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Dendritic cells

Use of anti-BDCA-2 antibody for detection of dendritic cells type-2 (DC2) in allogeneic hematopoietic stem cell transplantation

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Summary:

TH2-inducing dendritic cells (DC2) are commonly identified as negative for lineage markers and positive for HLA-DR and CD123 expression. More recently, normal blood DC2 were shown also to be positive for BDCA-2 and BDCA-4 antigens. The aim of this study was to evaluate whether BDCA-2 expression on DC2 is impaired in patients undergoing an allogeneic hematopoietic stem cell transplantation (HSCT) and in healthy donors treated with G-CSF for HSC mobilization. Flow cytometry assays for DC2 detection using either a triple staining with anti-HLA-DR PerCP, anti-Lin$^+$ anti-CD34 FITC and anti-CD123 PE monoclonal antibodies (mAbs), or a double staining with anti-HLA-DR PE and anti-BDCA-2 FITC mAbs were compared in blood samples from patients who underwent an allogeneic HSCT ($n = 30$) or from healthy donors before ($n = 11$) and after ($n = 8$) G-CSF mobilization, as well as in healthy donors' leukapheresis products ($n = 12$) or bone marrow ($n = 4$). Staining of BDCA-2$^+$ cells with other markers such as anti-CD38, anti-CD54 and anti-CD58 were also performed. Median values of CD123$^+$ DC2 and BDCA-2$^+$ DC2 were not statistically different in the blood of patients previously treated with chemotherapy, nor in the blood or bone marrow of heathy donors. Also, a 5 day G-CSF treatment did not affect BDCA-2 or adhesion molecule expression on healthy donors' blood DC2 significantly. A correlation between all the results ($n = 65$) obtained with the two assays was demonstrated in a linear regression curve ($r = 0.914$) ($P = 0.00001$). BDCA-2 is a marker highly specific for DC2 that is not downregulated by chemotherapy or G-CSF treatment. Therefore, the anti-BDCA-2 mAb can be efficiently combined with other mAbs and used in studies addressing the role of DC2 in the allogeneic HSCT setting.
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Human DC blood precursors, which are also present in tissues, mature and acquire potent T cell stimulating activity after migrating into secondary lymphoid organs.[1] However, different types of circulating DC have been described according to their phenotype and capacity of inducing T helper 1 (Th1), or Th2 responses. Th1-inducing DC (DC1) are of myeloid origin, express CD11c and are dependent on GM-CSF for their survival. Instead Th2-inducing DC (DC2), that have been previously recognized as plasmacytoid T cells, plasmacytoid dendritic cells (PDC), plasmacytoid monocytes, interferon-α/β producing cells (IPC), or type 2 pre-DC (pDC2) are likely to be of lymphoid origin, are HLA-DR$^+$, lin$^-$, CD4$^+$CD3$^-$CD11c$^-$ and also express the IL-3Rα (CD123).[2–7] Despite much experimental data that has been provided in recent years about DC1 and DC2 physiology, further information is needed in order to establish their role in the pathogenesis or in the control of immune diseases.[8] In particular, DC2 might be relevant to allogeneic hematopoietic stem cell transplantation in case they could regulate donor and/or recipient T cell cytotoxic responses across HLA barriers.[9,10]

Flow cytometry detection of DC2 requires a combination of multiple reagents that makes these studies time-consuming and difficult to standardize. Recently, normal blood DC2 were shown to express specific antigens, such as BDCA-2 and BDCA-4.[11] In this study we addressed the question of whether BDCA-2 may be a reliable marker of DC2 in normal bone marrow or G-CSF-mobilized blood stem cell allogeneic transplantation, as well as in the blood of patients who received chemotherapy and underwent an allogeneic stem cell transplant. Our results allow extension of the use of BDCA-2 mAb to a large number of patients in any type of transplant setting and facilitate the standardization of DC2 analysis in clinical studies.

