-h treatment with CD40L, abundant veils on the cell surface typical of mDCs were observed (Fig. 5c). Sorted ITD$^+$ pDCs cultured in the presence of IL-3 for 5 days resulted in conspicuously large cells, and subsequent 24-h treatment with CD40L resulted in cells with high granularity (Fig. 5d). Put together, these results demonstrated that circulating ITD$^+$ DCs have characteristics of leukemic blasts, which upon ex vivo, supra-physiological stimulation with cytokines

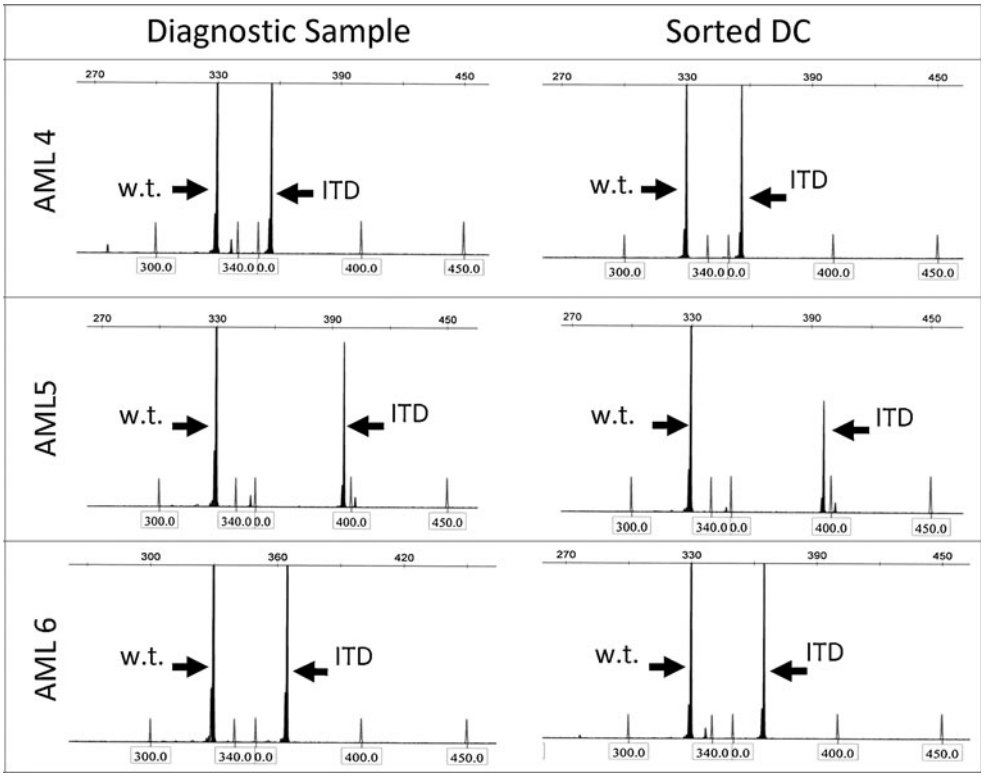

Fig. 4 Sequencing of PCR-ITD mutational insert product from ITD$^+$ patients. In addition to the w.t. *FLT3* amplification product, the ITD mutational insert is detectable in the original AML patient samples and in the sorted DCs

