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Barbara Morandi; ... et. al

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Distinctive Lack of CD48 Expression in Subsets of Human Dendritic Cells Tunes NK Cell Activation[1]

Barbara Morandi,* Roberta Costa,† Michela Falco,‡ Silvia Parolini,§ Andrea De Maria,¶ Giovanni Ratto,‖ Maria Cristina Mingari,*¶ Giovanni Melioli,‡ Alessandro Moretta,¶# and Guido Ferlazzo*[2]**

CD48 is a glycosyl phosphatidylinositol anchor protein known to be virtually expressed by all human leukocytes. Its ligand, 2B4, is a signaling lymphocyte activation molecule-related receptor involved in NK cell activation. Because dendritic cells (DCs) are strong inducers of NK cell functions, we analyzed the expression of CD48 in different human DC subsets. We observed that monocytes differentiating in DCs promptly down-regulate CD48. Similarly, DCs isolated from inflamed lymph nodes generally do not express CD48. Plasmocytoid DCs do not express CD48 either, whereas myeloid DCs harbored in blood, bone marrow, and thymus express it. In addition, we showed that CD48 expression in DCs affects NK cell functions during NK/DC cross-talk, because NK cells obtained from normal donors and from X-linked lymphoproliferative disease patients are, respectively, triggered or inhibited by DCs expressing surface CD48. Remarkably, IFN-γ production by lymph node NK cells, in contrast to blood NK cells, can be negatively modulated by 2B4/CD48 interactions, indicating a 2B4 inhibitory pathway in lymph node NK cells. Therefore, the CD48 deficiency of DCs harbored in inflamed lymph nodes that we report in this study might be relevant to successfully activate lymph node NK cells in the early phase of the immune response. Our results show that distinct subsets of human DCs, differently from all other mononuclear hemopoietic cells, specifically do not express CD48. Moreover, the expression of CD48 depends on the anatomic location of DCs and might be related to the tissue-specific 2B4 function (activating or inhibitory) of the NK cells with which they interact. *The Journal of Immunology,* **2005, 175: 3690–3697.**

The molecule CD48 is a glycosyl phosphatidylinositol anchor protein of the CD2 family member widely expressed on human leukocytes (1). In lymphocytes, the expression of its ligand, 2B4, is confined to all NK cells and a subpopulation of T cells (2). In T cells, ligation of 2B4 does not lead to cell activation (2), and the role of CD48/2B4 interactions seems to be limited to a costimulatory-like function for the survival of memory $CD8^+$ cells (3). On the contrary, the engagement of 2B4 on human NK cells results in the delivery of an activating signal, sufficient to trigger cytolytic activity and cytokine secretion (2, 4, 5). Exceptions to this rule are represented by NK cells at an early stage of differentiation (6) or NK cells derived from patients affected by X-linked lymphoproliferative disease (XLP)[3] (7, 8), where the signaling lymphocyte activation molecule-associated protein (SAP) is missing, and 2B4 delivers an inhibitory signal when engaged by CD48. SAP, also known as SH2D1A, is a regulator of the 2B4-associated signal transduction pathway (9, 10).

Dendritic cells (DCs) have recently been identified as strong activators of NK cells in the early phase of the immune response, before an adaptive immune response is evoked and T cell-derived cytokines become available (11–14). Both DC-derived soluble mediators and cell-to-cell contact seem able to act upon NK cell functions. Among the former, IL-12, IL-18, and type I IFNs are important for IFN-γ production or cytolytic functions of human NK cells, whereas a role in NK cell triggering has been advocated for mouse DC-derived IL-2 (15, 16). Some recent data would also suggest that IL-15 produced by human mature DCs has a major role in NK cell proliferation and long-term survival (17, 18). The contribution of cell contact during NK/DC cross-talk has also been investigated, as has triggering of NK cell-activating receptors by DCs expressing the putative ligands (12, 17–20). In particular, we have previously demonstrated that the activating receptor NKp30 is mainly involved in the recognition and lysis of human immature monocyte-derived DCs (mono-DCs) by autologous NK cells, whereas other activating receptors and coreceptors expressed by NK cells, including 2B4, play a limited role in DC recognition (14). This prompted us to analyze the expression of its ligand, the pan-leukocyte Ag CD48, in in vitro-derived DC subsets and in DCs directly isolated from different human lymphoid organs.

We report in this study that opposite expression of CD48 occurs in distinct subsets of human DCs, and that this expression impacts on DC-dependent NK cell activation.

Materials and Methods

Tissue samples

Reactive, swelled, nonmetastatic lymph nodes were obtained from 17 patients, who underwent thoracic surgery for non-small cell lung cancer.

*Istituto Nazionale Ricerca sul Cancro, Genoa, Italy; †Ospedale Santi Antonio e Biagio, Cesare e Arrigo, Alessandria, Italy; ‡Istituto Giannina Gaslini, Genoa, Italy; §University of Brescia, Brescia, Italy; ¶University of Genoa, Genoa, Italy; ‖Ospedale Santa Croce e Carle, Cuneo, Italy; #Centro di Eccellenza Ricerca Biomedica, Genoa, Italy; and **University of Messina, Messina, Italy

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[2] Address correspondence and reprint requests to Dr. Guido Ferlazzo, Laboratory of Immunology, Istituto Nazionale per la Ricerca sul Cancro, L.go R. Benzi 10, 16132 Genova, Italy. E-mail address: guido.ferlazzo@istge.it

[3] Abbreviations used in this paper: XLP, X-linked lymphoproliferative disease; DC, dendritic cell; mono-DC, monocyte-derived DC; MPB, G-CSF-mobilized peripheral blood; SAP, signaling lymphocyte activation molecule-associated protein; ILT, Ig-like transcript.

