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Comprehensive Phenotyping of Dendritic Cells in Cancer Patients by Flow Cytometry

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Dendritic cells (DCs) play a crucial role in the complex interplay between tumor cells and the immune system. During the elimination phase of cancer immunoediting, immunostimulatory DCs are critical for the control of tumor growth. During the escape phase, regulatory DCs sustain tumor tolerance and contribute to the development of the immunosuppressive tumor microenvironment that characterizes this phase. Moreover, increasing evidence indicates that DCs are also critical for the success of cancer immunotherapy. Hence, there is increasing need to fully characterize DC subsets and their activatory/inhibitory profile in cancer patients. In this review, we describe the role played by different DC subsets in the different phases of cancer immunoediting, the function exerted by different activatory and inhibitory molecules expressed on DC surface, and the cytokines produced by distinct DC subsets, in order to provide an overview on the DC features that may be useful to be assessed when dealing with the flow cytometric characterization of DCs in cancer patients. © 2020

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• Key terms

human DCs; conventional DCs (cDCs); plasmacytoid DCs (pDCs); peripheral blood DCs (PBDCs); tumor-associated DCs (TADCs); cancer immunoediting; costimulatory molecules; immune checkpoints; flow cytometry

DENDRITIC cells (DCs) are professional antigen-presenting cells (APCs) with a very central role in the activation, regulation, and polarization of all immune responses. They originate in the bone marrow, circulate in the blood, and spread to peripheral tissues, where they act as sentinels that sample the environment for antigens. DC ability to activate different arms of adaptive immunity relies on their state of activation, and their belonging to different subsets that are each endowed with functional specialization (1). Beyond their crucial ability to control adaptive immune responses, DCs also play an important role in shaping the innate immune response in the peripheral tissues where they detect and capture antigens, as they can locally produce high amounts of cytokines that can affect other innate immune cells present in the microenvironment (2,3).

Because of their properties, DCs play a relevant role in the complex interaction between tumor cells and the immune system. This complex interaction is described in the concept of cancer immunoediting, which is composed of three phases: elimination, equilibrium, escape (4,5). During the elimination phase, innate, and adaptive immune cells destroy developing tumors before they become clinically detectable. Here, DCs are essential for the induction of robust tumor-specific cytotoxic immune responses, as they have the unique ability to activate naive T cells (6). DCs capture tumor antigens in their microenvironment, undergo maturation and, while homing to the draining lymph nodes, they upregulate costimulatory molecules that are required to complete T-cell activation (7). If tumor cells are not completely

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eliminated, the tumor enters the equilibrium phase, in which tumor cells that develop resistance to effector immune cells survive and become dormant cells under the effects of immune mechanisms that prevent tumor cell outgrowth. In this phase, DCs contribute to maintain the relative balance between activated antitumor effector cells and immunosuppressive cells, needed to keep tumor cells in a state of immune-mediated dormancy (4). The progression to the escape phase occurs when the equilibrium characterizing the previous phase is lost because tumors acquire new properties that circumvent immune recognition and destruction (8). Tumor cells increase their resistance to cytotoxic mechanisms and establish an immunosuppressive tumor microenvironment (TME). These changes favor the progressive growth of the tumor that becomes clinically evident. In this phase, DCs contribute to the tumor escape from immune surveillance by several mechanisms, including the promotion of tumor-specific immune tolerance, and the participation in the development and maintenance of an immunosuppressive TME (9).

Because of their pivotal role in antitumor immunity, DCs are an important target of immunotherapeutic strategies aimed at reverting the mechanisms of tumor escape and potentiating tumor-killing effector mechanisms. Based on the immunological knowledge gained on DCs and the mechanisms of tumor escape, existing first- and second-generation DC vaccines are being implemented toward the next-generation DC vaccines, endowed with optimized activity (10). Other developing strategies aimed at potentiating the effects of DCs in cancer immunotherapy include using DC vaccines in combination with other anticancer therapies, and reprogramming tumor-infiltrating DCs in order to switch their behavior from tumor-induced immunosuppression to promotion of tumor rejection (6,11,12). In order to achieve these goals, a detailed characterization of DCs in cancer patients is needed.

In this review, we provide an overview of the flow cytometric strategies used for the identification of DC subsets, the characterization of their activatory/tolerogenic profile, and their production of relevant cytokines. We also provide an overview of the reported features of DCs in different types of human cancer.

CHARACTERIZATION OF PERIPHERAL BLOOD DCs IN CANCER PATIENTS

Because peripheral blood is the most accessible source of DCs, flow cytometric analysis of peripheral blood DCs (PBDCs) is a convenient procedure for the characterization of DCs in cancer patients. Moreover, it can also be serially repeated in patient follow-up.

Human blood contains three main subsets of DCs that differ from each other in origin, phenotype, and functional specialization. Conventional DCs (cDCs) are myeloid in origin. They are the most important APCs to T cells and are further subdivided in two subsets: cDC1s and cDC2s. cDC1s have a higher cross-presentation ability than other DC subsets, making them highly efficient in priming cytotoxic T cells (13,14). Accordingly, they are associated with better survival in cancer patients (15). cDC2s are the major subset of cDCs in blood. They are a heterogeneous population of potent stimulators of naive T-helper (Th) cells. Through the expression of a wide range of toll-like receptors (TLRs) they can be differentially activated to produce different cytokines that in turn promote different Th polarization (16). Plasmacytoid DCs (pDCs) lack myeloid markers and are characterized by the ability to produce huge amounts of type I interferon (IFN) upon recognition of viral patterns, thus conferring these cells a primary role in antiviral defenses (17). In their immature state, pDCs are mainly tolerogenic. Accordingly, in the context of tumor immunity, their presence in the TME is associated with a bad prognosis (18,19).

