

Nitric Oxide Confers Therapeutic Activity to Dendritic Cells in a Mouse Model of Melanoma

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Abstract

Susceptibility of dendritic cells (DCs) to tumor-induced apoptosis reduces their efficacy in cancer therapy. Here we show that delivery within exponentially growing B16 melanomas of DCs treated *ex vivo* with nitric oxide (NO), released by the NO donor (α)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO), significantly reduced tumor growth, with cure of 37% of animals. DETA-NO-treated DCs became resistant to tumor-induced apoptosis because DETA-NO prevented tumor-induced changes in the expression of Bcl-2, Bax, and Bcl-xL; activation of caspase-9; and a reduction in the mitochondrial membrane potential. DETA-NO also increased DC cytotoxic activity against tumor cells and DC ability to trigger T-lymphocyte proliferation. All of the effects of DETA-NO were mediated through cGMP generation. NO and NO-generating drugs may therefore be used to increase the anticancer efficacy of DCs.

Introduction

The function of dendritic cells (DCs), professional antigen-presenting cells involved in the initiation of immune responses, is impaired in tumors (1). This is due to a reduced ability of DCs to migrate to lymphoid organs and prime immune responses, and to tumor-induced apoptotic death of DCs, which correlates to the grade of the tumors and their prognosis (1–5). Indeed, preclinical studies and clinical trials have shown that intratumor delivery of DCs, modified *ex vivo* to increase their function, is a suitable therapeutic approach to limit tumor progression (6–8), whereas strategies to specifically enhance resistance of DCs to tumor-induced apoptosis have been studied to only a limited extent (5, 9–12).

A candidate molecule to improve DC ability to withstand the toxic tumor environment is nitric oxide (NO). Physiological concentrations of NO exert antiapoptotic effects in many cells (13). All of the specific events mediating tumor-induced apoptosis of DCs, including activation of caspases, reduction of the mitochondrial membrane potential and of the expression/activity of antiapoptotic Bcl-2, and increased expression of proapoptotic Bax (4, 5, 10, 14), were shown to be targets of NO, although in other cell types (13, 15). In addition, NO increases DC cytotoxic, endocytic, and antigen-presenting functions (16–18).

In this study we show that the treatment *ex vivo* of DCs with a pulse of the NO donor (α)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO) before delivery within a s.c.-

growing B16 mouse melanoma confers persistent antitumor action to DCs. We also provide the mechanisms responsible for the effect of NO.

Materials and Methods

Animals. Female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Calco, Italy) and treated in accordance with the European Community guidelines and with the approval of the Institutional Ethical Committee.

Cells. B16-F1 (H-2^b) melanoma cells were cultured as described previously (14). The culture-conditioned medium (B16SN) was prepared by culturing 1×10^6 cells in 20 ml of medium for 4 days and was used at a final concentration of 20% (14).

DCs were obtained from mouse bone marrow precursors, cultured as described previously (19). DCs were characterized by flow cytometry measuring plasma membrane expression of MHC class I and II, CD11c, CD80, CD83, and CD86 (17). The purity of DCs was in all experiments no less than 80%. The remaining CD11c-negative cells mostly comprised macrophages and lymphocytes. cGMP concentrations were measured by a radioimmunoassay (18) in samples (1×10^6 cells in 0.5 mM phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine) treated for 30 min with or without DETA-NO in the presence or absence of *H*-(1,2,4)-oxadiazolo[4,3- α]quinoxalin-1-one (ODQ; both from Alexis Italia, Florence, Italy; Ref. 18).

Intratumoral DC Injection. C57BL/6 mice (five animals/group) received 1×10^5 B16-F1 cells, *i.e.*, 10-fold the minimum tumorigenic dose (19), s.c. in the lower right flank on day 0 (19). Tumors reached the appropriate size (~ 10 mm²) after 6 days. DCs (1×10^6) or vehicle (PBS) were injected in the tumors on day 6, 12, 18, and 24, and tumor growth was monitored. Mice were sacrificed when their tumors reached 15 mm in size on either diameter. Mice received injections of vehicle; untreated DCs, DETA-NO-treated DCs, 8-Br-cGMP-treated DCs, and DCs treated with DETA-NO and ODQ. Treatments of DCs were for 2 h, and the compounds were removed by washing before injection. To reveal injected DCs at tumor sites, we labeled the DCs with fluorescent dye the 5-chloromethylfluorescein (CMFDA; 2 μ M; Molecular Probes, Leiden, the Netherlands) for 30 min at 37°C (19). Tumors were collected 24 and 72 h after DC injection. Single-cell suspensions (19) were labeled with a phycoerythrin-conjugated anti-CD11c monoclonal antibody and analyzed by flow cytometry.

