The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 15 2000

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J Immunol (2000) 165 (8): 4202–4208.
https://doi.org/10.4049/jimmunol.165.8.4202

A Subset of Cytolytic Dendritic Cells in Rat[1]

Benjamin Trinité,[2]* Cécile Voisine,[2]* Hideo Yagita,[†] and Régis Josien[3]*[‡]

Dendritic cells (DCs) are a rare population of leukocytes specialized in Ag processing and presentation to T cells. We have previously shown that cultured rat splenic DCs exhibit a cytotoxic activity against selected target cells. In this study, we analyzed this function in DCs freshly prepared from lymphoid organs using the DC-specific OX62 mAb and magnetic beads. Freshly extracted splenic DCs, but not lymph node and thymic DCs, exhibited a strong and moderate cytotoxic activity against YAC-1 and K562 target cells, respectively. FACS analyses showed that spleen contained a minor subset (10–15%) of $CD4^+$ and class II^{int} DCs that also expressed the OX41 Ag and the lymphoid-related Ags CD5 and CD90 (Thy-1) and a major (80–85%) subset of $CD4^-$/$OX41^-$/$CD5^-$ and class II^{int} DCs. The cytotoxic activity of splenic DCs was strictly restricted to the $CD4^-$ DCs, a subset poorly represented in LN and thymus. Contrasting with our previous report using cultured splenic DCs, freshly isolated splenic DCs killed YAC-1 cells using a Ca^{2+}-independent mechanism, but this function did not appear mediated by Fas ligand, TNF-related apoptosis-inducing ligand, or TNF-α. Therefore, rat DCs contain a subset of naturally cytolytic cells that could play a role in both innate and acquired immune responses. Together with our previous report, these data suggest that rat DCs can use two mechanisms of cytotoxicity depending on their maturation/activation state. ***The Journal of Immunology*****, 2000, 165: 4202–4208.**

Dendritic cells (DCs)[3] are a subset of leukocytes found in almost all tissues and more abundantly in the T cell areas of lymphoid organs whose major function is Ag presentation to T cells (1). Despite their rarity, subsets of DCs have been described based on the expression of surface markers that could be related to a different ontogeny (for review, see Ref. 2). In mouse spleen, DCs are primarily $CD8\alpha^-$, whereas a smaller subset expresses the CD8α molecule together with high levels of the DEC-205 receptor (3). Recent reports indicate these DC subsets differ in their Th cell stimulatory activity. $CD8^+$ splenic DCs induced the differentiation of Th1 cells, whereas $CD8^-$ DCs promoted the differentiation of Th2 cells (4). $CD8^+$ splenic DCs have also been shown to exhibit a negative regulatory function on $CD8^+$ and $CD4^+$ T cells (5, 6). In humans, different subsets of DCs have been described in blood. DC1s are monocyte-derived DCs that produce large quantities of IL-12 upon stimulation and promote the differentiation of Th1 cells (7). DC2s are a less abundant subset of DCs that display features of the lymphoid lineage and express high levels of IL-3Rα. They promote the differentiation of Th2 cells (7). More recently, it was shown that a subset of blood DCs, closely related to the myeloid-related DC1s cells, exhibited cytotoxic activity against tumor cells using the TNF-related apoptosis-inducing ligand (TRAIL)-TRAIL-R pathway (8).

Previously, we showed that overnight cultured splenic, but not lymph node (LN) or thymic DCs exhibited an NK-like cytotoxic activity in rats, as demonstrated by their ability to kill the YAC-1 cell line in vitro (9). Subsets of rat DCs have been described in pseudo-afferent intestinal lymph. $CD4^+$ DCs that also express the Ag recognized by the OX41 mAb are better stimulators of T cell proliferation than the $CD4^-$/$OX41^-$ subset (10). Thymic DCs also vary in their expression of markers such as CD5 and CD4 (11). In this work, we further define the phenotype and function of the cytotoxic subset of rat DCs in lymphoid organs. DCs were purified from lymphoid organs (spleen, LN, and thymus) using the OX62 mAb that recognizes an integrin expressed on rat DCs (12). This procedure allowed us to use freshly extracted DCs without the overnight culture step, which could result in the stimulation of nonphysiological functions. We identified the cytotoxic subset of DCs in rat spleen as the $CD4^-$/$OX41^-$ cells that are poorly represented in LN and thymus. The killing activity was Ca^{2+}-independent, but did not appear to be mediated by TNF family members Fas ligand (FasL), TRAIL, or TNF-α.

Materials and Methods

Animals

Sprague Dawley and Lewis rats were obtained from the Center d'Elevage Janvier (Le genest-Saint-Isle, France) and were used at 6–10 wk old.

Reagents

The Fas.Fc fusion molecule was kindly provided by Dr. Yongwon Choi (The Rockefeller University, New York, NY). Concanamycin A and EGTA were obtained from Sigma Aldrich (St. Louis, MO). Collagenase D was from Boehringer Mannheim (Mannheim, Germany).

Monoclonal Abs

The following mouse anti-rat mAbs obtained from the European Collection of Cell Culture (Salisbury, UK) were used in cytofluorometric studies and cell sorting after coupling to FITC, biotin, or PE (Bioatlantic, Nantes, France): OX6 (MHC class II), W3/25 (CD4), R7.3 (TCR$\alpha\beta$), OX7 (Thy-1, CD90), OX19 (CD5), OX33 (CD45R on B cells), OX42 (CD11c), OX41 (glycoprotein 110 on macrophages, granulocytes, and some DCs), OX8 (CD8α), OX62 (an integrin on DCs), and ED3 (sialoadhesin). FITC-conjugated anti-CD3 (clone G4.18), anti-CD86/B7-2 (clone 24F), and hamster anti-mouse CD40 (cross-reacting with rat CD40) mAbs were purchased from PharMingen (San Diego, CA). FITC anti-CD161a (NKRP1A) was purchased from Serotec (Oxford, U.K.). MFL4, a neutralizing anti-mouse

*Institut National de la Santé et de la Recherche Médicale Unité 437 and Institut de Transplantation et de Recherche en Transplantation, Nantes, France; [†]Department of Immunology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo, Japan; and [‡]Department of Nephrology and Clinical Immunology, Nantes University Hospital, Nantes, France

Received for publication March 31, 2000. Accepted for publication July 19, 2000.