Due to their lack of unique lineage markers, PBDCs can be identified using combinations of positive and negative markers. The most common strategy relies on identifying PBDCs as cells that constitutively express major histocompatibility complex class II (MHC-II) molecules on their surface, while lack monocyte, B-, T-, and NK-cell lineage-associated antigens. Gated on these cells, cDCs are identified based on the expression of the myeloid marker CD11c. Within cDCs, cDC1s are characterized by the expression of the C-type lectin-like receptor (CLEC) 9A and the Cell adhesion molecule 1 (CADM1), and express high levels of CD141 and XCR1 (13,20); cDC2s are commonly identified based on the expression of CD1c, and they also express the alpha subunit of the high-affinity IgE receptor (FCER1A), CLEC10A, CD301, and signal regulatory protein alpha (CD172a) (21–23). pDCs lack myeloid markers and are commonly identified based on the expression of CD123. Other typical pDC markers are CD303 and CD304 (20). Concise overviews of the markers used for human DC phenotyping have recently been published in the Optimized Multicolor Immunofluorescence Panel (OMIP) and Phenotype Report series on this journal (24,25). Table 1 summarizes the main changes of PBDCs reported in cancer patients. As shown in the table, in most cases the information is limited to numerical changes, with a reduction of one or more PBDC subsets being the most common observation, likely related to the inhibitory effects of tumor-derived factors on the production of DCs by the bone marrow (28,31). Beyond DC count, the flow cytometric analysis of PBDCs allows the phenotypic characterization of DC subsets. Particularly relevant to cancer immunity and to the comprehension of the role

Table 1. Reported changes of peripheral blood DCs (PBDCs) in cancer patients

TYPE OF CANCER	COUNT CHANGES OF CDCs	COUNT CHANGES OF PDCs	PHENOTYPIC CHANGES	FUNCTIONAL CHANGES	REFERENCES
Acute myeloid leukemia (AML)	↑cDC2s (diagnosis M4eo, inv(16) or t(16;16)) or ↓ ↓cDCs	↓			(26,27)
Breast cancer		= or ↓	↑CD83; ↓HLA-DR; ↓CD119	↓IL12; ↑IL10	(28–32)
Chronic myeloid leukemia (CML)		↓	↑CD40; ↑CD80; ↑CD86; ↑HLA-DR	= IRF8	(33)
Chronic myelomonocytic leukemia (CMML)		↓			(34)
Chronic lymphocytic leukemia (CLL)		↓	↓CD40; ↓CD80; ↓CD86; ↓CD83; ↓HLA-DR		(35)
Classic Kaposi's Sarcoma		↓	= CD80; = CD86; = CD83; ↑CD49c; ↑CD91	= IFNα; ↓IL12; ↑IL10	(36)
Colorectal cancer (CRC)	= or ↓	= or ↓	↓CD83; ↓HLA-DR		(29,37,38)
Gastric cancer	↓cDCs; ↑cDC2s	↑	↓CD80; ↓CD83		(39–41)
High Grade Gliomas (HGG)	↓ or absent	↓ or absent			(32,42–44)
Melanoma	=	=	= CD40; = CD80; = CD86; = CD83; = CXCR3; = CXCR4; ↑CCR6; = CCR7; = CCR10; ↑CD62L; ↑OX40L = PD-L1		(19,32,45)
Multiple myeloma					(46)
Myelodysplastic syndromes (MDS)	↓cDC1s; ↓cDC2s	↓	cDC1s: ↑CD141, ↑CD86, ↑ILT2, ↓TIM-3	Impaired responsiveness of all subsets to TLR stimulation	(47,48)
Non-small-cell lung cancer (NSCLC)		=			(49,50)
Ovarian cancer	↓cDCs, ↓cDC1s; = cDC2s; ↓CD141 ^{bright/dim} DCs	= or ↓	cDC2s: = CD40; ↑CD80; ↑/= CD86; ↑CD83; = ICOS-L; = PD-L1. CD141 ^{bright/dim} DCs: = CD40; ↑CD80; = CD86; ↑CD83; = PD-L1 ↑Annexin V		(51–54)
Pancreatic ductal adenocarcinoma (PDAC)	↓cDCs; also in bone marrow	= or ↓		↓IRF8 in pre-DC1s of bone marrow	(31,55–58)
Prostate cancer	= cDC1s; = cDC2s; ↓CD141 ^{bright/dim} DCs		cDC2s: ↓CD40; ↑CD80; = CD86; = CD83; = PD-L1. CD141 ^{bright/dim} DCs: = CD40; ↑CD80; ↓CD86; ↑CD83; = PD-L1	↓response to TLR3 stimulation	(54)

DC changes reported compared with matched healthy donors.

Table 2. Activatory and inhibitory molecules and maturation markers expressed on DC surface and their functions

