Differential expression of major histocompatibility complex class Ia, Ib and II molecules on monocytes and monocyte-derived dendritic and macrophagic cells

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Differential Expression of Major Histocompatibility Complex Class Ia, Ib and II Molecules on Monocytes and Monocyte-Derived Dendritic and Macrophagic Cells

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ABSTRACT: Blood monocyte derived antigen presenting cells (APC) such as dendritic cells and macrophages are considered as major promising tools for antitumoral immunotherapy. In order to contribute to their phenotype characterization, we have precisely investigated their levels of expression of MHC class Ia, Ib (HLA-G) and II molecules using mainly flow cytometry quantification assays. APC were generated from monocytes cultured for 7 days in the presence of GM-CSF and IL-4 or M-CSF. These cells, which exhibited known morphological and immunological features of dendritic cells and macrophages respectively, were evidenced to display high expression of MHC class Ia and class II antigens in comparison to that found in monocytes. Dendritic cells and macrophages thus expressed 2-fold more and 4-fold more MHC class Ia molecules and 5-fold and 3-fold more MHC class II DR molecules than parental monocytes. In addition, expression of MHC class II DP and DQ molecules, not or only barely detected in monocytes, was clearly demonstrated in the two kinds of APC. In contrast, monocytes, dendritic cells and macrophages failed to express MHC class Ib HLA-G antigen. The up-regulation in monocyte-derived APC of MHC class Ia and II molecules mediating the presentation of antigen peptides to lymphocytes fully supports the interest of such APC in antitumoral immunotherapy. *Human Immunology 60, 591–597 (1999).* © American Society for Histocompatibility and Immunogenetics, 1999. Published by Elsevier Science Inc.

KEYWORDS: dendritic cells; macrophages; quantification; MHC; HLA-G

ABBREVIATIONS

APC	antigen presenting cell
MHC	major histocompatibility complex
MoAb	monoclonal antibody
IFN	interferon
CTL	cytotoxic T lymphocyte
GM-CSF	granulocyte macrophage-colony stimulating factor
M-CSF	macrophage-colony stimulating factor
IL	interleukin

INTRODUCTION

Dendritic cells and macrophages are professional antigen presenting cells (APC) that play a major role in the initiation and development of the immune response [1]. Dendritic cells have thus the capacity of presenting antigenic peptides to naive T lymphocytes whereas macrophages can stimulate memory or activated T cells. These cellular functions critically require involvement of membrane proteins belonging to the major complex histocompatibility (MHC) system. Such a system comprizes a set of polymorphic genes; most of them encodes surface glycoproteins that present antigenic peptides to T cells. The three products of MHC class Ia genes (HLA-A, -B and -C) and those of MHC class II genes (HLA-DR, -DP and -DQ) thus interact with CD8+ and CD4+ lympho-

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cytes, respectively [2]. HLA-G, the product of a class Ib gene, is also thought to display a capacity of presenting antigenic peptides [3]. In addition, HLA-G can inhibit the function of NK cells directly [4] or indirectly through induction of cell surface expression of HLA-E [5], another MHC class Ib molecule; it therefore likely plays a major role in the maternal immune tolerance towards semi-allogenic foetal tissues. Both dendritic cells and macrophages have been demonstrated to express the products of MHC class Ia and II genes. HLA-G, initially described in placenta, has also been found in monocytic U937 cells treated by some cytokines such as granulocyte macrophage- colony stimulating factor (GM-CSF) and interferon-γ (IFNγ) [6].

Owing to their immunological function, dendritic cells and macrophages can be considered as major actors of the immune anti-tumoral response. Indeed, they can present tumor-related antigens to autologous T cells [7], thus allowing the development of a CD8+ T lymphocyte-mediated cytotoxicity (CTL) towards tumor cells. Recently, the use of dendritic cells or macrophages has been recognized in clinical immunotherapy programs against malignancies [8, 9]. The basis of such protocols is to pulse APC cells of patients *ex vivo* with tumor-associated proteins and thereafter to reinject these cells in order to favor the induction of a major specific anti-tumor response [10]. Preliminary positive results of this vaccinal therapy have already been reported in some cancers [8, 11].

