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Plasmacytoid dendritic cells represent a major dendritic cell subset in sentinel lymph nodes of melanoma patients and accumulate in metastatic nodes

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Abstract Plasmacytoid dendritic cells (pDC) represent the main source of interferon-α, a cytokine with antitumor activity. However, *in vitro* studies point to pDC as a key subset for induction of tolerance. Herein, we investigated pDC in sentinel lymph nodes (SLN) of melanoma patients. We report that pDC were constantly found in SLN and represented, with Langerhans cells, the most frequent dendritic cell subset. Their frequency in positive (with metastasis) SLN was significantly higher than in negative (without metastasis) SLN. PDC were observed in the T cell-rich areas of lymph nodes, particularly around high endothelial venules and, in metastatic nodes, they accumulated in close vicinity with melanoma nests. Finally, pDC capability to produce interferon-α *in situ* was impaired. Consistently, pDC expressed CD86, but neither CD80 nor CD83, suggesting a not complete activation in melanoma-draining lymph nodes. These results are consistent with the hypothesis of a tolerogenic role played by pDC in tumor immunology.

Abbreviations LN, Lymph node; SLN, Sentinel lymph node; IFN, Interferon; DC, dendritic cells; LC, Langerhans cells; pDC, plasmacytoid dendritic cells; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells.

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Introduction

Dendritic cells (DC) are highly specialized antigen presenting cells essential to generate primary immune responses [1]. It is now clear that DC are not an unique homogeneous cell population, but rather a pool of subsets with different origins, phenotypes and functions [2–5]. However, two are the most important DC subsets: myeloid DC and plasmacytoid DC (pDC), the latter considered to be of lymphoid origin [2–5]. Myeloid DC reside in immature state in peripheral tissues where actively capture and process antigens. Following exposure to pro-inflammatory cytokines or pathogen-derived products they undergo a maturation process and migrate via the afferent lymphatics to the draining local lymph nodes [6]. A well known myeloid DC subset is represented by Langerhans cells (LC) which are located in the epidermis and characterized by the expression of CD1a molecules and Langerin [7]. In contrast to myeloid DC, pDC do not reside in peripheral tissues during homeostasis, but are encountered in the peripheral blood and lymphoid organs [8–12]. The hallmark of pDC is their unique capability to produce large amount of interferon-α and -β (Type I IFN) in response to viruses [12,13]. Furthermore, pDC can differentiate into mature DC when stimulated by viruses [8–10,12]. For these reasons pDC represent key effectors in innate immunity and the ideal cell population in connecting innate and adaptive immunity.

PDC are rarely detected in healthy lymph nodes, in contrast they are abundant in inflamed lymph nodes where they migrate through the high endothelial venules [13,14].

The sentinel lymph node (SLN) represents the first node on the lymphatic draining way from a tumor and corresponds to the preferential site of early metastasis [15]. Similarly, primary immune responses against tumors are expected to occur first in the SLN, where DC present tumor antigens to naive T cells generating tumor-specific cytotoxic and/or helper T cell [16]. However, there is evidence showing that DC have phenotypic and functional defects in the SLN of cancer patients [17–19]. These studies analyzed classical myeloid DC, but the role of pDC in the SLN remains to be assessed. Indeed it seems that pDC may be of some relevance in human tumor immunity and in particular in melanoma immune responses: pDC have been recently described as an important component of the lymphoid infiltrate in primary and metastatic melanoma lesions [20] and a role of these cells in priming naive melanoma specific CD8+ T cells as well as in IFN-α/β production has been described [20,21].

Myeloid DC subsets and pDC can now be traced by the expression of specific molecules [4,5]. Taking advantage of these tools, we sought to analyze the DC repertoire within SLN of melanoma patients focusing on the presence and function of pDC with the aim to investigate whether pDC play a role in immune response to melanoma *in vivo*.

Material and methods

Patients and human samples

Melanoma patients undergoing wide local excision of the primary lesion plus sentinel node biopsy and patients undergoing complete regional lymphadenectomy for clinically evident lymph node metastasis were enrolled in the study after obtaining informed written consents. The study was conducted according to the Declaration of Helsinky Principles and the Institutional Ethics Committee approved all described studies. In total we have investigated n = 39 SLN from n = 27 patients and n = 9 clinically metastatic LN from n = 9 patients. SLN was detected and excised as previously described [22]. Briefly, all patients underwent pre-operative lymphoscintigraphy using 99mTc-labelled nanocolloid albumin (Nycomed, Amersham, Sorin, Milan, Italy), performing early dynamic and static views. pre-operative perilesional intradermal injections of 0.5–1 ml of blue dye (Patent Blue V 2.5%, Laboratoire Guebert, Aulnay-Sous-Bois, France) were performed 15–30 min before surgery. All blue nodes and radioactive nodes with an *in vivo*- or *ex vivo*-background count ratio of 2:1 or more were removed using gamma probe (ScintiProbe MR 100, Pol.Hi.Technologies, Rome, Italy). For research purposes SLN processing was performed as previously described with minor modifications [23]. Briefly, SLN was bisected crosswise and one cutting surface was scraped 5 times with a scalpel blade by the pathologists. The cells were collected in a 15 ml tube by rinsing the blade with phosphate buffered saline (PBS-EuroClone, Whetherby, UK). Cells were centrifuged and the pellet was re-suspended several times vigorously in 1 ml of complete medium and then passed through a cell strainer (70 μm pore diameter; BD Falcon, BD Biosciences, Bedford, MA, USA) in order to remove fragments and end up with a single cell suspension. SLN were processed for routine histology with hematoxylin and eosin and immunohistochemistry [22]. The immunohistochemical study was performed using the peroxidase-antiperoxidase immune complex method and monoclonal antibodies for S100 protein (clone 4C4.9) and for anti-melanosome (clone HMB-45); the reaction product was visualized by 3,3′ diaminobenzidine (DAB) chromogen, weakly counterstained with hematoxylin, washed and mounted with Permount media. All material was from Ventana, Tucson, AZ. Befory surgery, 30 ml of blood was collected from each patients. PBMC were isolated from blood by Lymphoprep™ (Axis-Shield, Oslo, Norway) gradient.