MOLECULE	RECEPTOR FAMILY	RECEPTOR/LIGAND	FUNCTIONS	REFERENCES
B7-1 (CD80)	B7 family	CD28; CTLA-4 (CD152)	Activatory molecule: CD28 delivers costimulatory signals in T cells; CTLA-4 delivers inhibitory signals in T cells	(60,63,64)
B7-2 (CD86)	B7 family	CD28; CTLA-4 (CD152)	Activatory molecule: CD28 delivers costimulatory signals in T cells; CTLA-4 delivers inhibitory signals in T cells	(60,63,64)
B7-H1 (PD-L1/CD274)	B7 family	PD-1 (CD279)	Inhibitory molecule: it binds PD-1 that contains cytoplasmic immunotyrosine-based inhibitory motifs (ITIMs)	(60,63,65)
B7-DC (PD-L2/CD273)	B7 family	PD-1 (CD279)	Inhibitory molecule: it binds PD-1 that contains cytoplasmic immunotyrosine-based inhibitory motifs (ITIMs)	(60,63)
B7RP1 (ICOS-LG/CD275)	B7 family	ICOS (CD278)	Activatory molecule: it delivers costimulatory signals for T-cell proliferation and activation	(60,66)
CD40	TNF superfamily	CD40L	Activatory molecule: it is engaged by CD40L, it licenses DCs to activate cytotoxic T cells and counteract immune tolerance	(67–69)
CD70	TNF superfamily	CD27	Activatory molecule: it promotes Th-independent expansion of primary cytotoxic T cells	(69–71)
4-1BBL (CD137L)	TNF superfamily	CD137	Activatory molecule: it promotes T-cell development, survival, proliferation, activation, and memory development	(69,72)
OX40L (CD252)	TNF superfamily	OX40 (CD134)	Activatory molecule: it promotes T-cell proliferation, survival, and production of cytokines	(69,72)
GITRL	TNF superfamily	GITR (CD357)	Inhibitory molecule: it induces the upregulation of indoleamine-pyrrole 2,3-dioxygenase (IDO) in pDCs	(69,72)
CD83	Immunoglobulin superfamily of receptors	CD83, forming homodimers	Maturation marker: it upregulates HLA class II molecules and CD86 on DCs	(63,73)
MHC class I	Immunoglobulin superfamily of receptors	TCR	Maturation marker: it is needed for the activation of cytotoxic T cells	(60,64,74)
MHC class II	Immunoglobulin superfamily of receptors	TCR	Maturation marker: it is needed for the activation of Th cells	(60,64,74)
CCR7 (CD197)	G protein-coupled receptor family	CCL19/CCL21	Maturation marker: it is needed for the recruitment of mature DCs to lymphoid tissues	(64,74,75)
DC-SIGN (CD209)	C-type lectin	High-mannose-containing glycoproteins	Maturation marker: it induces adhesion, migration, signaling, and antigen uptake/presentation in DCs	(76,77)
ILT2 (CD85j)	ILT family of leukocytes receptors	Nonclassical MHC Class I	Inhibitory receptor: upon interaction with its ligands, it inhibits cell function through ITIM signaling	(48,62,78,79)

(Continues)

Table 2. Continued

MOLECULE	RECEPTOR FAMILY	RECEPTOR/LIGAND	FUNCTIONS	REFERENCES
ILT3 (CD85k)	ILT family of leukocytes receptors	Unknown	Inhibitory receptor: upon interaction with its ligands, it inhibits cell function through ITIM signaling; it is required for the induction of CD4+Foxp3+ regulatory T cells by 1,25-dihydroxyvitamin D3	(62,78–80)
ILT4 (CD85d)	ILT family of leukocytes receptors	Nonclassical MHC class I	Inhibitory receptor: upon interaction with its ligands, it inhibits cell function through ITIM signaling	(62,78,79)
ILT5 (CD85a)	ILT family of leukocytes receptors	Unknown	Inhibitory receptor: upon interaction with its ligands, it inhibits cell function through ITIM signaling	(62,79)
LIR-8 (CD85c)	ILT family of leukocytes receptors	Unknown	Inhibitory receptor: upon interaction with its ligands, it inhibits cell function through ITIM signaling	(62,79)
TIM-3 (CD366)	T cell immunoglobulin (TIM) family	Galectin-9; HMGB1	Inhibitory receptor: it suppresses nucleic acid-mediated antitumor immune responses through HMGB1 binding	(81,82)

CCL, C–C motif chemokine ligand; CCR7, C–C Motif Chemokine Receptor 7; CTLA-4, cytotoxic T-lymphocyte antigen 4; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; GITR, glucocorticoid-induced tumor necrosis factor receptor-related protein; GITRL, glucocorticoid-induced tumor necrosis factor receptor-related protein ligand; HMGB1, high mobility group Box 1; ICOS, inducible T-cell costimulator; ICOS-LG, inducible T-cell costimulator ligand; ILT, immunoglobulin-like transcript; LIR-8, leukocyte immunoglobulin-like Receptor 8; MHC, major histocompatibility complex; PD-1, programmed cell death Protein 1; PD-L1, programmed death-Ligand 1; PD-L2, programmed death-Ligand 2; TCR, T-cell receptor; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; TNF, tumor necrosis factor.

played by DCs in the complex interaction between tumor cells and the immune system is the possibility to assess the activatory/inhibitory profile of DC subsets. Immunostimulatory DCs, able to activate robust anti-tumor responses during the elimination and equilibrium phases of cancer immunoediting, are characterized by high expression of class I and class II human leukocyte antigen (HLA) molecules, as well as of costimulatory molecules. Conversely, tolerogenic DCs that contribute to tumor escape by promoting tumor tolerance and participating in the immunosuppressive TME are characterized by low expression of costimulatory molecules and high expression of inhibitory receptors (59). Many costimulatory and inhibitory molecules expressed on DCs belong to the B7 family and to the tumor necrosis factor (TNF) family (reviewed in Ref. 60). Other inhibitory receptors expressed on DCs belong to the immunoglobulin-like transcript (ILT) family, contain a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) that transduces a negative signal, and include ILT2 (CD85j), ILT3 (CD85k), ILT4 (CD85d), and ILT5 (CD85a) (61,62). A complete list of activatory and inhibitory molecules expressed on DC surface and their functions is provided in Table 2. Although only few studies have investigated so far the activatory/inhibitory profile of PBDCs in cancer patients (Table 1), there is an increasing interest in using PBDC phenotype for developing biomarkers to be used in cancer patients undergoing checkpoint inhibition therapy (83). In fact, as

