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Peroxynitrite-Dependent Killing of Cancer Cells and Presentation of Released Tumor Antigens by Activated Dendritic Cells

Jennifer Fraszczak,* Malika Trad,* Nona Janikashvili,† Dominique Cathelin,* Daniela Lakomy,* Virginie Granci,* Alexandre Morizot,* Sylvain Audia,*‡ Olivier Micheau,* Laurent Lagrost,* Emmanuel Katsanis,‡,§ Eric Solary,* Nicolas Larmonier,†,§,‖ and Bernard Bonnotte,*¶

Dendritic cells (DCs), essential for the initiation and regulation of adaptive immune responses, have been used as anticancer vaccines. DCs may also directly trigger tumor cell death. In the current study, we have investigated the tumoricidal and immunostimulatory activities of mouse bone marrow-derived DCs. Our results indicate that these cells acquire killing capabilities toward tumor cells only when activated with LPS or Pam3Cys-SK4. Using different transgenic mouse models including inducible NO synthase or GP91 knockout mice, we have further established that LPS- or Pam3Cys-SK4-activated DC killing activity involves peroxynitrites. Importantly, after killing of cancer cells, DCs are capable of engulfing dead tumor cell fragments and of presenting tumor Ags to specific T lymphocytes. Thus, upon specific stimulation, mouse bone marrow-derived DCs can directly kill tumor cells through a novel peroxynitrite-dependent mechanism and participate at virtually all levels of antitumor immune responses, which reinforces their interest in immunotherapy. The Journal of Immunology, 2010, 184: 000–000.

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Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; CM, complete medium; DC, dendritic cell; Fas, Fas ligand; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III); chloroform; iNOS, inducible NO synthase; MHC-II, MHC class II; NMMA, N G-methyl-L-arginine; Pam, Pam3Cys-SK4; ROS, reactive oxygen species; SNAP, (S)-S-nitroso-N-acetylpenicillamine.
peroxynitrite production by activated killer DCs was dependent on the expression of inducible NO synthase (iNOS) and on the NADPH oxidase flavocytochrome b558. Importantly, after killing of tumor cells, cytolytic DCs were capable of engulfing released tumor Ags and of triggering Ag-specific T lymphocyte activation. Furthermore, administration of activated killer DCs to tumor-bearing mice significantly delayed growth of established tumors.

Materials and Methods

Animals

C57BL/6 and BALB/c were bred in the University of Burgundy animal facility (Dijon, France). C57Bl6/Cybβ<sup>tm1Absc</sup>, iNOS<sup>−/−</sup> (C57Bl6-Nos2<sup>−/−</sup>), perforin<sup>−/−</sup> (C57BL6-Prf1<sup>−/−</sup>), FasL<sup>−/−</sup> (B6Smn.C3-Fasl<sup>−/−</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animal use and handling were approved by the local veterinary committee and were performed according to the European laws for animal experimentation.

Cell lines and transfections

The mouse melanoma cell line B16F10, the fibrosarcoma cell line L929, the colon carcinoma cell line CT26, mammary tumor cell 4T1, and renal tumor cell Renca were obtained from American Type Culture Collection, Manassas, VA. The human cell lines, cervix carcinoma cell line Hela and colorectal carcinoma cells SW480, were purchased from the American Type Culture Collection. Human glioma cells were provided by Dr. Krishnan (Department of Pediatrics, Steele Children’s Research Center, Tucson, AZ). The PROb cell line derives from a chemically induced colon carcinoma obtained in our laboratory in BD-IX rats (28). B16F10 expressing the GFP (B16-GFP) or OVA (B16-OVA) peptide was obtained by retroviral infection or transfection, respectively. The generation of the virus constructs used in this study has been previously described (29). GP2-293 cells (HEK-293 cells with gag and pol plasmids) were transfected using a standard calcium phosphate technique with 10 μg murine EGF and 5 μg plasmid expressing the vesicular stomatitis virus G protein (envelope glycoprotein). GP2-293 cells produce the virus containing GFP, which is released in the supernatant. B16F10 cells were transduced for 16 h with viral supernatants containing polybrene (8 μg/ml), washed in PBS, and cultured in complete medium. The percentage of GFP-expressing cells was determined by flow cytometry. B16F10 cells were transfected with a plasmid encoding the OVA peptide SIINFEKL and neomycin resistance gene to select the cells.

Reagents

N<sup>4</sup>-methyl-L-arginine (NMMA), apocynin, LPS, flaggellin, gadiniquimod, and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO). z-VAD-fmk was purchased from Biomol (Le-Perray-en-Yvelines, France), 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride (FeTPPS) and (z)-(S)-nitroso-N-acetylpenicillamine (SNAP) were obtained from Calbiochem (San Diego, CA). Mouse IFN-γ and TNF-α were purchased from PeproTech (Rocky Hill, NJ), CD40L and recombinant mouse IL-2 from R&D Systems (Minneapolis, MN), polynucleosine-polytetylic acid and CpG-oligo(deoxy)xynucleotide 1826 from Invitrogen (Carlsbad, CA), and Pam from EMC (Tubingen, Germany).