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[1] This work was supported in part by a grant from La Ligue Nationale Centre le Cancer (to C.V).

[2] B.T. and C.V. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Régis Josien, Department of Nephrology and Clinical Immunology, Nantes University Hospital, 30 boulevard Jean Monnet, 44093 Nantes Cedex 1, France. E-mail address: rjosien@nantes.inserm.fr

[4] Abbreviations used in this paper: DC, dendritic cell; FasL, Fas ligand; LN, lymph node; TRAIL, TNF-related apoptosis-inducing ligand; mTRAIL, murine TRAIL.

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and rat FasL mAb (13), and DR5-Ig fusion molecule (14) were previously described, and a neutralizing anti-rat TNF-α mAb was purchased from R&D Systems (Minneapolis, MN).

Cell lines

Murine TRAIL (mTRAIL)- and mock-transfected 2PK-3 cells were described previously (14) and cultured in complete RPMI. To obtain stable transfectants for rat FasL, RT-PCR-generated rat FasL cDNA was cloned in pMKITNeo vector and transfected in L5178Y cells, as described (13). YAC-1, P815, K562, A20, and L929 target cells were obtained from the European Collection of Cell Culture and cultured in complete RPMI.

Cell preparation

DCs. Lymphoid organs (spleen, LN, thymus) were minced and digested in 2 mg/ml collagenase D in RPMI, 1% FCS for 30 min at 37°C. EDTA at 10 mM was added during the last 5 min, and the cell suspension was then pipetted up and down several times and filtered. Cells were washed once in PBS/EDTA, 2 mM/1% FCS, and low density cells were obtained after centrifugation on 14.5% Nycodenz gradient, as previously described (9). Cells were then incubated with a saturating concentration of biotin-conjugated OX62 mAb at 4°C for 20 min, washed twice, and then mixed with streptavidin-conjugated microbeads, following manufacturer's instructions (Miltenyi Biotec, Paris, France). Positive selection was performed on MiniMacs type positive selection columns (Miltenyi Biotec). Although positive selection of DCs was also effective from bulk collagenase-digested lymphoid suspensions (data not shown), the first step of DC enrichment was performed to reduce the volume of magnetic beads needed.

T cells. $CD4^+$ and $CD8^+$ T cells were prepared from LN cells by negative selection of class II^+, Ig^+, $CD161^+$, and $CD8^+$ or $CD4^+$ cells, respectively, followed by anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway). Purity was routinely $\geq$98% and $\geq$90% for $CD4^+$ and $CD8^+$ T cells, respectively.

NK cells. Spleen cells were depleted of class II^+ and surface Ig^+ cells (Dynal), and NK cells were sorted by FACS using the 3.2.3 mAb.

Cytofluorometry and cell sorting

For cytofluorometric analyses, 5×10^4 cells were incubated with PE-conjugated OX6 or W3/25 mAb together with another FITC-conjugated mAb for 20 min at 4°C. Cells were washed twice and analyzed on a FACScalibur (Becton Dickinson, Mountain View, CA). For cell sorting of $CD4^+$ and $CD4^-$ subsets of splenic DCs, $OX62^+$ cells were incubated with OX6-PE- and W3/25-FITC-conjugated mAbs and sorted on FACSvantage (Becton Dickinson).

Mixed leukocyte reaction

Increasing numbers of allogenic DCs were cultured with 5×10^4 purified total, $CD4^+$ or $CD8^+$ T cells in round-bottom 96-well plates in a final volume of 200 μl for 3 days at 37°C, 5% CO_2, and for the last 8 h in the presence of 0.5 μCi [^{3}H]TdR (Amersham, Les Ulis, France). The cells were then harvested on glass fiber filters, and [^{3}H]TdR incorporation was measured using standard scintillation procedure (Packard Instruments, Meriden, CT).

Cytotoxicity assays

Cytotoxic activity of DC populations was assessed in a standard 6-h ^{51}Cr release assay using the YAC-1, P815, K562, L929, and A20 cell lines as targets. Briefly, target cells were labeled with sodium ^{51}Cr for 45 min at 37°C in complete medium. Serial dilutions of effector cells in complete medium were mixed with 3000 target cells in V-bottom 96-well plates in triplicates, centrifuged 3 min at $1500 \times g$, and incubated for 6 h at 37°C, 5% CO_2. The supernatants were harvested and ^{51}Cr release was determined (Packard Instruments) using standard scintillation procedures. Specific release was calculated as $100 \times$ (experimental release $-$ spontaneous release)/(maximum release $-$ spontaneous release).

Results

Purification of fresh DCs from lymphoid organs

Because the overnight culture period that is used during the usual DC purification procedure promotes DC maturation (15), we focused in this study on freshly extracted DCs. Highly purified DC populations were prepared by positive selection using the OX62 mAb, which recognizes the αE2 subunit of an integrin (16) specifically expressed on rat DCs (12). Although it has been found that the OX62 mAb also recognized some $\gamma\delta$ T cells (12, 16), we found that these cells in LN were OX62 negative (data not shown). In four preliminary experiments, we found that 80%, 55%, and 70% of splenic, LN, and thymic DCs, respectively, express the integrin recognized by the OX62 mAb (data not shown). Whether $OX62^-$ DCs represent a specific subset or whether the OX62 integrin is sequentially regulated during DC differentiation and maturation is still unknown. However, preliminary experiments have shown the same distribution of DC subsets (see below) in both $OX62^+$ and $OX62^-$ DCs (data not shown).

