The Vascular Disrupting Agent, DMXAA, Directly Activates Dendritic Cells through a MyD88-Independent Mechanism and Generates Antitumor Cytotoxic T Lymphocytes

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Abstract
5,6-Di-methylxanthenone-4-acetic acid (DMXAA) is a small molecule in the flavanoid class that has antitumor activity. Although classified as a "vascular disrupting agent," we have recently conducted studies showing that DMXAA has remarkable efficacy in a range of tumors, working primarily as an immune modulator that activates tumor-associated macrophages and induces a subsequent CD8+ T-cell-mediated response. To more completely analyze the effect of DMXAA on CD8+ T-cell generation, we treated mice bearing tumors derived from EG7 thymoma cells that express the well-characterized chicken ovalbumin neotumor antigen. Treatment with DMXAA led to cytokine release, tumor cell necrosis, and ultimately reduction in tumor size that was lymphocyte dependent. Within 24 h of administration, we observed dendritic cell activation in tumor-draining lymph nodes (TDLN). This was followed by a rapid and marked increase in the number of tetramer-specific CD8+ T cells in the spleens of treated animals. In contrast, the vascular disrupting agent combretastatin A4-phosphate, which caused a similar amount of immediate tumor necrosis, did not activate dendritic cells, nor induce an effective antitumor response. Using in vitro systems, we made the observation that DMXAA has the ability to directly activate mouse dendritic cells, as measured by increased expression of costimulatory molecules and proinflammatory cytokine release via a pathway that does not require the Toll-like receptor adaptor molecule MyD88. DMXAA thus has the ability to activate tumor-specific CD8+ T cells through multiple pathways that include induction of tumor cell death, release of stimulatory cytokines, and direct activation of dendritic cells. [Cancer Res 2007;67(14):7011-9]

Introduction
5,6-Di-methylxanthenone-4-acetic acid (DMXAA) is a small molecule in the flavanoid class that has antitumor activity thought to be due to its ability to induce high local levels of tumor necrosis factor-α (TNF-α) that disrupt established blood vessels within tumors (1, 2). The drug has completed phase I testing in humans and is currently in phase II trials in combination with chemotherapy (3–5). Although often characterized as a "vascular disrupting agent," we have recently conducted studies showing that DMXAA has remarkable efficacy in a range of tumors, but primarily as an immune modulator that requires a biphasic effect for efficacy (6). First, DMXAA activates tumor-associated macrophages to release a variety of immunostimulatory cytokines and chemokines. This leads to vascular necrosis and some initial tumor regression. However, for effective tumor treatment, a subsequent CD8+ T-cell-mediated response is required.

The exact nature and mechanisms by which this antitumor CD8+ T cell response is generated by DMXAA is unknown. Tumor necrosis induced by vascular disruption and subsequent ischemia/reperfusion might lead to enhanced cell death, antigen release, and subsequent cross-priming. Macrophage activation and release of cytokines within the tumor might allow direct antigen presentation by macrophages or cytokine-mediated activation of dendritic cells (DC). However, given the effect of DMXAA on macrophages, we also explored the possibility that it might directly activate another myeloid-derived cell line, DCs.

The purpose of this study was to more carefully analyze the effect of DMXAA on CD8+ T cell generation. To facilitate this, we chose to study the EG7 thymoma system. EG7 cells express the chicken ovalbumin neotumor antigen (7). The dominant MHC class I peptide from ovalbumin (SIINFEKL) has been well characterized in C57B6 mice, and tetramers are available to specifically track these cells.