extensively discussed elsewhere (48), although the introduction of immune checkpoint inhibitors in cancer immunotherapy has revolutionized cancer immunotherapy, remarkable, and long-term responses to these treatments are obtained only in a proportion of patients, and there is an urgent need to develop biomarkers that allow to predict patients who are likely responsive to checkpoint blockade (84). In this respect, it is worthy to note that a positive response to anti-PD-L1 therapy has been associated with high expression of PD-L1 on tumor-infiltrating immune cells in many types of cancer, and that PD-L1-positive DCs may contribute to tumor escape by suppressing antitumor responses, which can be reinvigorated on PD-L1 blockade (85,86). Accordingly, PD-L1 expression on DCs may predict response to treatment and may be developed as a biomarker for patients undergoing anti-PD-L1 treatment. As the array of molecular targets of checkpoint inhibitors is growing, the expression of other inhibitory receptors on DCs, or even the ratio between activatory and inhibitory molecules may provide valuable information. Notably, according to DC heterogeneity, the expression of these molecules and the effect of their blocking may greatly differ among different DC subsets. Yet, our knowledge on the expression of activatory and inhibitory molecules on human DC subsets is fragmentary. By using a six-color flow cytometric panel dedicated to the analysis of DCs, we demonstrated that pDCs are characterized by a lower expression of the costimulatory molecules CD40, CD80, and