The phenotype of OX62-sorted cells was analyzed by FACS after double staining using PE-conjugated anti-class II mAb (OX6-PE) and FITC-conjugated mAbs. As shown in Fig. 1, more than 90% of cells expressed high levels of class II MHC molecules and the integrin recognized by the OX62 mAb (dot plots at the *bottom left*), and are therefore DCs. Contaminant cells that routinely represented 1–8% of total cells were mostly T and B cells, whereas sialoadhesin-positive cells (macrophages), NK cells ($CD161a^-$ and class II^- cells), never exceeded 2%. Marginal expression of CD3 was observed on DCs expressing very high levels of MHC class II molecules in LN and thymus (Fig . 1, *B* and *C*). The restricted isoform of CD45 recognized by the OX33 mAb and expressed by rat B cells was also detected on the surface of LN and thymic DCs (Fig. 1, *B* and *C*). Whether these molecules are really expressed or just picked up by DCs is unknown.

Freshly extracted splenic, but not thymic or LN DCs exhibit a NK-like cytotoxic activity

We previously reported that splenic, but not LN or thymic DCs exhibited a cytotoxic activity against the NK-sensitive cell line YAC-1 (9). However, these experiments were performed after a step of overnight culture whose physiological significance is not clear. We therefore analyzed the cytotoxic activity of freshly extracted DCs. As shown in Fig. 2*A*, splenic DCs very efficiently killed YAC-1 cells, whereas LN and thymic DCs were poorly cytotoxic. Interestingly, this cytotoxic activity against YAC-1 cells was close to that exhibited by purified spleen NK cells at the same E:T cell ratio (see Fig. 6). The T cell stimulatory activity of DCs was assessed in allogenic MLR. As shown on Fig. 2*B*, LN and thymic DCs were slightly better stimulator of allogenic T cells proliferation than splenic DCs. Several target cells commonly used to test NK cytotoxicity were also assessed against DCs. Freshly extracted splenic DCs were also moderately cytotoxic toward NK-sensitive K562 cells, and A20 cells but not LAK-sensitive P815 cells and the L929 fibroblast cell line (Fig. 3).

The phenotype of DC subsets in spleen, LN, and thymus

These previous results suggest that the cytotoxic activity of rat DCs was dependent on a subset of DCs present in spleen, but not LN and thymus. We therefore double stained freshly extracted DCs for MHC class II and several other markers allowing for the definition of subpopulations (Fig. 1). Several DC subsets could be defined based on levels of expression of MHC class II, CD4, CD5, OX41, CD90, CD161a, and CD11c molecules (Figs. 1 and 4). Unlike in mice (3), the expression of the CD8α molecule did not discriminate DC subpopulations, as all splenic DCs in rats were $CD8^-$ (Fig. 1*A*). In contrast and as previously shown previously in rat lymph (10), two subsets of splenic DCs could be defined based on the expression of CD4 (Figs. 1*A* and 4). The main subset (80%) was class II^{++}, $CD4^-$, $CD5^-$, $OX41^-$, $CD90^{-/+}$, $CD11c^{+++}$, and $CD161a^+$, and the minor subset (15%) was class II^+, $CD4^{++}$, $OX41^{++}$, $CD90^{+/+++}$, $CD11c^{+++}$, and $CD161a^{++}$ (Figs. 1*A* and 4). The cells of the minor subset were smaller (data not shown) and expressed lower levels of OX62 than the $CD4^-$ subset. A third

