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Dendritic Cells Trigger Tumor Cell Death by a Nitric Oxide-Dependent Mechanism

Alexandra Nicolas,2* Dominique Cathelin,2* Nicolas Larmonier,† Jennifer Fraschczak,* Pierre-Emmanuel Puig,* André Bouchot,‡ Andrew Bateman,§ Eric Solary,* and Bernard Bonnotte*§

Dendritic cells (DCs) are well known for their capacity to induce adaptive antitumor immune response through Ag presentation and tumor-specific T cell activation. Recent findings reveal that besides this role, DCs may display additional antitumor effects. In this study, we provide evidence that LPS- or IFN-γ-activated rat bone marrow-derived dendritic cells (BMDCs) display killing properties against tumor cells. These cytotoxic BMDCs exhibit a mature DC phenotype, produce high amounts of IL-12, IL-6, and TNF-α, and retain their phagocytic properties. BMDC-mediated tumor cell killing requires cell-cell contact and depends on NO production, but not on perforin/granzyme or on death receptors. Furthermore, dead tumor cells do not exhibit characteristics of apoptosis. Thus, intratumoral LPS injections induce an increase of inducible NO synthase expression in tumor-infiltrating DCs associated with a significant arrest of tumor growth. Altogether, these results suggest that LPS-activated BMDCs represent powerful tumoricidal cells which enforce their potential as anticancer cellular vaccines. The Journal of Immunology, 2007, 179: 812–818.

Their unique capacity to induce and modulate T cell responses places dendritic cells (DCs) at the center of the adaptive immune response (1). As APCs, DCs constitute the targets of numerous cancer immunotherapy protocols aimed at priming tumor-specific CD4+ or CD8+ T lymphocytes. The emerging concept that DCs may also foster antitumoral immunity by triggering cancer cell death has been evidenced by an increasing number of reports.

Splenic rat DCs may exhibit tumoricidal activity against a wide range of tumor cells. Such an effect was proposed to be mediated in part by NK cell receptor protein 1 (NKR-P1) (2). Mouse and human DCs may also become cytotoxic against tumor cells, either spontaneously or after activation with IFN-γ (3–6). However, in all of the above-mentioned reports, the studied DCs do not originate from the same lineage. Indeed, cytotoxic DCs identified in rat spleen exhibit a CD4+CD103+ MHC class IIlow phenotype (7), whereas splenic and bone marrow-derived cytotoxic DCs (BMDCs) identified in mice either express CD8α+ or B220 and NK1.1 (8). In humans, the subsets of cytotoxic DCs include M-DC8+ (9), peripheral blood CD141–derived DCs (4), or umbilical cord blood CD34+ cells (10). It has also been reported that immature DCs isolated from an ovarian carcinoma patient ascites may be cytotoxic against autologous tumor cells (6, 11).

The type of target cell death induced by cytotoxic DCs remains an additional controversial issue. Some reports indicate that Fas ligand (FasL) or TRAIL expressed at the DC cell surface could trigger tumor cell apoptosis after engagement with their death receptors (5, 6, 11–13). DC release of TNF-α has also been proposed as a killing mechanism against TNF-sensitive cancer cells (3, 4). In addition, human M-DC8+ DCs have been described to act indirectly through enhancing the tumoricidal potential of NK cells (9).

In the present study, we reported the ability of rat BMDCs to induce the death of several rat, mouse, and human tumor cells. We demonstrate that tumor cells exposed to LPS-activated rat BMDCs die by a nonapoptotic process that involves NO. Furthermore, intratumoral injection of LPS activates NO secretion by tumor-infiltrating DCs, which impedes tumor progression. Altogether, these data provide further support on the cytotoxic potential of activated DCs and indicate that, beside the previously reported death receptor-mediated apoptosis, cytotoxic DCs may also activate a non-apoptotic pathway within tumor cell death.

Materials and Methods

Animals

BD-IX rats used in this work were bred in our laboratory by brother-sister mating. Animal use and handling were approved by the local veterinary committee and were performed according to the European laws for animal experimentation.