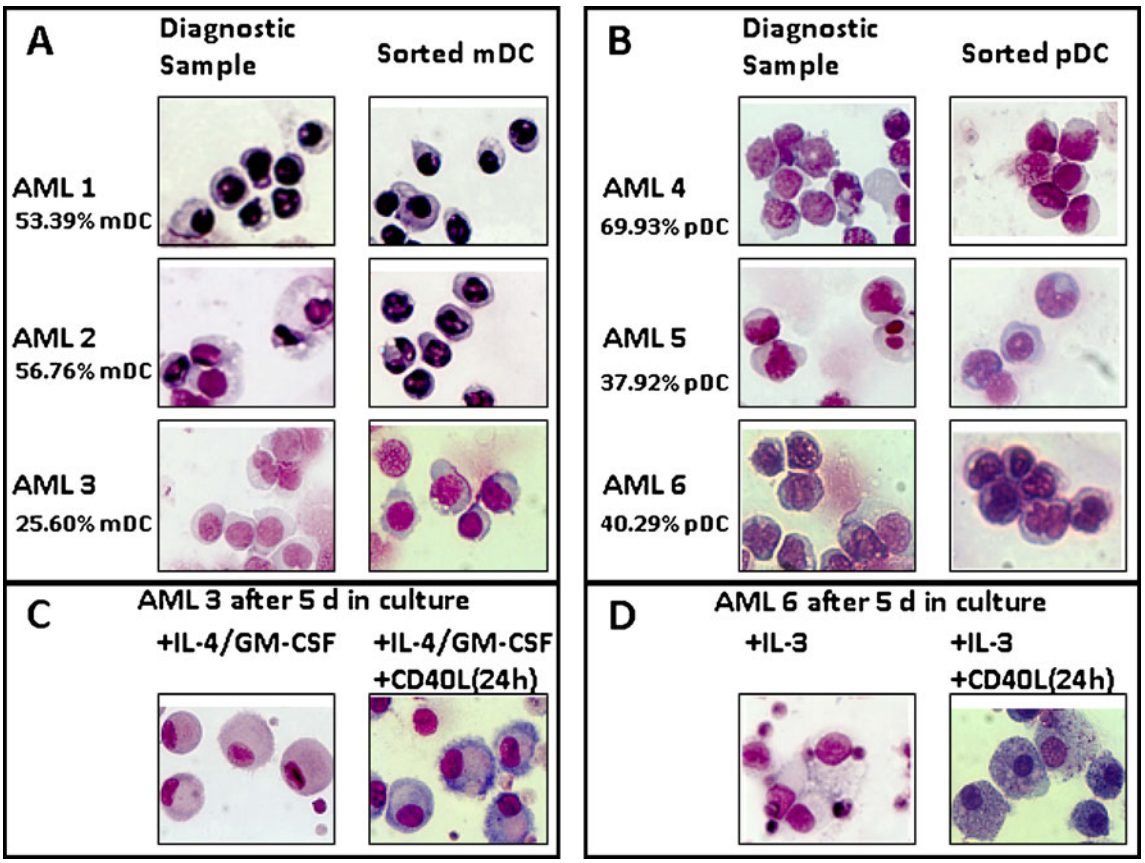

Fig. 5 Morphological analyses of cytospin/Giemsa preparations of *FLT3*-ITD$^+$ AML diagnostic samples prior and post-sorting of mDCs and pDCs. AML samples obtained from three patients and containing high frequencies of mDCs (**a**) or pDCs (**b**) before and after sorting resemble leukemia blasts. **c** ITD$^+$ AML-mDCs after sorting and culture with GM-CSF/IL-4 and maturation with CD40L show cell enlargement and veils. **d** ITD$^+$ AML pDCs after sorting and culture with IL-3 and maturation with CD40L show cell enlargement and high granularity

and maturation factors could drive the cells to acquire more differentiated characteristics.

General occurrence of a mixed lineage population of mDCs ($CD11c^+$)/pDCs ($CD123^+$) in ITD^+ and ITD^- AML samples

Previous work describing the occurrence of high frequencies of DCs in AML samples considered the $CD11c^+$ mDC and $CD123^+$ pDC populations as mutually exclusive events [16]. Since we had observed that some AML samples had high frequencies of both mDCs and pDCs and since the expression of markers of various hematopoietic lineages is common in leukemogenesis, we evaluated whether mixed mDCs/pDCs lineages could also be found in ITD^+ and/or ITD^- AML samples. The subsequent flow cytometry analyses consisted in the negative selection of non-DC lineage markers, positive selection of HLA-DR^+ DCs and, within this defined DC population, we analyzed the frequencies of single or double $CD11c^+$ and $CD123^+$ cells (Fig. 6a). For a subset of ITD^+ patients, we also included the selection of $CD4^+$ cells for a more stringent characterization of DCs (three representative examples are shown in Fig. 7).

Surprisingly, double positive mDC/pDC populations were observed in all ITD^+ and ITD^- AML samples analyzed, and the frequency of double positive DCs corresponded to an average of 58% for ITD^+ and 67% of ITD^-, indicating their preponderance (Fig. 6b, Tables 2 and 3). In addition to the double positive mDCs/pDCs, single mDCs and pDCs cell populations were also detectable in the samples in different distributions (Tables 2 and 3 A–C). Of note, $CD11c^+/CD123^+$ cells have recently been described as early precursors of myelocytic DCs derived from $CD34^+$ progenitors [20–22]. In fact, CD123 is the IL-3 receptor (IL-3R) alpha chain, which is a well-established stem cell marker in healthy and leukemic $CD34^+$ stem cells, and is known to be downregulated only late in myeloid differentiation.[23–25]. IL-3R/ CD123 expression in ITD^+ AML blasts has been described as a frequent event [26, 27], which here seems to be associated with the accumulation of DC precursors that are not terminally differentiated towards mDC or pDC. Of note, in this study, we also observed that a subset of ITD^+ AML samples showed a significantly higher expression of CD123 when compared with ITD^- AML samples (data not shown).