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After removal of necrotic tissues, lymph nodes were dissociated mechanically and enzymatically. Cells were collected and filtered through a coarse wire grid to exclude undissociated fragments. Debris and dead cells were then eliminated using a Ficoll-Hypaque discontinuous gradient. Single-cell suspensions obtained at the gradient interface were extensively washed and then analyzed by flow cytometry or used for functional experiments.

Aliquots of bone marrow and thymus samples were obtained, respectively, from normal donors for transplantation and from thymectomy associated with heart surgery and were processed as described above.

All tissue sample collections were obtained after informed consent of the donors, and all procedures were approved by our institutional ethic committee.

Cell isolation and culture

Whole blood from healthy donors and leukocyte concentrates served as sources of PBMC isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech). Mono-DCs were generated from $CD14^+$ PBMC isolated by positive selection using anti-CD14 microbeads and a magnetic separator (Miltenyi Biotec). Briefly, 5×10^5 $CD14^+$/ml were cultured in 24-well plates with RPMI 1640/10% FCS (Cambrex) or 2% autologous serum and recombinant human IL-4 and recombinant human GM-CSF (Euroclone) were added to final concentrations of 500 and 1000 U/ml, respectively, on days 0 and 3. In some experiments human recombinant Flt3 ligand, c-Kit ligand, GM-CSF, TNF-α (25 ng/ml), and IL-4 (100 IU/ml; Euroclone) were added, and medium was changed by demidepletion twice a week. $CD34^+$ peripheral blood hemopoietic precursor cells represented another source of DC. Peripheral blood hemopoietic precursor cells were isolated from chemotherapy and G-CSF-mobilized peripheral blood (MPB) samples obtained by leukapheresis from metastatic stage IV breast cancer patients undergoing collection of MPB for hemopoietic rescue after high dose chemotherapy. MPB were thawed in the presence of DNase (100 U/ml; Genentech) to minimize clumping and optimize recovery (the procedure does not affect the cell surface phenotype). $CD34^+$ cells were sorted by Qbend10 anti-CD34 mAb (Immunotech), goat anti-mouse IgG microbeads, and magnetic separator (Miltenyi Biotec). Purified $CD34^+$ cells (purity of the cell population was routinely >85%) were plated at 10^5 cells/ml in 24-well plates in medium supplemented with human recombinant Flt3 ligand, c-Kit ligand, GM-CSF, TNF-α (25 ng/ml), and, in some experiments, IL-4 (100 IU/ml; Euroclone). Medium was changed by demidepletion twice a week. After 2 wk, $CD34^+$cell-derived DCs (CD34-DCs) were sorted using anti CD1a mAb (American Type Culture Collection), goat anti-mouse IgG microbeads, and magnetic separator (Miltenyi Biotec) from the whole $CD34^+$ cell-derived population.

NK cells were negatively selected by the NK Cell Isolation kit (Miltenyi Biotec). The percentage of NK cells in the isolated population was then evaluated using anti-CD3 and anti-CD56 mAbs (Coulter Scientific) and flow cytometry. Recombinant IL-2 (100 IU/ml; Proleukin; Chiron) and PHA (1 μg/ml) were added to obtain a polyclonal NK cell population.

Flow cytometric analysis

Analysis of cell surface markers was performed using the following mAbs in immunofluorescence assays: anti-Ig-like transcript (ILT) 3, anti-CD56, anti-CD3, anti-CD14, anti-CD33 (Immunotech Coulter), anti-CD123 (Miltenyi Biotec), anti-CD11c and anti-CD48 (IgM; BD Pharmingen), anti-2B4 (MA344, IgM; produced in our laboratory).

Direct immunofluorescence procedure was performed by adding 1 mg/ml human γ-globulin (human therapy grade) to cell suspension to block nonspecific FcR binding. Cells were then labeled with fluorochrome-conjugated mAb for 30 min at 4°C, washed, and analyzed by flow cytometry. Indirect immunofluorescence assays were performed as follows. Cell-nonspecific binding sites were saturated with human γ-globulin, then the relevant mAb was added and incubated for 30 min at 4°C. After extensive washings, FITC- or PE-conjugated, isotype-specific, goat anti-mouse Abs (Southern Biotechnology Associates) were added and incubated for 30 min at 4°C. Negative controls included directly labeled or unlabeled, isotype-matched, irrelevant mAbs. Cells were then washed and analyzed by flow cytometry.

^{51}Cr release assay

To evaluate the role of CD48 expression in DC-dependent NK cell activation, we tested IL-2-activated NK cells from normal donors and XLP patients for their ability to lyse CD34-DCs and mono-DCs (as a negative control) in the presence of blocking mAb directed against CD48 or 2B4. Briefly, 10^6 target cells were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ for 90 min at 37°C, extensively washed, and then cultured for 4 h with effector cells at the indicated ratios. Supernatants were collected, and radioactivity counted by gamma counter (Beckman Coulter). Specific ^{51}Cr release was calculated on the bases of the ratio (sample release − spontaneous release)/(total release − spontaneous release). Assays were performed in triplicate at the indicated E:T cell ratios. To analyze the role of NK cell-activating receptors in the lysis, anti-NKp30 (IgM, F252; provided by D. Pende, Istituto Nazionale Ricerca sul Cancro, Genova, Italy), anti-2B4 (IgM, MA344), and anti-CD48 (IgM; BD Pharmingen) blocking mAbs were added in saturating amounts.