Reagents

LPS, EGTA, Mg2+, Nω-methyl-l-arginine (NMA), cycloheximide, propidium iodide, and crystal violet were purchased from Sigma-Aldrich and z-VAD-Ala-Ala-FL-fluoromethylketone was purchased from BIO-MOL. Bacillus Calmette-Guérin was purchased from Sanofi-Pasteur. IFN-γ from Peprotech and daunorubicin were purchased from Calbiochem. Supernatants of LPS-transfected Neuro-2a and TNF-α-transfected PROB cells were respectively used as a source for FasL (14) and TNF-α and were used...
at half dilution. Flagellin (500 ng/ml), CpG-oligodeoxynucleotide 1826 (100 μg/ml), and Gardiquimod (5 μg/ml) were purchased from InvivoGen; Pam3CysSK4 (5 μg/ml) was purchased from EMC; and poly(I:C) (125 μg/ml) was purchased from Sigma-Aldrich.

Antibodies

Anti-CD8α (OX-8), CD4 (W3/25), CD11c (8A2), NKR-P1 (3.2.3), MHC class II (OX-17), CD163 (ED2) mAbs and IgG isotype-matched control were obtained from Serotec and anti-CD80 and anti-CD86 Abs were obtained from BD Biosciences. Anti-inducible NO synthase (iNOS) Ab used in the histological study was purchased from Upstate Biotechnology and rabbit anti-cleaved caspase-3 was purchased from Cell Signaling Technology. Anti-human soluble TNF-RI and soluble TNF-RII blocking Abs were purchased from R&D Systems, anti-CD95 Fas-blocking Ab was purchased from Beckman Coulter. Blocking TRAIL-R2Fc Ab was provided by Dr. O. Micheau (Institut National de la Santé et de la Recherche Médicale, Unité 866, Dijon, France). 12C is a mAb raised against PROb cells (15).

Cell lines

The human colorectal cancer cell lines HT29, SW480, and HCT-8R, the rat glioma VIAI, and the mouse melanoma cell line B16F1 were purchased from the American Type Culture Collection. The PROb cell line derives from a chemically induced colon carcinoma obtained in our laboratory in BD-IX rats (16).

Cell isolation from rat tissues

Bone marrow precursor cells were obtained as previously described (17). The RBC were lysed in 150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA, and the cell suspension was passed through a 100-μm filter. Cells were then seeded in 6-well plates in RPMI 1640 (BioWhittaker) supplemented with 10% FBS (complete medium (CM)) supplemented in GM-CSF (AbCys) and IL-4 (AbCys) (5 μg/ml each). After 3 days, 3 ml of CM was added in each well. Five days after the start of the culture, all of the medium was removed and replaced with 3 ml of CM supplemented in GM-CSF and IL-4. After 7 days, floating and semiadherent cells were depleted of macrophages using ED2 mouse Ab and sheep anti-mouse Ig magnetic microbeads (Dynabeads; Dynal Biotech) according to the manufacturer’s protocol. Splenocytes from Brown-Norway rats were enriched for T cells by a nylon wool column to obtain a suspension containing at least 95% T cells after FACScan analysis (BD Biosciences).

Flow cytometry analysis of BMDCs

Five × 10^6 cells were washed in PBS supplemented with 0.5% BSA and 0.1% sodium azide and incubated with the appropriate mAb for 1 h. The cells were then washed in PBS and incubated with sheep anti-mouse Ig magnetic microbeads (Dynabeads; Dynal Biotech) according to the manufacturer’s protocol. Splenocytes from Brown-Norway rats were enriched for T cells by a nylon wool column to obtain a suspension containing at least 95% T cells after FACScan analysis (BD Biosciences).

Cytokine assays

Culture supernatants were collected at day 7 of BMDC generation and 48 h after the coculture with PROb and LPS. IL-12 (BioSource International), IL-6 (R&D Systems), and TNF-α (AbCys) concentrations were quantified using ELISA kits according to the manufacturer’s instructions.
Cytotoxicity assays

After 48 h of BMDC-target tumor cell coculture, the number of residual adherent cells was evaluated by crystal violet staining as previously reported (18). Data were presented as the percentage of relative absorbance calculated from the formula: \( \frac{A_{\text{test}}}{A_{\text{control}}} \), where \( A_{\text{test}} \) is the absorbance of tumor cells cultured with DCs after different stimulations and \( A_{\text{control}} \) is the absorbance of tumor cells cultured alone.