We show that DMXAA is effective in EG7 tumors, and that this effect is T cell dependent. Treatment with DMXAA led to rapid cytokine release and tumor cell necrosis. Within 24 h, we also observed DC activation in both the tumor-draining lymph nodes (TDLN) and in non-TDLNs. This was followed by a marked increase in the number of tetramer-specific CD8+ T cells in the spleens of treated animals. However, tumor cell death alone, as induced by the vascular disrupting agent combretastatin A4-phosphate (CA4P), did not induce DC activation in the TDLN, did not increase the number of tetramer-positive cells, and was not an effective antitumor agent. Using in vitro systems, we made the observation that DMXAA has the ability to directly activate mouse DC, as measured by increased expression of costimulatory molecules and proinflammatory cytokine release via a pathway that does not require the Toll-like receptor (TLR) adaptor molecule MyD88 and has different kinetics than known DC activators such as lipopolysaccharide (LPS) or stimulatory oligonucleotides. DMXAA thus has the ability to activate tumor-specific CD8+ T cells through multiple pathways that include induction of tumor cell death, release of stimulatory cytokines, and direct activation of DCs.
Materials and Methods

Cell lines. EG7 is a murine thymoma cell line transfected with an OVA cDNA construct on a C57/B6 background (7) and was provided by Wolfgang Weninger (Wistar Institute, Philadelphia, PA). EG7 cells were cultured and maintained in G418 selection media consisting of RPMI supplemented with 100 mg/mL G418, 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 100 mmol/L sodium pyruvate, and 2.5% β-mercaptoethanol. This cell line was regularly tested and maintained negative for Mycoplasma contamination.

Mice. Pathogen-free female C57/B6 mice (6–8 weeks old) were purchased from Taconic Laboratories or the Jackson Laboratory. Severe combined immunodeficiency (SCID) mice were purchased from the Wistar Institute. MyD88–/– mice were kindly provided by Dr. S. Akira (Research Institute for Microbial Diseases, Osaka, Japan; ref. 8) and were on the B6 background, backcrossed at least seven generations. Tlr3–/– knock-out mice were a kind gift from Dr. Richard Flavell at Yale University. Animals were housed in the animal facility at the Wistar Institute (Philadelphia, PA) or the University Laboratory Animal Resources facility of the University of Pennsylvania. The Animal Use Committees of the Wistar Institute and University of Pennsylvania approved all protocols in compliance with the care and use of animals.

Animal tumor models. Tumors were established with s.c. flank injections of 1 × 10^6 single cell suspensions in 100 µL PBS. Tumors were measured twice weekly, and volumes were estimated using the formula 3.14 × (largest diameter × (perpendicular diameter)^2)/4. Treatment was administered when tumors were > 200 to 300 mm^3 in size. Tumors were harvested in some studies at specified times (see below) for analysis. In other studies, mice were followed for tumor growth and sacrificed when the tumors became >10% body weight or the animals showed signs of distress. All experiments had at least five mice per group and were repeated at least once.

Reagents. The sodium salt of DMXAA was synthesized at the Auckland Cancer Society Research Center (9). DMXAA was formulated in normal saline and (unless otherwise stated) administered by i.p. injections at a dose of 18 mg/kg in 200 µL saline. CA4P was provided by OXiGene, Inc. The CpG oligodeoxynucleotide TCCATGAACGTT, known as CpG 1668, was synthesized on a phosphorothioate backbone (Integrated DNA technologies).

Protein studies for cytokine and chemokine levels. To compare changes in cytokine and chemokine protein levels within tumors, mice bearing EG7 flank tumors (n = 3 in each group) that were >200 to 300 mm^3 in size were treated with DMXAA. Tumors were harvested after 24 h and sonicated for 30 s in 1 mL of complete buffer [50 mM PBS containing one tablet of antiprotease cocktail (Roche, Indianapolis, IN)]. Tissues were then spun at 3000 rpm for 10 min and filtered through a 1.2-µm syringe filter unit. Total protein in each sample was determined. Mouse cytokine expression was measured using a mouse cytokine bead Lincoplex kit using LumineX 100 IS instrument as previously described (6). Samples were analyzed for TNF-α, IL10, IL12, IL18, IFN-γ, interleukin-12 (IL-12), IL-10, and IL-6. The concentration of cytokines was determined from a standard curve assayed at the same time with known amounts of recombinant cytokines.

