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Haematologica 2020 [Epub ahead of print]

Citation: Wei Wang, Joseph D. Khoury, Roberto N. Miranda, Jeffrey L. Jorgensen, Jie Xu, Sanam Loghavi, Shaoying Li, Naveen Pemmaraju, Than Nguyen, L. Jeffrey Medeiros, and Sa A. Wang. neoplastic plasmacytoid dendritic cells permits establishment of a 10-color flow cytometric panel for initial workup and residual disease evaluation of blastic plasmacytoid dendritic cell neoplasm. Haematologica. 2020; 105:xxx

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Immunophenotypic characterization of reactive and neoplastic plasmacytoid dendritic cells permits establishment of a 10-color flow cytometric panel for initial workup and residual disease evaluation of blastic plasmacytoid dendritic cell neoplasm

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Main Text Word Count: 3379

Number of Figures: 6

Number of Tables: 2

Number of Supplementary Figures: 3

Number of Supplementary Tables: 2

Number of References: 21

Keywords: Blastic plasmacytoid dendritic cell neoplasm (BPDCN), plasmacytoid dendritic cells, immunophenotype, flow cytometry, minimal residual disease

Running title: A novel flow cytometric panel to distinguish reactive and neoplastic PDCs

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ABSTRACT

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare hematopoietic neoplasm whose immunophenotype remains incompletely characterized, particularly in terms of distinction from reactive plasmacytoid dendritic cells (PDCs). This limitation complicates detection of low-level involvement by BPDCN as well as minimal residual disease (MRD) assessment following therapy. We conducted the current study to characterize the immunophenotype of BPDCN in a cohort of 39 patients, and compared it to reactive PDCs. We found that, in addition to CD56 expression (97%), BPDCN showed a number of aberrancies, including decreased/negative CD38 (82%), positive CD7 (64%), negative CD2 (81%), negative CD303 (56%), increased HLA-DR (69%) and decreased CD123 (78%). Although BPDCN cells were characterized by CD56 expression, reactive PDCs consistently included a CD56-positive subset, ranging 1.3%-20% (median 4.5%) of total PDCs, challenging MRD detection. These CD56+ reactive PDCs, however, were consistently positive for CD2 and CD303, brightly positive for CD38, and negative for CD7, distinctively different from BPDCN. Based on these findings, we set up a 10-color flow cytometry assay for BPDCN and validated it to a sensitivity of 0.01%. This panel was prospectively tested in 19 bone marrow samples from 7 BPDCN patients, and it effectively distinguished BPDCN cells from background reactive PDCs in all cases. In summary, by understanding the immunophenotype of reactive and neoplastic PDCs, BPDCN can be effectively detected by flow cytometry to a very low level using a panel of markers in addition to CD56, and such assay can be used for initial bone marrow workup as well as MRD detection after therapy.

INTRODUCTION

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare, clinically aggressive neoplasm derived from non-activated precursors of plasmacytoid dendritic cells (PDCs)(1, 2). Patients often present with a widespread disease involving multiple anatomic sites, most commonly the skin, followed by bone marrow (BM), peripheral blood and lymph nodes(1, 3-5). BPDCN can occur at any age, but mostly affects patients in their seventh decade. The diagnosis of BPDCN relies on morphology in combination with immunophenotypic studies(6). BPDCN cells are medium-sized with immature chromatin resembling lymphoblasts or myeloblasts. They often show cytoplasmic vacuoles and pseudopodia, but these features are neither sensitive nor specific as they may overlap with a variety of other hematolymphoid neoplasms(7). Immunophenotypically, BPDCN cells are typically positive for CD4, CD56, CD123, HLA-DR, TCL1, and TCF4, and are negative for lineage-specific antigens for B cells (e.g. CD19), T cells (surface and cytoplasmic CD3) as well as myeloid cells (myeloperoxidase)(3, 8). Monocytic markers such as CD64 are also negative. Our group has recently demonstrated that co-expression of CD123 and TCF4 by immunohistochemistry constitutes a highly reliable marker for BPDCN(9).
Despite significant advances in immunophenotypic characterization of BPDCN at baseline, data regarding the distinction between reactive/normal PDCs and BPDCN cells remain limited. Such a limitation presents diagnostic challenges particularly in the evaluation of BM samples with a minimal disease burden, either at presentation in patients with predominantly extramedullary disease or at post-therapy in patients evaluated for measurable/minimal residual disease (MRD). With advances in treatment options for BPDCN patients and the importance of achievement of disease remission for allogeneic stem cell therapy, the need for reliable and reproducible criteria to assess bone marrow samples for potential low-level BPDCN involvement have gained increased attention. An assay that can reliably distinguish neoplastic PDCs from background reactive PDCs becomes important. A variable numbers of reactive PDCs are detected routinely in the bone marrow by flow cytometry immunophenotyping (FCI) and/or immunohistochemistry. Similar to their neoplastic counterparts, reactive PDCs are positive for CD4, CD123, CD303, HLA-DR and TCF4, and they lack expression of lineage-specific antigens. CD56, a marker frequently expressed in BPDCN, has been the only marker to date being used to distinguish neoplastic from reactive PDCs. However, CD56 expression can be found in a small subset of reactive PDCs(10-12). Thus, distinguishing CD56+ BPDCN from CD56+ reactive PDCs becomes quite challenging in the assessment of post treatment BMs, which often contains reactive PDCs.