Reagents and antibodies for flow cytometry

Complete medium was prepared using RPMI 1640 (EuroClone) supplemented with 1% penicillin/streptomycin (EuroClone), 2% glutamine (Sigma-Aldrich, Irvine, UK), 1% sodium pyruvate and 10% heat inactivated FCS (EuroClone). The following unconjugated or FITC-, or PE-, or PE-Cy5 conjugated mouse anti human monoclonal antibodies were used: anti CD4 (SK3, IgG1), anti CD11c (S-HCL-3, IgG2b), anti CD36 (CB38-NL07-, IgM), anti CD80 (L307.4, IgG1), anti CD83 (HB15e, IgG1), anti CD86 (2331 FUN-1, IgG1), anti HLA-DR (L243, IgG2a), and anti-Lineage Cocktail 1(lin-1) from Becton Dickinson (San Josè, CA); anti CD14 (M5E2, IgG2a) and anti CD123 (9F5, IgG1) from BD-Pharmingen (San Diego, USA). Anti BDCA1(AD5-8E7, IgG2a), anti BDCA2 (AC144, IgG1) and anti BDCA3 (AD5-14H12, IgG1), from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti MxA mAb was kindly provided by J. Pavlovic (Department of Virology, University of Zurich, Switzerland). Anti CD1a (BL6, IgG1) and anti CD207 (Langerin, DCGM4,IgG1) were from Immunotech (Marseille, France). All mAb dilutions and washing steps were done in PBS containing 2% FCS and 2 mM EDTA. IsotyPE-matched Ab

were used as negative control. Cells were acquired using FACSort and the CellQuest softwer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Cell sorting and cultures

PDC cells were isolated by direct magnetic labeling with BDCA2 conjugated-beads (Miltenyi Biotec) followed by enrichment of labeled cells using a high-gradient magnetic cell sorting device (Mini-MACS; Miltenyi Biotec). Cells were then spun down on cytospins and stained with Giemsa. For activation experiments, blood pDC were sorted by BDCA4 conjugated-beads (BDCA2 was not used because its binding has been shown to inhibit IFN-α production [4]) and cultured at a concentration of 1×10^4 cells/ml for 24 hours in presence of IL-3 (R and D Sistems, Inc. Minneapolis, USA) and 5 hemagglutinating units /ml influenza (virus strain PR8). After 24 hours activated cells were processed for flow cytometry studies, and IFN-α production was measured by ELISA in the supernatant (R and D Sistems, Inc). In order to measure IFN-α production from fresh SLN we cultured the entire SLN cell suspension in presence of IL-3 without virus stimulation (the number of pDC purified from SLN is too low to perform direct measurements).

Immunohistochemistry and immunofluorescence

Representative serial sections from paraffin embedded SLN (n = 5 negative, n = 5 positive) and clinically metastatic nodes (n = 5) were stained with anti CD123 mAb in order to localize pDC within lymph nodes. CD123 stains also classical myeloid DC and macrophages, although less strongly, but pDC were distinguishable as CD123 positive cells with plasmacytoid shape. Anti BDCA2 mAb was not used because is not suitable for paraffin embedded tissues.

Sections were immunohistochemically studied using the peroxidase-antiperoxidase immune complex method; the reaction product was visualized by DAB chromogen, weakly counterstained with hematoxylin, washed and mounted with Permount media (Ventana).