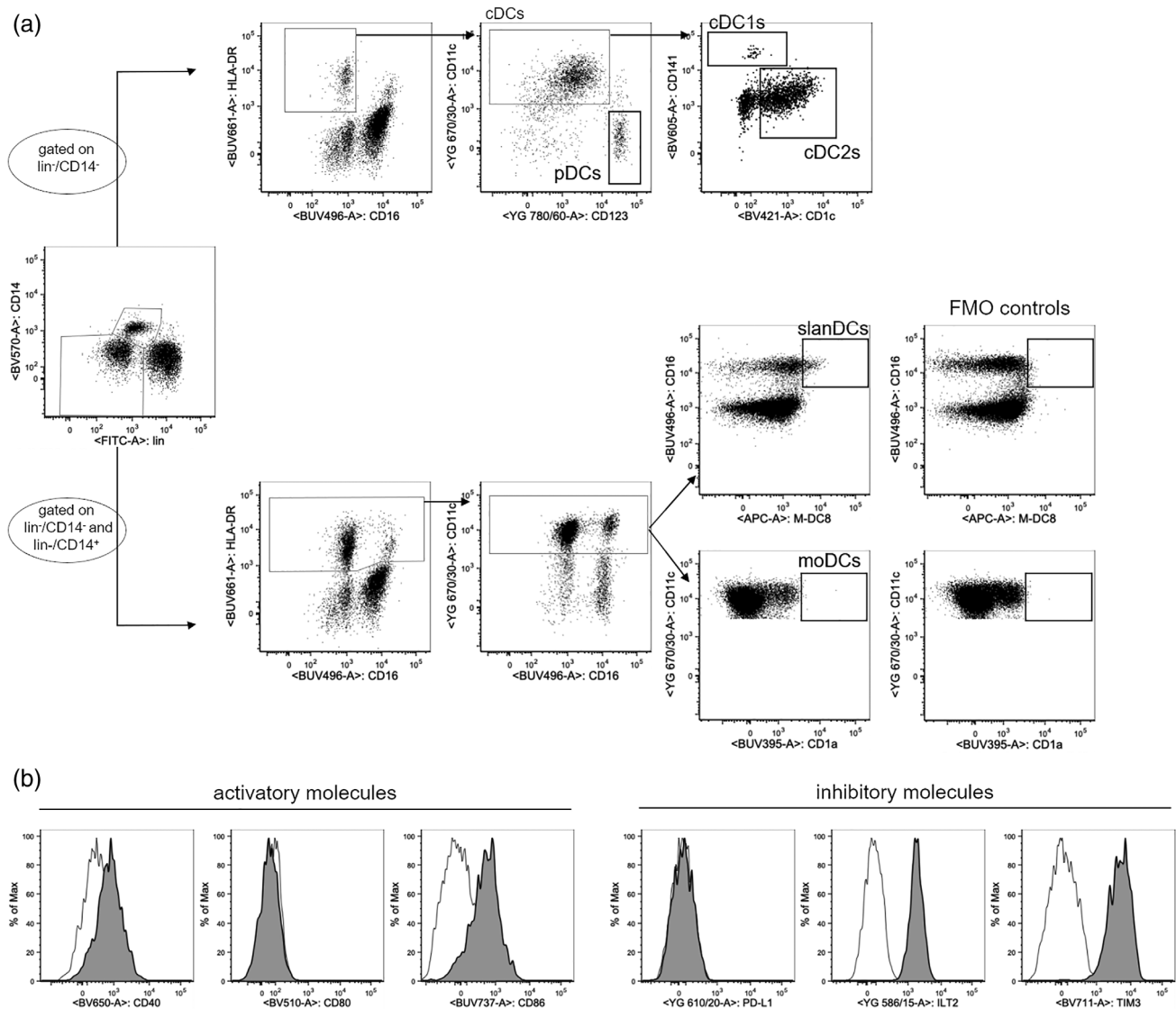


Figure 1. Representative 18-color flow cytometric analysis of PBDCs from a healthy donor, performed as reported in (48). **(A)** Gating strategy used to identify different DC subsets. Gated on single, live CD45 $^+$ mononuclear cells (not shown), DC-lineage DCs were identified within the gate of lin^+ (CD3,CD19,CD20,CD56)/CD14 $^-$ /CD16 $^-$ /HLA-DR $^+$ cells. Gated on these cells, pDCs were identified as CD11c $^-$ /CD123 $^+$ cells, whereas cDCs were identified as CD11c $^+$ /CD123 $^-$ cells. Gated on cDCs, cDC1s and cDC2s were identified based on the expression of CD141 and CD1c, respectively. Inflammatory DCs were identified as lin^+ /HLA-DR $^+$ /CD11c $^+$ cells that could be either negative or positive for CD14 and CD16 expression. Within inflammatory DCs, slanDCs, and moDCs were identified based on positive staining of M-DC8 and CD1a, respectively. Fluorescence minus one (FMO) controls stained with all reagents but lacking either M-DC8 or CD1a are shown on the right. **(B)** Histograms showing the expression of costimulatory and inhibitory molecules. Gated on cDC2s, chosen as an exemplificative DC subset, the expression of the costimulatory molecules CD40, CD80 and CD86, and the inhibitory molecules PD-L1, ILT2 and TIM-3 was shown. Each graph shows the overlay of the stained sample (full histogram) and the appropriate FMO control (empty histogram).

CD86, and a lower expression of the DC maturation marker CD83, compared with cDCs (87). More recently, we developed an 18-color flow cytometric panel dedicated to the analysis of DCs (a representative analysis showing the gating strategy is reported in Fig. 1) and demonstrated that cDC1s, cDC2s, and pDCs have also a differential expression of inhibitory molecules, with cDC1s having a unique immune checkpoint repertoire characterized by high expression of T-cell immunoglobulin and mucin-domain containing-3 (TIM-3,

CD366), low PD-L1 expression, and lack of ILT2 (48). Notably, this unique repertoire was subverted in patients affected by myelodysplastic syndromes, suggesting that the analysis of PBDC phenotype may be helpful to provide new insights in the comprehension of the role played by distinct DC subsets in the pathogenesis and progression of different types of cancer (48).

The flow cytometric analysis of PBDCs can also be used for assessing DC production of cytokines. The heterogeneous

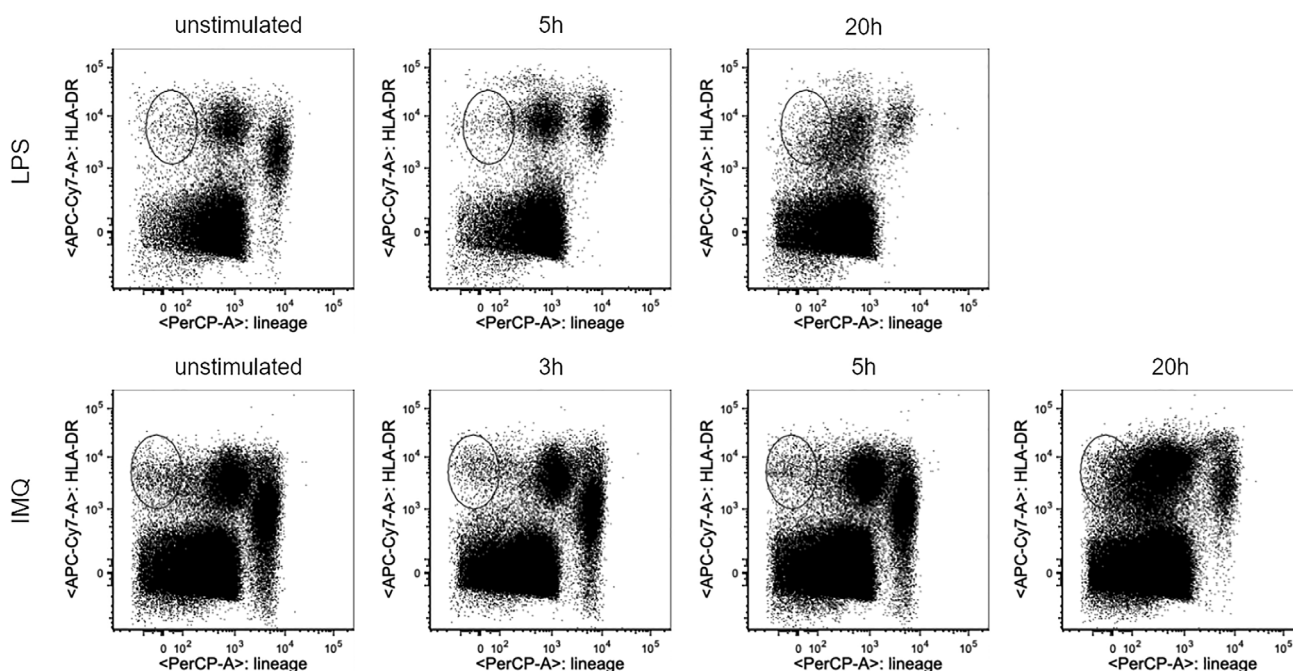


Figure 2. Representative flow cytometric analysis of PBDCs from two different healthy donors, showing the time-dependent changes whole blood samples undergo upon incubation with TLR ligands. Blood samples were incubated with either lipopolysaccharide (LPS, upper row) or imiquimod (IMQ, lower row) for the indicated times; at the end of incubation, samples were stained and analyzed by six-color flow cytometry as reported in (93). For each culture condition, only the lineage (CD3,CD14,CD16,CD19,CD20) versus HLA-DR plot is shown. As evident in these representative experiments, in most cases stimulated cells undergo a time-dependent change in the surface expression of molecules used as markers for gating on lineage[−]/HLA-DR⁺ DCs, making it advisable to keep the incubation time of blood samples with TLR ligands as short as possible.