In this study, our aim was to characterize the immunophenotype of BPDCN, with particular focus on the differences between BPDCN and normal/reactive PDCs. By understanding these differences, we developed and validated a 10-color clinical-grade FCI panel and compared its performance to orthogonal tools for residual disease evaluation.

METHODS

Study group

We identified all patients with BPDCN diagnosed at The University of Texas MD Anderson Cancer Center between 2010 and 2019. All patients fulfilled the diagnostic criteria of BPDCN as defined in the World Health Classification. Patients with available FCI performed on bone marrow specimens were included in this study. A control group of patients who had bone marrow evaluation by FCI for were also included to study reactive PDCs; this group included patients who underwent BM staging for lymphoma or had hematologic diseases other than BPDCN in complete remission with or without stem cell transplantation. This study was approved by the University of Texas MD Anderson Cancer Center Institutional Review Board and conducted in accordance with the Declaration of Helsinki.

Flow cytometric immunophenotypic analysis:
BM aspirate specimens were collected in EDTA anticoagulant tubes, and processed within 12 hours of collection using a standard lyse/wash technique (PharmLyse™, BD Biosciences, San Diego, CA). For each analysis minimum of 200,000 events was acquired on FACSCanto II instruments (8-color and 10-color, BD Biosciences).

At the time of initial diagnosis, a comprehensive panel designed for acute leukemia work-up was routinely performed (Panel #1 in Table 1). This panel included lineage-defining markers for B-, T-, myeloid, and monocytic cells, as well as markers (CD4, CD123, HLA-DR, CD56) necessary for initial screening of BPDCN. When a diagnosis of BPDCN was suspected from Panel # 1 analysis, an additional panel (Panel #2 in Table 1) was performed for further characterization and confirmation.

Based on the findings in the current study, a one-tube 10-color assay (Panel #3 in Table 1) was subsequently constructed and validated for distinguishing BPDCN cells from reactive PDCs.

Immunohistochemistry:

Immunohistochemical (IHC) studies were performed using formalin-fixed, paraffin-embedded BM core biopsy or aspirate clot specimens(13). TCF4/CD123 double stain was performed following the protocol described previously(9).

RESULTS

Immunophenotype of BPDCN

A total of 39 patients with a diagnosis of BPDCN were studied, including 30 men and 9 women with a median age of 69 years (range, 3-87 years). FCI analysis was performed using Panel #1 (Table 1) and the more recent cases were also tested using Panel #2 (Table 1). The median number of BPDCN cells detected by FCI was 18% (range, 0.1-91%). The immunophenotype of BPDCN in these 39 cases is summarized in Figure 1 and Supplementary Table 1.