IFN-γ production assay

IFN-γ production by NK cells isolated from peripheral blood and autologous lymph nodes was evaluated by culturing mononuclear cells for 48 h in the presence of recombinant human IL-2 at 500 U/ml (Proleukin; Chiron) and IL-12 at 20 ng/ml (PeproTech). Anti-CD48 mAb (IgM; BD Pharmingen) or isotype-matched irrelevant mAb as a control was added at 10 μg/ml. GolgiStop (containing monensin; BD Pharmingen) was added for the final 6 h of stimulation. Cells were subsequently stained for CD3 and CD56, then fixed using *p*-phormaldeid (4% *p*-phormaldeid in PBS). Cells were permeabilized using saponin (0.1% saponin/1% BSA in PBS) and stained for intracellular IFN-γ (anti-IFN-γ; BD Pharmingen) according to the manufacturer's instructions.

Statistical analysis

To compare IFN-γ production by lymph nodes or peripheral blood NK cells in the presence of anti-CD48 mAb, statistical analysis was performed using the StatView 4.2 program (Abacus Concepts). Statistical significance was evaluated by Wilcoxon signed rank test.

Results

CD48 is not expressed in DCs derived from monocytes, but is partially expressed in DCs derived from $CD34^+$ hemopoietic cell precursors

Human DCs can be derived in vitro from monocytes or hemopoietic cell precursors (21, 22). When monocytes were cultured in GM-CSF and IL-4, they promptly down-regulated CD48 surface expression (Fig. 1*A*). After addition of stimuli that induced complete DC maturation, such as LPS or proinflammatory cytokines, CD48 was not re-expressed (not shown).

Culturing $CD34^+$ cells isolated from peripheral blood in the presence of Flt-3 ligand, c-Kit ligand, GM-CSF, TNF-α, and IL-4 (FKGT4) gives rise to a cell population that includes 30–40% DCs (21, 23). Differently from mono-DCs, DCs obtained from hemopoietic cell precursors partially expressed CD48 (Fig. 1*B*, *upper panel*). DCs obtained using the FKGT4 mixture are expected to derive in part directly from $CD34^+$ hemopoietic precursor cells and in part from cells differentiating toward the monocytic lineage, which, in turn, are driven by IL-4 in DCs. The latter should be closely related to mono-DCs and should be expected to represent the $CD48^{neg}$ fraction of DCs obtained from $CD34^+$ cells. Indeed, omitting IL-4 in the culture resulted in a decreased yield of DCs, but a significantly higher percentage of $CD48^+$ DCs (Fig. 1*B*, *middle panel*). To rule out that the different culture conditions might account for different CD48 expression in DCs, monocytes were also cultured in the presence of FKGT4. As shown in Fig. 1*B*, *lower panel*, mono-DCs cultured in FKGT4 failed to express CD48. Thus, mono-DCs, but only a fraction of CD34-DCs, do not express CD48, indicating heterogeneity of CD48 expression in human DC subsets.

DCs expressing CD48 can trigger NK cells via 2B4

NK cells are able to recognize and lyse immature DCs (24). We have previously reported that recognition and lysis of mono-DCs by NK cells is mainly mediated by NKp30 (12), a natural cytotoxic receptor expressed in NK cells (25, 26), whereas 2B4 as well as other activating receptors are barely or not involved (12). These previous data are consistent with our present findings that the ligand of 2B4, CD48, is not expressed in mono-DC. Because CD34-DCs partially expressed CD48, we investigated whether this expression might have functional consequences in DC-mediated NK

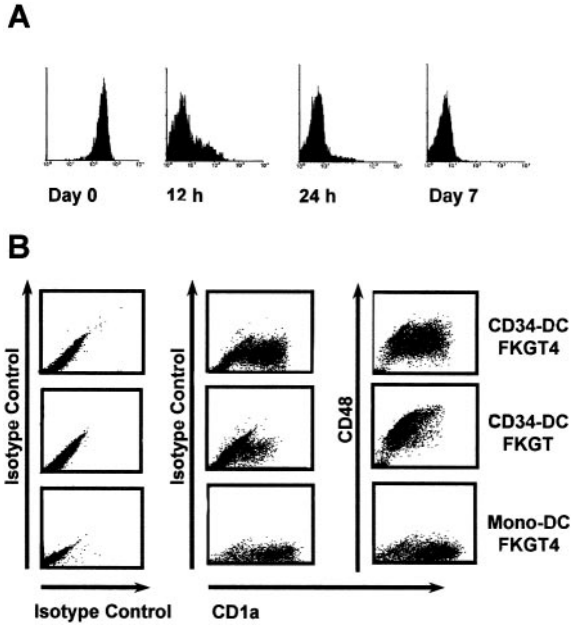

FIGURE 1. CD48 is expressed in CD34-DCs, but not in mono-DCs. *A*, DCs are derived from PBMC in the presence of GM-CSF and IL-4. Monocytes differentiating in DCs promptly down-regulate the expression of CD48 on their surface. Histograms represent CD48 expression of cultured cells at the indicated times. *B*, DCs are derived from peripheral blood $CD34^+$ hemopoietic cell precursors in the presence of FKGT4 (*upper panels*). DCs derived from peripheral blood $CD34^+$ stem cells partially express CD48, and the number of $CD48^+$ cells increases when IL-4 is not used for DC differentiation (FKGT; *middle panels*); under these latter culture conditions, several adherent cells with macrophage morphology were detectable at the end of the culture, indicating that monocytes derived from $CD34^+$ cells were not differentiated in DCs. As a control, monocytes were also cultured in FKGT4 to rule out that the different cytokines used in monocyte and $CD34^+$ cell cultures might account for the differences in CD48 expression (*lower panels*). Results are representative of seven independent experiments with cells obtained from different donors.