Immunofluorescence studies were performed on frozen sections from negative SLN (n = 19), positive SLN (n = 5), clinically metastatic nodes (n = 5). Sections were mounted on slides, air-dried at room temperature, fixed in acetone for 10 minute and stored at $-20\ ^\circ C$. Before staining slides were pre-incubated 1 h with PBS/Triton X-100 0.3% containing 20 mg/ml bovine serum albumin. Sections were stained with anti BDCA2 PE (Miltenyi Biotec) anti CD3 APC (IgG1, UCHT1, BD PharMingen), and anti CD19 FITC (IgG1, HD37, Chemicon Europe, Hampshire, UK), for 2 h to study localization of pDC within the SLN. Fluorescein signal was amplified with anti-FITC Alexa Fluor 488 conjugated (Molecular Probes Invitrogen, Milan, Italy) for 1 h. To study type I IFN production *in situ,* sections were stained with anti MxA mAb for 2 h, then slides were washed extensively with PBS and a subsequent incubation (1 h) with a PE-conjugated secondary antibody allowed visualization of the binding. A subsequent incubation (2 h) with a FITC-conjugated anti BDCA2 mAb was performed for double labeling. Frozen sections from varicella skin lesions (n = 5) were used as positive control for MxA staining. Specificity of immune detection was established by omission of the primary antibody. Sections were observed with a Leica DM-IRBE confocal microsopy (Leica, Glattbrugg, Switzerland). PDC express MxA [24], thus, in case of type I IFN secretion by pDC, the neighboring cells would also stain positive for MxA [25]. Therefore we would have a subset of MxA+ BDCA2+ cells representing pDC and a subset expressing only MxA representing the neighboring cells. To quantify type I IFN production by pDC *in situ*, the numbers of MxA and BCDA2 positive cells was assessed on recorded digital images calculating the mean values of cell numbers counted in three different fields (magnification x400). Thereafter, the ratio between MxA and BDCA2 positive cells was calculated to correct for the density of infiltrating pDC and to make possible comparison with varicella skin lesions.

Statistical analysis

Among SLN, pair wise frequency differences between 4 DC subsets (BDCA1, 2, 3 and LC) were assessed using the Wilcoxon non parametric test for matched samples. The same test was used to assess pair wise frequency differences between 3 DC subsets (BDCA1, 2, 3) for blood samples. In order to take into account the multiple comparison problem we fixed type I error at 0.008 and 0.017, respectively.

Among SLN and blood samples, for each of the 4 DC subsets (BDCA1, 2, 3 and LC), the Kruskal-Wallis non parametric analysis of variance was used to test the null hypothesis of no distribution differences among positive SLN, negative SLN and clinically metastatic nodes; the pair wise comparison of this group were performed with the Mann-Whitney non parametric test for unmatched samples setting at 0.017 type I error. The Kruskal-Wallis non parametric analysis of variance was also used to test the null hypothesis of no distribution differences for the ratio between MxA and BDCA2+ cells among positive SLN, negative SLN, clinically metastatic nodes and varicella skin lesions; the pair wise comparison of this group were performed with the Mann-Whitney non parametric test for unmatched samples setting at 0.008 type I error.

All tests were two-sided. All the analyses were performed using Stata Statistical Software: Release 9.

Results

Identification of plasmacytoid dendritic cells in sentinel node of melanoma patients

To assess the presence of pDC in sentinel lymph node of melanoma patients, we performed flow cytometry analysis on single cell suspension using anti BDCA2 - an antibody specific for human pDC – and anti CD123. A BDCA2+ CD123+ cell population was detected in all the n = 39 samples studied (Fig. 1a). Further analysis showed that BDCA2+ pDC expressed CD4, CD45 RA (Fig. 1b), HLA-DR , CD36 but did not express CD11c (data not shown), corresponding to the typical pDC immunophenotype [13]. Forward and side scattered properties of SLN pDC were similar to those of blood pDC being located between lymphocytes and monocytes (data not shown). Isolated pDC exhibited a plasmacytoid, roundish morphology without dendritic processes (Fig. 1c). Similarly, *in situ* pDC, detected as CD123+cells in paraffin embedded