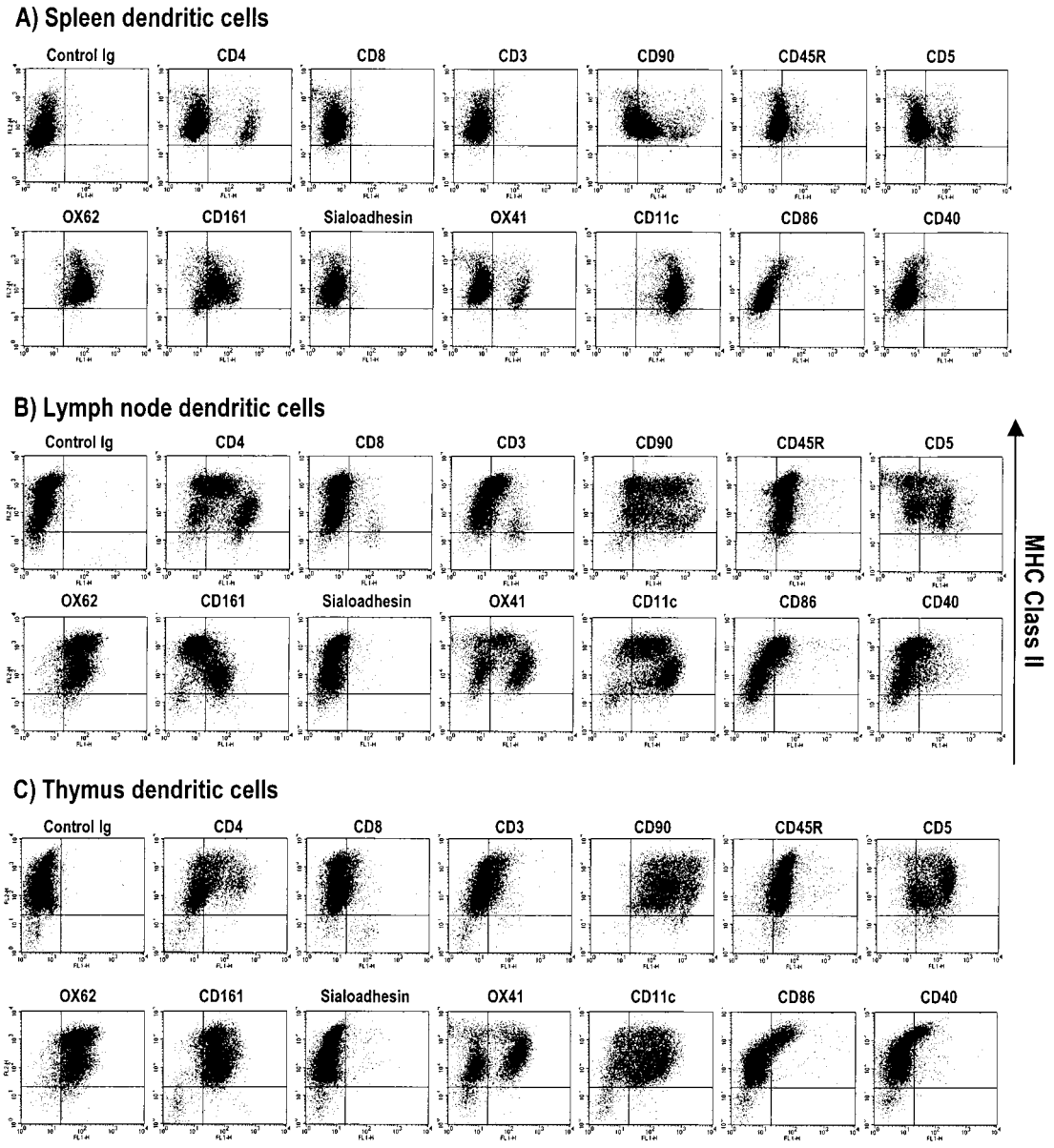

FIGURE 1. The phenotype of rat DCs freshly extracted from spleen, LN, and thymus. Lymphoid tissues (spleen, LN, and thymus) were digested in collagenase, and low density cells were selected on 14.5% Nycodenz gradient. Cells were incubated with biotin-conjugated OX62 mAb, followed by streptavidin microbeads (Miltenyi Biotec), and $OX62^+$ cells were harvested from a positive selection column. Splenic (*A*), LN (*B*), and thymic (*C*) were then double stained with a PE-conjugated anti-MHC class II mAb (*y*-axis), and one of the FITC-conjugated mAbs indicated on the *x*-axis. Cells were then analyzed on a FACScalibur after exclusion of dead cells using propidium iodide, and 10,000 events were acquired using the CellQuest software. Quadrants were set using both a PE and a FITC control mAb (not shown). The experiment is representative of five performed with similar results. For LN DCs, dot plots for CD5 and OX41 staining were obtained in a separate experiment in which the relative percentage of class II^{high} DCs was lower (compare with CD4 staining). Contaminants accounted for 1–8% of total cells depending on the experiments, and were mostly B and T cells.

and minor (<5%) subset was defined as class II^{+++}, $CD4^-$, $CD90^-$, $CD11c^{++}$, and $CD161a^{-/+}$ (Fig. 1*A*). Unlike the two other subsets that display an immature phenotype (no CD86 expression), the class II^{+++} cells express low levels of costimulatory molecules CD86 and CD40. However, levels of class II, CD86, and CD40 molecule expression were strongly up-regulated on all spleen and LN DCs upon spontaneous maturation that occurs during overnight culture (data not shown).

In LN, three subsets of $OX62^+$ DCs were identified using MHC class II and CD4 markers (see panel CD4 in Fig. 1*B*). The $CD4^-$/$OX41^-$/$CD5^-$ and $CD4^+$/$OX41^+$/$CD5^+$ DC subsets that we found in spleen represent 19% and 33% of LN DCs, respectively. The third and major subset (46%) of LN DCs expresses very high levels of MHC class II molecules (Fig. 1*B*), does not express CD161a, expresses lower levels of CD11c than other DCs, and expresses low levels of CD86 and CD40. This subset could also be separated in $CD4^+$/$CD90^{+++}$ and $CD4^-$/$CD90^{-/+}$ cells. In thymus, previously described subsets were not clearly observed (Fig. 1*C*). All thymic DCs express CD90, but most of the cells were $CD4^-$, indicating that, unlike in spleen and LN, these two markers are not coexpressed (see Fig. 4). However, thymic DCs segregated in $OX41^+$ and $OX41^-$ cells. They were also homogeneously $CD161a^+$ and $OX62^+$, and the cells expressing the higher level of MHC class II also express CD86 and CD40.

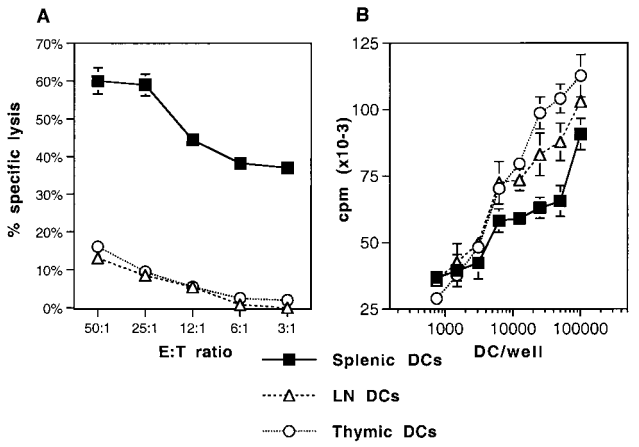

FIGURE 2. The cytotoxic and T cell stimulatory activities of fresh DCs. *A*, Splenic, but not LN or thymic DCs exhibit a NK-like cytotoxic function in vitro. Freshly purified splenic (full square), LN (empty triangle), and thymic (empty circle) DCs were mixed at the indicated E:T cell ratios with 3000 ^{51}Cr-labeled YAC-1 cells in triplicate in V-bottom 96-well plates in complete medium. Plates were centrifuged and incubated 6 h at 37°C, 5% CO_2, and centrifuged again, and supernatants were harvested. The percentage of specific lysis was determined using standard scintillation procedures. Similar results were obtained in three independent experiments. *B*, The T cell stimulatory activity of splenic, LN, and thymic DC in MLR. Increasing numbers of fresh splenic, LN, and thymic DCs (legend as in *A*) were mixed with 50,000 allogenic T cells in triplicate in round-bottom 96-well plates and cultured for 3 days with the last 8 h in the presence of [^{3}H]TdR. The cells were then harvested on glass fiber filters, and [^{3}H]TdR incorporation was measured using standard scintillation procedure. Similar results were obtained in two independent experiments.