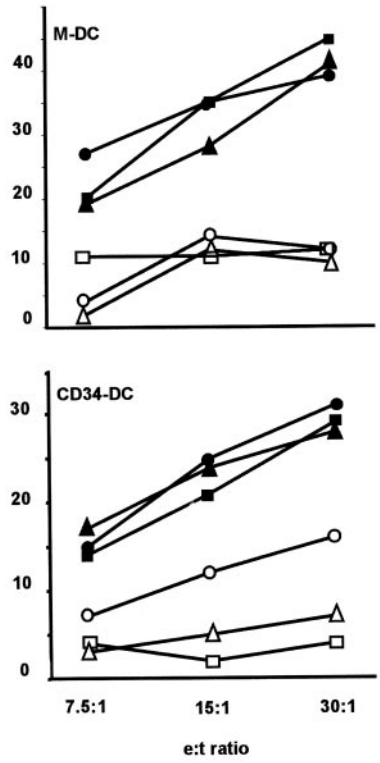

FIGURE 2. Expression of CD48 in DCs increases peripheral blood NK cell activation during NK/DC cross-talk. To evaluate whether the expression of CD48 was functional during NK/DC cross-talk, polyclonal NK cell lines were tested for their cytolytic activity against mono-DCs (not expressing CD48; M-DC) and CD34-DCs (expressing CD48; CD34-DC) in the absence or the presence of the indicated blocking Abs. ●, Isotype control; ○, anti-NKp30 (IgM); ■, anti-2B4 (IgM); ▲, anti-CD48 (IgM); □, anti-NKp30 plus anti-2B4; △, anti-NKp30 plus anti-CD48. As expected, blocking of NKp30 decreased NK cell lysis triggered by both mono-DCs and CD34-DCs. Conversely, NK cell activity was reduced by anti-2B4 or anti-CD48 mAbs (synergizing with anti-NKp30) when tested against CD34-DCs, but not mono-DC. The results shown are representative of three independent experiment, and data are the mean of triplicate determinations.

cell triggering. Monocytes and $CD34^+$ cells were isolated from the same donors, and DCs were derived as described above. CD34-DCs were purified from contaminating cells obtained during $CD34^+$ cell differentiation by magnetic bead sorting using anti-CD1a mAb. Polyclonal NK cells isolated from peripheral blood were then analyzed for their ability to lyse allogeneic DCs in the presence of mAbs specifically blocking NKp30, CD48, and 2B4 receptors. As shown in Fig. 2, DCs derived from either monocytes or $CD34^+$ cells were efficiently lysed by NK cells, and the lysis was mediated primarily by NKp30, but not 2B4. However, when anti-CD48 or anti-2B4 mAbs were combined with anti-NKp30, the two mAb-mediated blocking synergized only in CD34-DCs. This is consistent with previous observations showing that 2B4 can synergize with natural cytotoxic receptors, acting as a coreceptor in human NK cell activation (27). These data demonstrate that the expression of CD48 in DCs affects peripheral blood NK cell triggering during NK/DC cross-talk.

CD48 is expressed in circulating myeloid, but not plasmacytoid, DCs

Because CD48 was detectable in DCs derived in vitro from total hemopoietic cell precursors, we analyzed its expression in ex vivo isolated peripheral blood human DCs. To this end, samples from different donors were stained with fluorochrome-conjugated mAbs directed against ILT-3, CD14, and CD48. DCs have previously been reported to share ILT-3 expression only with monocytes (28, 29). Thus, gating on $CD14^{neg}ILT3^+$ cells enables us to assess CD48 expression in all circulating DCs. It is well established that DCs harbored in human blood can be divided into at least two subsets: myeloid DCs (expressing CD11c, but not CD123) and plasmocytoid DCs (expressing CD123, but not CD11c) (30). Most circulating blood DCs consistently expressed CD48 in the different donors analyzed (Fig. 3*A*). Interestingly, $CD48^+$ DCs were predominantly of myeloid origin, expressing CD11c and the myeloid marker CD33; conversely, cells expressing CD123 (IL-3R) were $CD48^{neg}$ or expressed very low levels (enlargement of detail in Fig. 3*A*).