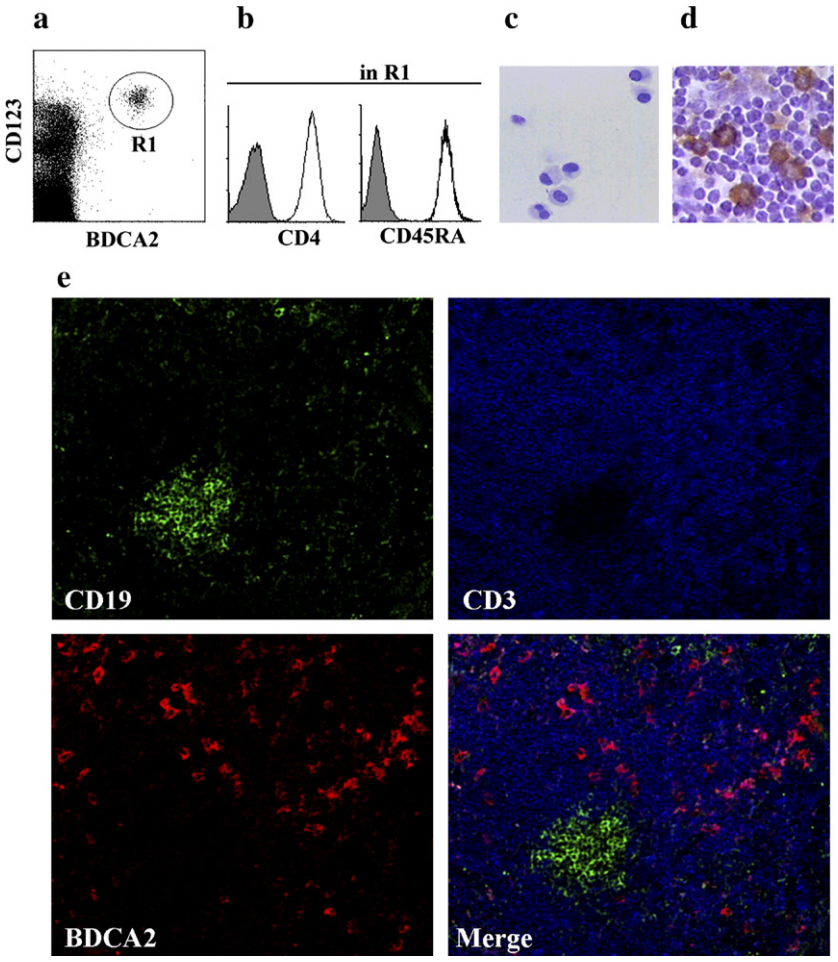

Figure 1 Plasmacytoid dendritic cells characterization in sentinel lymph nodes of melanoma patients. (a-b) Single cell suspension was triple stained with anti-BDCA2 / CD123 and CD4 or CD45RA and analyzed by flow cytometry. a) A BDCA2/CD123 double positive population was observed. b) Gating in R1 plasmacytoid dendritic cells resulted positive for CD4 and CD45RA. c) BDCA2 positive cells were sorted by magnetic beads, cytocentrifuged and stained with Giemsa *(Original magnification x400)*. d) Immunohistochemical staining with mAb anti-CD123 on paraffin embedded SLN of melanoma patients revealed the presence of roundish, plasmacytoid CD123+ cells *(Original magnification x400)*. e) Frozen sections of SLN were triple stained with anti-CD19 to identify B cells (green), anti-CD3 to identify T cells (blue) and anti-BDCA2 to identify pDC (red) and analyzed by confocal laser scanning microscopy. pDC were exclusively localized within the T-cells area *(Original magnification x200)*.

sections (Fig. 1d) and as BDCA2+ cells in frozen sections (Fig. 1e), showed a roundish, typical plasmacytoid feature and were observed within the T cell-rich area of LN (Fig. 1e).

Frequency of dendritic cell subsets in sentinel nodes of melanoma patients

Using the BDCA1, 2, 3 antibodies we investigated the presence of the different DC subsets in SLN of melanoma patients. Since these nodes drain lymph from the skin we also investigated LC by the expression of Langerin. As shown in Fig. 2, LC and BDCA2+ pDC were the most frequent DC type with median values of 0.50% (interquartile range: 0.32%–0.77%) and 0.37% (interquartile range: 0.24%–0.56%) of total SLN cells respectively (Wilcoxon test p value = 0.073). Lower frequencies (Wilcoxon test p values <0.008) were observed for BDCA1+ (median 0.29%; interquartile range: 0.15%–0.47%) and BDCA3+ DC (median 0.14%; interquartile range: 0.09%–0.20%) (Fig. 2b). In contrast, blood DC subsets frequency in melanoma patients were different (Wilcoxon test p values <0.017) with higher frequency observed for BDCA1+ cells (median 0.56%; interquartile range: 0.41%–0.65%), followed by BDCA2+ cells (median 0.33%; interquartile range: 0.21%–0.48%) and BDCA3+ cells (median 0.08%; interquartile range: 0.05%–0.10%) while LC were not detected (Fig. 2b). Mean values were not statistically different from those observed in blood of normal donors (BDCA1+ cells: 0.55 ± 0.17%, BDCA2+ cells 0.43 ± 0.14% and BDCA3+ cells 0.11 ± 0.05% - data not shown).

Plasmacytoid dendritic cells accumulation in metastatic lymph nodes

Among the SLN (n = 39) from melanoma patients (n = 27) melanoma cells were found in 8 SLN. In all cases, metastatic