BPDCN cells were positive for CD45, falling into the “blast” gate on CD45/SSC in all cases (39/39, 100%). CD45 expression was often present at a similar level, or slightly higher, than that of granulocytes (Fig 2A) with the exception of 3 cases (8%) which showed a significantly lower CD45 expression (dimmer than granulocytes) (Fig 2B). HLA-DR as well as CD123 expression was uniformly positive in all cases. CD4 was positive in all 38 cases assessed, uniform in 34 (89%) and partial in 4 (11%) cases. CD56 was positive in 97% (36/37) of cases, mostly (92%, 33/36) uniform and occasionally partial (3/36, 8%). The only case with negative CD56 expression was a 3-year-old girl who otherwise had a typical immunophenotype of BPDCN. CD303, a marker which showed a high diagnostic value for BPDCN in some previous studies(14, 15), was positive in 44% (7/16) of cases. Additionally, CD7 was positive in 64% (21/33) of cases, with a uniform expression in 11 and a partial expression in 10 cases. CD38 was variably positive in 88% (30/34) of cases. CD2 was positive in 5/27 (19%) cases and all
positive cases showed a bright expression. CD33 was positive in 48% (16/33) of cases. CD36 was positive in 57% (17/30) of cases, uniform in 7(41%) and partial in 10 (59%). CD117 was partially expressed in 3 of 34 (9%) cases. CD5 expression was uncommon, only observed in 1/30 (3%) case. Partial CD14 without CD64 expression was detected in 1 (3%, 1/34) case and TdT expression was detected in 25% (4/16) of cases. All cases were negative for CD3 (surface and cytoplasmic), CD13, CD15, CD19, CD22, CD25, CD34, CD41, CD64 and myeloperoxidase.

**Immunophenotype of normal/reactive PDCs**

The immunophenotype of normal/reactive PDCs was studied in 22 BMs from patients without BPDCN, including 11 cases that were submitted for lymphoma staging, 4 cases of B-lymphoblastic leukemia in remission and 7 post-transplant BMs of patients with B-lymphoblastic leukemia or acute myeloid leukemia. In these cases, normal/reactive PDCs represented 0.11% (median) of total nucleated cells with a range from 0.01% to 0.43% by flow cytometry.

Similar to BPDCN, normal/reactive PDCs were consistently positive for CD123 and HLA-DR and negative for CD64. They were all positive for CD4, CD45, and CD303. CD38 was positive and bright in all cases. CD33 was also positive in all cases, uniform in 12/21 (57%) and partial in the rest (43%, 9/21). Of note, although positive, CD33 expression in normal/reactive PDCs was lower than that of monocytes and basophils (Fig 3D). CD2 expression by normal/reactive PDCs showed a bimodal pattern with a spectrum from completely negative cells to positive cells in all cases (Fig 4A). CD7 was consistently positive in a small subset of normal/reactive PDCs with a median of 13% (range, 0.3% to 21%). Of note, these CD7 positive PDCs were negative for CD56 (Fig 4A).

**The immunophenotype of CD56+ normal/reactive PDCs**

CD56 expression was observed in a subset of normal/reactive PDCs in all 22 non-BPDCN cases described above, with a median of 4.5% (range 1.3% to 20%) of total PDCs. This CD56+ subset of PDCs showed substantial immunophenotypic overlap with BPDCN in PDC-defining markers, including being positive for CD4, CD123, HLA-DR, and CD303; and the panel (Panel #2, Table 1) designed initially for BPDCN was incapable of distinguishing these cells from BPDCN.

This population of CD56+ PDCs was further studied with an expanded panel of markers, and demonstrated a remarkably consistent pattern. They were positive for CD2 (100%), negative for CD7 (100%), and showed bright CD38 (100%) expression in all 22 cases tested (Table 2). CD303 was also positive in all cases (100%), uniform in 13 (59%) and partial in 9 (41%). A representative case of CD56-positive reactive PDCs is shown in Fig 4A.
Of note, the expression of CD56 in normal/reactive PDCs is not limited to BM samples. We analyzed a reactive PDCs proliferation using immunohistochemistry in a patient who had a self-limited skin lesion, likely an insect bite, which had CD56 expression and had presented a diagnostic challenge at initial encounter (Fig 5).