Abs and flow cytometry analysis

The following mouse Abs were purchased from eBiosciences (San Diego, CA): FITC-CD40, FITC-CD205, Alexa 647-CD11c (myeloid cell marker), PE-CD86, FITC-CD83, Alexa 647-CD11b, FITC-Gr-1, FITC-CD3, FITC-CD19, APC-F4/80, PE-MHC class II (MHC-II), and PE-NKG2D. Anti-mouse TNF receptor I (TNF-R1)- and TNF-R1-blocking Abs were purchased from BD Pharmingen (San Jose, CA), and anti-mouse TRAIL-blocking (CD253, clone N2B2) Ab was obtained from eBiosciences. Soluble anti-CD3 and anti-CD28 Abs were obtained from BD Pharmingen. For flow cytometry analysis, cells (8 x 10<sup>6</sup>) were washed in PBS with 0.5% BSA and 0.1% sodium azide and incubated with the appropriate conjugated Ab for 1 h, then washed and analyzed by FACSscan (BD Biosciences, San Jose, CA).

Generation of DCs

Total bone marrow cells were isolated from the femurs and tibias of C57BL/6 mice. Red cells were lysed in 150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, and 0.1 mM EDTA, and the cell suspension was passed through a 100-μm filter.

Cells (5 x 10<sup>6</sup>/ml) were seeded in six-well plates (3 ml/well) in RPMI 1640 (BioWhittaker, Basel, Switzerland) supplemented with 10% FBS (complete medium [CM]) and GM-CSF and IL-4 (AbCys, Paris, France) (10 ng/ml each) and were incubated in 5% CO<sub>2</sub> at 37°C. Three and 5 d after the beginning of the culture, the medium was completely replaced with 3 ml fresh CM supplemented with GM-CSF and IL-4. At day 7, CD11c<sup>+</sup> cells were selected from the culture using anti-CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured for an additional 2 d with GM-CSF and IL-4.

Detection of T lymphocyte proliferation

T lymphocytes were obtained from BALB/c mouse spleen cells incubated for 4 h in nylon wool columns. T cells were plated at 1 x 10<sup>5</sup> cells/well in 96-well round bottom plates in CM. DCs (1 x 10<sup>3</sup>) were seeded with T cells, and the coculture was prolonged for 5 d. [3H]Thymidine was added (1 μCi/well) for the last 24 h. [3H]Thymidine incorporation was measured using a liquid scintillation counter.

Ag presentation to specific T cells

BMDCs were cocultured for 48 h with B16 or B16-OVA tumor cells with or without LPS. At the end of the culture, DCs were selected using anti-CD11c microbeads and cocultured with B3Z cells (DC/B3Z ratio: 1:10). B3Z is a mouse CD8<sup>+</sup> T cell hybridoma that contains an Escherichia coli lacZ reporter gene driven by NF-AT elements from the IL-2 promoter. The specific recognition of the SIINFEKL peptide of OVA (OVA<sub>257-264</sub>) in the context of MHC class I by the TCR of B3Z results in the expression of the enzyme β-galactosidase. The activity of this enzyme is detected by subtracting the subsequent conversion of a chemoluminescent substrate measured by luminometry (Novagen kit, Madison, WI).

Cytokine assays

The concentrations of IL-12 in cell culture supernatants were determined using ELISA kits according to the manufacturer’s procedures (eBiosciences).

Cytotoxicity assays

DC-target tumor cell cocultures were cocultured for 48 h, and the number of residual adherent cells was evaluated by crystal violet staining as previously reported (30). Data were presented as the percentage of relative absorbance calculated from the formula A<sub>test</sub>/A<sub>control</sub>, where A<sub>test</sub> is the absorbance of tumor cells cultured with DCs in different conditions, and A<sub>control</sub> is the absorbance of tumor cells cultured alone.

Western blotting

DCs stimulated or not with LPS or Pam were incubated at 4°C for 20 min in lysis buffer (1% SDS, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM Tris [pH 7.4]) containing protease inhibitors (2.5 μg/ml pepstatin, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 0.1 mM PMSF). After centrifugation, protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Forty micrograms of proteins were separated by SDS-PAGE (12%) polyacrylamide gel for granzyne and perforin proteins and 7% for iNOS. Proteins were electroblotted to a nitrocellulose membrane and incubated with the appropriate primary Abs. The ECL Western blotting analysis system (tebu-bio, Cambs, U.K.) was subsequently used for protein detection.

NO quantification

Culture supernatants were collected and incubated (100 μl) with an equal volume of Griess reagent. After incubation (15 min) at room temperature, the absorbance was read at 550 nm against 690 nm as recommended, as previously reported (31).