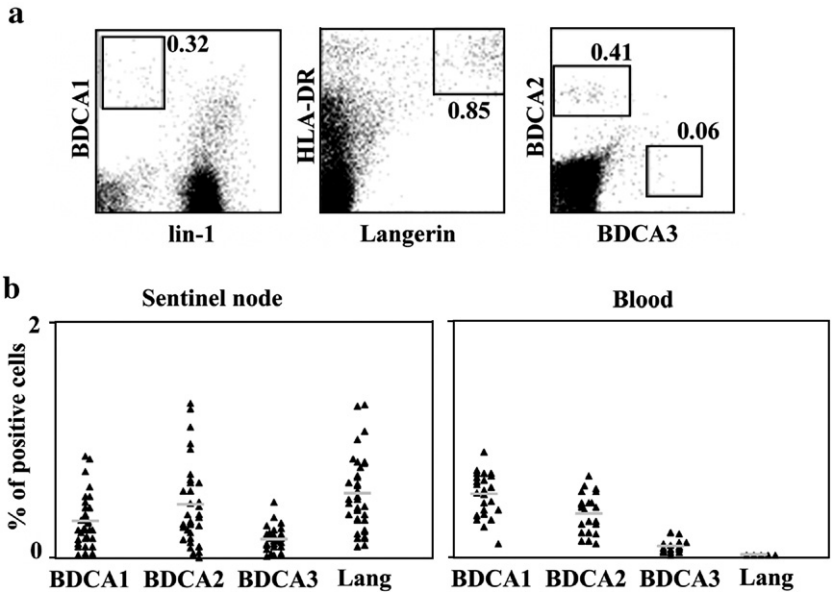

Figure 2 Plasmacytoid dendritic cells represent a major DC subset in sentinel lymph nodes of melanoma patients. Dendritic cells subsets were identified by flow cytometry analysis using mAb anti-BDCA1, -BDCA2 and -BDCA3. Langerhans cells were identified by the expression of the specific marker Langerin. Since BDCA1 is also expressed by some B lymphocytes, DC were identified by the expression of BDCA1 and the negativity for the lineage markers (Lin-1). a) A representative flow cytometry analysis is shown. The numbers represent the percentage of cells within the total cell population. b) Quantification of dendritic cell subsets in sentinel lymph nodes and blood from multiple donors. Horizontal bars represent the median.

lesions were micrometastases (clinically unapparent and detected in sentinel lymph nodes, as defined in the American Joint Commettee of Melanoma Staging Cancer System) [26]. The frequency of pDC in positive SLN (median 0.84%; interquartile range: 0.46%–1.18%) was significantly higher (Mann-Whitney test p value = 0.006) than in negative SLN (median 0.34%; interquartile range: 0.21%–0.47%) (Fig. 3a). Interestingly, the higher percentage of pDC was observed in the SLN with the larger metastasis. This data suggested that the frequency of pDC may be related to the presence of melanoma cells in the SLN. To address this question we further extended our investigation to melanoma patients with clinically evident metastatic LN undergoing total local lymphanedectomy. In metastatic LN the presence of pDC was slightly higher (median 0.90%; interquartile range: 0.70%–1.30%) than that observed in positive SLN although the increase was not statistically significant (Mann-Whitney test p value = 0.596) (Fig. 3a). Notably, in one case it reached up the percentage of 6.1 (Figs. 3a and b).

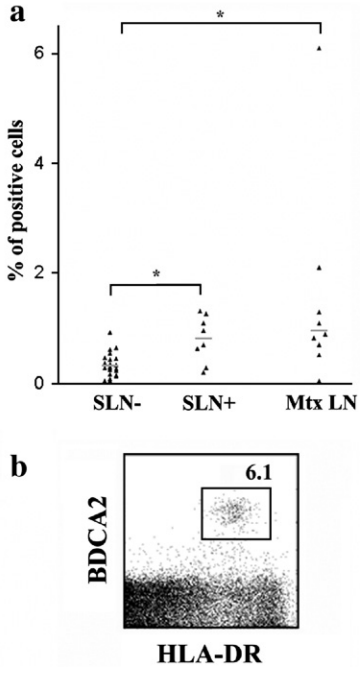

Figure 3 Plasmacytoid dendritic cells accumulation in metastatic lymph nodes of melanoma patients. a) BDCA2+ pDC identified by flow cytometry analysis in negative sentinel lymph nodes (n = 31), metastatic sentinel lymph nodes (n = 8) and clinically metastatic lymph nodes (Mtx) (n = 9). * indicate a statistically significant difference between the groups. Horizontal bars represent the median. b) Dot plot of flow cytometry analysis showing the highest number of BDCA2/HLA-DR double positive pDC within one of the clinically metastatic lymph node.

Plasmacytoid dendritic cell distribution within metastatic lymph node

As described above, the highest percentages of pDC were found within metastatic LN, raising the question of whether melanoma cells may somehow enhance pDC migration in the LN. Therefore, the localization within the LN and their position respect to melanoma cells was studied by immunohistochemistry on paraffin embedded sections stained with anti CD123 mAb. PDC appeared as aggregates of numerous cells in close vicinity with melanoma nests (Figs. 4a and d)