Because cell culture can affect cell functions and phenotype, we tried to determine whether the in vitro culture of peripheral blood myeloid DCs might affect CD48 expression to the same extent as DCs derived in vitro from monocytes, which down-regulate CD48 within the first 24 h of culture in the presence of GM-CSF and IL-4. Peripheral blood monocytes were removed before culture from mononuclear cells by anti-CD14 magnetic beads. Because circulating DCs were identified as $ILT\text{-}3^+CD14^{neg}$ cells, eliminating monocytes ruled out possible contamination by monocytes down-regulating CD14 during the 48 h of culture in GM-CSF and

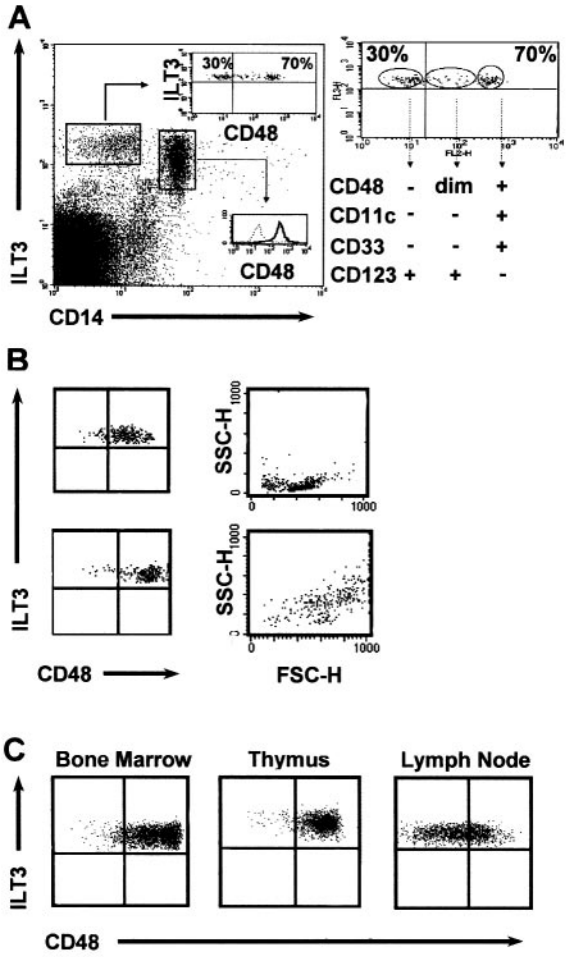

FIGURE 3. CD48 is differentially expressed by subsets of DCs isolated directly from distinct tissues. *A*, PBMC were stained with fluorochrome-conjugated mAbs specific for the indicated markers, which allow distinguishing $CD14^+$/$ILT3^+$ monocytes (homogeneously expressing CD48) and $CD14^{neg}ILT3^+$ DCs. Gating on DCs, the expression of CD48 was evaluated, as was the expression of markers specific for myeloid or plasmocytoid DCs (*upper right inset* and its enlargement). Circulating myeloid DCs express CD48, whereas plasmocytoid DCs mainly do not. The data shown are representative of results obtained in five different donors. *B*, CD48 expression in peripheral blood DCs did not change upon cell culture. CD48 expression in peripheral blood DCs was evaluated, as described in *A*, before (*upper panels*) and after 48 h of culture in the presence of GM-CSF and IL-4 (*lower panels*). For this purpose, monocytes were removed from mononuclear cells before culture to avoid possible contamination by monocytes down-regulating CD14 in the presence of GM-CSF and IL-4. $ILT3^+$ DCs became larger and more granular (*right panels*), but did not down-regulate their CD48 expression (*left panels*). *C*, Single-cell suspensions of mononuclear cells were obtained from human bone marrow, thymus, and reactive lymph nodes. Analysis of CD48 expression was performed by gating on $ILT3^+CD14^{neg}$ cells as shown in *A*. DCs harbored in bone marrow and thymus show surface expression of CD48, whereas only a fraction of DCs isolated from inflamed lymph nodes express it.

IL-4. Upon culture, blood DCs underwent activation: they became larger, more granular, and heterogeneous, as assessed by their cytofluorometric physical parameters (Fig. 3*B*, *right panels*). Nevertheless, culturing blood myeloid DCs for 48 h did not cause down-regulation of CD48 expression (Fig. 3*B*, *left panels*).

These results confirm that circulating myeloid DCs represent a subset of DCs distinct from mono-DCs and that these two subsets of myeloid origin may express different functional molecules, indicating that they might also differ in their NK cell-activating properties.

CD48 is expressed in DCs from primary lymphoid organs, but not from reactive lymph nodes

At early stages of NK cell differentiation, 2B4 functions as an inhibitory, rather than an activating, receptor. This has been identified as a fail-safe mechanism that prevents the killing of normal autologous cells, because developing NK cells express major activating receptors and acquire cytolytic activity before the HLA class I-specific inhibitory receptors are functional (6). Thus, it is conceivable that the expression of CD48 in DCs is an effective mechanism that blocks an undesired lysis of DCs in regions of lymphocyte ontogeny (e.g., bone marrow). Moreover, given the intimate cross-talk between DCs and NK cells, and the ability of these two cell types to rapidly cluster (12, 19, 20), it is also plausible that it is an important mechanism for inhibiting a still unbalanced activity of developing NK cells. We therefore assessed CD48 expression in DCs contained in primary lymphoid organs by analyzing mononuclear cells, isolated from human bone marrow and stained as described above, for circulating blood DCs. As shown in Fig. 3*C*, all DCs present in bone marrow homogeneously expressed CD48, and similar results were obtained in thymic DCs. On the contrary, when the same analysis was performed in inflammatory lymph nodes, most of the DCs isolated from these tissues did not display CD48 (Fig. 3*C*). Similar to peripheral blood, CD48 was not detectable in $CD123^+CD11^{neg}$ plasmocytoid DCs isolated from lymph nodes (not shown). These results show that in primary lymphoid tissue, where undesired activation of immature NK cells should be averted through 2B4 engagement, DCs are well equipped to accomplish this task.