distribution of TLRs among DC subsets endows each subset with the ability to sense different pathogen recognition patterns (PRRs) and produce a different array of cytokines. cDC1s express high levels of TLR3 (CD283) and TLR9 (CD289) that allow them to sense intracellular dsRNA and DNA, inducing these cells to produce IL12 and type I IFN (13,88,89). cDC2s express a wide range of TLRs, and upon stimulation they correspondingly produce a wide range of cytokines and chemokines, including IL1, IL6, IL8, IL12, IL18, TNF α , CCL3, CCL4, CXCL8 (reviewed in Ref. 90). pDCs express high levels of endosomal TLR7 and TLR9 that allow them to sense ssRNA and dsDNA, respectively and produce huge amounts of type I and type III IFNs (91,92). They also produce TNF α and IL6. All DC subsets can also produce immunoregulatory cytokines, such as IL10 and TGF β . TLR ligands are optimal stimulators of PBDCs in whole blood assay and can be used for the assessment of cytokine production by DC subsets by intracellular flow cytometry (93). When performing these assays, it is recommended to keep the incubation time of blood samples with TLR ligands as short as possible, because stimulated cells change the surface expression of molecules used as markers for gating on DCs, making the identification of these cells difficult. A representative experiment showing the time-dependent phenotypic changes of whole blood samples upon stimulation with the TLR ligands lipopolysaccharide and imiquimod is shown in Figure 2. In our hands, incubation times no longer than 5 h

are preferred. A complete list of the cytokines produced by DC subsets is provided in Table 3. As shown in Table 1, few studies addressed the production of cytokines by PBDCs in cancer patients, mainly demonstrating an impaired ability to produce the immunostimulatory cytokine IL12, and an increased production of the immunosuppressive cytokine IL10 (28,36).

CHARACTERIZATION OF TUMOR-ASSOCIATED DCs

Although the study of PBDCs in cancer patients have several advantages as mentioned above, a deep characterization of tumor-associated DCs (TADCs) can provide further important information, useful to better understand the role of distinct DC subsets in tumor escape and in responsiveness to immunotherapeutic treatments. For example, as expected based on the superior ability of cDC1s to activate cytotoxic T cells, the presence of these cells in the TME is associated with better survival across multiple types of human cancer (15). However, evidence has been provided that the antitumor activities of cDC1s are not limited to their ability to activate naive T cells in the tumor-draining lymph nodes, but also to their actions exerted directly in the TME, including the ability to attract T cells through the production of the chemokines CXCL9 and CXCL10, to locally activate other immune cells through the production of cytokines, and to present antigens and activate cytotoxic T cells within the TME (reviewed in

Table 3. Activatory and inhibitory soluble factors produced by DCs and their functions

SOLUBLE FACTOR	PRODUCED BY	FUNCTIONS	REFERENCES
IL1 α /IL1 β	cDC1s, cDC2s	Pro-inflammatory cytokines	(90)
IL6	cDC1s, cDC2s	It drives: plasma cell responses; TReg cell and Th17 cell responses	(90,94,95)
IL8 (CXCL8)	cDC1s, cDC2s, pDCs	Pro-inflammatory cytokine; immune cell recruitment	(90,95)
IL10	cDC2s	It drives TReg cell and Th17 cell responses	(1,95)
IL12	cDC1s, cDC2s, pDCs	It promotes: effector CD8+ T-cell and Th1-cell responses; NK cell activation; cDC1-cDC2 crosstalk	(69,90,95–97)
IL15	cDC1s, Langerhans cells	NK-cell activation	(97,98)
IL18	cDC2s, pDCs	NK-cell activation	(90,95)
IL23	cDC1s	It drives Th17 immune responses	(90,96)
CCL3	cDC2s, pDCs	Immune cell recruitment	(90,95)
CCL4	cDC2s, pDCs	Immune cell recruitment	(90,95)
CCL5	pDCs	Immune cell recruitment	(90,95)
CXCL10	pDCs	Immune cell recruitment	(90,95)
CXCL11	pDCs	Immune cell recruitment	(90,95)
FGF23	pDCs	Pro-inflammatory cytokine; pro-angiogenic factor	(99,100)
GzmB	pDCs	Immune regulation via induction of cell death or suppression of T cells	(95)
TGF β	pDCs	It drives TReg cell and Th17 cell responses	(94,95,101)
TNF α	cDC1s, cDC2s	Pro-inflammatory cytokine	(90)
TRAIL (CD253)	pDCs	Immune regulation via induction of cell death or suppression of T cells	(95)
type I IFNs	cDC1s, pDCs	They promote: effector CD8+ T-cell and Th1-cell responses; NK-cell activation; antiviral responses; cDC-pDC crosstalk. They drive plasma cell responses	(69,90,95,97)
type III IFNs	cDC1s, pDCs	It drives Th1-cell responses	(96,102)
VEGF	cDC2s	Pro-angiogenic factor	(1,96)

CCL, C–C motif chemokine ligand; CXCL, C–X–C motif chemokine ligand; FGF, fibroblast growth factor; GzmB, Granzyme B; IFN, Interferon; IL, interleukin; TGF β , transforming growth factor β ; TNF α , tumor necrosis factor α ; TRAIL, TNF-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor.

Ref. 103). Notably, it has been recently demonstrated that, upon uptake of tumor antigens, tumor-infiltrating cDC1s can upregulate PD-L1 and become tolerogenic by activating a regulatory program (104). The acquisition of similar detailed information on the different subsets of TADCs across different types of human cancers at different clinical stages will be needed in order to deeply understand the biology of DCs in the interaction between tumor cells and the immune system, and to improve our ability to treat cancer patients.