Analogous to our findings, Ma et al. [28] have also demonstrated clonal involvement in circulating myeloid and lymphoid precursors of dendritic cells in peripheral blood of patients with myelodysplastic syndromes. Altogether, these observations provide evidence that ITD^+ and also ITD^- leukemic or progenitor stem cells differentiate towards common $CD11c^+/CD123^+$ mDC/pDC precursors which are accumulated prior to subsequent split between differentiated mDCs and pDCs.

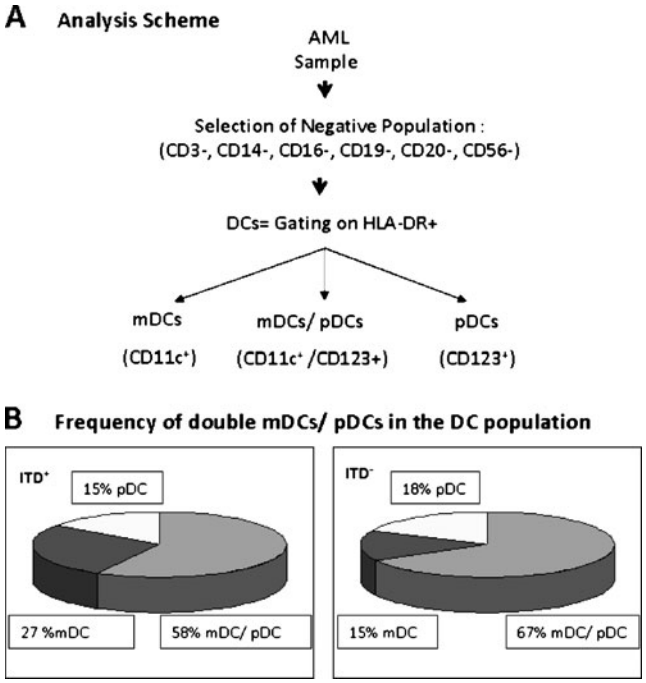

Fig. 6 Immunophenotypic detection of mDCs/pDCs' mixed lineages. **a** Schematic presentation of flow cytometry analyses. **b** Average frequency of mixed lineage mDCs/pDCs, single mDCs and pDCs obtained for ITD^+ and ITD^- patients

Stimulation of mDCs/pDCs with CD40L or CpG results into partial activation of the cells for production of inflammatory cytokines

ITD^+ AML samples were further analyzed for mDC/pDC frequencies based on detection of the surface markers Lin^-/HLA-$DR^+/CD4^+$. Using these more stringent analyses, 5.2–20.5% of the DCs present in the ITD^+ AML samples corresponded to mDC/pDCs (Fig. 7a).

In order to correlate the immunophenotypic characteristics of the mDC/pDC mixed lineage population with DC function, we performed intracellular staining of inflammatory cytokines [interferon (IFN)-α, TNF-α, IL-1α] after stimulation of the AML blasts with CD40L or CpG (Fig. 7b). We used healthy donors as controls for these analyses, which confirmed upregulation of IFN-α production by pDCs after CpG stimulation (Fig. 7). IFN-α and IL-1α were detectable in all mDC/pDC populations, but their regulation through CpG and CD40L was extremely variable. Production of TNF-α by mDCs/pDCs was hardly detectable in comparison to healthy mDCs and pDCs