Measurements of Apoptosis. The mitochondrial membrane potential and DNA content were analyzed by flow cytometry in DCs (1×10^6 cells/sample) stained with the potential-sensitive fluorescent dye tetramethylrhodamine ethyl ester (500 nm; Molecular Probes; Ref. 20) or propidium iodide (50 μ g/ml in permeabilized DCs; Ref. 15), respectively. We assessed caspase-9 activity in DC lysates as described previously (15), measuring the cleavage of the fluorogenic caspase-9 substrate Ac-LEHD-7 amino-4-trifluoromethyl coumarin with a Perkin-Elmer LS50 fluorometer. Western blot analyses were carried out on 50 μ g of DC lysates (17). Relevant bands were immunolabeled with rabbit polyclonal primary antibodies specific for either Bcl-xL or Bax (Cell Signaling Technology, Beverly, CA) or mouse monoclonal antibodies recognizing Bcl-2 (Upstate Biotechnology, Lake Placid, NY), followed by incubation with horseradish peroxidase-conjugated goat polyclonal antirabbit or antimouse IgGs (Transduction Laboratories, Lexington, KY). Immunoreactive bands were visualized by the enhanced chemiluminescence procedure and

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quantified by microdensitometry using a Molecular Dynamics Imagequant apparatus (17).

Measurements of DC Functions. For mixed-lymphocyte reactions, DCs from C57BL/6 mice were irradiated (2500 rad) and cocultured at different ratios with spleen-derived BALB/c lymphocytes (10^5 cells/well; Ref. 14). Incorporation of methyl- ^3H thymidine by T cells was evaluated at day 5 (18). Cytotoxic activity of DCs was assessed by measuring the specific ^{51}Cr release in a standard assay in which serial dilutions of DCs were mixed with 3000 ^{51}Cr -labeled B16-F1 cells or syngeneic splenocytes (19).

All solutions used were endotoxin free as determined by the *Limulus* test (PBI, Milan, Italy).

Statistical Analysis. All results are expressed as means \pm SE, and n represents the number of individual experiments. Statistical analyses were performed with Student's t test and the log-rank statistic. All data were considered statistically significant at $P < 0.05$. In the figure panels, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Results

Treatment with NO Enhances DC Antitumor Activity *in Vivo* and Their Persistence at the Tumor Site through a cGMP-Dependent Pathway. DCs were treated in the presence or absence of the NO donor DETA-NO (10 μM), the cell permeable analog of cGMP 8-Br-cGMP (3 mM), or DETA-NO combined with ODQ (1 μM), a selective guanylate cyclase inhibitor that prevents NO-dependent cGMP generation (18). DETA-NO at 10 μM yielded a steady-state concentration of 11 ± 0.1 nM NO ($n = 4$) as measured by a NO electrode (18). All treatments were for 2 h, and agents were removed after treatments. Vehicle or the DC preparations were injected intratumorally in s.c.-growing B16 melanomas 6, 12, 18, and 24 days after injection of B16-F1 cells (1×10^5 cells; Fig. 1A). In the group of mice receiving untreated DCs, tumor growth was slightly delayed compared with vehicle-injected mice. All of the animals eventually died because of the tumor. Conversely, in the groups receiving injections of DETA-NO- or 8-Br-cGMP-treated DCs, tumor growth was significantly reduced (Fig. 1A), and animal survival was 33 ± 4 and $37 \pm 3\%$, respectively ($P < 0.001$ versus untreated DCs; $n = 6$; Fig. 1B). Tumor regression was observed in all surviving animals. Injection of DCs treated with DETA-NO together with the guanylate cyclase inhibitor ODQ neither inhibited tumor growth nor cured any animals.