DCs expressing CD48 can inhibit NK cells displaying a 2B4 inhibitory pathway

To verify whether DCs expressing CD48 might provide an inhibitory signal for NK cells displaying a 2B4 inhibitory pathway, polyclonal NK cell lines were derived from XLP patients. NK cells from these immunodeficient patients, as well as NK cells at early stages of development, lack the adaptor protein SAP, which is involved in 2B4 signal transduction. As a consequence, in SAP-deficient NK cells, 2B4 delivers an inhibitory signal when engaged by CD48 (6, 7).

Mono-DCs and CD34-DCs isolated from the same donors were analyzed for their ability to trigger cytolytic activity of XLP-NK cells. CD34-DCs were sorted by anti-CD1a magnetic beads, and as a control, mono-DCs (cultured in FKGT4), which mostly expressed CD1a, were also similarly sorted. XLP-NK cells efficiently lysed mono-DCs (Fig. 4*B*), whereas lower cytolytic activity was consistently observed against CD34-DCs, which express CD48 (Fig. 4*A*). Notably, disrupting CD48/2B4 interactions by the addition of blocking mAb resulted in a significant increase in CD34-DC, but not mono-DC lysis. These experiments demonstrated that CD48 expression in DCs can control the activity of NK cells displaying 2B4 inhibitory pathway.

NK cells isolated from human lymph nodes display a 2B4 inhibitory pathway

Human secondary lymphoid organs have recently been identified as the main sites of interactions between DCs and NK cells (18, 31–33). NK cells harbored in secondary lymphoid organs differ from peripheral blood NK cells in function and phenotype, but homogeneously express surface 2B4 molecules (32). Because we demonstrated in this study that most DCs isolated from reactive

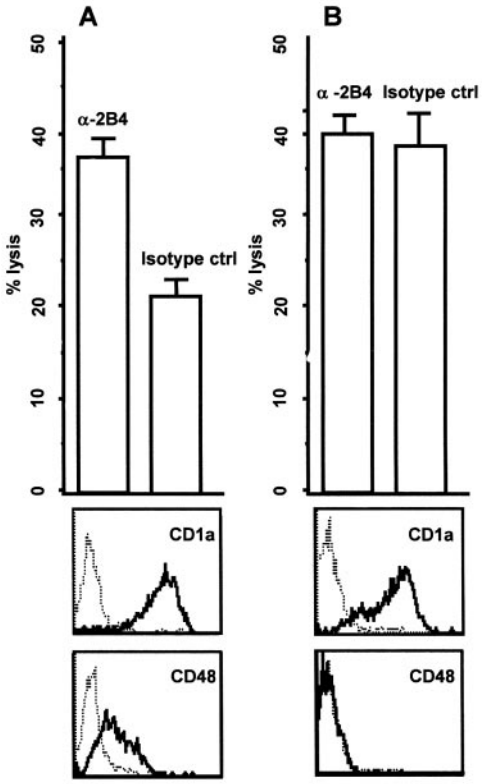

FIGURE 4. DCs expressing CD48 inhibit NK cells characterized by a 2B4 inhibitory pathway. Polyclonal NK cell lines were derived from XLP patients, whose NK cells lack SAP and receive an inhibitory signal when 2B4 is engaged. XLP NK cell lines were tested for their ability to exert cytolytic activity against allogeneic mono-DCs (not expressing CD48) and CD34-DCs (partially expressing CD48). α-2B4, anti-2B4; isotype ctrl, isotype-matched mAb as negative control. Blocking the interactions between CD48 on DCs and 2B4 on NK cells by the addition of blocking Abs resulted in an increase in CD34-DC lysis, reflecting an increase in NK cell activity. As expected, mono-DC lysis was not affected by mAb-mediated blocking. The E:T cell ratio was 20:1. The experiment shown is representative of four independent experiments with two different NK cell lines obtained from two distinct XLP patients. Data shown are the mean of triplicate determinations.