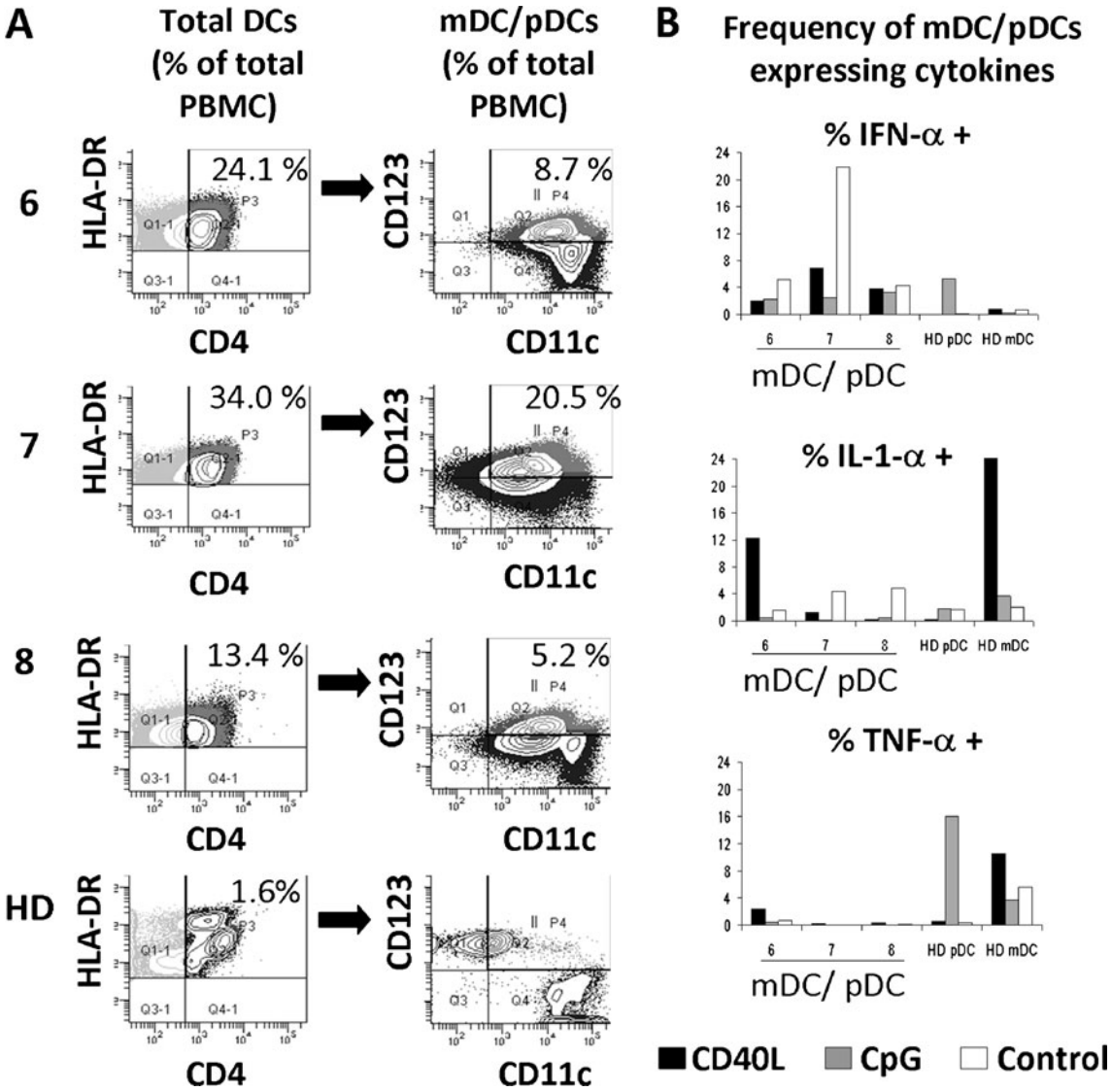

Fig. 7 Functional analyses of double positive CD11c/CD123 mDCs/pDCs in ITD$^+$ AML samples of three patients. **a** Gating approach for detection of mDCs/pDCs. **b** Frequency of mDC/pDCs with detectable intracellular cytokines after stimulation with CD40L or CpG

(Fig. 7). Thus, these functional analyses indicated a deregulated baseline cytokine production and stimulatory responses in mDCs/pDCs.

Based on long-standing clinical expertise that graft versus leukemia (GVL) effect is an immunologic factor positively influencing leukemia eradication, immunotherapeutic approaches to enhance GVL have been sought. During past years, several groups have pointed to the fact that AML cells can express antigens and can be "corrected" as antigen-presenting cells through cytokine stimulation leading to their terminal differentiation [29]. This approach was however not shown practical for ITD$^+$ AML cultures maintained in vitro, which failed to generate ITD$^+$ AML-DCs in the presence of GM-CSF, IL-4, and TNF-α [13]. In our work, we did obtain a partial correction of the DC morphology and cytokine production, but this was mainly noticeable in the presence of CD40L or CpG, which drive terminal maturation of DCs.