APC-related cancer immunotherapy needs high numbers of dendritic cells or macrophages. Macrophages can be obtained from blood monocytes whereas dendritic cells can be generated using cultures from bone marrow [12] or peripheral CD34+ progenitor cells or from peripheral blood mononuclear phagocytes [13]. This last source is probably the most convenient and thus allow to get functional dendritic cells from monocytes cultured in the presence of both GM-CSF and interleukin 4 (IL-4) for 7 days. Precise phenotypic characterization of dendritic cells and macrophages generated from blood monocytes in view of vaccinal therapy is however required. In this context, the present study was designed to carefully investigate levels of MHC class Ia and II molecules in monocytes and monocyte-derived APC using flow cytometric quantification assays since these MHC antigens play a major role in the function of APC as reported above. In addition, HLA-G expression, that has already been reported in cytokine-treated monocytic U937 cells, was also analysed in monocyte-derived macrophages and dendritic cells at basal state and after stimulation with some cytokines.

MATERIAL AND METHODS

Reagents

Cytokines. GM-CSF (specific activity: 1.82 10^8 U/mg), IL-4 (specific activity: 4.1 10^7 U/mg), Macrophage colony stimulating factor (M-CSF) (specific activity: 1.75 10^6 U/mg) and tumor necrosis factor (TNFα) (5.88 10^7 U/mg) were purchased from Genzyme (Cambridge, MA).

Monoclonal antibodies (MoAbs). The MoAbs used in this work are listed in Table 1 and were from commercial sources except MoAb D1/12 directed against HLA-DR proteins and MoAb 87G recognizing membrane bound and soluble HLA-G molecules, which were kindly provided by ETS (Rennes, France) and Dr. D.E. Geraghty (Fred Hutchinson Cancer Research, Seattle,Washington, USA), respectively.

Cell Culture

Macrophage and dendritic cell cultures were processed as previously described [14]. Briefly, mononuclear cells were obtained by Ficoll gradient (d = 1.077) (Nycomed, Pharma AS, Norway), centrifugation of blood buffy coat from healthy volunteers. They were then plated (1.10^7/3 ml per well) into 6-well culture plates (Costar, Cambridge, MA) in RPMI 1640 medium supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol and 10% fetal calf serum. After 2 h-incubation at 37°C in humidified 5% CO_2, nonadherent cells were removed and adherent cells were further cultured during 7 days with 800 U/ml GM-CSF and 500 U/ml IL-4 or with 50 U/ml M-CSF.

Flow Cytometry Staining

Direct and indirect immunofluorescence labelling were performed as previously decribed [6]. Prior labelling, in order to block Fc receptors of immunoglobulins, cells were first incubated for 60 min in 5% human AB serum. The secondary antibody used for indirect staining was a $F(ab')_2$ goat anti-mouse IgG antibody conjugated with

TABLE 1 List of the MoAb used for flow cytometry analysis

Marker	MoAb	Source
CD1a	T6/OKT6	Ortho
CD14	RM052	Immunotech
CD80	MAB104	Immunotech
MHC class I antigens	W6/32	Sigma
HLA-G antigens	87G	Dr. D.E. Geraghty (Seattle)
HLA-DR antigens	D1/12	ETS (Rennes)
HLA-DP antigens	B7/21	Becton Dickinson
HLA-DQ antigens	HK19	Sigma