Annexin V/propidium iodide

The percentage of apoptotic cells and necrotic cells was determined by using the FITC-conjugated Annexin V Apoptosis Detection Kit I according to the manufacturer’s recommendations (BD Pharmingen).

Statistical analysis

Unless specified otherwise, all experiments were reproduced three times and performed in triplicate. For all of the experiments, the error bars represent the SD from triplicate cultures. Student t tests were used to determine significant differences between groups.
Results

Induction of mouse BMDC tumoricidal activity with selected ligands

The nature of inducers and regulators of DC cytotoxic potential is still unclear. We investigated the tumoricidal activity of mouse BMDCs (32), selected based on CD11c expression. Consistent with previous results (6, 14, 15, 19, 20), our data indicate that BMDCs were not endowed with inherent cytotoxic activity against B16F10 melanoma cells except for the highest tumor cell/BMDC ratio (1:20) (Fig. 1A), but acquired a strong killing activity when exposed to the TLR-4 ligand LPS (Fig. 1B). Although most TLR mRNAs were detected in BMDCs (Supplemental Fig. 1A), the TLR-2 agonist Pam, a synthetic bacterial lipopeptide, was the only one among the other tested TLR ligands to also promote DC killing activity (Fig. 1B). IFN-γ or CD40L were not capable of inducing BMDC tumoricidal potential (Fig. 1B). DC killing activity depended on the dose of LPS and Pam (Fig. 1B) and on the DC/tumor cell ratio (Fig. 1C). Based on these data, subsequent investigations of killer DC functions were performed using a concentration of 1 μg/ml for LPS and Pam and a 5:1 effector:target ratio.

LPS or Pam may act directly on tumor cells, sensitizing them to DC killing. The results depicted in Fig. 1D indicate that LPS or Pam had no direct cytotoxic effects on B16F10 melanoma cells, and exposure of the tumor cells to these TLR ligands did not alter their susceptibility to killing by LPS- or Pam-activated BMDCs (Fig. 1D). In addition, BMDCs pretreated with LPS or Pam for 24 h and washed off to eliminate the residual ligand were still capable of inducing B16F10 killing (Fig. 1E).

The death of B16F10 cells upon contact with killer BMDCs was responsible for their detachment from the culture flask and was confirmed by the inability of these detached cells to form colonies when subcultured in fresh culture medium for 4 d (not shown). Elimination of tumor cells by activated killer DC was further evidenced by videomicroscopy (Supplemental Video 1). Dead B16F10 melanoma cells were detected as early as 6 h after the initiation of the coculture, and most of the tumor cells were eliminated by 28 h. Further characterization of the mechanism of tumor cell death induced by killer DCs highlighted a caspase-independent necrotic-like process as evidenced by propidium iodide and Annexin V staining (Supplemental Fig. 1B), the absence of DNA internucleosomal fragmentation in the dead tumor cells (not shown), and the inability of the broad-spectrum caspase inhibitor z-VAD-fmk to impair DC-induced B16F10 tumor cell death (Supplemental Fig. 1C). Additionally, LPS- or Pam-activated DCs were able to kill a variety of allogeneic and xenogeneic cells, including melanoma, kidney, and colon cancer cell lines (Fig. 1F).

Killer BMDCs exhibit phenotypical and functional features of mature DCs

Mouse bone marrow was cultured for 7 d with GM-CSF and IL-4, and BMDCs were selected based on CD11c expression. This enriched population of cells was further cultured for 2 d. Day 9 BMDCs expressed a high level of CD11c, and CD40, CD83, CD205, CD86, and MHC-II molecules were also detected (Fig. 2A). To some extent, BMDCs also expressed F4/80, a characteristic marker of macrophage BMDCs differentiated in the presence of GM-CSF (33, 34) and of some DC subpopulations (35, 36). These data indicate that, as a result of a spontaneous maturation process, the cells generated under such conditions exhibited the phenotypic characteristics of mature DCs as previously described (37). Exposure of these BMDCs to LPS or Pam for 24 h resulted in the upregulation of CD86 and, to a lesser extent, CD40. Expression of CD11c, CD83, CD205, and MHC-II molecules was maintained at similar levels compared with