In vitro DC activation. Tumor-bearing mice were treated with saline, DMXAA, or CA4P for 24 h. Draining and nondraining lymph nodes were isolated and harvested from euthanized mice and made into single cell suspensions by straining through a 70-µm filter with PBS with 10% FBS. Lymphocytes were washed, counted, and pooled. Fc receptors were blocked with antimonouse unconjugated CD16/32 antibody (BD Pharmingen) for 15 min following by washing. Lymph node cells were then incubated for 50 min at 4°C with anti-CD45, CD11c, CD86, CD80, CD40, and intercellular adhesion molecule-1 (ICAM-1) antibodies (BD Pharmingen). Stained cells were analyzed on a FACs Calibur (BD Biosciences), and data analysis was done using FlowJo software (Tree Star, Inc.). Histogram plots for CD86, CD80, CD40, and ICAM-1 expression was established for CD45^-/CD11c^+ DC.

Tetramer staining. To investigate the number and distribution of antigen-specific CD8^+ T cells, tumor-bearing mice were treated with saline, DMXAA, or CA4P. Spleen and draining lymph nodes were harvested 5 days after treatment. Tissues were crushed and strained through a 70-µm filter with PBS with 10% FBS. Tissues were then incubated 30 min at room temperature in APC-labeled ovalbumin iTAG tetramers (Beckman-Coulter Immunomics) loaded with the OVA peptide SIINFEKL. After 30 min, cell surface antibodies for CD8 were added for an additional 30 min. Samples were washed and fixed in 2% paraformaldehyde, and flow cytometry was done. The percentage of antigen-specific cells was determined by first gating on lymphocytes (based on size) and then gating on CD8^-/tetramer^- lymphocytes.

Pathologic studies. Animals bearing flank tumors were treated with i.p. injections of PBS, DMXAA, or CA4P at the doses described above. Three mice per group were euthanized 24 h after treatment, tumors harvested and immediately placed in formalin and later embedded in paraffin. Sections of 5 µm were cut followed by H&E histologic staining done according to established protocols. Tumor necrosis was quantified using image analysis to determine the percentage of necrotic versus viable tumor in multiple sections (>8) per tumor by a blinded observer.

DC culture. Bone marrow–derived DCs (BMDC) were cultured using modifications to a previously described protocol (10): In 24-well plates, 1 × 10^6 total bone marrow cells per well were seeded in 1 mL Iscove's modified Dulbecco's medium (IMDM) with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L l-glutamine, 1.5 µmol/L 2-ME, 3 ng/mL granulocyte macrophage colony-stimulating factor, and 3 ng/mL IL-4. On day 2, another 1 mL of supplemented media was added, and every other day thereafter, 1 mL of old media was removed and replaced with fresh media. After 7 days of culture, the cells were harvested, washed thoroughly, and used. In some experiments, BMDCs were enriched using magnetic beads to a purity of at least greater than 93% CD11c^-/CD11c^+ cells. Briefly, 1 µL biotinylated anti-CD11c antibodies (BD Pharmingen) per 15 × 10^6 cells was added to cells suspended in at least 500 µL buffer comprised of PBS with 2 mMol/L EDTA and 0.5% bovine serum albumin. After 30 min at 4°C, cells were washed with buffer and labeled with MACS Streptavidin MicroBeads (Miltenyi Biotech) per manufacturer's instructions. Cells were then positively selected using MACS LS columns (Miltenyi Biotech) and washed before use in experiments.