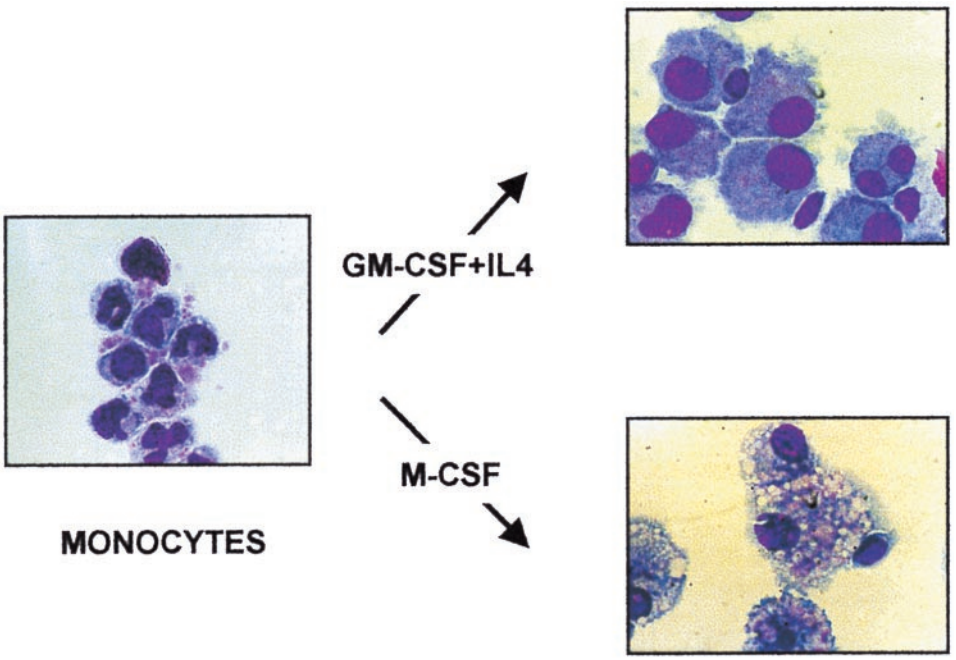

FIGURE 1 Microscopic analysis of monocytes (on the left) and monocyte-derived cells (on the right) stained with May-Grünwald Giemsa coloration. At day 7, monocytes cultured with GM-CF and IL-4 or M-CSF present morphologic features characterizing dendritic cells and macrophages, respectively.

phycoerythrin (Immunotech, Marseille, France). For each labelling, a negative control was performed in parallel using an isotypic irrelevant antibody. Fluorescence was detected by a Cytoron Absolute flow cytometer (Ortho Diagnostic, Roissy, France).

Flow cytometric quantitative analysis of MHC class I and II molecules (DR, DP, DQ) was performed using the "quantitative immunofluorescence indirect kit" (Qifikit) (Dako, Glostrup, Denmark). The procedure consisted to label cell samples with primary mouse monoclonal antibody directed against the antigen of interest at saturating concentrations. An irrelevant mouse monoclonal antibody was used in parallel as negative control. Then cell samples and calibration beads, precoated with different, well defined quantities of mouse antibodies directed against CD5, were labelled with fluorescein- conjugated anti-mouse secondary antibody and analyzed by flow cytometry. The mean fluorescence intensity recorded from cells and from beads are correlated with the number of bound primary antibodies molecules. The results concerning calibration beads were used for the construction of the calibration curve (mean fluorescence intensity (MFI) versus antibody binding capacity units), which allowed us to calculate by interpolation the number of antigenic sites present at the surface of cells. This method enables to detect a minimum threshold of 4000 sites/cell [15].

Immunocytochemical Staining

Cells were centrifuged onto glass slides and fixed for 10 min in cold acetone. Cells were then incubated in 5% human AB serum to eliminate non specific binding. Endogeneous peroxydase was inhibited in 3% hydrogen peroxyde for 5 min. Cells were then incubated with 87G MoAb (10 μg/ml). An IgG2a MoAb was used as negative control. Staining was then done using an avidin biotin peroxydase immunocytochemistry kit (LSAB2 kit peroxydase, Dako).

RESULTS

Morphologic and Phenotypic Characterization of Monocyte-Derived Cells

Adherent cells cultured in the presence of GM-CSF and IL-4 for 7 days differentiated in non-adherent clusters of cells with typical morphological features of dendritic cells, i.e. cytoplasmic projections on the periphery and large irregular shaped nuclei (Fig. 1). Flow cytometry analysis of these cells demonstrated that they poorly expressed CD14 (13.7% ± 14.2% of positive cells), and strongly expressed CD1a (84.4% ± 12.9%) as previously described for monocyte-derived dendritic cells. In contrast, initial mononuclear adherent cells were CD14 positive (77% ± 6.2) and CD1a negative (2.5% ± 2.6%).