FIGURE 1. Cytotoxic activity of BMDCs against tumor cells. A, B16F10 tumor cells were cocultured for 48 h with day 9 BMDCs (DC) at the indicated B16F10/DC ratio. The viability of tumor cells was determined using a crystal violet assay. The data represent the mean ± SD from triplicate cultures. NS, no significant difference when compared with B16F10 cells cultured alone. B, B16F10 tumor cells were cultured with day 9 BMDCs (B16F10 tumor cells/DC ratio = 1:5) in the presence of the indicated TLR ligands (LPS, TLR-4 agonist); Pam (ligand of TLR-1, -2, and -6); Poly (polyinosinic-polycytidylic acid, TLR-3 agonist); Fla (flagellin, TLR-5 agonist); Gard (gardiquinom, TLR-7 agonist); CpG (TLR-9 agonist), CD40L, or IFN-γ. Tumor cell survival was evaluated after 48 h. Data are mean ± SD from triplicate cultures. *Significant difference when compared with control B16F10 cultured in CM with the corresponding ligands (p < 0.001). C, BMDCs were cultured for 48 h with B16F10 cells in the presence of LPS (1 μg/ml, DC+LPS) or Pam (1 μg/ml, DC+Pam) at the indicated B16F10/DC ratios, and killing of tumor cells was evaluated. Data are mean ± SD from triplicate cultures. *Significant difference when compared with tumor cells cultured with nonactivated DCs (DC) (p < 0.05). D, B16F10 melanoma cells were pretreated for 24 h with LPS (1 μg/ml) or Pam (1 μg/ml), washed with PBS, and cultured for 48 h alone (Control), with nonactivated DCs (DC), or with DCs in the presence of LPS or Pam as indicated (Activated DC). Tumor cell survival was determined as described above. Data are mean ± SD from triplicate cultures. NS, no significant difference when compared with pretreated B16F10 cells cultured in absence of DCs. E, BMDCs were preactivated for 24 h with LPS (1 μg/ml) or Pam (1 μg/ml), washed twice, and cultured with B16F10 cells without LPS or Pam (DC/B16F10 tumor cell ratio = 5:1). BMDCs that were not preactivated with the TLR ligands were used as control (Medium). The viability of B16F10 was determined using a crystal violet assay. Data are mean ± SD from triplicate cultures. *Significant difference when compared with control tumor cells cultured alone (p < 0.005). F, BMDCs were cultured with different tumor cell lines in the presence of LPS or Pam (target tumor cells/DC ratio = 1:5), and tumor cell killing was evaluated after 48 h. Data are mean ± SD from triplicate cultures. *Significant difference when compared with tumor cells cultured in absence of DCs (p < 0.05).
nonactivated cells (Fig. 2A). The phenotype of CD11c⁺ BMDCs remained unchanged after their coculture with B16F10 tumor cells (not shown). Importantly, we did not detect any conventional cytotoxic immune cells (cytotoxic T lymphocytes [CD3], B cells [CD19], or NK cells [CD49b]) in CD11c⁺ BMDC cultures (Fig. 2B). Incubation of BMDCs with B16F10 tumor cells did not significantly modify their phenotype (not shown).

To further characterize the nature of killer DCs generated from mouse bone marrow, a functional analysis of these cells was conducted. Immature DCs obtained after 5 d of culture were not capable of triggering allogeneic T cell proliferation (not shown). Conversely, day 9 BMDCs induced the proliferation of allogeneic T lymphocytes, and this effect was neither enforced nor reduced by exposure to LPS or Pam (Fig. 2C) or by coculture of the DCs with tumor cells (data not shown). Day 9 BMDCs produced a high level of the proinflammatory cytokine IL-12, which was only slightly increased by LPS or Pam (Fig. 2D). These data indicate that day 9 BMDCs exhibit the phenotypical and functional features of mature DCs and that further stimulation with LPS or Pam and/or coculture with tumor cells does not substantially modify these features.

**BMDC killing activity requires direct cell contact but not NK2G2D or CD40L**

Previous studies have indicated that DC killing activity may depend on a direct cell contact (6, 15, 18, 20). The data depicted in Fig. 3A indicate that when LPS- or Pam-activated BMDCs were separated from B16F10 melanoma cells by a microporous membrane, they lost their ability to kill these tumor cells (Fig. 3A). Consistent with this observation, supernatants of LPS- or Pam-activated DCs did not significantly affect the viability of B16F10 cells (Fig. 3A), indicating that a cellular contact between killer DCs and their targets is required. To further study the mechanism(s) underlying DC-mediated cytotoxic activity, we used LPS-activated DCs in the next experiments because this ligand was the most potent at promoting their killing potential.

It has been reported that the mode of action of some subsets of killer DCs may involve NK2G2D (38) or CD40L (9), both requiring direct cell contact. NKG2D was not detected on DCs activated with LPS or Pam, and CD40 was not expressed by B16F10 tumor cells (Fig. 3B). Consistently, blocking anti-NKG2D and anti-CD40 Abs did not inhibit BMDC killing activity (not shown).

**BMDC killing activity does not depend on perforin/granzyme or on the death receptor ligands TNF-α, FasL, or TRAIL**

The perforin/granzyme system has been described as one of the mechanisms by which some DC subsets may kill tumor cells (21). However, compared with activated splenocytes used as positive control (splenocytes stimulated with IL-2, anti-CD3, and anti-CD28 Abs), killer DCs expressed low levels of granzyme B and did not express a detectable amount of perforin (Supplemental Fig. 2A). Similar results were observed using flow cytometry (29.39 ± 5% of untreated CD11c⁺ DC and 34.8 ± 5% of LPS-activated CD11c⁺ DC expressed granzyme B) (Supplemental Fig. 2B). In addition, BMDCs generated from perforin⁻/⁻ mice and activated with LPS displayed similar killing potential against B16F10 tumor cells compared with DC generated from wild-type animals (Fig. 4A).