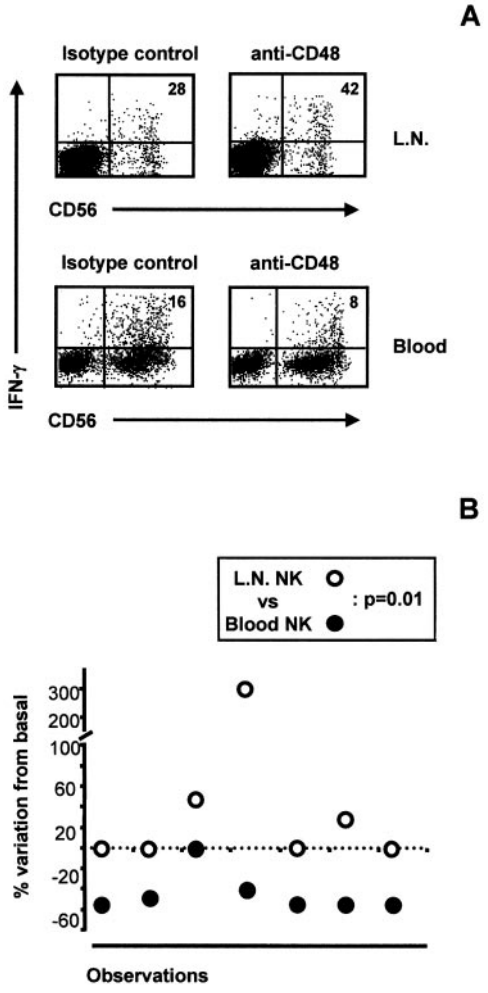

FIGURE 5. 2B4 acts as an inhibitory receptor in NK cells isolated from human lymph nodes. Lymph node and autologous blood mononuclear cells were cultured in the presence of IL-12 and IL-2 for 48 h with isotype-matched irrelevant mAb (isotype control) or anti-CD48 blocking mAb (anti-CD48). IFN-γ production by NK cells was evaluated by intracytoplasmic staining. *A*, A representative experiment of IFN-γ intracytoplasmic staining is shown. Analysis was performed gating on $CD3^{neg}$ cells, and the numbers shown represent the percentage of IFN-γ-producing NK cells. *B*, Analysis of IFN-γ-producing NK cells in lymph node and peripheral blood upon disruption of CD48/2B4 interactions by anti-CD48-blocking mAb. All experiments performed are represented. The percent variation of IFN-γ-producing NK cells in the presence of anti-CD48 mAb compared with isotype control (percent variation from basal) was calculated in blood (●) and lymph node (○) NK cells.

lymph nodes failed to express CD48, we explored whether 2B4 delivers an activating or an inhibitory signal in NK cells isolated from lymph nodes. Freshly isolated lymph node NK cells are not cytolytic, but are highly effective in cytokine production (32). For this reason, cytokine secretion was used as a read-out for 2B4-mediated lymph node NK cell activity. Mononuclear cells, which express CD48, were isolated from human inflamed lymph nodes and autologous blood, then cultured for 48 h in the presence of IL-12, IL-2, and blocking mAb against CD48. Then IFN-γ production was assessed by intracytoplasmic staining in $CD3^{neg}CD56^{+}$ NK cells (Fig. 5*A*). We confirmed that lymph node NK cells are characterized by high IFN-γ production (18, 31–34), comparing for the first time the abilities of lymph node and peripheral blood NK cells to produce IFN-γ. Indeed, the median proportion of lymph node NK cells producing IFN-γ was significantly higher than that of peripheral blood NK cells (33 ± 26 vs 14 ± 14%; $p = 0.0166$). Moreover, 2B4 engagement in lymph node and blood NK cells resulted in opposite outcomes (Fig. 5*B*). In fact, blocking 2B4 engagement in lymph node NK cells did not result in inhibition of IFN-γ production in any sample analyzed, whereas in three of seven experiments, a consistent increase in the proportion of IFN-γ-producing NK cells was observed (Fig. 5*B*). As expected, and in contrast to lymph node NK cells, blocking CD48/2B4 interactions in peripheral blood NK cells always resulted in a decreased proportion of IFN-γ-producing cells, with the exception of one donor in whom no variation was detected (Fig. 5*B*). As shown in Fig. 5*B*, the difference in IFN-γ production between lymph node and blood NK cells upon disruption of the CD48/2B4 engagement had statistical significance ($p = 0.01$).

These results indicate that, differently from peripheral blood NK cells, at least a part of NK cells harbored in human lymph nodes receive an inhibitory signal upon 2B4 engagement. Thus, the above-described absence of CD48 in lymph node DCs might account for a critical tuning of lymph node NK cell functions.

Discussion

CD48 has to date been considered a pan-leukocyte Ag (1). In this study we show that this surface molecule is indeed specifically not expressed in distinct subsets of ex vivo isolated human DCs, namely plasmocytoid DCs and a large part of myeloid DCs harbored in lymph nodes. In addition, CD48 is rapidly down-regulated in monocytes differentiating in vitro toward the DC lineage.

DCs play a pivotal role in the initiation of adaptive immunity, and they have recently also been identified as early activators of the NK cell response (11–18). DCs are highly specialized immune cells, and the specific lack of CD48 expression, unique among hemopoietic cells, suggests functional outcomes in cells expressing the related ligand. In this study we focused on the consequences of CD48 expression in DCs for NK cell functions and found that this expression has relevant effects during NK/DC interactions.

Because DCs are known to be important for the initiation of both T and NK cell responses, the lack of CD48, a ligand for a lymphocyte-activating receptor, may appear paradoxical. Indeed, it is well established that human 2B4 might also deliver an inhibitory signal (6–8), and emerging evidence demonstrates a prevalent inhibitory role of 2B4 in mouse NK cells (35). It should be borne in mind that $CD48^{neg}$ DCs are represented by mono-DCs, which are known to efficiently migrate to secondary lymphoid organs (36–38), by DCs harbored in inflamed lymph nodes, and by plasmocytoid DCs that are also enriched in inflamed secondary lymphoid organs (29). On the contrary, a large fraction of DCs derived from pluripotent hemopoietic stem cells express CD48, and this is consistent with the observation that blood myeloid DCs also express CD48.

The physiological significance of the observed variance of CD48 expression in distinct subsets of DCs may find an explanation in the different functions of 2B4 in distinct NK cell compartments. For example, differently from peripheral blood mature NK cells, engagement of 2B4 by CD48 in NK cells at an early stage of development delivers an inhibitory signal. This has been elucidated as a mechanism to block potentially harmful cells; in developing NK cells, the expression of 2B4 precedes the expression of HLA class I-specific inhibitory receptors and is presumed to replace these receptors until the stage at which they are surface expressed (6). The delivery of an inhibitory signal is traceable to late expression of SAP, an adaptor molecule whose expression is mandatory for an activating signal mediated by 2B4 (10). In line with this view, in vivo treatment with anti-CD48 mAb after bone marrow transplantation significantly delayed hemopoietic recovery, highlighting an important function for CD48 in the hemopoiesis of the bone marrow compartment (39). Consistent with these previous reports, we found that DCs harbored in human bone marrow, i.e., where immature NK cells are developing, express CD48. At the same time, we demonstrated that the cytolytic activity of NK cells derived from XLP patients, which lack SAP as do immature NK cells, could be inhibited by CD34-DCs expressing CD48. Given the recently described intimate cross-talk between NK cells and DCs, bone marrow DCs would be well suited to block unwanted activation of NK cells during the early steps of their differentiation.