Cells cultured in the presence of M-CSF for 7 days differentiated in adherent clusters. These cells have bean-

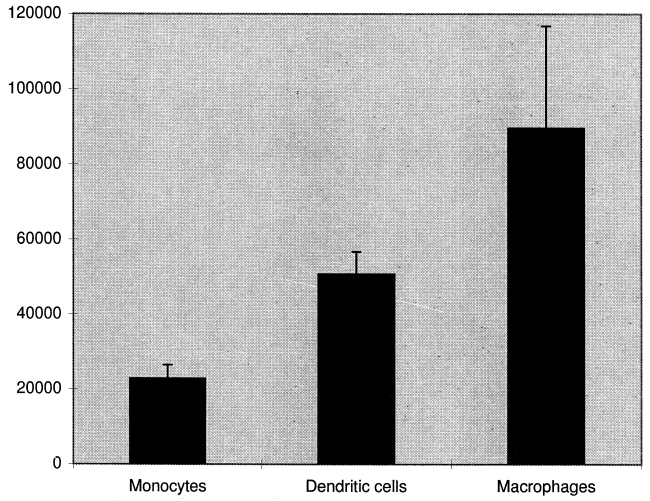

FIGURE 2 Up-regulation of MHC class I molecules on dendritic cells and macrophages. Columns represent the mean ± SD (bar) of at least three independent experiments in each group.

shaped nuclear and finely granular cytoplasm containing phagocytic vacuoles and therefore exhibit features consistent with a macrophage morphology (Fig. 1). In addition, they remained CD14 positive (73.8% ± 22.4%) and CD1a negative (4.1% ± 4.1%). Moreover CD80, a macrophage differentiation marker [1], not expressed in monocytes (2.7% ± 0.7% of positive cells), was strongly expressed in M-CSF-treated cells (72.4.% ± 15.6%).

Taken together, the data of the morphology and phenotypic analysis of monocyte-derived cell cultures therefore clearly indicate that, in agreement with previous results, addition of GM-CSF and IL-4 yielded to dendritic cells whereas that of M-CSF resulted in macrophagic cells.

Expression of MHC Class I Antigens

Monocytes, dendritic cells and macrophages were found to express MHC class I molecules as indicated by immunostaining with the antibody W6/32 that recognizes the three types of HLA class Ia molecules. Quantification of these antigens demonstrated that their amounts were augmentated in dendritic cells and macrophages by a 2-fold and 4-fold factor in comparison with the levels found in initial monocytes (Fig. 2).

The class Ib HLA-G antigen was not detected in both monocytes, dendritic cells and macrophages using flow cytometry (Fig. 3 B, C, D) or immunocytochemical staining (data not shown). In contrast, it was clearly evidenced in choriocarcinoma Jeg 3 cells (Fig. 3A) used here as positive controls [16]. In addition, treatment of dendritic cells or macrophages for 48 h with different cytokines alone (IFNγ, IL2, GM-CSF), or in combination (IFN+IL2, IFN+GM-CSF) was not found to induce HLA-G expression in both immunocytochemistry and flow cytometry assays.

Expression of MHC Class II Antigens

MHC class II DR antigens were present at substantial levels in monocytes whereas the amounts of DP and DQ molecules were at the limit or below the threshold of the method used (4000 antigen sites/ cell) (Table 2). Dendritic cells showed enhanced levels of HLA-DR, -DP and DQ molecules. Indeed, the amount of HLA-DR molecules detected at the surface of dendritic cells was about 5-fold more elevated than that present on monocytes whereas HLA-DQ and HLA-DP antigen site numbers were about 4 and 7 fold more higher than the threshold of the method. Macrophagic cells were also found to display enhanced expression of HLA-DR, -DQ and -DP antigens when compared to the monocytes from which they derived (Table 2); in particular, the expression of HLA-DR molecules was about 3-fold increased in the macrophagic cells. Comparison of the number of HLA-class II molecules between dendritic cells and macrophages revealed however that the former cells displayed about 1.5 for DR , 3 for DP and 2 for DQ more antigen sites.

DISCUSSION

The use of APC like dendritic cells and macrophages is thought to represent a promising tool in the immunotherapy of cancers. A common source of such APC consists in peripheral blood monocytes *in vitro* exposed to cytokines [13] and reinfused to patients. In the present study, in order to precisely characterize the phenotype of these APC, we have carefully investigated levels of MHC molecules in dendritic cells and macrophages generated from blood monocytes, using mainly a quantitative immunofluorescence indirect assay. In agreement with previous studies, the cytokines used for the obtention of APC were GM-CSF and IL-4 (for dendritic cells) and M-CSF (for macrophages). Analysis of the cells we obtained demonstrated that they displayed morphological and immunological features that characterized monocyte-derived APC (i.e., high expression of CD1a and down-regulation of CD14 for dendritic cells and elevated expression of both CD14 and CD80 for macrophages).