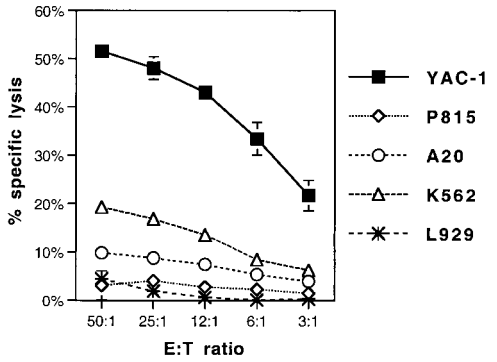

FIGURE 3. The sensitivity of different target cells to DC-mediated killing. Freshly purified splenic DCs were mixed at the indicated E:T cell ratios with ^{51}Cr-labeled YAC-1, P815, A20, K562, and L929 cells, and cytotoxic assays were performed as in Fig. 2*A*. The experiment is representative of three with similar results.

Identification of cytotoxic DCs in spleen as the CD4$^-$ subset

To determine which subset of splenic DCs exhibits a killing activity, splenic DCs were double labeled with PE-conjugated OX6 mAb (MHC class II) and FITC-conjugated W3/25 mAb (CD4) and sorted on a FACSvantage in OX6^{++} CD4$^-$ and OX6^{++} CD4$^+$ subsets (Fig. 5). Fig. 6 shows clearly that the CD4$^-$, but not the CD4$^+$ subset exhibited a cytotoxic activity. To investigate whether this cytotoxic function could play a role during Ag presentation to T cells by DCs, we performed MLR. Interestingly, CD4$^+$ splenic DCs were slightly better stimulator of both CD4$^+$ and CD8$^+$ T cell proliferation than CD4$^-$ cells (Fig. 7). This was mostly observed at high ratio of stimulator:responder cells. However, FACS-sorted CD4$^-$ splenic DCs were unable to kill resting or activated, syngenic or allogenic, CD4$^+$ or CD8$^+$ T cells in vitro (data not shown).

The cytolytic activity of splenic DCs is not perforin, FasL, TRAIL, or TNF-α mediated

In our previous report, we showed that the cytotoxic activity of overnight cultured splenic DCs was strictly dependent on Ca^{2+} (9), suggesting a mechanism of exocytosis of perforin- and granzyme-containing lytic granules, as described in NK and CD8$^+$ T cells (17, 18). Mechanisms of cytotoxicity of freshly extracted splenic DCs were therefore studied and compared with those of purified splenic NK cells. Surprisingly, the cytotoxic activity of freshly extracted splenic DCs was not inhibited by EGTA, indicating a Ca^{2+}-independent mechanism (Fig. 8*A*). Previous studies have shown that concanamycin A, an ATPase inhibitor, specifically inactivated perforin by increasing pH in intracellular lytic granules (19). Despite the absence of an effect of EGTA, concanamycin A strongly, but not completely, inhibited the cytotoxic activity of splenic DCs (Fig. 8*A*). In contrast, the cytotoxic activity of purified fresh spleen NK cells against YAC-1 was inhibited to the same extent by EGTA and concanamycin A, indicating that rat spleen NK cells mainly used the perforin pathway to kill the YAC-1 cells (Fig. 8*B*). Moreover, the cytolytic activity of splenic DCs against YAC-1 cells was strongly inhibited by cycloheximide, indicating that the killing mechanism requires protein synthesis in DCs (data not shown).

The role of FasL and TRAIL in DC-killing activity was then assessed. Indeed, a role for these molecules in the cytolytic activity of murine (5) and human DCs (8), respectively, has been shown previously. Kayagaki et al. (14) recently described that YAC-1 cells were not sensitive to FasL- and TRAIL-induced apoptosis. Accordingly, we also found that YAC-1 cells, as well as K562 cells, were not killed by an agonistic anti-Fas mAb (JO_2) (Fig. 9*A*). The killing of YAC-1 by splenic DCs was not significantly inhibited by a neutralizing mAb against FasL (Fig. 9*B*), nor by the fusion molecule Fas.Fc (data not shown). Moreover, only a small subset of A20 cells that are highly sensitive to Fas-induced apoptosis (Fig. 9*A*) was killed by splenic DCs (see Fig. 3), and this killing was not inhibited by Fas.Fc or the anti-FasL MFL4 mAb

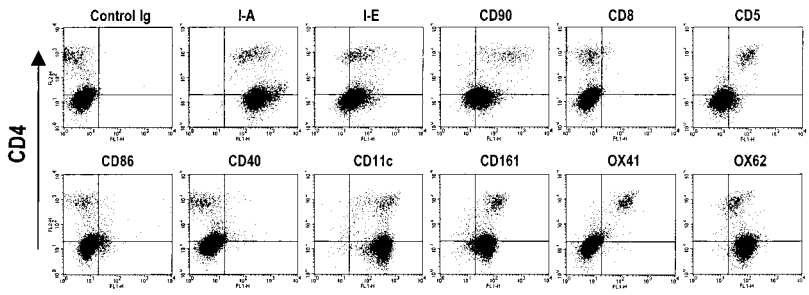

FIGURE 4. The phenotype of CD4$^+$ and CD4$^-$ subsets of splenic DCs. Freshly purified splenic OX62$^+$ DCs were double stained with PE-conjugated anti-CD4 mAb (W3/25, *y*-axis) and several FITC-conjugated mAbs (*x*-axis). Cells were analyzed on a FACScalibur. Note that the CD4$^+$ subset of splenic DCs also expresses the molecule recognized by the OX41 mAb and the lymphoid markers CD90 (Thy-1.1) and CD5.