To evaluate their intratumor survival, we stained DCs with the dye CMFDA (19) before their *ex vivo* treatment and injection into tumors. Tumors were recovered 24 and 72 h after injection, and cells were dispersed and analyzed by flow cytometry after staining for the DC marker CD11c. Treatment *ex vivo* of DCs with DETA-NO or 8-Br-cGMP resulted in the recovery of significantly higher numbers of CMFDA $^+$ /CD11c $^+$ cells at the tumor site (Fig. 1C). The effect of DETA-NO was abrogated by ODQ. CMFDA $^-$ CD11c $^+$ cells accounted for $<0.1\%$ of the recovered cells and did not change significantly with the various treatments (not shown).

All NO effects were mimicked by 8-Br-cGMP and prevented by ODQ, indicating that NO acts through cGMP. Indeed, treatment with DETA-NO increased generation of cGMP. Values measured in the first 30 min were 4.39 ± 0.11 , 0.55 ± 0.07 , and 0.69 ± 0.08 pmol/mg/min in DETA-NO-treated DCs, controls, and DETA-NO-plus ODQ-treated DCs, respectively ($P < 0.001$ in DETA-NO-treated cells versus control; $n = 4$). The rate of cGMP generation did not change significantly with time up to 2 h (not shown).

NO Protects DCs from Apoptosis Induced by B16-F1 Cells in a cGMP-Dependent Manner. DC apoptosis is induced by tumor cells through the paracrine release of as yet unidentified factors (4, 10, 14). DCs cultured for 48 h in the presence of B16SN died via apoptosis, as demonstrated by the appearance of a hypodiploid DNA peak and by the decrease in the mitochondrial membrane potential (Fig. 2A).