We demonstrated that both MHC class Ia and class II molecules were strongly up-regulated in monocyte-derived dendritic cells and macrophages. In particular, the number of antigenic sites was increased by an approximatively 5-fold factor for HLA-DR molecules on dendritic cells when compared to parental monocytes; similarly, class Ia molecules in macrophages were augmentated by a 4-fold factor in comparison with

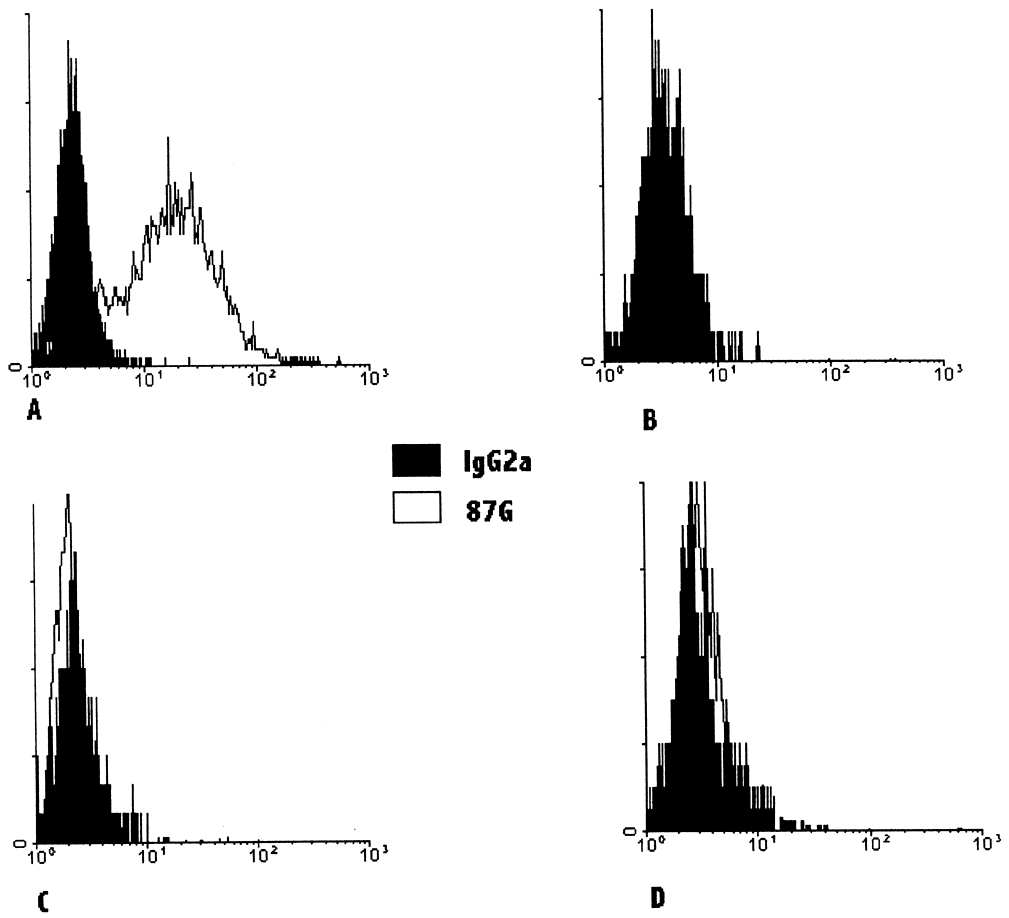

FIGURE 3 Flow cytometric analysis of HLA-G molecules on cell surface on Jeg 3 cells (A), monocytes (B), dendritic cells and macrophages (D). Staining was performed using the MoAb 87G. Results are expressed as histograms of cell number on the ordinate (linear scale) versus fluorescence intensity (log scale) on the abscissa. The data shown are representative of five independent experiments.