**Differential Immunophenotypic Characteristics of BPDCN and Reactive PDCs**

The immunophenotype of BPDCN was compared to reactive PDCs. In addition to “positive” and “negative”, the markers of expression were also scored as “increased” or “decreased/partial” if the intensity difference was greater than one-third log scale (Fig 1). This comparison was facilitated by the presence of reactive PDCs in some cases of BPDCN at initial diagnosis and in many cases of BPDCN following therapy. Compared with reactive PDCs, BPDCN cells showed brighter HLA-DR expression in 25/36 (69%) (Fig 3A), and lower CD123 expression in 28/36 (78%) (Fig 3A). Of the latter, although decreased, CD123 levels in BPDCN were still higher than that of monocytes (Fig 3A). CD303, a marker that is consistently positive in normal/reactive PDCs, was only positive in 7 of 16 (44%) BPDCN cases, of which 6 showed decreased expression and only 1 (6% in total) showed a normal level of CD303 (Fig 3B). In contrast to a bright CD38 expression in reactive PDCs, CD38 expression was frequently down-regulated in BPDCN cells, decreased in 24/34 (70%) and negative in 4/34 (12%) (Fig 1, Fig 3C). While CD33 was positive in all cases of reactive PDCs, it was only positive in 48% (16/33) of BPDCN cases.

We next focused on the difference between BPDCN and reactive CD56+ PDCs. Unlike CD56+ reactive PDCs that were uniformly positive for CD2, bright for CD38 and consistently negative for CD7, BPDCN cells were frequently CD2 negative (81%), CD7 positive (64%) with decreased or negative (82%) CD38 (Fig 4B) (Table 2). In contrast to 100% of reactive PDCs positive for CD303, only 44% of BPDCN cases were positive. Using a combination of markers (CD2, CD7, CD56, CD303, CD38), none of the 39 BPDCN cases showed an immunophenotype exactly as CD56+ reactive PDCs which were CD56+/CD2+/CD7-/CD303+/CD38+ bright.

**Establishment and validation of a flow cytometry assay for minimal residual disease**

Based on these findings, a one-tube 10-color assay CD2/CD7/CD38/CD303/CD123/HLA-DR/CD64/CD4/CD45/CD56 was constructed (Panel#3, Table 1). Detailed information including the antibody clones and the fluorochrome attached to each antibody is listed in Supplementary Table 2. CD123, HLA-DR, CD45, and CD64 were included to identify PDCs that were CD123 bright/HLA-DR+/CD64-/CD45dim+. In patients who received targeted therapy to CD123, an alternative gating strategy was also used to examine PDCs that were CD4+CD64-CD56+HLA-DR+CD45dim+. Representative cases to illustrate our gating strategy are shown in supplementary Figures 1 and 2. The sensitivity of this panel was validated to be 0.01% according to the MRD testing guideline by the College of American Pathologists (supplementary Fig 3).

The 10-color MRD panel was tested prospectively in 19 BM samples from 7 patients who had a confirmed diagnosis of BPDCN. These 19 samples included 1 for initial BM diagnosis and 18 samples for evaluation of
residual disease during the course of treatment. Using this flow cytometry panel, 12 (63%) samples were positive for BPDCN and the median number of aberrant cells was 0.05% of total nucleated cells with a range of 0.008% to 56.5%. Of the 12 positive samples, 1 was detected as early relapse after stem cell transplant, with 0.01% of aberrant PDCs. Of note, all samples had mixed reactive PDCs in the background, serving as an internal comparison. All positive cases showed a similar immunophenotype to that identified in the original diagnostic specimen and no significant immunophenotypic shift was observed. For patients who received anti-CD123 target therapy, CD123 expression was still maintained in BPDCN as well as normal PDC.