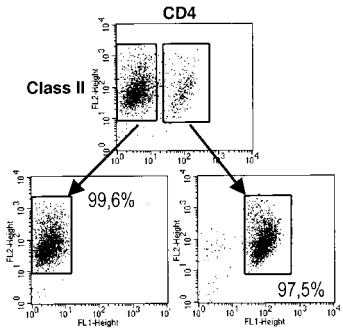

FIGURE 5. FACS sorting of $CD4^+$ and $CD4^-$ spleen DCs. $OX62^+$ splenic DCs were double stained with PE anti-MHC class II and FITC anti-CD4 mAbs and sorted on a FACSvantage in $CD4^-$ and $CD4^+$ splenic DCs. Purity was ≥98.5% for $CD4^-$ DCs and ≥95% for $CD4^+$ ones.

(data not shown). As previously shown (14), YAC-1 cells were not killed by mTRAIL-transfected 2PK-3 cells (Fig. 9*A*). Moreover, spleen DC-mediated killing of YAC-1 cells was not inhibited by DR5-Ig (Fig. 9*B*). In contrast, TRAIL-sensitive L929 cells were killed by mTRAIL transfectants (Fig. 9*A* and Ref. 14), but not efficiently by splenic DCs (Fig. 3).

YAC-1 cells did not appear to be sensitive to soluble rat TNF-α in a 5-h assay (Fig. 9*A*), and neutralizing TNF-α during the assay did not inhibit cytotoxicity (Fig. 9*B*). Taken together, these data suggest that the cytotoxic activity of fresh splenic DCs is not dependent on FasL, TRAIL, or TNF-α. Moreover, it was not mediated by a soluble molecule, as no cytotoxic activity against YAC-1 cells was detected in the supernatant of a splenic DC cytotoxic assay (data not shown). Finally, paraformaldehyde-fixed splenic DCs lost their cytotoxic activity, arguing against the involvement of a preformed membrane molecule (data not shown).

Discussion

The major function of DCs is to process and present Ag to T lymphocyte in a stimulatory or a tolerogenic fashion (1). Several recent reports (8), including ours (9), indicate that, besides their Ag-presenting function, DCs have also the capacity to kill some tumor target cells, such as the NK-sensitive YAC-1 or K562 cell lines. In this work, we showed that this unusual function in rat is restricted to a peculiar subset of DCs exhibiting an immature and $CD4^-/OX41^-/CD5^-$ phenotype that represents the main subset of DCs in spleen, but a minor one in LN and thymus. This cytotoxic activity was natural, as it did not require any stimulation, was Ca^{2+}

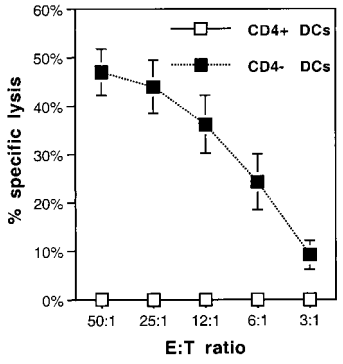

FIGURE 6. $CD4^-$, but not $CD4^+$ splenic DCs exhibit a cytotoxic activity. FACS-sorted $CD4^+$ and $CD4^-$ splenic DCs were immediately tested in a cytotoxic assay against YAC-1 cells, as described in Fig. 2. *A*, Experiment representative of three with similar results.

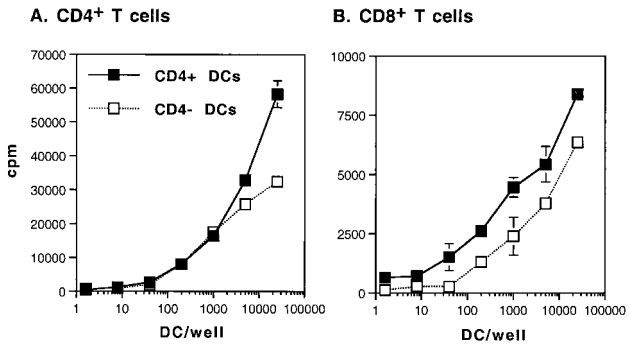

FIGURE 7. $CD4^+$ splenic DCs are better stimulator of allogenic T cell proliferation than $CD4^-$ DCs. To perform MLR, increasing numbers of FACS-sorted $CD4^+$ and $CD4^-$ splenic DCs were mixed with 50,000 purified $CD4^+$ and $CD8^+$ LN T cells. Proliferation of allogenic T cells was assessed as described in Fig. 2*B*. In five independent experiments, $CD4^+$ splenic DCs were found to be better stimulator of allogenic T cell proliferation than $CD4^-$ ones, and the major difference was always observed with $CD4^+$ T cells.

independent, but probably not mediated by the FasL/Fas, TRAIL/TRAIL-R, or TNF/TNF-R systems.

DCs with cytotoxic activity have been described previously in several species, suggesting that this function is conserved and might play a role in immune responses. In mouse, a subset of splenic DCs expressing the CD8α molecule and exhibiting lymphoid feature has been shown to mediate apoptosis of $CD4^+$ T cells through the expression of FasL (5). However, these data have proven difficult to reproduce (our unpublished observation). Lu et al. (20) also found that murine DCs derived in vitro from marrow