Another NK cell compartment where 2B4 might deliver an inhibitory signal is represented by uterine decidua NK cells. A recent report comparing uterine decidual NK cells with peripheral blood NK cells by microarray analysis showed that SAP is expressed in blood, but not in decidua, NK cells (40). NK cells constitute 50–90% of lymphocytes in human uterine decidua in early pregnancy, are characterized by a $CD56^{bright}CD16^{neg}$ phenotype (40, 41), and have been thought to play a role in implantation and pregnancy, at least in early gestation. Remarkably, this subset of decidua NK cells has been shown to share intimate contact with $CD14^{+}$/HLA-DR^{+}/DC-$SIGN^{+}$/$CD83^{neg}$ APCs, which can further differentiate into $CD14^{neg}CD25^{+}CD83^{+}$ mature DCs in the presence of inflammatory cytokines (42). It is noteworthy that, among human DC subsets, DC-SIGN is expressed only in mono-DCs (30), which promptly down-regulate CD48 expression during their differentiation (Fig. 1*A*). Together, these findings appear to constitute another fail-safe mechanism to avoid unwanted NK cell activities in the decidua. This inhibitory effect would subsequently be removed during an inflammatory response causing differentiation of decidua $CD14^{+}CD83^{neg}$ cells in $CD14^{neg}CD83^{+}$ mono-DCs, which do not express CD48.

We have recently characterized NK cells harbored in human secondary lymphoid organs (32) and identified these regions as a main site for DC-mediated NK cell activation (18, 31). NK cells isolated from human normal lymph nodes show a phenotype reminiscent of decidual NK cells, i.e., $CD56^{bright}CD16^{neg}$ (31, 32, 41). Interestingly, our present results also show that the activity of human lymph node NK cells can be down-regulated by the engagement of their 2B4 with CD48, suggesting that at least a part of these NK cells display an inhibitory 2B4 pathway. Additional investigations, beyond the aims of this study, will be needed to elucidate the physiological significance of this observation. Nevertheless, based on our present data, it is conceivable that secondary lymphoid organs may also represent a site for immature NK cell differentiation. This claim would be consistent with the absence of Ig-like killer inhibitory receptors in human lymph node and tonsil NK cells (32), i.e., similar to immature NK cells obtained in vitro from hemopoietic cell precursors (6).

Because NK cells are particularly enriched in the parafollicular area of the T cell zone (18, 34), where they colocalize with DCs, the absence of CD48 in a fraction of DCs isolated from inflamed lymph nodes is also suggestive of a regulatory mechanism based on the down-regulation of CD48 in DCs along the course of an immune response. This would help to sustain early NK cell cytokine production, which can be effectively mediated by intimate NK/DC interactions in secondary lymphoid organs (18, 31, 33, 43) and therefore affect the subsequent T cell polarization.

NK cells displaying a 2B4 inhibitory pathway have been associated, at least in humans, with the absence of SAP; given the present results, analysis of lymph node NK cells at the clonal level for the expression of SAP and other proteins necessary for signaling through signaling lymphocyte activation molecule-related receptors may prove to be an interesting avenue of investigation.

Our findings that DCs migrated or resident in inflamed lymph nodes do not express CD48 together with the evidence that subsets of T cells also express 2B4 (44) might call into question the consequences of CD48 down-regulation in DCs migrating in secondary lymphoid organs to induce an effective adaptive immune response. Nevertheless, 2B4 is expressed only in memory T cells (3, 44), and it is, hence, conceivable that T cell priming would hardly be affected by the absence of CD48 in Ag-presenting DCs.

Finally, the physiological role of 2B4 is still a puzzling issue in view of the conflicting information on its function emerging from human and mouse systems. Indeed, the phenotype caused by lack of 2B4 expression in mice was recently reported by

Kumar et al. (45). Unexpectedly, both cell-mediated cytotoxicity and IFN-γ secretion were enhanced, rather than diminished, in 2B4$^{-/-}$ NK cells (35), indicating that in the mouse system, 2B4 acts as a negative regulator of NK cells (45–48). Most studies on 2B4 functions in human NK cells have been conducted using peripheral blood NK cells, where 2B4 is a well-established activating receptor (30). Our recent observation that most NK cells in the human body are contained in secondary lymphoid tissues (31, 32) along with our present data suggesting a 2B4 inhibitory pathway in lymph node NK cells might help to reconcile this apparent discrepancy between mouse and human 2B4 activities.

In conclusion, our present findings demonstrate that DCs from different tissues specifically express or do not express CD48, and that CD48 expression by DCs can affect NK cell activation. Because NK cells from distinct compartments can express either activating or inhibitory 2B4, the different expression of CD48 in DCs might be related to the regulation of specific NK cell functions. Consistent with this view, we also show that NK cells isolated from human lymph nodes might display an inhibitory 2B4 pathway, thereby providing a functional explanation for the absence of CD48 in DCs isolated from inflamed lymph nodes, i.e., where an immune response requiring activated NK cells is most likely unfolding.

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Disclosures

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