Materials and methods

Cell samples

Peripheral blood before and after 5 days of treatment with G-CSF, leukapheresis or bone marrow samples were obtained from healthy donors of hematopoietic stem cells. Blood samples from patients with hematological neoplasms previously treated with chemotherapy who underwent allogeneic peripheral blood or marrow stem cell transplantation were also collected at different times during the 12 months after transplant. Informed consent was obtained from patients and donors using the protocols approved by our hospital's ethics committee. Peripheral blood stem cell (PBSC) donors were treated with G-CSF (Lenograstin, Aventis, Milan, Italy) 10 μg/kg/day s.c. on days 0–5 and leukaphereses were performed on days 5–7 to collect $\geqslant 4 \times 10^6$ $CD34^+$ cells/recipient body weight, and then cryopreserved in liquid nytrogen until transplantation as previously described.[12]

Monoclonal antibodies

Monoclonal antibodies used in this study were: phycoerythrin (PE)- or peridin chlorophyll protein (PerCP)-conjugated anti-HLA-DR, fluorescein isthiocyanate (FITC)-conjugated anti-Lin, anti-CD34 FITC, anti-CD45 FITC, anti-CD123 PE, anti-CD38 PE and appropriate isotype controls from Becton Dickinson (San Jose, CA, USA), anti-CD54 PE from Exalpha (Boston, MA, USA) and anti-CD58 PE (Tema Ricerca, Bologna, Italy). BDCA-2 FITC mAb was kindly provided by Dr Juergen Schmitz (Miltenyi Biotec, Bergisch Gladbach, Germany).

DC2 immunophenotype

Fresh whole blood, leukapheresis or marrow samples were used for direct immunofluorescence staining. Circulating DC2 were identified as positive for HLA-DR, negative for Lin (that is a mixture of anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and anti-CD56 mAbs) and for anti-CD34, and positive for anti-IL3Rα (CD123), as previously described.[6] Alternatively, an anti-BDCA-2 mAb was employed and cells that were positive for anti-BDCA-2 were considered DC2, as previously shown in steady-state normal peripheral blood.[11] Double staining with anti-BDCA-2 and HLA-DR, or in selected experiments with anti-CD38, anti-CD54, or anti-CD58 mAbs was also performed. Briefly, appropriate amounts of mAbs were added to 100 μl of whole blood or 10^6 marrow or G-CSF mobilized blood cells, followed by a 15 min incubation at room temperature. Red cells were then lysed using FACS lysing solution (Becton Dickinson) according to the manufacturer's instructions. After washing twice the samples were acquired on a FACS Calibur instrument (Becton Dickinson) and analyzed by CellQuest software. Cells were initially gated as CD45-positive, based on isotypic negative control, to set a second gate on forward and side scatter parameters. Finally, a further gate was set in order to acquire at least 1000 events $HLA\text{-}DR^+Lin^-$ or $HLA\text{-}DR^+BDCA\text{-}2^+$ cells. Analysis of CD123 expression was then performed in $HLA\text{-}DR^+Lin^-$ selected cells.

Statistical analysis

Statistical analyses were performed using Wilcoxon test. Student's *t*-test was used to evaluate the statistical significance of the correlation coefficient (r).

Results

BDCA-2 is expressed in circulating DC2 of patients undergoing an allogeneic blood stem cell transplantation

Since it has been previously demonstrated that BDCA-2 is a marker of normal blood DC2,[11] we initially addressed whether patients with hematologic disorders previously undergoing chemotherapy and receiving an allogeneic peripheral blood or marrow stem cell transplantation may be efficiently monitored for their circulating DC2 number using an anti-BDCA-2 mAb. Thirty samples were collected at different times after transplant to compare the proportion of DC2 detected by using a double staining with anti-HLA-DR PE and anti-BDCA-2 FITC mAbs *vs* a triple staining with anti-HLA-DR PerCP, anti-Lin + anti-CD34 FITC and anti-CD123 PE mAbs. In the 30 samples analyzed the difference observed between the overall median proportion of $HLA\text{-}DR^+Lin^-CD123^+$ DC: 0.092% (range 0.002–0.65%) and the overall median proportion of $HLA\text{-}DR^+BDCA\text{-}2^+$ DC: 0.078% (range 0.005–0.99%) was not statistically significant ($P = 0.3$). One representative sample from a patient evaluated for $BDCA\text{-}2^+$ and $CD123^+$ DC2 3 months after PBSC transplant is shown in Figure 1. Notably, HLA-DR is expressed in all $BDCA\text{-}2^+$ cells (Figure 1b).