**Flow cytometry versus Immunohistochemistry in the Assessment of Minimal Residual Disease**

We compared FCI and dual-color immunohistochemistry for TCF4/CD123 to determine the relative performance of these assays in BM evaluation in the context of BPDCN post-therapy. To achieve this, we first assessed systematically the number, distribution, and morphologic characteristics of TCF4/CD123 dual-positive cells in 18 bone marrows of patients without BPDCN. In such cases, PDCs were few and often scattered, with a broad range of morphologic characteristics that ranged from mature plasmacytoid forms to others with increased nucleus-to-cytoplasm ratio and occasional nuclear membrane convolutions. Although occasional loose PDC aggregates were identified, none of the cases had tight PDC aggregates or sheets of PDCs. Next, we also performed TCF4/CD123 double-stain immunohistochemistry in 14 cases with a history of BPDCN who had been evaluated for residual disease by FCI. In such cases, TCF4/CD123 highlighted scattered PDCs but could not reliably distinguish reactive from neoplastic PDCs. As shown in Fig 5, TCF4/CD123 immunostain highlighted scattered PDCs in a case of BPDCN prior to (Fig 6A) and post stem cell transplant in remission (Fig 6B), both around 1-2% of total cells in BM. It is uncertain whether they are aberrant or not by TCF4/CD123 immunostain. Flow cytometry, on the other hand, was capable of differentiating them apart; it detected neoplastic PDCs mixed with normal PDCs in pre-transplant specimen (Fig 6C), whereas only reactive PDCs but no aberrant PDCs were detected in post-transplant specimen (Fig 6D).

**DISCUSSION**

In this study, we investigated the immunophenotype of BPDCN in a large cohort of 39 patients and compared to reactive PDCs cells. This study is the first to go beyond a simple characterization of the BPDCN immunophenotype, but to understand the immunophenotypic aberrancy/alterations of BPDCN. Of particular interest, we show that CD56 is expressed in a small subset of normal/reactive PDCs and therefore, CD56 alone is insufficient to differentiate BPDCN from reactive PDCs especially when the tumor burden is low. Through further characterization of these CD56+ normal PDCs, we identified a combination of markers that can detect BPDCN and distinguish neoplastic from non-neoplastic PDCs in BMs with a sensitivity of 0.01%.
The diagnosis of BPDCN at the time of initial presentation, typically with a high tumor burden, is often straightforward as BPDCN cells show a distinct immunophenotype, positive for HLA-DR, CD123 (bright), CD4, CD56, and absence of myeloperoxidase and monocytic markers as well as B- and T-cell lineage defining markers. The neoplastic infiltrate can be further confirmed by immunohistochemical studies using CD123, TCL1 or a more specific TCF4/CD123 double stain. Basophils often have a similar level of CD123 expression but they are negative for HLA-DR. Monocytes, some hematopoietic precursors and AML blasts are positive for both CD123 and HLA-DR(16), but their CD123 expression level is substantially lower than that of PDCs.

More challenging is the evaluation of MRD status post treatment or the assessment of staging BMs with a low tumor burden involvement. This challenge is attributable to immunophenotypic overlap between BPDCN cells and reactive PDCs, especially, the CD56+ subset of reactive PDCs. In fact, an initial panel designed for BPDCN MRD (Panel #2, Table 1) failed to distinguish BPDCN from normal PDCs. Immunohistochemical study with TCF4/CD123 was able to highlight PDCs, but incapable to differentiate BPDCN from reactive PDCs. These challenges prompted us to study the immunophenotype of reactive PDCs, and explore the immunophenotypic difference between neoplastic and reactive PDCs.

Although both reactive PDCs and BPDCN cells were uniformly positive for CD123 and HLA-DR, BPDCN cells tend to have brighter HLA-DR and lower CD123. CD33, while was positive in all reactive PDCs, became negative in 52% of BPDCN cases. All reactive PDCs were positive for CD2 with a bimodal pattern, whereas only 19% of BPDCN cases were positive for CD2. For other lymphoid antigens, CD7 expression in BPDCN is very frequent (64%), whereas CD5 was only observed less than 5% of cases. CD303, a marker considered specific for PDCs, was reported positive in 90%(14), 63%(17) and 53%(18) of BPDCN cases by immunohistochemistry. By flow cytometry, CD303 was positive in 75% (15) and 64%(19) of cases. Of note, various anti-CD303 antibodies have been used in previous studies, including clone DDX0043(Dendritics, Dardilly, France) (14) (17), rabbit anti-cytoplasmic CD303(18), and AC144(15). In our study, using clone 201A from Biolegend, CD303 was positive in all reactive PDCs, whereas it was only positive in 44% of BPDCN cases. Of the CD303 positive BPDCN cases, many showed a decreased expression level comparing to internal normal PDC. Recently, Huang and colleagues reported decreased or absent CD303 expression in early stages of plasmacytoid maturation in their series of myeloid neoplasms with plasmacytoid dendritic cell differentiation(20). The lower CD303 intensity in BPDCN might reflect the immaturity of tumor cells as they derived from less mature/precursors of PDCs. Nonetheless, this altered expression level of CD303 in BPDCN facilitates the identification of neoplastic cells from background normal PDCs, and contributes to the MRD detection.