In vivo DC activation. About 2.6 × 10^5 BMDCs were cultured in 96-well round-bottom plates in complete IMDM, with or without the addition of 0.5 µmol/L CpG DNA, 100 ng/mL LPS (Ultrapure LPS, InvivoGen), or graded concentrations of DMXAA. In some experiments, 1 µg/mL polymyxin B (Sigma) was added to all cultures to neutralize LPS activity. After 24 h, cell-free supernatants were collected, and cells were harvested. Cytokine concentrations in supernatants were determined by commercial ELISA kits (OptEIA, BD Pharmingen) according to the manufacturer’s instructions. Cells were washed, FcγRIII receptors were blocked (Fc Block, BD Pharmingen), followed by staining with surface antibodies to CD11c and CD86 for flow-cytometric analysis.

RNA isolation and real-time, reverse transcription-PCR. Quantitation of BMDC mRNA levels was done as previously described (6). BMDCs were prepared as described above, and on day 7 of culture, LPS (100 ng/mL), CpG DNA (0.5 µmol/L) DMXAA (150 µg/mL), or PBS (control) was added. After 1 and 6 h, at least 1 × 10^6 cells were harvested from each condition and total RNA isolated using the RNeasy Mini Kit/Qiashredder spin homogenizer (Qiagen Inc.). RNA (3 µg) from each condition was reversed transcribed using 5-µg oligo(dT) (Promega) and 1 unit PowerScript reverse transcriptase in First-Strand Buffer and 10 mmol/L DTT (Clontech) for 60 min at 42°C. Equal amounts of cDNA from each condition was pooled. Primers were obtained from the literature or designed using standard protocols. Primer sequences can be obtained from the authors upon request. Semi-quantitative analysis of gene expression was done using a Cepheid Smart Cycler following the...
manufacturer’s protocol for SYBR Green kit supplied by Roche. cDNA concentrations from each gene pool were normalized using β-actin as a control gene. Relative levels of expression of each of the selected gene (fold change versus saline control) were determined. Each sample was run in triplicate or quadruplicate.

**Statistical analysis.** Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student’s t tests. Multiple comparisons were made using ANOVA with appropriate post hoc testing. Differences were considered significant when P value was <0.05.

**Results**

DMXAA is effective in EG7 tumors, induces cytokines, and causes tumor necrosis. To study specific mechanisms involved in the immune response generated by DMXAA, we used a tumor system with a well-characterized tumor antigen. EG7 thymoma cells express the chicken ovalbumin neotumor antigen (7) and grow well in the flanks of C57/B6 mice. The dominant MHC class I peptide (SIINFEKL) has been well characterized, and tetramers are available to specifically track antigen-specific CD8+ T cells.

To determine if DMXAA was effective in this tumor model, mice bearing large tumors (300–400 mm³) were injected with 18 mg/kg DMXAA via the i.p. route. Similar to our previous studies with mesothelioma and lung cancer cell lines (6), this dose of DMXAA (a) induced substantial tumor necrosis in the center of the tumors (Fig. 1A versus B); (b) markedly up-regulated a number of tumor cytokine/chemokine proteins such as MCP-1, TNFα, RANTES, MIP1α, KC, IL-6, and IP-10 (Table 1); and (c) was effective in reducing tumor size with a number of cures (Fig. 1D and F).

DMXAA stimulates a CD8+-dependent antitumor immune response. To confirm the role of lymphocytes, C57/B6 or SCID mice were injected s.c. with 1 × 10⁶ tumor cells and were later injected i.p. with DMXAA when tumors reached 300 mm³. Tumor growth was followed and measured twice a week. In the SCID animals (Fig. 1E), there was an initial reduction in tumor size, but a