Yet, the flow cytometric analysis of TADCs (an overview of the main changes of TADCs is reported in Table 4) is hampered by several technical issues. The first issue is represented by the paucity of DCs in the TME that may render sometimes difficult the analysis of an adequate amount of cells. The second issue is represented by the complexity of DC subsets in the TME. In fact, TADCs are composed not only by the DC-lineage subsets present in the peripheral blood, but can also contain inflammatory DCs that are heterogeneous across tissues and local

inflammatory microenvironments (1,117,118). Monocyte-derived DCs (moDCs) represent a highly heterogeneous major population of inflammatory DCs (1,119–121). Although they have long been considered monocytic in origin, their ontology is at present under debate, as recent data obtained by high dimensional single-cell protein and RNA analysis suggest that moDCs may be related to cDC2s rather than monocytes (21). Other inflammatory DCs are represented by 6-sulfo-LacNAc (slan)-positive cells (1,122). Slan⁺ cells in the peripheral blood have a transcriptional profile that overlaps with the profile of CD16⁺ nonclassical monocytes, thus suggesting a monocyte origin of these cells (122–124). Nevertheless, slanDCs in the peripheral tissues are endowed with DC-specialized functions including efficient antigen presentation, capacity to activate naive T cells and promote Th1/Th17 immune responses (125,126). CD1a and MDC-8 are commonly used to identify moDCs and slanDCs, respectively, within myeloid DCs (48). However, additional markers including CD89 and CD163 may be useful for

Table 4. Reported changes of tumor-infiltrating DCs (TADCs) in cancer patients

TYPE OF CANCER	TADC CHANGES VERSUS DCs IN HEALTHY TISSUE	TADC CHANGES VERSUS DCs IN METASTASIS/ASCITES	TADC CHANGES VERSUS PDCs	REFERENCES
Breast cancer	↑pDCs; ↑cDC1s		↑CD40; ↑CD86; ↑CD83; ↑HLA-DR; ↓BDCA-2	(30,105)
Colorectal cancer (CRC)	*cDC1s, cDC2s, moDCs			(106)
Gastric cancer	↓ or ↑pDCs; = or ↓cDCs	↓cDCs	↓CD80; ↓CD83	(39)
Head and neck squamous cell cancer (HNSCC)			↑OX40, OX40 ⁺ pDCs; ↑CD40, ↑CD80, ↑CD86, ↑Siglec6, ↑CD25, ↑4-1bb, ↑ICOS(L), ↑OX40(L), ↑Axl, ↑TRAIL; ↑IFNα, ↑IL-12p70, ↑GzmB [> cDCs, >OX40-pDCs]	(107–109)
High Grade Gliomas (HGG)	*↑ pDCs from I to III grade HGG, absent in IV grade; cDC1s absent; cDC2s absent			(32,110)
Melanoma and metastatic melanoma	*cDC1s, cDC2s	↑pDCs, ↑CD40; ↑CD80; ↑CD86	↑ pDCs, ↑CD40; ↑CD80; ↑CD86	(15,19,111–113)
Non-smallcell lung cancer (NSCLC)	↑pDCs; ↑cDC2s, ↑CD33; ↑HLA-A1; ↑HLA-D; ↑PD-L1, ↑IFNα; ↑GzmB			(49,106,114,115)
Ovarian cancer		= cDC2s, ↓IFNα (TIpDCs)	↓ pDCs; = cDCs, ↑CD40, = CD86, ↑PD-L1; ↑CD49e; = CD11a; = CD49d; = CD54; = CD62P; = CD62E; = CD62L; = CXCR3; = CXCR4; = CCR5; = CCR6; ↑IFNα/β/ω	(51,52,116)

Asterisk symbol (*) denotes the presence of the reported DC subsets, but no comparison with a healthy counterpart available.