We further confirmed that CD56 was normally expressed in a subset of normal PDCs, ranging from 1.3% to 20% of total PDCs. A similar observation was previously reported in peripheral blood and BMs from healthy persons(10-12). These CD56+ PDCs have been proposed to be precursors as well as the cell of origin of BPDCN. We show here that the CD56+ subset of normal PDCs are positive for CD2 and CD303, negative for CD7 (10-12),
and retained a high level of CD38. This immunophenotypic pattern is distinctively different from CD56+ BPDCN cells, which are often CD2 negative (81%), CD7 positive (64%), with negative CD303 (56%), and decreased or negative CD38 (82%). Based on the immunophenotypic difference, we designed a new flow cytometric panel including these markers that was capable of detecting BPDCN cells to a level of 0.01%. This panel was prospectively tested in 19 bone marrow samples from 7 patients, and was able to reliably distinguish BPDCN cells from reactive PDCs in all samples. Of note, every BM sample contained reactive PDCs that served as internal controls for comparison. Other markers that could be explored in the future to distinguish BPDCN cells from normal PDCs include CD5, CD13, CD22, and CD33. BCL2 is also potentially valuable as it is expressed in BPDCN but often negative in reactive PDCs(5).

In this study, the flow cytometry assay for MRD detection was not compared to mutational analysis due to a number of reasons. First, not every case of BPDCN had detectable mutations using our current next-generation sequencing (NGS) analysis covering 81 frequently mutated genes in myeloid/lymphoid neoplasms. Second, the mutations frequently found in BPDCN, such as TET2, ASXL1, TP53 and NRAS, are also commonly found in myeloid neoplasms. It has been well known that myeloid neoplasms such as myelodysplastic syndrome and chronic myelomonocytic leukemia frequently co-occur with BPDCN(8, 21). Thus the detection of these mutations by NGS cannot differentiate BPDCN clone and myeloid clone in such cases. Last, the sensitivity of NGS is about 1%, unable to reach the sensitivity level of 0.01% by flow cytometry.

In summary, we have provided the immunophenotypic characteristics of BPDCN in detail in this study. We also have defined “immunophenotypic aberrancies” of BPDCN in comparison with normal/reactive PDCs. It is imperative to recognize that reactive PDCs usually contain a small subset of CD56+ cells, which should not be misinterpreted as BPDCN. These CD56+ PDCs cells have an immunophenotypic profile distinctively different from BPDCN, which allow us to develop a flow cytometric assay that has a high sensitivity and specificity in the detection of MRD. Such laboratory tests are much in need in the era of targeted therapy and precision medicine. This flow cytometry panel is valuable for disease monitoring during treatment and also enables early detection of relapse in BPDCN patients who underwent allogeneic stem cell transplant, allowing for early intervention. The significance of positive MRD in pre- and post-SCT is of great interest in BPDCN, and deserves future studies.

REFERENCES

Table 1: The list of antibodies used in our flow cytometric panels

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<tr>
<td></td>
<td>Tube 2: HLA-DR/CD117/CD4/CD34/CD123/CD38/CD45</td>
</tr>
<tr>
<td></td>
<td>Tube 3: CD41/CD36/CD56/CD34/CD64/HLA-DR/CD14/CD45</td>
</tr>
<tr>
<td></td>
<td>Tube 4: CD5/CD25/CD22/CD34/CD38/CD15/CD45</td>
</tr>
<tr>
<td><strong>Panel #2</strong></td>
<td>HLA-DR/CD64/CD4/CD33/CD56/CD45/CD303/CD123</td>
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<tr>
<td><strong>Panel #3</strong></td>
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Table 2: The major immunophenotypic difference between CD56-positive reactive PDCs and BPDCN

<table>
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<tr>
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<th>Positive CD2</th>
<th>Positive CD7</th>
<th>Bright CD38</th>
<th>Positive CD303</th>
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<td><strong>CD56-positive reactive PDCs</strong></td>
<td>100%</td>
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<td>100%</td>
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<td><strong>BPDCN</strong></td>
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<td>64%</td>
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**FIGURE LEGEND**

**Fig 1:** The summary of BPDCN immunophenotype in 39 patients. Different colors represent different levels of expression.