![Figure 1. Effect of DMXAA and CA4P on EG7 tumors. Histologic effects: mice with established EG7 tumors were treated i.p. with either saline (Control, A), 18 mg/kg of DMXAA (B) or 200 mg/kg of CA4P (C). Twenty-four hours later, tumors were harvested, and sections were stained using H&E. Both DMXAA (B) and combretastatin (C) induced hemorrhagic necrosis and microscopic tumor cell death in the center of the tumors. Effects on tumor growth: D, C57/B6 mice were injected s.c. in the flank with 1 × 10⁶ EG7 tumor cells. Once the tumors were 200 mm³, they were treated with DMXAA (18 mg/kg) i.p. ( ■), CA4P (200 mg/kg; ▲) or saline (control; ●). Tumor volumes were measured twice per week. *, P < 0.05 compared with control; **, P < 0.05 compared with control and CA4P. E and F, Scid mice (E) or C57/B6 mice (F) were injected s.c. with 1 × 10⁶ tumor cells and were later injected i.p. with DMXAA (25 mg/kg) when tumors reached 300 mm³. Tumor growth was followed and measured twice a week. In the Scid animals (E), there was an initial reduction in tumor size, but a rapid regrowth as opposed to the much stronger and prolonged antitumor effect in the C57/B6 mice (F). *, P < 0.05 compared with control.](https://www.aacrjournals.org/doi/abs/10.1158/0008-5472.CAN-07-0627)
rapid regrowth as opposed to the much stronger and prolonged antitumor effect in the C57/B6 mice (Fig. 1F). This is consistent with the biphasic effects of DMXAA seen previously (ref. 6; see Introduction).

Using the EG7 system, we could more carefully characterize this immune response. Five days following DMXAA injection, the spleens and TDLN from animals were isolated and subjected to fluorescence-activated cell sorting (FACS) analysis to identify CD8+ T cells reactive with the ovalbumin-specific peptide tetramer. Although DMXAA did not increase the number of CD8+ T cells in TDLNs (Fig. 2A), it did significantly (P < 0.01) increase the number of SIINFEKL-tetramer–positive CD8+ T cells by more than 4-fold (Fig. 2B). After DMXAA treatment, the percentage of CD8+ T cells in the spleens of tumor-bearing animals significantly increased (P < 0.01) from 15% to 24% (Fig. 2C). More importantly, the percentage of splenic CD8+ T cells that were tetramer positive went from 0.6% to 3.9% (P < 0.01; Fig. 2D). These data show that DMXAA treatment was highly effective in generating antigen-specific antitumor CD8+ T cells.

DMXAA activates TDLN DCs in vivo. Given this impressive CD8+ T cell response, we postulated that DMXAA was rapidly activating DCs within the TDLNs. Accordingly, 1 day after i.p. administration of DMXAA or saline into tumor-bearing mice, we dissected out TDLNs, combined the nodes from three animals and subjected the cells to multiparameter FACS. DCs were identified by CD11c+ staining. There were no differences in the size of the lymph nodes or the percentage of CD11c+ cells within the lymph nodes of DMXAA-treated or control-treated animals. However, as shown in Fig. 3, the DC in the mice treated with DMXAA had a marked up-regulation of the costimulatory molecule CD86 (mean fluorescent intensity of 96.7 versus 330; Fig. 3A). ICAM-1 (mean fluorescent intensity of 61 versus 123; Fig. 3B) and MCP-1

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<th>MIP-1α</th>
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<th>IFN-γ</th>
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<td>1.5</td>
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<td>44</td>
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<tr>
<td>DMXAA (pg/mL)</td>
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<td>47</td>
<td>13</td>
<td>175</td>
<td>94</td>
<td>478</td>
<td>0.9</td>
<td>2.3</td>
<td>20</td>
<td>476</td>
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<td>Fold change</td>
<td>4.5*</td>
<td>6.1*</td>
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<td>5.9</td>
<td>6.4</td>
<td>3.0</td>
<td>1.5</td>
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Table 1. Effect of DMXAA on cytokine concentrations within EG7 tumors

NOTE: Flank EG7 tumors were established by s.c. injection in mice. The mice were treated with 18 mg/kg DMXAA i.p. when the tumor size was ~300 mm3. To assess cytokine/chemokine levels, tumors (n = 3) were harvested 24 h after i.p. injection of DMXAA and homogenized, and cytokine levels were measured using a Luminex bead assay. Data in the last line of the table shows the fold change (DMXAA treated versus control).