Detection of DNA cleavage and chromatin modifications

Chromatin condensation and fragmentation were monitored after staining with Hoechst 33258 fluorochrome (Sigma-Aldrich) as previously reported (19).

Western blotting analysis

PROb cells were incubated at 4°C for 10 min in lysis buffer (1% SDS, 1 mM Na3VO4, and 10 mM Tris (pH 7.4)) and protease inhibitors (2.5 µg/ml pepstatin, 10 µg/ml aprotinin, 5 µg/ml leupeptin, and 0.1 mM PMSF) and ultrasonicated. Protein concentration was measured in supernatants using a Bio-Rad protein assay. Forty micrograms was separated by SDS-PAGE using a 12% polyacrylamide gel and were electroblotted to a polyvinylidene difluoride membrane (Bio-Rad). The Amersham ECL Western blotting analysis system was subsequently used for protein detection.

Detection of iNOS expression and NO quantification

PROb cells were removed by selective depletion using sheep anti-mouse Ig magnetic microbeads (Dynal Biotech) after labeling with 12C Ab as described above. Western blotting analyses were performed as reported above with rabbit polyclonal anti-iNOS Abs (BD Transduction Laboratories) and peroxidase-conjugated anti-rabbit IgG. Diaminofluorescein-2 diacetate (10 µM) was added 24 h after the beginning of the coculture. After 30 min at 37°C, cells were removed and the fluorescence of noninternalized bacteria was quenched with trypan blue during 1 min at room temperature. Then, DCs were washed three times and analyzed on a FACScan.

Results

Evidences of BMDC tumoricidal properties

To determine whether rat BMDCs could act as effector tumor cell killers, several cancer cell lines were established as a monolayer and cultured with rat BMDCs for 48 h. BMDCs exhibited a cytotoxic effect against four of six tested cell lines by eliminating 25–30% of tumor cells. Interestingly, this killing activity overcame the species barrier, because xenogeneic tumor cell lines were also eliminated by BMDCs (Fig. 1A). We then reasoned that the tumoricidal function of BMDCs could be modulated by activating DCs. Indeed, LPS and IFN-γ significantly enhanced the cytotoxic potential of BMDCs (Fig. 1A). BMDCs activated with either LPS or IFN-γ eliminated >50% of PROb tumor cells, which were not susceptible to nonactivated BMDC-mediated killing. Coactivation with both LPS and IFN-γ did not demonstrate any synergistic effect (Fig. 1B). Bacillus Calmette-Guérin and TNF-α, two other DC activators, did not enhance BMDC cytotoxicity (Fig. 1B).
To further analyze the mechanisms by which BMDCs trigger tumor cell death, we focused our investigations on the rat PRO tumor model that has been widely characterized in our laboratory for its cellular and immunological properties (16, 19–22). The cytotoxic effect of LPS-stimulated BMDCs correlated with the DC:target cell ratio (Fig. 2A). In addition, PROb cells, which were not sensitive to nonstimulated BMDCs at a 5:1 ratio (Fig. 2A), became sensitive at a 50:1 ratio. Tumor cells killed by activated DCs detached from the culture flask, which was visualized as large holes in the cell monolayer (Fig. 2B). The death of detached cells was confirmed by their inability to generate colonies when suspended in fresh CM and cultured for 5 days (data not shown). Since LPS could directly interact with tumor cells, we demonstrated that LPS do not have direct antitumor activity and that LPS did not enhance the susceptibility of the tumor:DC-mediated killing (Fig. 2C). Interestingly, only the TLR-4 ligand LPS was able to promote BMDC cytotoxic potential. PamCysSK2 (TLR-1 and -2), poly(I:C) (TLR-3), flagellin (TLR-5), Gardiquimod (TLR-7), and CpG (TLR-9) exposure of BMDCs did not enhance their tumoricidal properties (Fig. 2D).