Some subsets of killer DCs were reported to use death receptor ligands of the TNF superfamily, such as TNF-α, FasL, or TRAIL (5, 8, 12–14, 17–19, 21, 22, 25, 26, 38). Mouse BMDC cytotoxicity was not inhibited with anti-TNF-R1 and -R2 blocking Abs (Fig. 4B). In addition, FasL expression by killer DCs was low and similar to control nonkiller DCs (Supplemental Fig. 3). Moreover, CD11c⁺ DC generated from FasL⁻/⁻ mice induced B16F10 tumor cell killing to a similar extent when compared with DC from wild-type mice (Fig. 4C). Furthermore, activated killer DCs did not express TRAIL (Supplemental Fig. 3), and blocking anti-TRAIL Ab did not alter DC killing activity (Fig. 4D), excluding the role of this molecule in BMDC cytotoxic activity.

**BMDC cytotoxic activity depends on peroxynitrites**

NO, reactive oxygen species (ROS), and their products are potential effector molecules produced by immune cells to eliminate pathogens or tumor cells. We (15) and others (20) have documented that NO may play a critical role in DC killing function. The concentration of nitrates, principal NO metabolites, was increased in the culture supernatants of LPS- and Pam-activated
BMDCs (Fig. 5A), and the expression of iNOS was upregulated by LPS and Pam (Fig. 5B). A nitrite donor, SNAP, was able to induce B16F10 cell death in a nitrite dose-dependent manner (Fig. 5C). Consistently, the tumoricidal activity of these killer DCs was significantly impaired by NMMMA, an inhibitor of iNOS (Fig. 5D).

The toxicity of NO may be related to the chemical reaction of NO· with superoxide ion (O2·-), leading to the secondary oxidants, peroxynitrites, endowed with a high cytotoxic activity (39). BMDC-mediated cytotoxic effects were significantly impaired by the peroxynitrite metabolite accelerator (40) FeTPPS (Fig. 5E). Moreover, apocynin, an inhibitor of NADPH (an enzyme playing a key role in ROS) oxidase, led to the secondary oxygen species (O2·-) (42). O2·- production is thus hampered in gp91(phox)−/− mice. The cytotoxicity of activated DCs generated from iNOS−/− or gp91(phox)−/− mice was significantly impaired (Fig. 5F). The killing potential of these cells was, however, not completely abrogated, suggesting a possible role for additional mechanisms of cytotoxicity. Nevertheless, these results indicate that NO and ROS, particularly in the form of peroxynitrite, critically contribute to the killing activity of mouse bone marrow-derived DC toward cancer cells.

Killer DCs take up and present tumor Ags to specific T cells in vitro and delay tumor growth in vivo

To further confirm the role of NO and ROS in DC killing activity, we evaluated the tumoricidal effects of day 9 BMDCs generated from iNOS−/− or gp91(phox)−/− mice. Gp91 is the glycosylated subunit of the NADPH oxidase flavocytochrome b558, responsible for the production of superoxide ion (O2·−) (42). O2·− production is thus hampered in gp91(phox)−/− mice. The cytotoxicity of activated DCs generated from iNOS−/− or gp91(phox)−/− mice was significantly impaired (Fig. 5G). The killing potential of these cells was, however, not completely abrogated, suggesting a possible role for additional mechanisms of cytotoxicity. Nevertheless, these results indicate that NO and ROS, particularly in the form of peroxynitrite, critically contribute to the killing activity of mouse bone marrow-derived DC toward cancer cells.

BMDC killing activity depends on a direct cell contact but not on NKG2D or CD40. A, B16F10 cells were cultured alone (Control), with LPS- or Pam-activated BMDCs (DC), with LPS- or Pam-activated BMDCs separated by a transwell insert (DC/Trw), or with the supernatant of LPS- or Pam-activated BMDCs and B16F10 cocultures (Sup). Tumor cell killing was determined after 48 h. Data are mean ± SD from triplicate cultures. *Significant difference when compared with B16F10 tumor cells cultured with DC and LPS or DC and Pam (p < 0.05). B, Expression of NKG2D by nonactivated (DC) or LPS-activated (DC+LPS) BMDCs. Spleen NK cells isolated by magnetic cell sorting (DIX microbeads) and activated with IL-12 (1 ng/ml) and IL-2 (10^3 M) (NK) for 24 h were used as a positive control (black line, isotype control). CD40 receptor expression by B16F10 melanoma cells was assessed by flow cytometry.