G-CSF mobilization does not affect BDCA-2 expression in hematopoietic blood stem cells of healthy donors

The proportion of DC2 was first evaluated in healthy donors before treatment with G-CSF both in peripheral blood and in marrow. Consistent with previous findings,[6] an overall greater number of DC2 was found in mobilized blood samples (post G-CSF) ($n = 8$) and leukapheresis products ($n = 12$), as compared to steady-state blood (pre-G-CSF) ($n = 11$) or marrow ($n = 4$), as may be observed from the median cellularity and $CD123^+$ DC2 proportions of the different cell sources analyzed (Table 1). Also, to test whether G-CSF or the leukapheretic procedure may induce internalization of BDCA-2 we compared the two cytofluorimetric assays for DC2 detection in peripheral blood after 5 days of treatment with G-CSF and in leukapheresis products of healthy donors. Data on Lin-$CD123^+$ and $BDCA\text{-}2^+$ DC in each type of cell source are shown in Table 1. The results clearly show that no significant differences in the median proportion of DC2 are observed using the BDCA-2-based or the CD123-based cytometric approach. Importantly, the proportion of DC2 and the expression of BDCA-2 were not modified in cryopreserved leukapheresis products analyzed after thawing, before infusion into patients (data not shown). Furthermore, in three separate experiments the expression of CD38, CD54 (ICAM-1) and CD58 (LFA-3) antigens was observed in BDCA-2 both from normal blood (not shown) and G-CSF-mobilized PBSC (Figure 2).

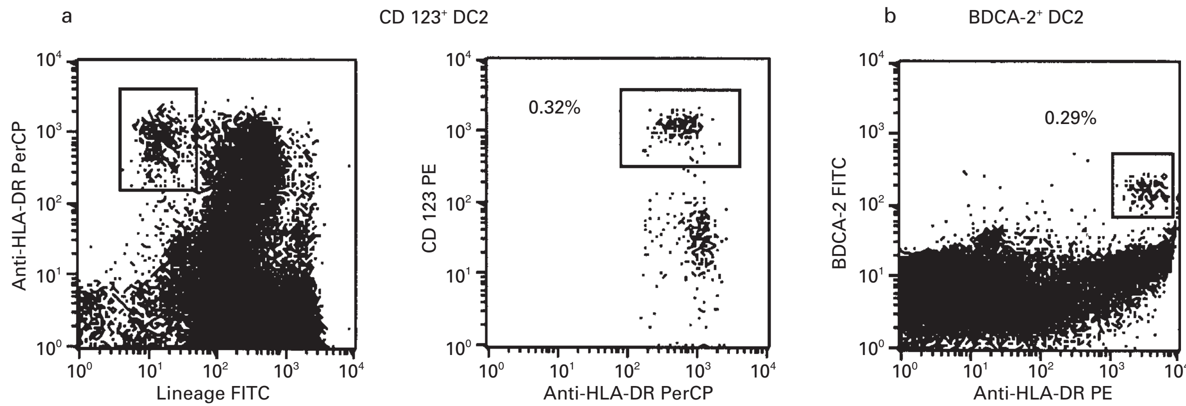

Figure 1 Expression of CD123 or BDCA-2 for detection of blood DC2. Phenotypic characterization of blood DC2 was performed in a patient who received an allogeneic PBSC transplant 3 months before. To evaluate the percentage of DC2 (**a**) a triple staining with anti-HLA-DR, anti-Lin and anti-CD123 mAbs was performed, and the cells that were Lin$^-$ and HLA-DR$^+$ (left scatter) were then analyzed for CD123 positivity (central scatter). In this case the proportion of DC2 was calculated by dividing the number of CD123$^+$ events by the total number of events acquired. Alternatively, (**b**) a double staining with anti-HLA-DR and BDCA-2 mAbs was performed, and the number of double positive cells (right scatter) was divided by the total number of events acquired to establish the proportion of DC2.