*P < 0.05.
†P < 0.07.
inducing danger signals released by tumor necrosis (10). To test this hypothesis, we took advantage of the availability of a second vascular disrupting agent (CA4P; refs. 12, 13). CA4P works through an entirely different mechanism that involves direct endothelial cell toxicity (via microtubular inhibition) rather than cytokine release. We have confirmed this difference by doing real-time reverse transcription-PCR (RT-PCR) on combretastatin-treated tumors and observed only minimal up-regulation of cytokine/chemokine messages (data not shown), quite unlike the robust response after DMXAA (see Table 1 and ref. 6). However, treatment of tumor-bearing mice with 200 mg/kg of CA4P led to marked necrosis in EG7 tumors (Fig. 1C). The degree of tumor necrosis was quantified using image analysis in multiple tumors. Tumors from untreated animals had 22 ± 11% of tumor area with necrosis. Tumors from DMXAA-treated tumors had 63 ± 30% of tumor area with necrosis. Tumors from CA4P-treated animals had 76 ± 3% of tumor area with necrosis. The values from CA4P and DMXAA tumors were statistically larger than control (P < 0.05), but not different from each other.

Using CA4P as a necrosis control, we compared DC activation in TDLNs after saline, CA4P, and DMXAA. As shown in Fig. 3 and Table 2A, CA4P did not increase CD86, ICAM-1, or CD80 expression on the DCs in the TDLNs or non-TDLNs. In contrast to DMXAA, CA4P did not increase the percentage of tetramer-positive cells in the spleen (Fig. 2D). Thus, tumor necrosis alone is not sufficient for DC activation. Consistent with the importance of immune activation, tumors treated with CA4P showed only a short growth delay (probably due to the initial central necrosis) with rapid regrowth, whereas the animals treated with DMXAA showed marked tumor regression with 3/5 animals being cured (Fig. 1D).

**DMXAA can directly activate DC.** To study the mechanism by which DMXAA activated DCs, we switched to an in vitro model. We have previously shown that tumor-associated macrophages can be activated to secrete cytokines in vitro after exposure to DMXAA (6). Similar studies were conducted using mouse bone marrow–derived DCs (BMDC). In our first set of experiments, BMDC were cultured with LPS and CpG DNA (as positive controls) and varying concentrations of DMXAA (1–90 μg/mL). Supernatants collected from the cultures showed clear dose-responsive increases in IL-6 and IL-12 production after DMXAA treatment (Fig. 4A). FACS of the CD11c+ cells showed up-regulation of the costimulatory molecule CD86 upon treatment with DMXAA in a dose-responsive manner (Fig. 4B). DMXAA was thus capable of activating DCs in vitro.

To determine if DMXAA was activating DCs directly or indirectly via potentially contaminating mononuclear cells, we repeated these experiments using an enriched DC preparation. With the use of magnetic cell separation, CD11c+ expressing cells were positively selected and consistently enriched to purity at least >93%. DMXAA-induced purified DCs to produce IL-6 and IL-12 (Fig. 4C) and up-regulate CD86 and CD40 (Fig. 4D). To determine if these effects were due to the possible contamination of DMXAA with LPS, experiments were repeated in the presence of the LPS-binding antibiotic polymyxin B. Polymyxin B blocked LPS-mediated production of IL-12 and DC activation as measured by CD86 expression, but had no effect on DMXAA-mediated activity (data not shown).

We also studied the effect of DMXAA on DC at the RNA level (Table 2B). Purified DC were treated as above, but harvested at 1 and 6 h for RNA extraction, cDNA preparation, and real-time RT-PCR. LPS, CpG, and DMXAA all caused rapid, and in some cases dramatic, increases in CD40 and IL-12 production after DMXAA treatment (Fig. 4). We have previously shown that tumor necrosis may indeed activate DCs in vivo (11). However, we found that the CD11c+/GR-1+/B220+ cell population was up-regulated in DC from draining lymph nodes in DMXAA-treated animals compared with control or animals treated with CA4P.