To investigate the capability of killer BMDCs to uptake tumor Ags from killed target tumor cells and to trigger tumor-specific T lymphocyte activation, we used B16F10 melanoma cells infected with GFP (B16-GFP). These tumor cells were cultured for 24 h with day 9 BMDCs. CD11c+ expressing cells were then positively selected by magnetic cell sorting and analyzed by flow cytometry. B16-GFP particles were detected within some killer CD11c+ DCs (Fig. 6A), which was confirmed by fluorescence microscopy (Fig. 6B).

To define whether BMDCs that had killed and captured tumor cells were capable of presenting and stimulating T cells, OVA-expressing B16 melanoma cells were cocultured with day 9 BMDCs with or without LPS for 48 h. Then DCs were purified by magnetic cell sorting and incubated with the B3Z hybridoma cell line as explained in Materials and Methods. B3Z cells express a TCR that specifically recognizes the SHINEKL peptide of OVA in the context of MHC class I molecules. These T cells have been engineered to express β-galactosidase following specific engagement of their TCR (43). Only DCs that had killed OVA-expressing B16 melanoma cells triggered activation of B3Z cells (Fig. 6C). These results therefore demonstrate that tumor-killer DCs are capable of engulfing, processing, and presenting tumor peptides to specific T lymphocytes.
FIGURE 5. BMDC-mediated cytotoxicity depends on peroxynitrites. A, Detection of nitrites in the supernatants of nonactivated BMDCs (DC) or BMDCs stimulated as indicated. B16F10 cultured with LPS and Pam were used as control (Control). Data are mean ± SD from triplicate cultures. *Significant difference when compared with untreated DCs (p < 0.05). B, Expression of iNOS determined by Western blotting in BMDCs from a coculture with B16F10 in the absence (DC) or presence of LPS (DC + LPS) or Pam (DC + Pam). iNOS expression was not detected in B16F10 exposed to LPS (B16 + LPS) or Pam (B16 + Pam). C, Effect of different concentrations of SNAP (NO donors) on B16F10. The viability of B16F10 tumor cells (black line) was determined using a crystal violet assay. The nitrite level (purple dashed line) was assayed in the same cultures using the Griess technique. D–F, Effects of different inhibitors on BMDC tumoricidal activity. B16F10 tumor cells were cultured without DC (Control) or with LPS- or Pam-activated DCs with or without the indicated inhibitor. NMMA (1 mM) (D), FeTPPS (75 μM) (E), or apocynin (300 μM) (F) inhibited BMDC-mediated killing of B16F10. Data are mean ± SD from triplicate cultures. *Significant difference when compared with the corresponding group without inhibitor (p < 0.05). G, BMDCs generated from wild-type (WT), iNOS−/− (iNOS−/−), or gp91phox−/− (gp91phox−/−) mice were cultured with B16F10 melanoma cells in the presence of LPS or Pam. Tumor cell killing was determined after 48 h. Data are mean ± SD from triplicate cultures. *Significant difference when compared with WT DC in the corresponding treated groups (p < 0.01).

To evaluate the antitumoral properties of activated BMDCs in vivo, mice bearing palpable established B16F10 tumors at day 8 were treated with LPS and Pam (1 μg/ml each, intratumoral injection), with DCs alone, or with LPS plus Pam-activated DCs. The combination of LPS plus Pam was considered for these in vivo experiments because BMDC cytotoxicity against B16F10 tumor cells in vitro was significantly enhanced when the two TLR ligands were used concurrently (Fig. 6D). Administration of DCs activated with LPS plus Pam significantly delayed tumor growth (Fig. 6E), but did not completely cure the animals. The direct intratumoral inoculation with LPS and Pam did not significantly impair tumor growth, indicating that these ligands displayed no significant effects on resident cells, such as tumor-infiltrating macrophages, NK cells, lymphocytes, or DCs.

Discussion

The Ag-presenting function of DCs has been the basis for their development as promising tools to induce and sustain antitumor immunity. However, objective clinical responses in patients with cancer treated with DCs loaded with tumor Ags have remained relatively rare, which in part may stem from the failure to appropriately trigger the full antitumor potential of these cells. The observation that DCs may also exert direct tumor killer properties has two fundamental implications in cancer immunotherapy. First, the direct killing of tumor cells by DCs may foster the release and thereby the immediate availability of specific tumor Ags for presentation to effector T cells, and second, DC may participate in the effector phase of the immune response, potentially augmenting the diversity of the killing mechanisms needed for tumor elimination. A more clear definition of DC killing activity as well as identification of specific signals that may trigger this cytotoxic function thus deserve further investigation.

