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

J Immunol published online 20 January 2010
http://www.jimmunol.org/content/early/2010/01/20/jimmunol.0900831

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/01/13/jimmunol.0900831.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 immunosuppressor 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.
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-CD81tm1lau, iNOS1tm1aSo, Fas1tmff/J (C57Bl6-Prf1tmlSdz), FasLtm29(J) mice were obtained from The Jackson Laboratory (Bar Harbor, AZ). The PROb cell line derives from a chemically induced colon carcinoma cell line. 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 pLXSN-EGFP 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

N4'-methyl-L-arginine (NMMA), apocynin, LPS, flaggandin, gudiquimod, and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO). z-VAD-fmk was purchased from Biomol (Le-Perray-en-Yvelines, France). 3,10,15,20-tetraakis(4-sulfonatophenyl)phloroglucuronoyl tri-III, chloride (FctTppS) and (c-)-5-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), polyinosinic:polycytidylic acid and CpG-oligodeoxynucleotide 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-CD40b, FITC-Gr-1, FITC-CD3, FITC-CD19, APC-F4/80, PE-MHC class II (MHC-II), and PE-NKGD2. 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 × 10^6) 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 NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA, and the cell suspension was passed through a 100-μm filter.
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 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 (gardiquimod, 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 twice, 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 control B16F10 cells cultured in absence of DCs. E, BMDCs were precoated 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 precoated 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 NKG2D 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 NKG2D (38) or CD40L (9), both requiring direct cell contact. NKG2D was not detected on BMDCs activated with LPS, 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 BMDCs 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 peroxynitrates**

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 an essential role in ROS and peroxynitrite production) (41), significantly reduced the cytotoxic activity of LPS- or Pam-activated BMDCs (Fig. 5F).

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−/− 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−/− mice. The cytotoxicity of activated DCs generated from iNOS−/− or gp91−/− 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.

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

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 SIINFEKL 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 3.** BMDC killing activity depends on a direct cell contact but not on NK2G2D 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 NK2G2D by nonactivated (DC) or LPS-activated (DC+LPS) BMDCs. Spleen NK cells isolated by magnetic cell sorting (DX5 microbeads) and activated with IL-12 (1 ng/ml) or IL-2 (10^5 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.

**FIGURE 4.** BMDC killing activity does not require perforin/granzyme or death receptor ligands TNF-α, FasL, or TRAIL. A, B16F10 melanoma cells were cultured alone (Control) or with LPS-activated BMDCs generated from wild-type (DC WT) or perforin−/− (DC perf−/−) mice. The viability of tumor cells was determined after 48 h. Data are mean ± SD from triplicate cultures. NS, no significant difference when compared with DC WT. B, TNF-α does not play a role in BMDC cytotoxic activity. TNFRI and -II blocking Abs (TNF R Ab) were added to B16F10 tumor cell and activated BMDC (DC+LPS) coculture as indicated. L929 cells cultured with recombinant TNF-α (50 ng/ml) with or without TNF-R1 and -II blocking Abs were used as control. Data are mean ± SD from triplicate cultures. NS, no significant difference when compared with cells cultured without TNF-R1 and –II blocking Abs. *Significant difference when compared with cells cultured without TNF-R1 and -II blocking Abs. C, FasL does not play a role in BMDC cytotoxic activity. B16F10 tumor cells were cultured with LPS-activated BMDC from wild-type (DC WT) or from FasL+/− (DC FasL+/−) mice, and their viability was determined after 48 h. Data are mean ± SD from triplicate cultures. D, BMDC killing potential is independent on TRAIL. B16F10 tumor cells and activated BMDCs (DC+LPS) were cultured with or without anti-TRAIL blocking Ab (TRAIL Ab, 10 μg/ml) as indicated. Tumor cell killing was evaluated after 48 h. L929 cells cultured with NK (activated as described in Fig. 3B) with or without anti-TRAIL blocking Ab were used as control. Data are mean ± SD from triplicate cultures. NS, no significant difference when compared with the corresponding group without TRAIL blocking Ab. *Significant difference when compared with the corresponding group without TRAIL blocking Ab (p < 0.05).
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 < 0.005). 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 same treated group (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 2d. 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细胞 ≤10:1). A slight spontaneous toxicity (~25%) was apparent only at a 20:1 ratio. Although the effector killer DC/target tumor cell ratio (5:1) used in 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