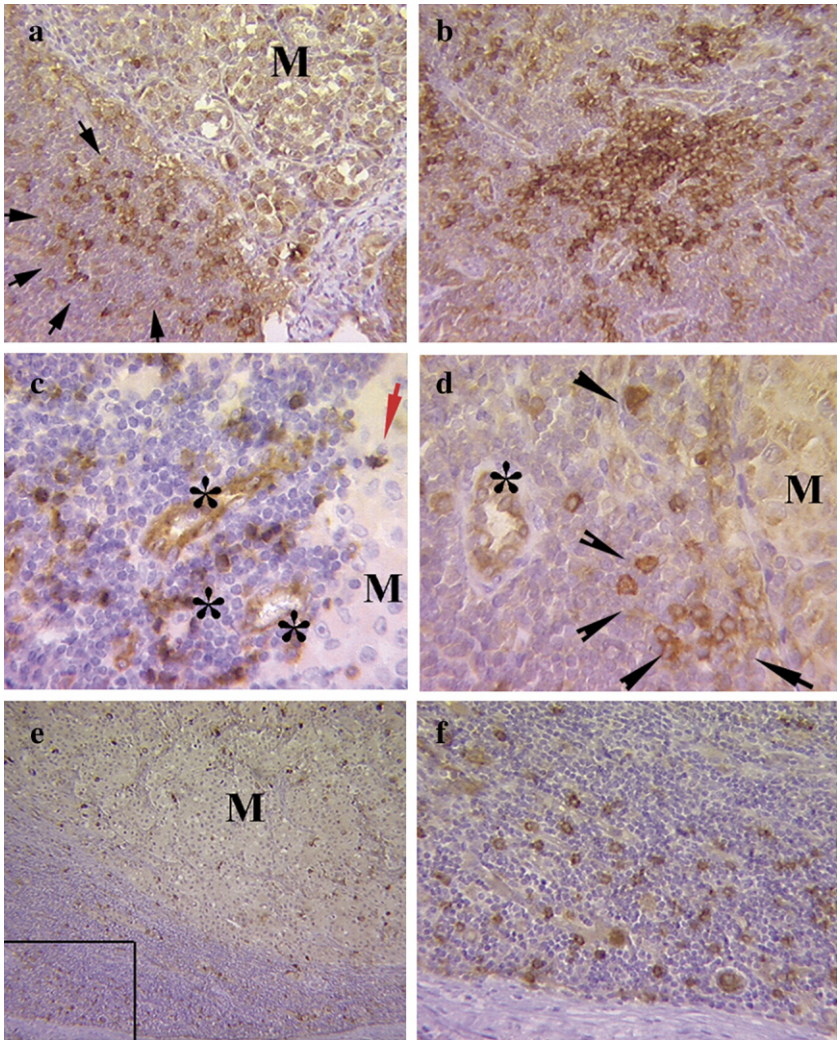

Figure 4 Plasmacytoid dendritic cells aggregate around melanoma cells in metastatic lymph nodes. Representative sections of positive sentinel lymph nodes and clinically metastatic lymph nodes were stained with mAb anti-CD123. a, c and d) Clusters of plasmacytoid dendritic cells around melanoma cells. b) A massive infiltration of plasmacytoid dendritic in a clinically metastatic lymph node. c and d) Plasmacytoid dendritic cells distribution around high endothelial venules. e) A large melanoma metastasis with a thin rim of lymph node. f) High magnification of the inset in e showing a high number of plasmacytoid dendritic cells. Black arrows indicate clusters of plasmacytoid dendritic cells and red arrow indicate single pDC within the melanoma mass. Melanoma cells are indicated by M and asterisks indicate high endothelial venules. a-c and f *(Original magnification x200)*, d *(x300)*, e *(x100)*.

reaching an impressive concentration in presence of clinically metastatic LN (Fig. 4b). Melanoma cells-pDC contact was a frequent finding at the edge of the tumor mass (Figs. 4a and d), while pDC within the melanoma mass were observed only occasionally (Fig. 4c, indicated by the red arrow). PDC, within the T cell-rich areas of LN, were observed around high endothelial venules (HEV) which also stained positive (Figs. 4c and d, indicated by the asterisks). PDC were frequently detected between HEV and melanoma cells (Figs. 4c and d) suggesting a migration from blood towards the tumor area. In a case of clinically metastatic lymph node we detect a very low percentage of pDC by flow cytometry (0.05%), indeed, histopathological examination of that case showed that the lymph node was almost completely replaced by melanoma cells, remaining only a thin peripheral rim of lymph node tissue (Fig. 4e), where a high number of CD123+ pDC were observed (Fig. 4f).

Type I interferon production by Plasmacytoid dendritic cells in sentinel lymph node

The main feature of pDC is the capability to produce type I IFN upon virus stimulation and there is evidence that even cancer cells may trigger a such production [27]. Furthermore, freshly isolated pDC from ovarian carcinomas express MxA [24], a type I IFN–inducible intracellular protein well established as a surrogate marker for local type I IFN production [25]. Therefore we asked whether pDC were able to secrete type I IFN in SLN and in clinically metastatic LN of melanoma patients. To assess this functional aspect we performed a double labeling on frozen sections of LN with anti MxA and anti BDCA2 mAbs. In case of type I IFN secretion by pDC, the neighboring cells, beyond pDC themselves, would stain positive for MxA [25]. Indeed, MxA expression was mostly limited to the BDCA2+ cells (Fig. 5a) and only a