(a) ORIGIN

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1  mfshlpfdcv lllllllltr sseveyraev gqnaylpcfy tpaapgnlvp vewgkgacpv
61 fecgnvvrlt derdvnwts rywlngdfrk gdvsltienv tladsgiycc riqipgimnd
121 ekfnlklvik pakvtpaptr qrdftaafpr mltrghgpa etqtlgslpd inltqistla
181 nelrdsrlan dlrdsatir igiyigagic aglalalifg alifkwysks kekiqnslsi
241 slnlpssgl anavaegirs eeniytieen vyeveepney ycyvssrqqp sqplgcrfam
301 p

```

cutting site of type IV
collagenase

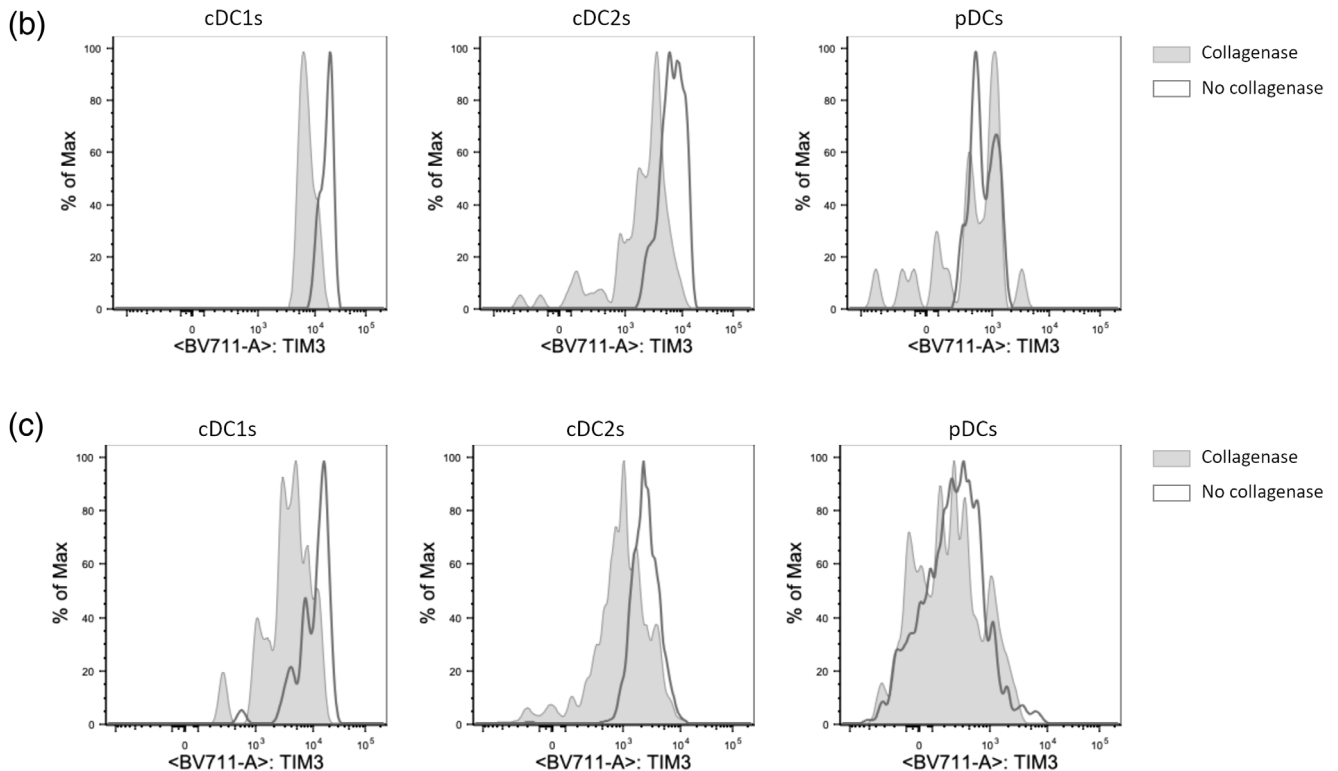


Figure 3. Effects of incubation with Collagenase type IV on TIM-3 detection by flow cytometry. (A) Protein sequence of TIM-3 and highlighting of the cutting site of collagenase, located in the extracellular portion of the molecule and thus supporting the possibility of TIM-3 cleavage upon collagenase treatment. Comparison of TIM-3 detection on cDC1s, cDC2s and pDCs obtained from (B) tumor tissue or (C) whole blood treated (shaded) or not treated (empty) with Collagenase type IV for 1 h. In both cases, the reduction of TIM-3 detection on cDC subsets after incubation with collagenase is evident.

discriminating between these cells and cDC2s (21,24). The third technical issue that can hamper the flow cytometric analysis of TADCs is represented by the use of reagents for tissue processing that may affect the detection of particular molecules of interest. As an example for this last issue, we report here the case of TIM-3.

ASSESSING THE EXPRESSION OF TIM-3 ON TADCs

TIM-3 is an immune checkpoint molecule that has recently come to the attention as an immunotherapeutic target. Initially identified as a negative regulator of Th1 cells after binding galectin-9, it is expressed also on myeloid cells, including cDCs (127,128). TADCs can upregulate their surface

expression of TIM-3 in response to the synergistic actions of multiple immunoregulatory factors released from tumor cells (82). The immunosuppressive action of TIM-3 on TADCs is exerted by interfering with the alarmin-mediated activation of nucleic acid-sensing systems, as binding of HMGB1 suppresses the immunogenicity of nucleic acids released from dying tumor cells (82). Therefore, there is increasing interest in investigating the relevance of TIM-3 to human cancer and in developing TIM-3-targeting checkpoint inhibitors. Indeed, a high expression of TIM-3 on the surface of myeloid TADCs has been reported in some human cancers (82,129), but most human cancer types remain so far poorly investigated.

When we applied our 18-color flow cytometric panel that allowed the detection of high levels of TIM-3 expression

on PBDCs to tissue cancer samples, we observed an unexpected low expression of TIM-3. Because tissue processing included the incubation of our samples with type IV collagenase at a final concentration of 1.6 mg/ml for 1 h at 37°C, we reasoned that the low detection of TIM-3 may be related to collagenase digestion. Indeed, type IV collagenase is a protease with a specificity for the X-Gly bond in the sequence R-Pro-(X-Gly-Pro), where X is most frequently a neutral amino acid (130). By using the protein database of National Center for Biotechnology Information (NCBI), we observed that the cutting site of type IV collagenase was indeed in the extracellular region of the amino acidic sequence of TIM-3 (Fig. 3A). In order to investigate whether in our experimental setting the treatment with type IV collagenase could be responsible for a reduction in TIM-3 expression, we compared the expression of TIM-3 in a tumor sample treated or not treated with Type IV collagenase, by using the same flow cytometric panel described in (48). As shown in Figure 3B we observed, indeed, that TIM-3 expression was downregulated on both cDC subsets by collagenase treatment. The same results were observed on PBDCs (Fig. 3C), supporting the hypothesis that the low expression of TIM-3 expressed on TADCs of tumor samples digested with Collagenase IV had likely to be ascribed to a technical artifact and suggesting the need for a careful setup of all experimental conditions, including the preanalytical phase, when planning the flow cytometric analysis of TADCs.

CONCLUSIONS

This report describes the main subsets of DCs involved in human cancer and the main changes they undergo during the complex interaction between tumor cells and the immune system. By providing detailed lists of surface molecules and cytokines that are involved in the process, this report may guide the planning of flow cytometric studies addressing DCs in different types of human cancer. It also provides some tips and tricks that may support the setting of experimental procedures.

AUTHOR CONTRIBUTIONS

Claudia Carenza: Conceptualization; data curation; writing-original draft. **Sara Franzese:** Conceptualization; data curation; writing-original draft. **Francesca Calcaterra:** Data curation; writing-original draft. **Domenico Mavilio:** Conceptualization; funding acquisition; writing-review and editing. **Silvia Della Bella:** Conceptualization; data curation; funding acquisition; writing-original draft; writing-review and editing.

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