monocytes. Such an marked increased expression of MHC antigens in APC has already been reported [17, 18] although, in contrast to our study, the other reports did not accurately quantify the number of MHC molecules. Interestingly, this overexpression well correlates with the known functional role of APC in the immune response. Indeed, both dendritic cells and macrophages have been evidenced to present exogenous or endogenous antigenic peptides to lymphocytes in association with MHC class Ia and class II molecules and are thus able to trigger MHC class Ia- and MHC class II-restricted immune responses [18, 19]. In contrast monocytes, that displayed lower expression of MHC class Ia and class II

TABLE 2 Comparative quantitation of MHC class II antigens on monocytes and monocyte-derived dendritic cells and macrophages

	MHC class II antigenic site numbers			Induction factor	
	Monocytes	Dendritic cells	Macrophages	DC	Mac
DR	20.6 (±1.8)	95.1 (±28.4)	60.8 (±16.0)	4.6	2.9
DP	5.0 (±1.8)	34 (±13.0)	12.6 (±4.8)	6.8	2.5
DQ	<4	17.1 (±7.6)	8.6 (±2.3)	>4.2	>2.1

Values represent the number of MHC class II antigen sites per cell. The data, expressed as 10^3 antigenic sites per cell, are the means ± SD of at least three independent determinations. Induction factor is defined as the ratio of MHC class II molecules on dendritic cells (DC) and macrophages (Mac) versus monocytes.

molecules, are less effective in inducing immune responses such as the stimulation of allogeneic lymphocytes or the generation of CTL [20]. Up-regulation of MHC antigens in monocyte-derived cells is therefore fully consistent with the potential role of these APC cells in the immunotherapy of cancers.

Comparison of the number of MHC molecules on dendritic cells and macrophages indicates that the higher levels of MHC class Ia and class II antigens were observed in macrophages and dendritic cells, respectively. These data favor the idea that MHC class Ia-restricted responses may be triggered in a more efficient way by macrophages whereas the MHC class II -related responses may be preferentially handled by dendritic cells. The fact that blood monocyte-derived dendritic cells have been recently demonstrated to be more effective than macrophages in inducing the MHC class II antigen-related mitotic response of allogeneic peripheral blood lymphocytes supports, at least in part, this hypothesis [18].

Molecular mechanisms involved in the up-regulation of MHC class Ia and class II molecules in monocyte-derived APC remain to be determined. Several factors acting on MHC gene expression have been described. In particular, CIITA encodes a non-DNA binding transactivator that controls the levels of DP, DQ and DR antigens and that is also thought to play a role in the regulation of MHC class Ia molecules [21]. Since MHC class Ia and class II antigens were evidenced to be coordinately induced in both dendritic cells and macrophages when compared to parental monocytes, it can be hypothesized that CIITA may be involved in such a regulation of MHC molecules. In addition, it is noteworthy that expression of CIITA has recently been shown to be increased in response to IFN through a JAK-STAT signaling pathway [22], that is also shared by GM-CSF, a cytokine used in this study for the obtention of monocyte-derived dendritic cells.

In contrast to MHC class Ia and class II molecules, the class Ib HLA-G antigen, involved in immunotolerance processes but also thought to be able to present antigenic peptides [3], was not evidenced at the protein levels in both monocytes and monocyte-derived APC. This absence of HLA-G expression was found both at the cell surface by flow cytometry assays and in the cytoplasm by immunocytochemistry. Similarly, we have recently failed to demonstrate the expression of HLA-G using the monoclonal antibody 87G in various hematological cells although basal levels of HLA-G mRNAs were found [6]. The absence of detectable HLA-G at the membrane level and in the cytoplasm in dendritic cells and macrophages argues against an important role of this MHC molecule in the function of these APC in the immune response. In addition, HLA-G was not found to be induced in monocyte-derived APC by several cytokines, including IL2 and IFN. In contrast, such cytokines have been found to up-regulate HLA-G levels in the monocytic U937 cells [6]. The reasons of such a discrepancy are rather unclear, but could be related to the tumoral nature of U937 cells.

In summary, accurate determination of MHC molecules in monocyte-derived APC using mainly a quantitative flow cytometric assay demonstrated that both dendritic cells and macrophages displayed marked induction of HLA class Ia and class II (DP, DQ and DR) molecules when compared to parental monocytes, whereas they failed to present detectable levels of the class Ib, HLA-G antigen. These results are fully consistent with the known role of such APC in the initiation of the immune response, including antitumoral response, that critically requires involvement of MHC class Ia and class II molecules.

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