**LPS-activated BMDCs exhibited features of mature DCs**

To rule out the possibility that non-DC-contaminating cells that could be present in the bone marrow cultures may account for the observed cytotoxic effects, we systematically eliminated CD163-expressing macrophages from BMDCs and further defined the phenotype of these cells. FACS analyses indicate that day 7 BMDC expressed the myeloid cell and DC marker CD11c, MHC class II molecules, and CD80 and CD86 at the cell surface. The obtained cells thus exhibited the phenotypic characteristics of mature DCs. NKR-P1 was not detected, suggesting the lack of contamination with NK cells. In addition, the cultures comprised <2% of CTLs. The 48-h incubation period in the presence of LPS and/or tumor cells did not significantly modify the BMDC phenotype (Fig. 3A).

Activated mature DCs are characterized by their ability to produce proinflammatory cytokines. As expected, LPS treatment promoted IL-12, IL-6, and TNF-α production by BMDCs (Fig. 3B). IL-10, IL-1β, or IFN-γ concentrations in the supernatant of BMDCs cultured alone or with LPS were not detectable (data not shown). Functionally, DCs are characterized by their capacity to prime and activate naïve T cells. To further define the nature of the generated cytotoxic BMDCs, we evaluated their potential to induce T lymphocyte proliferation. Our results argued that BMDCs stimulated the proliferation of allogeneic T cells, as indicated by thymidine incorporation assays (Fig. 3C). LPS-activated DCs demonstrated an enhanced T cell stimulation potential. In addition, cytotoxic BMDCs retained the capability to engulf Gram-negative bacteria. However, this property decreased over time in the presence of LPS (Fig. 3D). Cytotoxic BMDCs demonstrated the phenotypic and functional characteristics of mature DCs along with conserved phagocytosis capacity.
BMDCs induce tumor cell death by necrosis

Based on previous reports indicating that cytotoxic DCs trigger apoptosis of target cells, we reasoned that activated rat BMDC-induced tumor cell killing may also be related to such a death process. Condensation and fragmentation of nuclear chromatin was not detected in PROb cells killed by BMDCs (Fig. 4A). In addition, BMDC-induced tumor cell death was not prevented by the wide spectrum caspase inhibitor z-VAD-fmk (Fig. 4B). Confirming these results, caspase-3 cleavage was not detected by Western blotting in detached tumor cells after coculture with DCs and LPS (Fig. 4C). Furthermore, we determined that tumor cells killed by rat BMDCs rapidly became permeable to propidium iodide, suggesting the early loss of plasma membrane integrity (Fig. 4D). These data, therefore, suggested that tumor cells exposed to BMDCs died by necrosis.

BMDC cytotoxic activity requires a direct contact with target tumor cells

Separation of tumor cells from BMDCs by a microporous membrane strongly hindered the cytotoxic effect of BMDCs (Fig. 5A). In addition, supernatants from a 48-h culture of LPS-activated BMDCs and PROb cells were not cytotoxic against fresh PROb cells. These two observations indicated that a direct contact of BMDCs with tumor target cells was a prerequisite for their cytotoxic effect. Considering that BMDC tumoricidal potential is cell contact dependent, we hypothesized that the calcium-dependent perforin/granzyme cytotoxic pathway (typically the main NK or cytotoxic T cell effector killing mechanism) could contribute to the

FIGURE 6. BMDC-mediated cytotoxicity depends on iNOS expression. A, Effect of NMMA (1 mM) on the cytotoxicity of BMDCs toward the PROb cell monolayer during a 48-h culture in the presence of LPS. Crystal violet cytotoxicity assay was used for measuring tumor cell monolayer density; mean ± SD from triplicate cultures. B, Nitrite levels in the supernatants of PROb cells cultured with BMDCs and LPS for 48 h. Nitrite release was determined by spectrophotometry using the Griess assay (46). C, iNOS expression was assessed by Western blotting in BMDCs after a 48-h coculture with PROb and LPS. PROb cells were removed at the end of the culture using 12C mAb and Dynabeads. D, Diaminofluorescein-2 diacetate staining. Decay-accelerating factor was added to PROb and BMDCs cocultured with (filled histogram) or without LPS (open histogram) 24 h after the beginning of the coculture.