In our study, BMDCs were selected at day 7 of culture based on the expression of CD11c and further cultured for an additional 2 d. This specific protocol ensured a highly purified cell population exempt from contamination with other potentially cytolytic immune cells, characterized phenotypically and functionally as mature DCs. Other reports have documented that some subsets of DCs may display spontaneous cytotoxic effects against tumor cells without requiring prior activation (10, 14). However, these studies were conducted with a high DC/tumor cell ratio (20:1 to 50:1). In our study, we did not detect any significant cytotoxicity of nonactivated mouse BMDCs against B16F10 melanoma cells at low effector:target ratios (DC/tumor cells ≤ 10:1). A slight spontaneous toxicity (~25%) was apparent only at a 20:1 ratio. Although the effector killer DC/tumor cell ratio (5:1) used in our experiments to demonstrate cancer cell killing by LPS- or Pam-activated DCs is not compatible with a physiological situation where very few DCs are typically found at the tumor vicinity, it is still relevant for therapeutic purposes insofar as a high number of these killer DCs could be injected directly into the tumor site. These killer cells may foster the release of large quantities of tumor Ags, with two consequences: 1) an augmented source of tumor-specific Ags available for T cell priming; and 2) the establishment of a proinflammatory environment. This may significantly foster the induction and maintenance of antitumor immunity.

In an effort to delineate the optimal conditions for the induction of DC killing activity, we explored the effects of different TLR ligands. LPS (6, 14, 15, 19, 20), imiquimod (5, 21), or CpG (5, 7) have been reported as potent inducers of DC cytotoxic activity. Our results confirm that LPS significantly promotes mouse...
BMDC-mediated killing of B16F10 tumor cells and identify Pam as an additional molecule capable of triggering DC tumoricidal activity. Other tested TLR ligands failed at inducing BMDC killing function. This result is not surprising because the nature (type of cytokines produced) and level of DC activation may be different depending on the nature of the TLR ligand used to stimulate the cells (44, 45). Similarly, only LPS triggered CD11c+ splenic DC killing potential, but to a limited extent (not shown). We further excluded the possibility that LPS and Pam may directly sensitize tumor cells to BMDC cytolytic action. These data thus clearly demonstrate that tumor cell killing by LPS and Pam-stimulated BMDCs is solely due to CD11c+ cells that maintain the cardinal features of mature DCs.

The mechanisms underlying DC killing activity remain a controversial issue, as previous reports have documented the involvement of death receptors (5, 8, 12–14, 17–19, 21, 22, 25, 26, 38), the perforin/granzyme system, NK cell-related pathways (11, 38), or CD40/CD40L (9), and it is possible that this process may depend on the DC subsets, the species, the stimulation signals, or the mode of DC preparation. We were able to exclude the role of...
death receptors in DC killing activity. Indeed, blocking anti-TRAIL Ab did not hamper DC cytotoxic activity, and B16F10 tumor cells that were sensitive to BMDC killing resisted soluble TRAIL-mediated cytotoxicity. Similarly, FasL, TNF-α, or CD40L did not appear to play a major role in BMDC killing activity. Furthermore, killer BMDCs did not express NGK2D or CD49b, excluding an NK-associated cytotoxic process. We additionally demonstrate that the perforin/granzyme system was not involved in this mechanism. This discrepancy with the studies mentioned above may be explained by the possible presence of contaminating NK cells, macrophages, or monocytes in the BMDC preparation compared with the purified BMDCs used in our study.

Our results demonstrate that killing of tumor cells by Pam- or LPS-activated BMDCs requires a direct cell contact. Strong iNOS expression was detected, and a large amount of NO was released by Pam- or LPS-activated BMDCs, which argues for the role of NO in the killing process. However, NMMA only partially inhibited PAM or LPS-activated BMDCs, which demonstrates that the perforin/granzyme system was not involved excluding an NK-associated cytotoxic process. We additionally demonstrate that killer BMDCs did not express NKG2D or CD49b, did not appear to play a major role in BMDC killing activity.

Whether killer DCs can take up, process, and present tumor Ags to specific T cells remained to be addressed. However, due to the complex, heterogeneous nature of DC preparations that have been used in previous studies, this issue has been inadequately investigated. We demonstrated in this study that a highly purified CD11c+ DC population activated with LPS or Pam is endowed with multiple functions, including: 1) the killing of B16F10 tumor cells expressing the model Ag OVA; 2) the uptake of cellular fragments from B16F10-OVA cells; and 3) the presentation of OVA Ag to OVA-specific T lymphocytes resulting in the proliferation of these OVA-specific T cells. The fact that these killer cells are endowed with multiple functions is of therapeutic importance. Indeed, as underlined above, it is improbable that killer DCs alone completely eradicate tumors (particularly at low DC/tumor cell ratios), they may enhance the release of tumor-specific Ags and create a proinflammatory environment, which may promote the priming of efficient adaptive antitumor immunity.