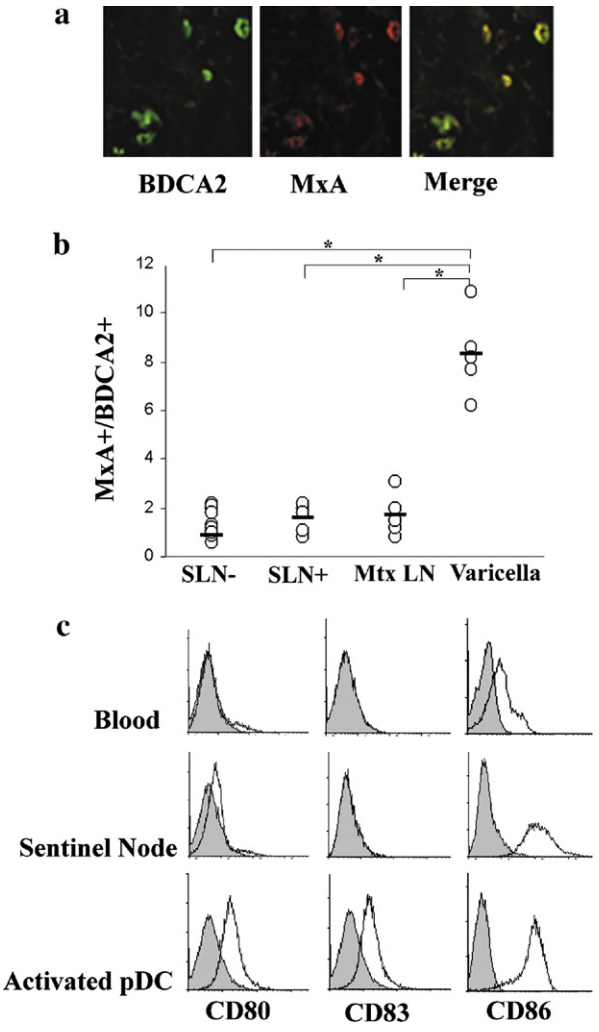

Figure 5 Type I IFN production and activation profile of Plasmacytoid dendritic cells a) SLN sections were stained with anti-BDCA2 and anti-MxA mAb and analyzed by fluorescence microscopy: MxA expression was limited to the BDCA2+ cells. b) Type I IFN production by pDC in negative SLN (n=19), in positive SLN (n=5), in clinically metastatic LN (Mtx LN) (n=5) and in varicella lesions (n=5), was assessed counting the numbers of MxA+ cells and BCDA2+ cells and calculating the mean values of cell numbers in three different fields (magnification, x400). Thereafter, the ratio between MxA+ and BDCA2+ cells was calculated to correct for the density of infiltrating pDC and to make possible comparison among the different conditions. Horizontal bars represent the median. * indicate a statistically significant difference between varicella and the melanoma groups. c) Representative data of flow cytometry analysis of CD80, CD83 and CD86 on fresh pDC from blood and SLN and on influenza virus-activated pDC derived from the same melanoma patient. Influenza virus-activated pDC expressed all the markers, while fresh pDC from SLN and blood expressed respectively high and moderate levels of CD86 molecules. Filled histograms represent isotype control. Data are representative of six independent experiments.

few scattered MxA single positive cells were sporadically detected around BDCA2 and MxA double positive cells (data not shown). These findings suggested that pDC in SLN produce low amount of type I IFN. To quantify this production *in situ*, the total numbers of MxA+ cells and BDCA2+ cells were counted. To correct for the density of infiltrating pDC, the ratio between MxA+ and BDCA2+ cells were calculated for each samples and compared with those obtained in varicella, a condition characterized by a strong type I IFN production by infiltrating pDC [28]. While the differences in the values of Mxa/BDCA2 ratios among the LN groups did not reach the level of statistical significance (Kruskall-Wallis p-value=0.419) (negative SLN: median 1.10%; interquartile range: 0.90%–1.80%; positive SLN: median 1.80%; interquartile range: 1.10%– 2.00%; clinically metastatic LN: median 1.50%; interquartile range: 1.20%; 2.00%), those obtained in varicella skin lesions were significantly much higher (median 8.20%; interquartile range: 7.70%– 8.60%; Mann-Whitnet test p-values<0.008) meaning that type I IFN production in LN is poor (Fig. 5b). Consistently, IFN-α was not detected in supernatant from fresh LN cell suspension as revealed by Elisa (data not shown).

Immunophenotype of Plasmacytoid dendritic cells in sentinel lymph node

The ability to produce type I IFN by pDC is directly related to their activation state [29]. In order to study this aspect we analyzed the expression of the costimulatory molecules CD80, CD86 and the maturation marker CD83 in blood and SLN pDC as well as in influenza virus-activated pDC. Blood pDC were negative for CD80 and CD83, while expressed low levels of CD86 molecules. Similarly, in both positive (n=2) and negative (n=4) SLN pDC did not expressed CD80 and CD83 but showed an upregulation of CD86. In contrast, influenza virus-activated pDC expressed all these markers (Fig. 5c). These findings suggest that pDC are only partially activated in SLN and are consistent with the low ability to produce type I IFN observed *in situ*.

Discussion

PDC are considered a key defensive mechanism of the innate immune system, crucial to limit viral infections spreading thanks to the capability to produce large amount of type I IFN [2,9,10,28]. Since type I IFN inhibit proliferation of certain tumors, their role has also been studied in the immune response to cancer cells [20,24,30–35].

Herein, we have identified pDC as an important DC subset localized in the SLN of melanoma patients. We have demonstrated that pDC are present in higher percentage in positive SLN than in negative SLN. Additionally, we have observed that pDC frequency was even higher in clinically metastatic nodes. It's worth to mention that pDC are rarely detected in normal lymph nodes, while they accumulate in certain inflammatory LN conditions [13,14,36,37]. As described in inflamed LN, pDC were often observed around high endothelial venules suggesting that they migrate from the blood. In certain pathological conditions characterized by accumulation of pDC in the lesions, i.e. psoriasis, systemic lupus erythematosus and varicella infection, the numbers of circulating pDC are decreased [28,29,38], in contrast, in melanoma patients the percentages of pDC in the blood were comparable with those observed in healthy

donors, indicating that pDC recruitment in SLN may occur gradually in the time.