FIGURE 7. Effects of LPS on established PRO tumors. A and B, Immunohistological analysis of 21-day tumor grafts in control (PRO) or LPS-treated rats (PRO/LPS) was performed on serial sections by using Abs that label tumor cells (12C), myeloid cells (CD11c), mature macrophages (ED2), and iNOS. Forty-eight hours after LPS injection, animals were sacrificed and tumors were resected. Similar results were observed in four pairs of tumor grafts from control or LPS-treated animals. A, Immunohistochemical study was performed on acetone-fixed 5-μm cryostat sections as previously described (21). B, Colocalization of iNOS and CD11c expressions in tumor sections. Tumors were cryopreserved with 30% sucrose, frozen in liquid nitrogen, and sectioned at 5-μm steps. Secondary Abs were the Alexa Fluor 488 goat anti-rabbit IgG for iNOS and the Alexa Fluor 568 goat anti-rabbit IgG for CD11c (Invitrogen Life Technologies). C, Tumor volume kinetics in 21-day-old PRO tumor-bearing rats receiving an intratumoral injection of LPS (1 μg/200 μl of Ham’s F10/rat) per week for 3 wk (■). A control group received medium only (200 μl of Ham’s F10/rat) (□). The tumor volumes were evaluated weekly using a caliper. Similar results were found in two independent experiments (three rats per group).
observed toxicity. The data depicted in Fig. 5B indicate that depleted of Ca\(^{2+}\) from the culture medium using EGTA in the presence of Mg\(^{2+}\) did not reduce the cytotoxic activity of BMDCs against PROb cells, making such a process unlikely. The role of CTLs in this cytotoxic mechanism was strongly weakened by the absence of cytotoxicity inhibition of anti-MHC class I Ab (data not shown). Because a high amount of IL-12 (Fig. 3B) may enhance NK cytotoxicity, we checked whether these activated BMDCs demonstrate any usual NK cytotoxic properties. We excluded a role of NK-like cells in this cytotoxic mechanism, because these activated BMDCs do not kill YAC-1 and P815 cells (Fig. 5C).

Finally, the addition of TNFR, Fas-blocking Abs, or blocking TRAIL-R2Fc Ab did not modify the cytotoxic effect of BMDCs against SW480 human cells (Fig. 5D), in which TRAIL-induced death was abrogated by TRAIL-R2Fc (data not shown). This observation suggested that a death receptor-ligand interaction did not account for the studied cytotoxic effect.

**BMDC cytotoxic activity depends on NO**

Because NO has been widely reported to mediate cell death (21, 23), we sought to define its role in the BMDC-mediated tumoricidal function. BMDC-induced tumor cell death was strongly reduced by NMMA, an inhibitor of iNOS (Fig. 6A). Moreover, we detected high levels of nitrates in supernatants of a 48-h DC-PROb-LPS coculture. NMMA strongly dampened this nitrite production (Fig. 6B). In addition, iNOS expression (Fig. 6C) and high amounts of intracellular NO were detected in DCs cocultured for 48 h in the presence of PROb cells and LPS, compared with nonstimulated DCs (Fig. 6D).

**LPS activation of intratumoral DCs correlated with tumor growth arrest**

To investigate the tumoricidal properties of LPS-activated DCs in vivo, s.c. PROb tumors were treated by intratumoral administrations of LPS. Immunohistological analysis performed 48 h after the first injection indicated that LPS fostered tumor infiltration by CD11c\(^{+}\) DCs, but not ED2\(^{+}\) macrophages (Fig. 7A). LPS also induced a dramatic increase in iNOS expression by CD11c\(^{+}\) DCs as visualized by immunofluorescence microscopy (Fig. 7B). These effects correlated with a significant decrease in tumor growth with 20% of the animals being cured (Fig. 7C).

**Discussion**

Although the role of DCs in tumor Ag presentation is well established, their ability to directly kill tumor cells is less defined. In the present study, we demonstrated that rat LPS-activated BMDCs are able to kill tumor cells of diverse origins through a NO-dependent mechanism.