**Fig 2:** The location of BPDCN cells on CD45/SSC plots: Most (92%) BPDCN cases show CD45 expression at a level similar to or slightly higher than that detected in granulocytes (A). A small subset (8%) of cases shows a lower CD45 expression (B). Red population represents BPDCN cells.

**Fig 3:** BPDCN cells often show increased HLA-DR, decreased CD123, decreased CD303, decreased CD38, and positive CD56. Pink: basophils; Blue: reactive PDCs; Red, neoplastic PDCs; and Gray: monocytes. A, both basophils and PDCs are bright for CD123. Basophils are negative whereas PDCs are positive for CD123. In comparison to reactive PDCs (blue), neoplastic PDCs (red) often show decreased CD123 and increased HLA-DR. Monocytes (gray) are also positive for CD123 and HLA-DR, but their CD123 level is much lower when compared to PDCs. B, neoplastic PDCs are positive for CD56 and negative for CD303. CD303 is positive in reactive PDCs. C, neoplastic PDCs often show decreased CD38 expression when compared to reactive PDCs. D, Reactive PDCs are positive for CD33, and approximately half of BPDCN cases are negative for CD33.

**Fig 4:** The representative cases of reactive (A) and neoplastic (B) CD56-positive PDCs (gray: CD56-negative reactive PDCs; blue: CD56-positive reactive PDCs; red: CD56-positive neoplastic PDCs). A, CD56-positive reactive PDCs are consistently positive for CD2 and CD303, negative for CD7. CD38 is bright. B, In contrast, neoplastic CD56-positive neoplastic PDCs are often negative for CD2 with decreased to negative CD303. CD7 is often positive and CD38 expression level is often decreased. When focused on CD56-negative PDCs (gray) in both panels A and B, they are positive for CD303 and CD38. CD2 shows a bimodal expression pattern (both negative and positive cells present). A small subset of reactive PDCs is CD7 positive and these CD7 positive reactive PDCs are negative for CD56.

**Fig 5:** A case of reactive PDCs proliferation with positive CD56 by immunohistochemistry. A, skin biopsy shows small clusters of PDCs, some with plasmacytid morphology in a self-limited skin lesion, likely caused by insect bite. Insert shows the low-power view of the skin biopsy. B, CD123/TCF4 double stain highlights scattered and loosely clustered PDCs. C, CD56 immunostain shows many PDCs are positive.

**Fig 6:** Immunostain and flow cytometric analysis of a BPDCN case before (A, C) and after transplant (B, D). Immunostain using dual-color TCF4/CD123 double stain showed scattered PDCs in both samples, before (A) and after (B) transplant. Flow cytometric analysis showed that a subset of PDCs cells (red) in pre-transplant sample (C) was aberrant (decreased CD38, negative CD2, decreased CD303) whereas all PDCs (D) in post-transplant sample showed a normal immunophenotype.
Fig 2:

A

92% of cases

B

8% of cases
Fig 4:
Supplementary Fig 1: A case illustration of BPDCN gating and analysis strategy using CD123. CD123/low SSC cells are 0.30% of total nucleated events after removing doublets, debris, platelets and red blood cells, and these cells are reflected on CD45/SSC plot. CD123+/low SSC and HLA-DR+ cells are plasmacytoid dendritic cells (PDC), comprising 0.07% of total events. The CD123+HLADR- cells are basophils. The neoplastic PDCs are highlighted in red and normal PDC are in blue.
Supplementary Fig 2: A case illustration of BPDCN gating and analysis strategy using CD56+/CD64-. An alternative gating strategy is also applied in all cases, considering the possibility of loss or down regulation of CD123 post anti-CD123 treatment. The CD56+HLADR+CD64-negative are analyzed. The CD56+HLADR- cells are mostly NK cells, as reflected on CD45/SSC. The neoplastic PDC are highlighted in red and other CD56+HLADR+CD64- cells are in blue. So far, we have not identified any cases with residual BPDCN showing CD123 loss.
Supplementary Fig 3: A representative case with serial dilutions shows the sensitivity of this panel is below 0.01%. Pink cells are neoplastic cells.
Supplementary Table 1: The immunophenotype of BPDCN