Several strategies may be envisioned to integrate killer DCs in cancer immunotherapy. First, killer DCs generated in vitro that are allowed to kill, capture, and process tumor cells in culture may be administered as DC vaccines. A second approach may consist of inducing the differentiation of tumor-infiltrating DCs into killer DCs by direct injection into the tumor site of TLR ligands (21). We have evaluated the therapeutic potential of this second approach by injecting the TLR ligands (LPS plus Pam) into s.c. melanoma in mice. No significant effect of these molecules on tumor growth was observed, which could be explained by the tolerogenic state of tumor-infiltrating DCs usually highly resistant to immune activation (48). A third possibility consists of activating DCs with the TLR ligands into killer DCs in vitro before intratumoral injection. We demonstrated that this approach significantly delays tumor growth but was not sufficient to cure all the mice. This may be due to the phenomena of cancer-induced immunosuppression, leading to effector antitumoral T lymphocyte inhibition. These results therefore highlight the need for combining killer DC-based therapy with strategies that aimed at reducing or eliminating the mechanisms of tumor-induced tolerance.

Our findings thus demonstrate that after tumor cell killing, the same subset of cytotoxic CD11c+ BMDCs is capable of engulfing, processing, and presenting specific tumor Ags to T lymphocytes. These cytotoxic DCs may thus represent a multitasking cell type that can act at virtually all levels of the antitumor immune response (tumor Ag release, induction, maintenance/regulation, and effector/tumor killing phases). These killer APCs can be exploited as more effective antitumor cells in immunotherapy approaches.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure S1:

S1A: Detection by RT-PCR of different TLR mRNA in non-activated or activated BMDC.

mRNA expression was examined by RT-PCR using cDNA from untreated BMDC (DC), LPS-activated BMDC (DC+LPS) and Pam-activated BMDC (DC+Pam). The expression of β-actin was determined using same the same cDNA. PCR products were run on 2% agarose gel.

S1B: Identification of the mechanism of tumor cell death induced by killer dendritic cells using annexin V/PI.

B16F10 tumor cells were stained with annexine-V and propidium Iodide (PI) (Fite annexin V apoptosis detection kit, BD pharmlingen) at different time points during the co-culture with killer DC. The number of apoptotic (PI-/annexine-V+ cells) or necrotic (PI+/annexine-V+ cells) tumor cells was determined by flow cytometry after 6, 12 or 24 hrs of culture with killer DC (bottom panel). Controls consisted in apoptotic tumor cells (B16F10 cells treated with cycloheximide (205 µg/mL) and FasL (5µg/mL) for 6 hrs) or necrotic tumor cells (B16F10 cells were alternatively treated 30 sec at 37°C and 30 sec at 4 °C 5 times) (top panel).

S1C: Identification of the mechanism of tumor cell death induced by killer dendritic cells using z-VAD-fmk.

Killer DC were cultured for 48 hrs with B16F10 cells (B16F10:DC ratio=1:5) in the presence of LPS (1µg/mL), with or without the caspase inhibitor ZVADfmk (10 µM). Crystal violet assays were then performed; mean ± SD from triplicate cultures.
Supplemental Figure S2: Expression of granzyme B and perforin by BMDC.

S2A: The expression of granzyme B or perforin was determined by western blotting in non-activated BMDC (DC) or in LPS-activated BMDC (DC+LPS). Cell extracts from splenocytes activated with IL-2 (0.1 µg/mL), anti-CD3 Ab (1 µg/mL) and anti-CD28 Ab (1 µg/mL) were used as positive control.

S2B: The expression of granzyme B was determinate by flow cytometry. We stained B16F10 in the presence or not of LPS (1µg/mL) and the BMDC from the coculture with B16F10 in the presence or not of LPS with anti CD11c Ab and with anti granzyme B Ab. The cells were analyzed by flow cytometry.
Supplemental Figure S3: Expression of FasL and TRAIL by killer BMDC.

The expression of FasL or TRAIL was determined by western blotting in non-activated BMDC (DC) or in LPS-activated BMDC (DC+LPS). Cell extracts from splenocytes activated with IL-2 (0.1 µg/mL), anti-CD3 Ab (1 µg/mL) and anti-CD28 Ab (1 µg/mL) were used as positive control.
Supplemental Video S1: Visualization of B16F10 melanoma cell killing by killer BMDC.

B16-GFP melanoma cells were cultured with LPS-activated DC in a humidified chamber maintained at a temperature of 37°C under 5% of CO2 and was subjected to pulsed microscopy video recording with a Gx20 objective for 72 hrs at a time lapse of 10 minutes, using a Cell Observer Station Zeiss (phase contrast analysis).
Figure S1

A

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<th>DC+LPS</th>
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Apoptotic B16F10

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Necrotic B16F10

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<td>71.5 %</td>
<td>95 %</td>
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B16F10 tumor cells isolated after culture with killer DC

C

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![Image](image23.png)
Figure S2

A

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B

![Graph](image13)

CD11c

- **DC**: 51.89%
- **DC/LPS**: 49.42%
Figure S3

-DC
-DC + LPS
-Splenocytes
-FasL
-TRAIL
-HSC 70