**Fig. 3.** DMXAA (but not CA4P) activates DCs in draining lymph node. Tumor-bearing mice were treated with DMXAA (18 mg/kg) i.p., CA4P (200 mg/kg) or saline control. Twenty-four hours after treatment, draining lymph nodes (n = 3) were isolated, harvested, and pooled for FACS analysis. DC cells were distinguished by their CD45- and CD11c-positive staining. MFI, mean fluorescence intensity in arbitrary units. A, histograms for CD86 expression. B, histograms for ICAM-1. C, histograms for CD80 expression. All three molecules were up-regulated in DC from draining lymph nodes in DMXAA-treated animals compared with control or animals treated with CA4P.
cases, very large increases in IL-6 and IL-12 mRNA levels. Clear changes were also seen in message levels for CD86, CD40, and ICAM-1. Interestingly, similar to the FACS data, the induction of the costimulatory molecules was much more sensitive to DMXAA than the induction of cytokines when compared with LPS and CpG.

These data indicate that DMXAA can directly activate DC to produce costimulatory molecules and cytokines. DMXAA similarly DMXAA activation of CD86 expression was also independent of MyD88 (Fig. 4F). In similar experiments using BMDC derived from Tlr3−/− mice, we found the effects of DMXAA on DC to be independent of TLR3 expression (data not shown). These experiments indicate that activation of DC with DMXAA occurs independently of the TLR adaptor molecule MyD88 and suggests that DMXAA does not activate DCs via TLRs.

**Discussion**

Although a range of immunotherapies have been successful in preventing the growth of tumors ( prophylactic therapy) or in treating early or small tumors, immunotherapy has generally been quite ineffective in treating large, established tumors in animal models or in clinical trials (19–21). This can likely be explained by the extensive requirements for a successful adaptive antitumor immune response, which include (a) release of relatively specific tumor-derived antigens, (b) uptake of these antigens by tumor-infiltrating DCs, (c) activation of these DCs with migration to draining lymph nodes, (d) presentation of tumor antigen by the DC

### Table 2. Expression levels of DC activation markers

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<th>A. Expression levels of activation markers (mean fluorescence intensity using FACS) on DC from TDLNs and non-TDLNs</th>
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<tr>
<td><strong>TDLNs</strong></td>
</tr>
<tr>
<td>CD86</td>
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<td>DMXAA</td>
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<td>Combretatin</td>
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<th>B. Expression levels of mRNA for DC activation markers in vitro</th>
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<td><strong>Activation marker</strong></td>
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<td>ICAM-1</td>
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NOTE: (A) Tumor-bearing animals were treated with saline (control), DMXAA, or combretastatin (n = 3 for each group). Twenty-four hours later, the TDLN and non-TDLNs were isolated, combined, processed, and subjected to FACS. The expression levels of activation markers on the CD11c+ cells are expressed as the mean fluorescence intensity. The experiment was repeated once with similar results. (B) Cultured DCs were exposed to saline, LPS (100 ng/mL), CpG (0.5 μmol/L), or DMXAA (150 μg/mL), mRNA extracted, cDNA prepared, and subjected to real-time RT-PCR. Samples were normalized for β-actin expression, and each sample was compared with saline controls. The mean fold increase in message level (from quadruplicate samples) is tabulated along with the SE.
to lymphocytes, (e) generation and expansion of CTLs (primarily CD8+ T cells), (f) trafficking of the CTL to the tumor, and (g) CTL-mediated destruction of tumors (by either direct killing or by activation of other cytotoxic cells, like macrophages) (22–24).