Table 1 BDCA-2 efficiently identifies DC2 in normal bone marrow, peripheral blood before and after G-CSF treatment and PBSC

	Cases	*Cell No./μl median (×10³)*	*BDCA-2$^+$ median (range)*	*Lin$^-$ CD123$^+$ median (range)*	*P value*
Bone marrow	4	17.3	0.13 (0.01–0.24)	0.15 (0.02–0.29)	0.4
Pre-G-CSF	11	7.2	0.09 (0.05–0.19)	0.12 (0.06–0.25)	0.08
Post-G-CSF	8	36.2	0.07 (0.04–0.09)	0.08 (0.06–0.14)	0.1
PBSC	12	250.5	0.24 (0.12–0.39)	0.23 (0.12–0.35)	0.8

Identification of DC2 as HLA-DR and BDCA-2-positive cells (BDCA-2$^+$), or as Lin-negative, HLA-DR and CD123-positive cells (Lin$^-$ CD123$^+$) in healthy donors' bone marrow, peripheral blood before (pre-G-CSF) and after (post-G-CSF) treatment with G-CSF and leukapheresis products (PBSC) was performed as described in Materials and methods. Data are shown as median percentages of positive cells. The overall median cell concentration of marrow or blood samples used for cytometric analysis is also indicated. Statistical analysis was performed using the Wilcoxon test.

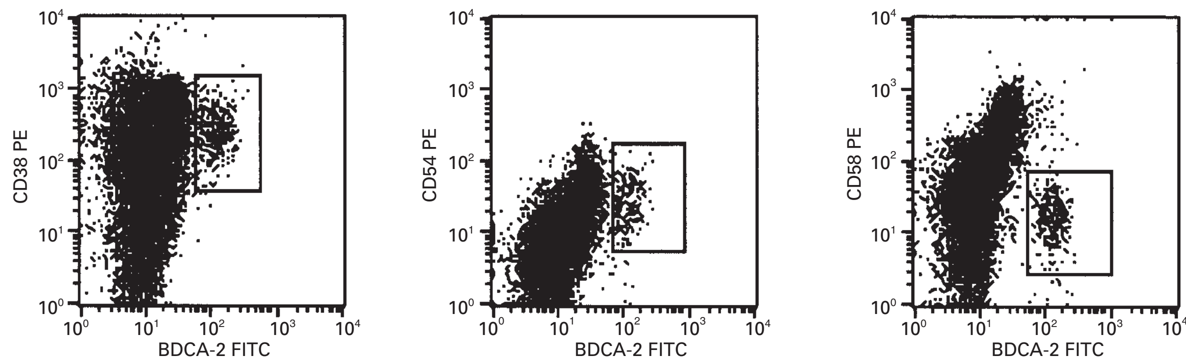

Figure 2 Co-expression of BDCA-2 and CD38 and adhesion molecules in DC2 from PBSC. A double staining with anti-BDCA-2 FITC and anti-CD38, or CD54, or CD58 PE mAbs was performed on healthy donor G-CSF-mobilized PBSC and all the BDCA-2$^+$ cells were positive for these markers. This result is representative of three separate experiments.

Statistical correlation between BDCA-2$^+$ and Lin$^-$CD123$^+$ cells

In order to definitively demonstrate if DC2 percentages obtained by using the anti-BDCA-2 antibody may significantly correlate with those achieved by CD123 staining, the results from all the 65 fresh samples were evaluated to measure the correlation coefficient (r), as shown in the linear regression curve in Figure 3. Our data clearly demonstrate a statistically significant correlation ($r = 0.914$) ($P = 0.00001$) between HLA-DR$^+$BDCA-2$^+$ and HLA-DR$^+$Lin$^-$CD123$^+$ cells both in samples with lower and higher concentration of DC2, thus further suggesting that BDCA-2 may be a reliable marker of DC2$^+$ in any hematopoietic stem cell transplant setting.

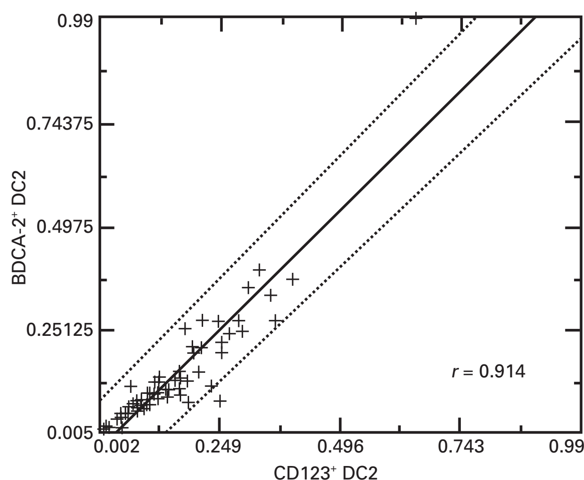

Figure 3 Correlation between CD123 and BDCA-2 expression in DC2. The proportions of DC2 evaluated by either a CD123-based or BDCA-2-based assay in all the 65 samples included in this study were plotted as a regression curve and a statistically significant correlation was observed ($P = 0.00001$).