The presence of a high number of pDC in melanoma bearing lymph nodes suggests that pDC may be recruited within the lymph nodes by the presence of melanoma cells or by soluble factors secreted by the tumor. Consistent with this hypothesis, a high number of pDC were found around melanoma metastasis as observed by immunohistochemical analysis. Furthermore, it has been shown that melanoma-secreted cytokines are widely detected in melanoma SLN [39]. This could explain why pDC are observed even in SLN devoid of melanoma cells, but draining lymph from cutaneous melanoma areas. A factor that can mediate a specific recruitment of pDC, as shown in ovarian carcinomas [20,24], is the Stromal Derived Factor (SDF)-1 which is largely produced by melanoma cells [20]. Notably, in the studies describing pDC in solid cancers, these cells have been often described in close vicinity with neoplastic cells, as we found in SLN with metastasis [20,24,40]. It could be hypothesized that the few resident pDC, after interaction with dangerous signals, in this case represented by a neoplastic process, start to produce inflammatory chemokines, the so called CXCR3 ligands, promoting the recruitment of circulating pDC at sites of constitutive SDF-1 expression as described in viral infections [41,42]. Notably, SDF-1 is widely expressed in LN, particularly on HEV [41]. Thereafter, the pDC positioning within the lymph node may be further re-directed by melanoma derived-SDF-1, thus explaining the cluster formation around tumor nests.

The role of pDC in the immune response in oncology is still largely controversial. Some findings suggest that pDC may have a potential role in inducing active immunity, but on the other hand a growing body of evidence strongly propose a tolerance role for these cells. Indeed, pDC may limit the tumor spreading by type I IFN, a well known cytokine with anti tumor properties and with immune regulatory functions. Type I IFN can protect CD8+ T-cells from antigen-induced cell death [43] and can promote final maturation of myeloid DC [44]. However, the capability to produce type I IFN in our study was rather poor. This was observed by direct measurements of IFN-α in the fresh SLN cell suspension (data not shown) and by the finding that a very few cells, apart pDC, expressed MxA protein, in contrast to varicella lesions where a diffuse MxA expression was observed. It has been shown that pDC infiltrating psoriatic lesions express CD80, CD83 and high levels of CD86 consistent with the idea of an *in situ* activation. In line with the activation profile, psoriatic pDC expressed large amount of IFN-α [29,38]. In contrast, we have found that pDC in SLN did not express neither CD80 nor CD83, but only CD86 suggesting a defective *in situ* activation. This was in accordance with the low ability to produce type I IFN observed *in situ*. The scattered MxA single positive cell sporadically found in this study might be due to a certain moderate production of type I IFN in basic conditions or, in the context of a mild inflammatory process in SLN. These findings suggest that pDC, although unable to produce constitutively type I IFN in SLN, might still be stimulated in certain conditions such as the presence of inflammatory cytokines. This phenomenon, however, does not seem to be specifically melanoma-related as demonstrated by the fact that no significant differences in MxA expression were found in negative SLN, positive SLN and in metastatic LN. Furthermore, *in vitro* experiments, with stimulation of pDC by melanoma cell lines, did not show any type I IFN secretion (data not shown). However, we cannot rule out the possibility that some melanoma cells *in vivo,* or stimulation with fresh melanoma cells *in vitro,* might induce type I IFN production by pDC. In previous study on solid tumors, type I IFN production by pDC has been described to be totally absent in breast cancer [40], moderate in primary melanoma [20] and very high in malignant ascites of ovarian carcinomas patients [24]. In favor of a positive role played by pDC in anti cancer immunity is the report showing that, *in vitro*, pDC can prime naive CD8+ T-cell specific for melan-A antigen to become IFN-gamma secreting cells [21].

In strong contrast, a series of data depict pDC as negative regulators of tumor immunity. In this regard it has been shown that infiltration of pDC in primary breast tumor correlates with an adverse outcome suggesting a role of pDC in the progression of cancer cells [40]. Negative effects have also been reported in ovarian carcinomas, where a high number of functionally defective pDC are described in malignant ascites, in the solid tumor and in the tumor draining LN [24]. Additionally, pDC can polarize T-cell response toward a Th2 profile [45], an immune response favoring the tolerance towards tumor. Furthermore pDC can generate CD8 T-regulatory cells which produce IL-10 [46], a strong immune suppressive cytokine, produced also by melanoma cells, capable of inhibiting the antigen presenting capacity of DC [47,48] and to induce anergy in melanoma specific CD8 T cells [49].

In conclusion, SLN of melanoma patients comprise different subsets of DC including pDC. PDC number is higher in melanoma bearing LN and, *in situ,* they are localized around melanoma cells, suggesting a specific recruitment. Several additional data are needed in order to uncover the role exerted by pDC in tumor immunity, however the findings of this study, together with the above mentioned observations in other tumors, are consistent with a role for pDC in cancer immunological tolerance.

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