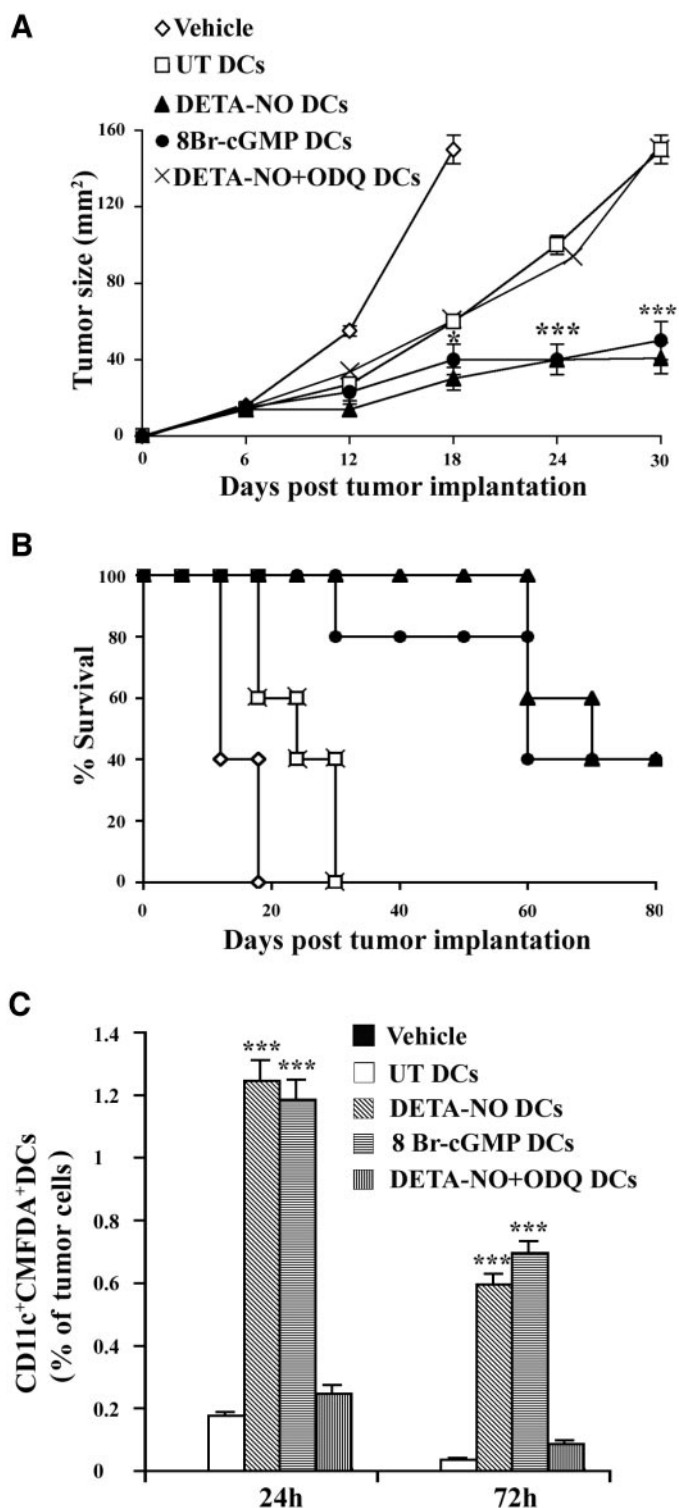


Fig. 1. Treatment with nitric oxide (NO)/cGMP enhances dendritic cell (DC) antitumor activity *in vivo*. C57BL/6 mice (5/group) received injections of 1×10^5 B16-F1 cells. Vehicle or DCs (1×10^6 cells) treated for 2 h in the absence (UT DCs) or presence of (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO; 10 μM), 8-Br-cGMP (3 mM), or DETA-NO plus *H*-(1,2,4)-oxadiazolo[4,3- α]quinoxalin-1-one (ODQ; 1 μM) were injected at days 6, 12, 18, and 24. A, tumor size \pm SE (bars; $n = 6$); B, survival of animals (in one of six reproducible experiments). C, C57BL/6 mice received injections of 1×10^5 B16-F1 cells. After 6 days, vehicle or 1×10^6 5-chloromethylfluorescein (CMFDA)-loaded DCs treated for 2 h in the absence (UT DCs) or presence of DETA-NO, 8-Br-cGMP, or DETA-NO plus ODQ were injected intratumorally. Tumors were recovered after 24 and 72 h, and cells were dispersed, stained with CD11c, and analyzed by flow cytometry. The values show the percentage \pm SE (bars) of double-positive DCs ($n = 5$). Statistical significance versus untreated DCs: *, $P < 0.05$; ***, $P < 0.001$.