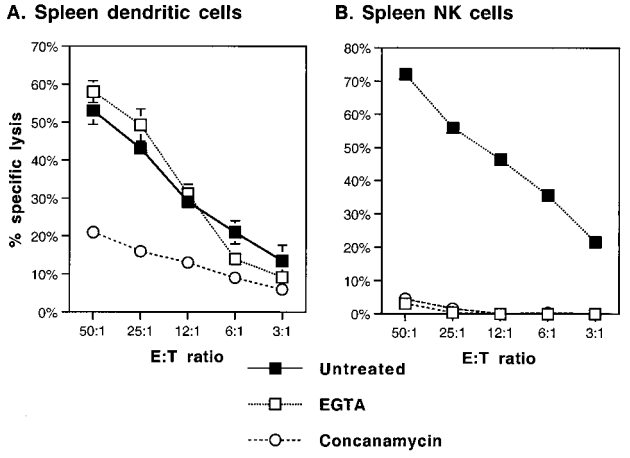

FIGURE 8. The effect of EGTA and concanamycin A on the cytotoxic activity of splenic DCs (*A*) and NK cells (*B*). *A*, Freshly purified splenic DCs were used in a cytotoxic assay against YAC-1 cells, as described in Fig. 2*A*. To test for the role of extracellular Ca^{2+} in the cytolytic function, EGTA was added to the culture medium during the assay at 2 mM. Concanamycin A is an inhibitor of vacuolar ATPase, and is known to inactivate perforin contained in granules by increasing their pH. Splenic DCs were preincubated with 10 μg/ml concanamycin A for 30 min at 37°C, and the cytotoxicity assay was also performed in the presence of 10 μg/ml concanamycin A. Similar results were obtained in three independent experiments. *B*, To obtain fresh NK cells, spleen cells were depleted of B cells and other class II^+ cells and stained with FITC anti-NKR-P1A (3.2.3) mAb. Small NKR-$P1A^+$ cells were then sorted on a FACSVantage and immediately used in a cytotoxic assay, as previously described. EGTA and concanamycin A were used as described in *A*. Similar results were obtained in four independent experiments.

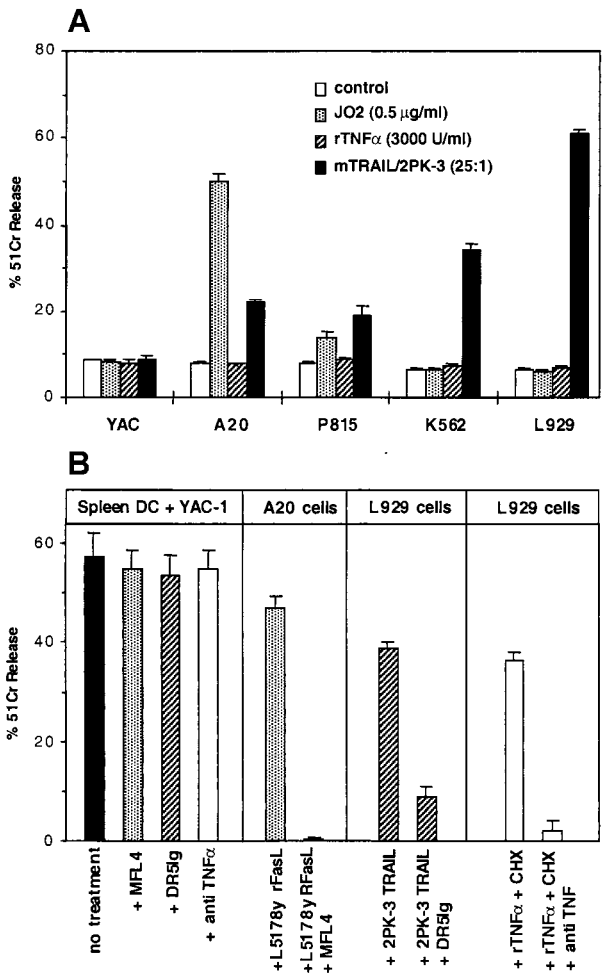

FIGURE 9. FasL, TRAIL, and TNF-α are not involved in the spleen DC-mediated killing of YAC-1 cells. *A*, YAC-1 cells are not sensitive to FasL-, TRAIL-, or TNF-α-induced cell death. ^{51}Cr-labeled YAC-1, A20, P815, K562, and L929 cells were incubated for 5 h at 37°C in round-bottom 96-well plate in the absence or the presence of the agonistic anti-Fas J02 mAb (0.5 μg/ml), rat rTNF-α (3000 U/ml), or mTRAIL-2PK-3 cells (ratio 2PK-3:targets, 10:1). ^{51}Cr release was assessed in the supernatant. Experiment representative of two with similar results. *B*, Absence of an effect of blocking FasL, TRAIL, or TNF-α on spleen DC-mediated killing of YAC-1 cells. In the *left histogram*, freshly purified splenic DCs were used in a cytotoxic assay against YAC-1 cells, as described in Fig. 2*A*, in the absence or in the presence of MFL4 mAb (10 μg/ml), DR5-Ig (20 μg/ml), or a neutralizing anti-TNF-α mAb (5 μg/ml). Control experiments showing the blocking efficiency of those reagents are shown in *right histograms*. MFL4 inhibited A20 cell death induced by rat FasL-transfected L5178Y cells (ratio E:T, 50:1) in a 6-h assay. DR5-Ig inhibited L929 cell death induced by murine TRAIL-transfected 2PK-3 cells (ratio E:T, 10:1) in a 6-h assay. Anti-TNF-α mAb inhibited L929 cell death induced by soluble rat TNF-α (3000 U/ml) in the presence of cycloheximide in a 6-h assay.

progenitors in the presence of GM-CSF and IL-4 express FasL and could induce apoptosis in Jurkat T cells. A subset of human blood DCs with myeloid feature has recently been shown to be cytotoxic toward several tumor cell lines through the expression of TRAIL, another apoptosis-inducing molecule (8). These human DCs need, however, to be activated by IFN-γ or IFN-α to express TRAIL and acquire the ability to kill. Interestingly, type I IFNs also up-regulated TRAIL expression on human T cells (21), suggesting that this effect could be involved in the antitumor activity of these cytokines. Unlike in human DCs, the cytotoxic activity of splenic DCs in rats did not require any stimulation, suggesting that this function could be instrumental in vivo, as described for NK cells. In addition, on a per cell basis, splenic DCs were almost as efficient as NK cells in killing YAC-1 cells in vitro (Fig. 8).

The major difference found in the cytotoxic activity of DCs prepared from spleen, LN, or thymus is likely to reflect the relative distribution of this $CD4^-$ cytotoxic subset in lymphoid organs. Indeed, these cells were abundant in spleen (>85% of total DCs), but rare in LN (<20% of total DCs) or thymus. Moreover, we could not detect such a cytotoxic activity in immature or mature bone marrow-derived or blood monocyte-derived DCs, despite a phenotype almost similar to the one we described in this work for the $CD4^-/OX41^-$ splenic DC subset (data not shown).