In summary, our work demonstrates that both ITD$^+$ and ITD$^-$ AML samples contain a high proportion of cells with DC progenitor characteristics, hindered in terminal differentiation, partially blocked for maturation, and with deregulated cytokine production characteristics. Thus, our study indicates immunosuppressive mechanisms of an immature mDC/pDC leukemic lineage, which might be operative during leukemogenesis and can potentially

Table 2 Frequency of double positive mDCs/pDCs and single mDCs and pDCs in 11 ITD$^+$ AML samples

AML sample	WBC	FAB	% DCs in PBMC	% mDC/pDC in DCs	% mDC in DCs	% pDC in DCs
A: High mDC/pDC and high mDC frequencies						
5	6.3	M4	23.28	79.30	18.17	2.53
6	56.2	M5a	39.2	34.95	64.79	0.25
7	30.3	M4/5	34.7	64.54	30.84	4.61
8	216	M0	34.1	39.00	60.41	0.59
9	107	M5a	39.1	69.80	29.92	0.25
10	1.5	M1	3.57	27.74	60.50	11.76
B: High mDC/pDC and high pDC frequencies						
1	69.9	M2	39.04	35.00	0.48	64.52
2	46.3	M5	52.02	64.28	6.62	29.10
11	65.5	M5	11.00	73.09	8.09	18.82
4	79.0	N.A.	11.55	55.16	10.32	34.52
C: High mDC/pDC and low mDCs or pDCs						
3	66.7	M5	45.97	90.12	5.21	4.67

Despite the fact that mDCs/ pDCs were observed in all AML samples, most of the samples showed high frequency of mDCs (A), and some samples showed also high frequencies of pDCs (B) or comparable frequencies of mDCs and pDCs (C)

AML acute myeloid leukemia, *FAB* French–American–British classification, *FLT3* FMS-like tyrosine kinase 3, *ITD* internal tandem duplication, *WBC* white blood cells, *N.A.* not applicable, *PBMC* peripheral blood mononuclear cell, *DCs* dendritic cells, *pDC* plasmacytoid dendritic cell, *mDC* myeloid dendritic cell

promote anergy induction, tolerance, and ultimately immune escape of leukemia cells.

These results suggest that by hindering the "AML-DC" differentiation and maturation, the occurrence of *FLT3*-ITD can potentially undermine anti-leukemia immune responses, which calls for more effective adjuvant immunotherapeutic strategies in ITD$^+$ leukemia in order to rebound the immune regeneration for ultimate leukemia elimination.

Table 3 Frequency of double positive mDCs/pDCs and single mDCs and pDCs in 12 ITD$^-$ AML samples

AML sample	WBC	FAB	% DCs in PBMC	% mDC/pDC in DCs	% mDC in DCs	% pDC in DCs
A: High mDC/pDC and high mDC frequencies						
1	4.7	M1	58.26	36.09	57.57	6.32
2	182	N.A.	9.9	87.36	10.20	2.41
3	76	M4	9.15	59.22	34.09	6.67
B: High mDC/pDC and high pDC frequencies						
4	5.2	N.A.	8.11	81.37	3.33	15.29
5	34	M5b	53.71	71.37	0.36	28.23
6	6.8	M4	27.44	58.71	1.75	39.54
7	129.5	N.A.	52.79	61.53	1.52	36.94
C: High mDC/pDC and high mDCs or pDCs						
8	0.7	M4eo	23.97	60.52	18.22	21.23
9	69	N.A.	31.8	62.33	18.21	19.47
10	18.4	M5	21.97	61.63	15.66	22.70
11	23.2	M1	8.08	77.60	13.24	9.16
12	115.7	M5	1.57	82.17	7.01	10.83

The samples varied relative to the majority of mDCs (A), pDCs (B), but for most of the cases comparable frequencies of mDCs and pDCs were observed (C)

AML acute myeloid leukemia, *FAB* French–American–British classification, *WBC* white blood cells, *N.A.* not applicable, *DCs* dendritic cells, *PBMC* peripheral blood mononuclear cell, *pDC* plasmacytoid dendritic cell, *mDC* myeloid dendritic cell

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