<table>
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<tr>
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<tr>
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<td>0% (0/15)</td>
</tr>
<tr>
<td>CD4</td>
<td>100% (38/38)</td>
</tr>
<tr>
<td>CD5</td>
<td>3% (1/30)</td>
</tr>
<tr>
<td>CD7</td>
<td>64% (21/33)</td>
</tr>
<tr>
<td>CD13</td>
<td>0% (0/30)</td>
</tr>
<tr>
<td>CD14</td>
<td>3% (1/34)</td>
</tr>
<tr>
<td>CD15</td>
<td>0% (0/28)</td>
</tr>
<tr>
<td>CD19</td>
<td>0% (0/30)</td>
</tr>
<tr>
<td>CD22</td>
<td>0% (0/26)</td>
</tr>
<tr>
<td>CD25</td>
<td>0% (0/22)</td>
</tr>
<tr>
<td>CD33</td>
<td>48% (16/33)</td>
</tr>
<tr>
<td>CD34</td>
<td>0% (0/27)</td>
</tr>
<tr>
<td>CD36</td>
<td>57% (17/30)</td>
</tr>
<tr>
<td>CD38*</td>
<td>88% (30/34)</td>
</tr>
<tr>
<td>CD41</td>
<td>0% (0/12)</td>
</tr>
<tr>
<td>CD45</td>
<td>100% (39/39)</td>
</tr>
<tr>
<td>CD56</td>
<td>97% (36/37)</td>
</tr>
<tr>
<td>CD64</td>
<td>0% (0/36)</td>
</tr>
<tr>
<td>CD117*</td>
<td>9% (3/34)</td>
</tr>
<tr>
<td>CD123*</td>
<td>100% (36/36)</td>
</tr>
<tr>
<td>CD303*</td>
<td>44% (7/16)</td>
</tr>
<tr>
<td>HLA-DR*</td>
<td>100% (36/36)</td>
</tr>
<tr>
<td>MPO</td>
<td>0% (0/16)</td>
</tr>
<tr>
<td>TdT</td>
<td>25% (4/16)</td>
</tr>
</tbody>
</table>
Notes: **CD38**: Although 88% (30/34) cases were positive, only 18% (6/34) showed a normal CD38 level, whereas 70% (24/34) showed decreased CD38 and 12% (4/34) showed negative; **CD117**: Only 9% (3/34) cases were positive and all were partially positive; **CD123**: Although all cases were positive, 78% (28/36) showed decreased expression; **CD303**: Although 7 out of 16 (44%) cases were positive, only 1 (6%) showed a relatively normal level when compared to reactive PDCs and overall, most cases showed decreased or negative CD303; **HLA-DR**: All cases were positive and 69% (25/36) showed brighter expression when compared to normal/reactive PDCs.

Supplementary Table 2: The fluorochromes and antibodies used in the newly designed panel (corresponding to Panel #3 in Table 1)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CD7</th>
<th>CD2</th>
<th>CD4</th>
<th>CD64</th>
<th>CD56</th>
<th>CD38</th>
<th>CD45</th>
<th>CD303</th>
<th>CD123</th>
<th>HLA-DR</th>
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<tr>
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<td>PE</td>
<td>PerCP-Cy5.5</td>
<td>PE-Cy7</td>
<td>APC</td>
<td>R700</td>
<td>APC-H7</td>
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<td>BV605</td>
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<td>SK3</td>
<td>10.1</td>
<td>NCAM16.2</td>
<td>HB7</td>
<td>2D1</td>
<td>201A</td>
<td>7G3</td>
<td>G46.6</td>
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