In our previous report, we showed that the cytotoxic activity of overnight cultured splenic DCs was Ca^{2+} dependent, and therefore probably mediated by exocytosis of perforin-containing lytic granules (9). The killing activity of freshly prepared splenic DCs was not inhibited by EGTA, which is known to antagonize both lytic granule exocytosis and perforin polymerization. However, DC-mediated cytotoxicity was strongly reduced by concanamycin A, a reagent known to inactivate perforin by increasing pH in lytic granules (19). It is possible that independent of its perforin inactivation properties, concanamycin A could have effects on other intracellular proteins involved in cytotoxic function and whose function might also be pH dependent. The possibility that concanamycin A could have an effect on YAC-1 target cells is excluded by the fact that it did not protect them against NK cell-mediated killing.

Calcium-independent mechanisms of cytotoxicity are mainly related to molecules of the TNF superfamily that induce apoptosis in target cells through interaction with a death domain-containing specific receptor (22). The fact that fixed splenic DCs were not cytotoxic indicated that the killing was not mediated by a preformed membrane molecule. However, apoptosis-inducing molecules could have been synthesized during the cytotoxicity assay. Indeed, the inhibition of DC-mediated cytotoxicity by cycloheximide strongly suggests that the molecule(s) involved in YAC cell killing needs to be synthesized during the in vitro assay. Although both FasL and TRAIL have been shown to mediate the induction of apoptosis in target cells by murine or human DCs, respectively (5, 8, 20), our results and those of Kayagaki et al. (14) strongly suggest that none of these molecules are involved in splenic DC-mediated cytotoxicity in rat. TNF-α was probably not involved, because the cytotoxicity was not inhibited by a neutralizing mAb against TNF-α. The role of recently cloned members of the TNF-R family containing a death domain such as DR3 (23) and DR6 (24) will be tested. Together with our previous report (9), our data suggest that rat splenic DCs have the capacity to kill YAC-1 cells using both a Ca^{2+}-dependent and a Ca^{2+}-independent mechanism. Interestingly, depending on their maturation stage, NK cells can also exhibit different mechanisms of cytolysis, and granule release-dependent cytotoxicity is present in mature, but not immature NK cells (25). Our preliminary results indicated that freshly extracted $OX62^+$ spleen DC lose their Ca^{2+}-independent cytotoxic activity upon overnight culture and that the residual cytolytic function turns to be Ca^{2+} dependent. However, the cytolytic function of overnight cultured $OX62^+$ spleen DC was much lower than the one we described in our previous report (9). This difference might be due to the very short $t_{1/2}$ of freshly extracted $OX62^+$ DC in culture. In contrast, these discrepancies could be related to the different DC purification procedures that we used in these two studies, and that might preferentially enrich for different DC subsets. These hypotheses are currently being tested.

Rat $CD4^+$ and $CD4^-$ DC subsets have been previously described by Liu et al. (10) in the pseudo-afferent intestinal lymph. The same authors suggested that the $CD4^+$ and $CD4^-$ subsets in rat exhibited lymphoid and myeloid features, respectively (10). In support of this hypothesis, we have shown that the $CD4^+$ and noncytotoxic subset of splenic DCs expressed the lymphoid-related markers CD90 and CD5. Our study identified another subset of DCs in LN expressing high levels of class II Ag, and low levels of CD11c and CD4 that might also be lymphoid derived. We have shown that the cytotoxic subset of DCs was the main subset in spleen, whereas these cells were rare in LN and thymus, in which lymphoid DCs are thought to reside. The potential in vivo role of these cytotoxic DCs remains unknown. One hypothesis is that these cells are involved in tolerance rather than immunity and act by inducing cell death in T cells. However, although $CD4^+$ splenic DCs are better stimulator of T cells than $CD4^-$ ones (Ref. 10 and this report), this is probably not related to the cytotoxic activity of $CD4^-$ splenic DCs, as they were unable to kill T cells in vitro.

Huang et al. (26) recently reported that a discrete subpopulation of DCs isolated from rat intestinal lymph was specialized in the transport of apoptotic cells from intestine to draining LN. DCs containing apoptotic bodies derived from injected allogenic lymphocytes have also been described previously in rat spleen by Fossum and Rolstad (27). This recent finding may provide a mechanism by which immature DCs tolerize T cells to self Ags derived from apoptotic cells throughout the body (28). It is important to note that this subset of DCs appears to be phenotypically identical with the cytotoxic subset of splenic DCs that we described in this study (i.e., $CD4^-/OX41^-$ cells). It would be interesting to test whether the $CD4^-/OX41^-$ subset isolated from rat intestinal lymph also exhibits a cytolytic function in vitro. We are currently investigating whether the killing of target cells by DCs is followed by the phagocytosis of their victims and efficient presentation of target cell-derived Ags to T cells (29). Cytotoxic DCs would therefore create an immediate link between innate and adaptive immunity and may be a tool for antitumor immunotherapy. For this purpose, a large panel of tumor cells is currently being tested for their sensitivity to the cytolytic activity of DCs. However, in vivo, this function may not result in the induction of an immune response. Indeed, DCs need to mature to acquire the capacity to stimulate naive T cells (1). Two recent papers have shown that the ingestion of apoptotic cells or bodies by immature DCs does not induce their maturation (30, 31), again suggesting that this source of Ag could be important for tolerance rather than immunity. Because spontaneous maturation of splenic DCs occurs in vitro after their extraction from lymphoid tissues, an in vivo model will be required to assess the role of this cytotoxic subset of DCs in tolerance and immunity.

Acknowledgments

We thank Dr. Yongwon Choi for providing Fas.Fc, Ignacio Anegon for providing TNF-R.Fc, Nelly Robillard for cell sorting, and Michèle Heslan for technical help. We also thank Brian Wong, Paul Szabolcs, Maria-Cristina Cuturi, and Jean-Paul Soulillou for critically reading the manuscript.

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