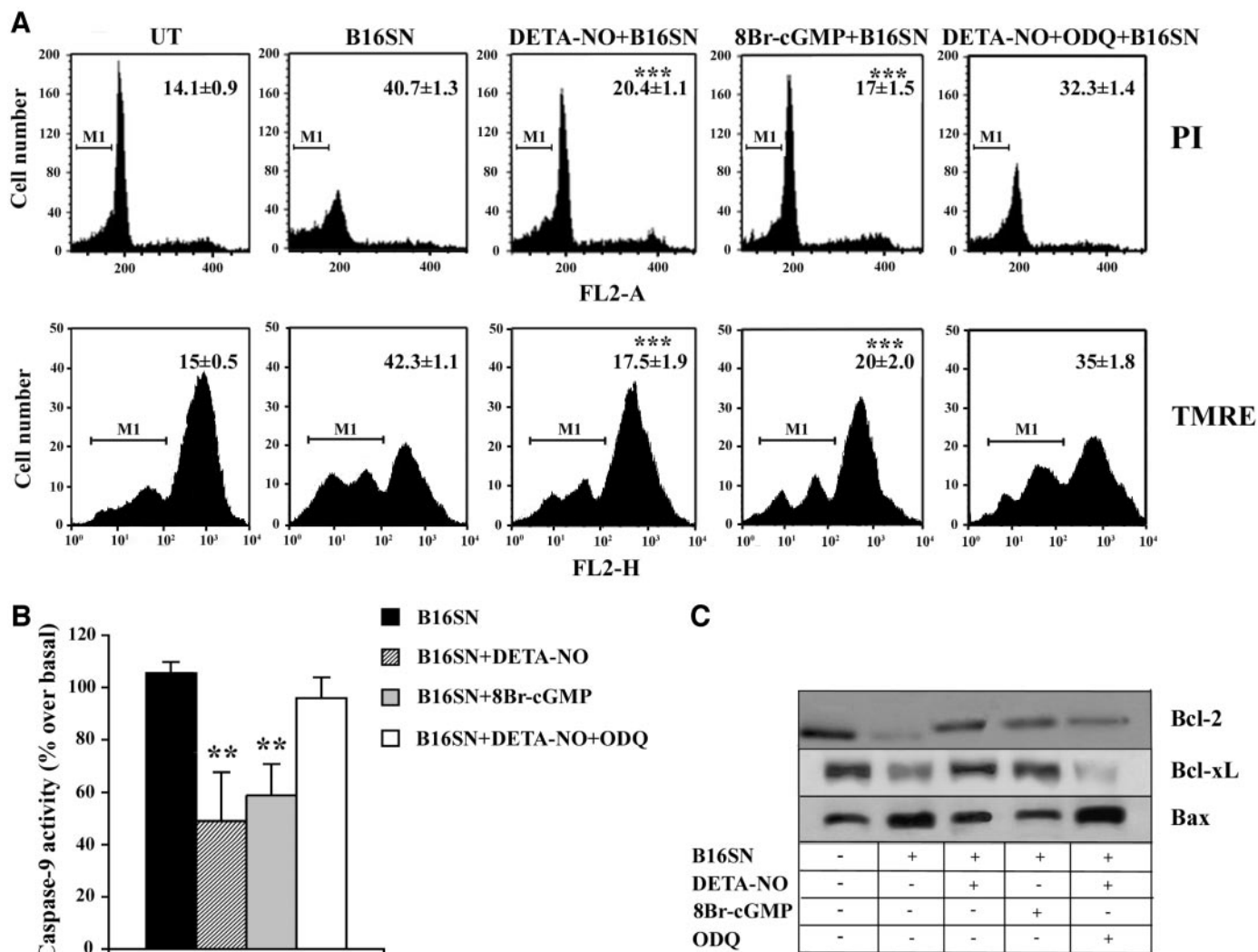


Fig. 2. Treatment with nitric oxide (NO) counteracts in a cGMP-dependent manner apoptogenic signals elicited in dendritic cells (DCs) by B16SN. DCs were treated for 2 h in the absence (UT) or presence of (z)-1-[2-(2-aminoethyl)-N-(2-aminoethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO; 10 μ M), 8-Br-cGMP (3 mM), or DETA-NO plus *H*-(1,2,4)-oxadiazolo[4,3- α]quinoxalin-1-one (ODQ; 1 μ M). The compounds were then removed, and DCs were exposed for 72 h to B16SN. A, evaluation by flow cytometry of the hypodiploid DNA peak, measured after staining with propidium iodide (PI), or of the mitochondrial membrane potential, measured using the potential-sensitive mitochondrial dye tetramethylrhodamine ethyl ester (TMRE). Results are from one of eight reproducible experiments. The numbers in the panels represent the percentage of fluorescence values \pm SE measured for the eight experiments in the region indicated by the horizontal bars labeled M1. B, caspase-9 activity values are reported as the percentage \pm SE (bars) of those observed in untreated, control DCs (11 ± 0.1 pmol/min/mg; $n = 5$). C, expression levels of Bcl-2, Bcl-xL, and Bax, revealed by immunoblotting. The panel shows one of five reproducible experiments. Statistical significance versus B16SN-treated DCs: **, $P < 0.01$; ***, $P < 0.001$.

Treatment with DETA-NO resulted in significant ($P < 0.001$; $n = 8$) protection of DCs, an action mimicked by 8-Br-cGMP and prevented when the NO donor was administered together with ODQ (Fig. 2A). In the absence of tumors, none of the treatments affected DC viability (not shown). These results indicate that NO protects DCs from B16SN-induced apoptosis in a cGMP-dependent manner.