Previous studies reporting the in vitro cytotoxic effect of nonactivated DCs against tumor cells were conducted at 25:1 to 50:1 DC:tumor cell ratio (3, 11, 24). We observed that rat BMDCs exhibit a spontaneous cytolytic effect against four of six tested tumor cell lines at a lower 5:1 ratio. Tumor cell lines that are resistant to this spontaneous killing effect at low DC:tumor cell ratio become sensitive at a 50:1 ratio. Thus, activation of BMDCs by either LPS or IFN-\(\gamma\) strongly enhances DC cytotoxic activity against tumor cells unlikely to TLR-2, -5, -7, -8, and -9 ligands. By eliminating any direct interaction of LPS with the tumor cells for enhancing their susceptibility to DC killing, we have confirmed the predominant role of LPS-activated DCs in the killing process.

It has been suggested that the mode of tumor cell death could affect the antitumor immune response (i.e., necrotic tumor cells could trigger DC maturation, whereas apoptotic tumor cells could not). However, the optimal approach for inducing cell death that would lead to effective endocytosis and activation of DCs remains controversial (22, 25, 26). The cytotoxic effect of DCs toward tumor cells was reported to be related to the activation of a caspase-dependent apoptotic pathway (5, 27) through activation of death receptors such as Fas, TNF-\(\alpha\) receptor, and TRAIL receptor (4, 6, 28). Contrasting with these reports, our results outline that BMDCs do not trigger the apoptosis of target tumor cells. We provide evidences that tumor cells killed by activated BMDCs die by necrosis, as indicated by the rapid permeabilization of the plasma membrane.

Tumor cell death induction requires a direct contact between LPS-activated DCs and target cells and involves NO production by BMDCs. The NO released by LPS-activated rat BMDCs is responsible for the cytotoxicity of BMDCs, as this effect is decreased by the addition of NMMA. NO was demonstrated to trigger either apoptosis or necrosis, depending on its concentration (29). The high concentration of NO released in the coculture by LPS-activated BMDCs may explain the necrosis-like mode of death of target tumor cells.

However, NO may induce the death of nontumor cells, such as T cells (30), and may also affect the priming of T cells by DCs (31). Nevertheless, our data demonstrate that NO-producing cytotoxic BMDCs are still able to induce allogeneic T cell proliferation even after LPS stimulation.

Another important issue to address before the use of these cytotoxic DCs in clinic is the difference in the NO pathway from humans and rodents (32–36). For example, iNOS activation increases the cytotoxicity of mouse (37) and rat (38) NK cells, unlike to their human counterparts (39). Moreover, mouse and rat macrophages synthesize large amounts of NO from the substrate L-arginine, via an enzyme system which is not functional in human macrophages (40, 41). Nevertheless, even though the induction of iNOS is more difficult in human than in rodent macrophages, it is well established that human monocytes produce NO (42). Furthermore, the quantity of NO which might exert immunological relevant functions is still debated.

Because BMDCs spontaneously secrete low amounts of NO, tumor cell death induced by nonstimulated BMDCs could also be mediated by NO. Because of its short half-life, NO action strongly requires proximity of producing cells and their targets, explaining the requirement for a cell-cell contact.

Using an in vivo model of tumor generated by s.c. injection of PROb cells to syngeneic rats, we demonstrated that tumor growth delay or suppression induced by intratumoral injection of LPS correlates with induction of iNOS expression in tumor-infiltrating CD11c\(^{+}\) DCs. We have previously demonstrated that experimental tumors (43), as well as human tumors (44), are infiltrated by DCs. However, these tumor-infiltrating DCs are blocked in an immature and nonfunctional stage (45). Activation of these DCs into cells secreting NO may be an attractive strategy in immunotherapy. The cytotoxicity of these activated DCs could play a role in the innate immune response against tumors, in a similar manner to Tip-DCs, a subset of spleen DCs involved in anti-infectious defense (23), or IKDCs, a subset of DCs implicated in tumor immunosurveillance (13). The current immunotherapy strategies aim to take advantage of the Ag-presenting function of DCs to induce specific antitumor T lymphocytes. Our study suggests that activation of DCs into cytotoxic cells that efficiently kill tumor cells may represent an alternative and complementary approach to future immunotherapy protocols.

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Disclosures
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