Discussion

New antigens, such as BDCA-2 or BDCA-4, characterizing DC2 in normal peripheral blood were recently described.[11] In this study we demonstrate that BDCA-2 is also expressed in DC2 of patients after chemotherapy and allogeneic stem cell transplantation, as well as in healthy donors' blood cells before and after G-CSF treatment for hematopoietic stem cell mobilization.

The role of host and donor DC in the immune events occurring after allogeneic stem cell transplantation, such as GVHD or graft rejection, or even immunological tolerance, is still a matter of investigation.[13,14] Nevertheless, while T cell alloreactivity is commonly demonstrated upon DC1 stimulation, the specific role of DC2 in T cell responses after transplantation is not yet defined. A growing interest in the role of DC2 in allogeneic transplantation has derived from the demonstration that G-CSF mobilization of hematopoietic $CD34^+$ cells into the peripheral blood of healthy donors also results in an increase in circulating $HLA\text{-}DR^+$ Lin^- $CD123^+$ $CD11c^-$ DC2, as compared to steady-state peripheral blood and bone marrow.[6] Interestingly, the difference between mobilized blood and bone marrow DC2 content has been hypothesized to be one of the potential explanations for a comparable rate of aGVHD in allogeneic transplants with PBSC and bone marrow as stem cell sources, despite significantly greater numbers of T cells infused with PBSC.[9] Therefore, it appears very important that future studies may address the question of whether DC2 included in the graft, as well as DC2 recovery after allogeneic transplantation of hematopoietic stem cells, may correlate with hematological and immunological reconstitution, infectious events, GVHD or graft rejection episodes after transplant. For example, an initial study by Waller *et al*[15] suggested that a higher content of $CD3^-CD4^{bright}$ DC2 in the graft would correlate with chronic GVHD and relapse after allogeneic bone marrow transplant. Monitoring of circulating DC2 numbers might also be of clinical importance in settings other than HSCT, as recently proposed for HIV patients.[16,17]

Standardization of cytometric assays for detection of DC2 is crucial when analyzing DC subsets because of the very small number of these cells. In fact, differences due to the technique or to the reagents are likely to result in incorrect comparisons of DC2 numbers among different studies. Triple staining with anti-HLA-DR, anti-Lin added with an anti-CD34 and anti-CD123 mAbs is considered to be the most appropriate assay for human DC2.[3,6] Other mAbs recognizing DC2-specific antigens, such as BDCA-2 and BDCA-4, have been produced to identify peripheral blood DC2, where BDCA-2 is a lectin that is likely to be internalized by the DC upon antigen capturing.[18]

In this study we tested the expression of BDCA-2 antigen on DC2 both in patients who received allogeneic stem cell transplantation, and in normal donors' marrow and peripheral blood before and after G-CSF stem cell mobilization, and we compared the results with those obtained in the same samples using the triple staining indicated above. Our data show that BDCA-2 antigen expression is not affected by chemotherapy. Also, since the proportions of $BDCA\text{-}2^+$ and $CD123^+$ DC2 in normal donors were not statistically different, we conclude that neither G-CSF treatment nor the leukapheresis procedure induce internalization of BDCA-2 receptor in blood DC2. Furthermore, by a double staining experiments of normal blood, both before and after G-CSF mobilization (PBSC), with anti-BDCA-2 and other mAbs, we demonstrated that G-CSF does not affect the expression of molecules such as HLA-DR, CD38, ICAM-1 (CD54) and LFA-3 (CD58) in $BDCA\text{-}2^+$ cells. Therefore, since the BDCA-2-based assay is highly specific for DC2 detection and is easy to standardize, the anti-BDCA-2 mAb can be included in studies addressing DC reconstitution after marrow or PBSC allo-transplantation on a routine basis, and, importantly, it can be combined with other mAbs that will allow a more complete characterization of the surface and cytoplasmic phenotype of DC2.

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