NO/cGMP Inhibits DCs Apoptosis by Reversing the Effects of B16SN on Activation of Caspase-9 and Expression of Members of the Bcl-2 Family of Proteins. Exposure of DCs to B16SN caused an increase in caspase-9 activity (Fig. 2B). Activation of caspase-9 was crucial to tumor-induced DC apoptosis as shown by the effects of the enzyme inhibitor Ac-LEHD-CHO (60 μ M), which reduced apoptosis by $79.3 \pm 6.8\%$ ($P < 0.001$ versus control; $n = 5$). Caspase-9 activation by B16SN was inhibited significantly ($P < 0.01$; $n = 5$) by treatment of DCs with either 8-Br-cGMP or DETA-NO, but not with DETA-NO plus ODQ (Fig. 2B). None of the treatments modified the basal activity of caspase-9 (not shown).

Upstream of caspase-9, altered expression of Bax, Bcl-xL, and Bcl-2 has been implicated in tumor-induced apoptosis of DCs (5, 10,

11, 14). Consistently, exposure of DCs for 48 h to B16SN resulted in increased expression of Bax and reduced expression of Bcl-2 and Bcl-xL (Fig. 2C). Treatment of DCs with DETA-NO reversed the effects of B16SN on expression of Bax, Bcl-2, and Bcl-xL (by 89 ± 4.9 , 87 ± 2.5 , and $97 \pm 3.4\%$, respectively; $P < 0.001$ in DETA-NO-treated cells versus control; $n = 5$). The effects of DETA-NO were mimicked by 8-Br-cGMP and prevented by ODQ (Fig. 2C). DETA-NO, 8-Br-cGMP, and ODQ had no effect on Bcl-2, Bax, and Bcl-xL expression levels when administered alone (not shown).

NO/cGMP Restores Mixed-Lymphocyte Reaction and Cytotoxic Functions of DCs Exposed to B16SN. DCs exert a direct cytotoxic effect against tumor cells (16, 21). DCs exposed for 16 h to B16SN displayed significantly reduced cytotoxicity against B16-F1 cells (Fig. 3A). DETA-NO restored DC cytotoxic activity. DCs did not kill syngeneic splenocytes used as controls (not shown). DCs exposed to tumors stimulate T-cell proliferation less efficiently (3, 14). Indeed, DCs incubated for 16 h with B16SN showed a reduced capacity to stimulate T cells in a mixed-lymphocyte reaction assay (Fig. 3B). DC