FIGURE 6. Killer BMDCs take up, present tumor Ag to specific T cells in vitro, and delay B16F10 tumor growth in vivo. A, B16F10-GFP melanoma cells obtained by infection of B16F10 with the GFP (B16-GFP) were cocultured for 24 h with nonactivated (B16-GFP+DC) or with activated BMDC (B16-GFP +DC+LPS) at a B16F10/DC ratio of 1:5. BMDCs were then selected using anti-CD11c microbeads and analyzed by flow cytometry. B, Activated BMDC were cocultured with B16-GFP for 24 h and analyzed using fluorescent microscopy. BMDC were stained with anti-CD11c (red membrane staining) and with DAPI (blue nucleus). The arrow indicates uptake of a fragment of B16-GFP (green intracellular spot) tumor cells by BMDC (red membrane staining, original magnification ×63). C, Activated BMDCs were cultured alone (DC), in presence of OVA (10 μg/ml) as positive control (DC+OVA), with B16 in presence or not of LPS (DC+B16+LPS and DC+B16, respectively), or with B16-OVA with or without LPS (DC+B16-OVA+LPS and DC+B16-OVA, respectively). BMDCs were then selected from the culture using CD11c microbeads and incubated with B3Z cells (BMDC/B3Z cells ratio = 1:10) as described in Materials and Methods. B3Z cells were cultured alone as a negative control (Medium). The activity of β-galactosidase as a readout for B3Z cell activation was then assessed by measuring the conversion of its substrate into a chemiluminescent product. *Significant difference when compared with B3Z hybridoma cells alone (p < 0.05). D, BMDC were cultured with B16F10 (tumor cell/DC ratio = 1:1 or 1:2) in the presence of Pam (1 μg/ml), LPS (1 μg/ml), or both. B16F10 cell viability was evaluated using crystal violet. Data are mean ± SD from triplicate cultures. *Significant difference when compared with BMDC stimulated with LPS alone or Pam alone (p < 0.005). E, C57BL/6 mice were injected (s.c) with 1.5 × 10^5 B16F10 tumor cells. Mice were then treated with Pam (1 μg/ml) plus LPS (1 μg/ml) (LPS/Pam), with 1 × 10^6 untreated BMDCs (BMDC), or with 1 × 10^6 BMDC and LPS plus Pam (BMDC+LPS/Pam) at days 8, 11, 14, and 17 following tumor cell injection. Tumors were palpable at day 8. Data are mean ± SD (n = 8 mice per group) of two independent experiments. *Significant difference when compared with BMDC injected alone or Pam/LPS administered alone (p < 0.05). RLU, relative luminescence unit.
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 NKG2D 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
BMDC-mediated cytotoxicity, suggesting that NO was not the final
effector molecule. NO can combine with ROS, such as the su-
doxytiranne (39, 46). The peroxide ion, partially generated by the enzyme NADPH, to form
the killing process. However, NMMA only partially inhibited
BMDC-mediated cytotoxicity, suggesting that NO was not the final
BMDC killing activity is consistent with the requirement for cell–cell
contact and explains the nonspecific killing of a wide range of
cancer cells.

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 in-
vestigated. 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, if it is improbable that killer DCs alone com-
pletely 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 ac-
tivation (48). A third possibility consists of activating DCs with
the TLR ligands into killer DCs in vitro before intratumoral injec-
tion. 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 immnosupression,
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 re-
sponse (tumor Ag release, induction, maintenance/regulation, and
effector/tumor killing phases). These killer APCs can be exploited as
more effective antitumor cells in immunotherapy trials.

Acknowledgments
We thank A. Fromentin and A. Bouchot for technical assistance and
N. Sassi, J.F. Jeannin, and A. Bettaiha for helpful discussion.

Disclosures
The authors have no financial conflicts of interest.

References
572.
Broekstedt, T. W. Dubensky, M. F. Stins, L. L. Lanier, et al. 2006. Interferon-
producing killer dendritic cells provide a link between innate and adaptive immu-
Plumas. 2006. Virus or TLR agonists induce TRAIL-mediated cytotoxicity of
broad spectrum of tumor cell lines by activated human dendritic cells. Blood
95: 2346–2351.
duces mature natural killer dendritic cells to produce IFN-γ and inhibit tumor
dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apo-
Georgopolous, D. M. O’Donnell, and A. A. Melcher. 2008. OK432-acti-
vated human dendritic cells kill tumor cells via CD40/CD40 ligand interactions.
11. Janjic, B. M., W. Zhang, Y. Wang, and X. Cao. 2001. The involvement of TNF-
5415.
12. Lu, G., B. M. Janjic, J. Janjic, T. L. Whiteside, W. J. Storkus, and J.
Vujanovic. 2002. Innate direct antitumor effector function of human immu-
nate dendritic cells. I. Involvement of an apoptosis-inducing pathway. J. Immu-
dritic cells express natural killer cell receptor protein 1 (NKR-P1) and have
hyctoxic activity to select targets via a C2±dependent mechanism. J. Exp.
Med. 186: 467–472.
14. Liu, S., Y. Yu, M. Zhang, W. Wang, and X. Cao. 2001. The involvement of TNF-
5415.
15. Lu, G., B. M. Janjic, J. Janjic, T. L. Whiteside, W. J. Storkus, and J.
Vujanovic. 2002. Innate direct antitumor effector function of human immu-
nate dendritic cells. II. Role of TNF, lymphotxin-α, β, Fas ligand, and TNF-
17. Nicolas, A., D. Cathelin, N. Larmornon, J. Fraszczak, F. P. Pang, A. Bouchot,
A. Bateman, E. Solary, and B. Bonnotte. 2007. Dendritic cells trigger tumor cell
18. Nouri-Shirazi, M., J. Banchereau, D. Bell, S. Burkeholder, E. T. Kraus,
J. Davoust, and K. A. Pulucu. 2000. Dendritic cells capture killed tumor cells
and present their antigens to elicit tumor-specific immune responses. J. Immunol.
165: 3797–3803.
19. Roux, S., L. Apetoh, F. Chalmain, S. Ladoire, G. Mignot, P. E. Puig, G. Lauvaux,
L. Zitvogel, F. Martin, B. Chauffert, et al. 2008. CD4+-CD25+ Tregs control the
TRAIL-dependent cytotoxicity of tumor-infiltrating DCs in rodent models of
20. Schmitz, M., S. Zhao, Y. Deuse, K. Schäkel, R. Wehner, H. Wöhner, K. Hölig,
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) (Fitc annexin V apoptosis detection kit, BD pharmingen) 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 determined 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

<table>
<thead>
<tr>
<th></th>
<th>DC</th>
<th>DC+LPS</th>
<th>DC+Pam</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-2</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>TLR-4</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>TLR-5</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>TLR-6</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>β-actine</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

B16F10 tumor cells isolated after culture with killer DC

C

![Graph showing viability of cells with and without zVAD](image16.png)
Figure S3