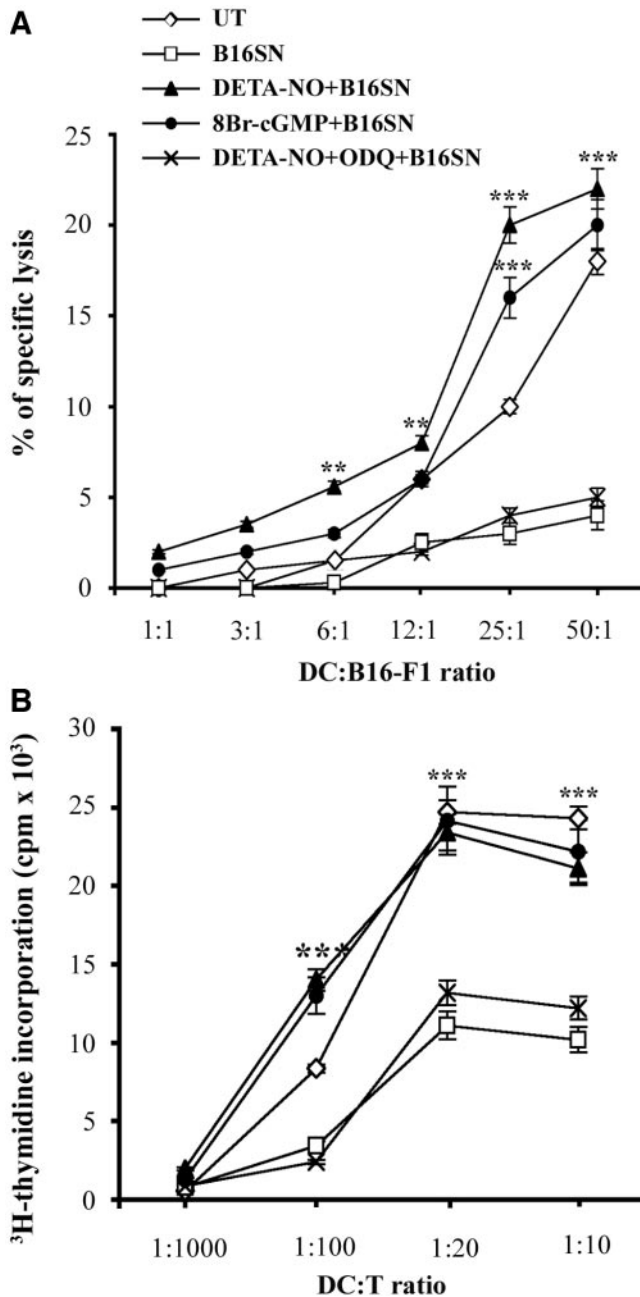


Fig. 3. Treatment with nitric oxide (NO) increases dendritic cell (DC) functions in a cGMP-dependent manner. DCs were treated for 2 h in the absence (UT) or presence of (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO; 10 μ M), 8-Br-cGMP (3 mM) or DETA-NO plus *H*-(1,2,4)-oxadiazolo[4,3- α]quinoxalin-1-one (ODQ; 1 μ M). The compounds were then removed, and DCs were exposed for 16 h to B16SN. A, DCs were mixed with ⁵¹Cr-labeled B16-F1 cells at the indicated ratios. Shown are the percentages of specific ⁵¹Cr release \pm SE (bars; $n = 5$). B, DCs were irradiated and cocultured at the indicated ratios with allogeneic T cells (10^5 cells/well). [³H]Thymidine incorporation in T cells was measured after 5 days ($n = 5$). Statistical significance versus B16SN-treated DCs: **, $P < 0.01$; ***, $P < 0.001$.

treatment with DETA-NO rescued the phenotype. The effects of NO on both DC cytotoxic activity and their ability to activate T cells were cGMP dependent because they were mimicked by 8-Br-cGMP and were prevented when DETA-NO was added together with ODQ.

Discussion

In the present study, we showed that a brief *ex vivo* treatment of DCs with NO increases their antitumor therapeutic efficacy. NO-treated DCs not only reduced the growth of the highly tumorigenic

and poorly immunogenic B16 melanoma, but also caused tumor regression and improved animal survival. In contrast, untreated DCs disappeared from the tumor site and failed to cure any animals. The action of NO was found to be persistent (brief exposure of DCs yielded long-lasting antitumor action) and to depend on the generation of cGMP after activation of guanylate cyclase, an enzyme activated by physiological (nanomolar) concentrations of the gas (13, 15, 17, 18). Thus, antitumor action was obtained with a short-term drug exposure, compatible with minimal manipulation of DCs *ex vivo*, and with concentrations of NO in the physiological range.

The antitumor action of NO/cGMP was associated with an increased persistence of DCs in the tumor. This suggests that *in vivo* DCs were protected against tumor-induced apoptosis. This conclusion is supported by *in vitro* experiments showing that a brief pulse with NO increases DCs resistance to the apoptogenic effects of the tumor environment. NO inhibited the tumor-induced reduction of the mitochondrial membrane potential and activation of caspase-9 and restored the expression levels of Bcl-2, Bax, and Bcl-xL, which are altered in tumor-exposed DCs (4, 5, 10, 11, 14). This, together with the observation that the NO action persists after its removal, strongly suggests that NO switches off the entire apoptotic program triggered by tumors in DCs rather than acting as a simple negative modulator of selected apoptotic signals. This explains the efficacy and the persistence of the protective action of NO. We found two additional effects of NO that might contribute to its antitumor effect. NO prevented the tumor-induced impairment of both DC cytotoxic activity and the ability of DCs to stimulate T-cell proliferation. Of importance, all of the *in vitro* effects of NO were found to depend on generation of cGMP, consistent with the cGMP dependence of its effects *in vivo*.

The treatment of DCs we propose does not require extensive cell manipulation, is not toxic, and appears worth pursuing in view of the new NO donors and compounds able to increase cGMP concentration undergoing validation for clinical use at present (22). Because of its simplicity, the treatment might be easily combined with other strategies to yield an enhanced therapeutic effect combining increased DC survival and efficacy in eliciting antitumor immune responses.

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