Although each of these steps occur successfully in response to most infectious insults, the ability of the immune system to eliminate tumors is not nearly as effective. This difficulty in generating a successful antitumor response is clearly illustrated in the experimental EG7 thymoma model used here, where the tumor cells express a very immunostimulatory foreign antigen (chicken ovalbumin), yet are still able to grow easily in an immunocompetent mouse (25). This failure to recognize and reject cells expressing this antigen is likely due to numerous immunosuppressive barriers generated by the tumors, including generation of suppressor cells (such as myeloid suppressor cells, M2 macrophages, T-regulatory cells), the presence of inactivating cytokines.
Because of these multiple barriers, it has become increasingly clear that successful immunotherapy must attack as many of these obstacles as possible (23). The small flavonoid compound DMXAA is especially attractive as an antitumor agent in this regard because it has the ability to target multiple points in the immunotherapeutic cascade by inducing tumor cell death (antigen release), as well as altering the tumor microenvironment to become more immunostimulatory (1, 2). Specifically, in previous work (6), we showed that DMXAA was able to induce tumor regression by activating tumor-associated macrophages resulting in the secretion of immunostimulatory cytokines that led to (a) vascular activation, tumor vessel occlusion, and ischemic necrosis; (b) initial recruitment of mononuclear cells; and (c) subsequent recruitment of CD8+ T cells. This immune mechanism is consistent with experiments using the related flavanoid acetic acid (FAA) showing that mice treated with FAA + IL-2 were immune to rechallenge (29).

The purpose of this study was to more completely dissect the immunologic mechanisms by which DMXAA exerted its effects by using a model with a well-characterized tumor antigen (EG7 cells). We were first able to show that DMXAA induced central tumor necrosis and strong antitumor activity in EG7 tumors (Fig. 1B). The antitumor effect of DMXAA was lymphocyte-dependent in EG7 tumors (Fig. 1E), as it was in a variety of other tumors (6). With the availability of peptide-specific tetramers, we now clearly show that DMXAA markedly increases the number of antigen-specific T cells in both TDLN and in the spleen 5 days after therapy (Fig. 2).

We had originally assumed that the ability of DMXAA to induce a CTL response was due to DC activation induced by the combination of tumor cell necrosis plus the production of effective danger signals (such as necrotic cell debris and cytokines, including MCP-1, TNF, MIP1α, IFNγ, and IL-12). Our animal model confirmed the presence of such activated DCs (evidenced by up-regulation of CD86, CD80, and ICAM-1) 24 h after DMXAA treatment within the TDLNs (Fig. 3). Interestingly, we also saw up-regulation of these activation markers on DC in the non-TDLNs (Table 2) suggesting a systemic effect. By using the drug CA4P, we were able to confirm that induction of widespread tumor necrosis via ischemia/reperfusion is not sufficient to activate DC, induce antigen-specific T cells, or induce a therapeutically effective immunologic response. CA4P is a true vascular disrupting agent that targets the microtubules of tumor-associated endothelium leading to a vascular collapse within the tumor and subsequent necrosis (Fig. 1C; refs. 12, 13), but does not activate tumor-associated macrophages and does not induce cytokine release within the tumor (data not shown). Although the amount of tumor necrosis induced by CA4P was equal to or greater than that of DMXAA (Fig. 1), CA4P did not up-regulate DC activation markers (Fig. 3 and Table 2) and did not induce tetramer-positive cells (Fig. 2). Consistent with this lack of immune activation, CA4P only transiently slowed tumor growth, in contrast to the marked reduction in size after treatment with DMXAA (Fig. 1D).

Our most important new observation from these studies was the unexpected finding that DMXAA can also directly activate DCs. Although DMXAA and its parent compound, flavone 8-acetic acid (FAA), have been shown to activate NFκB in tumor-associated macrophages and natural killer cells (14–16, 29, 30), to our knowledge, there has been no published data showing these compounds could affect DC. When we studied purified bone marrow–derived DCs in vitro, we found that DMXAA had the ability to induce DC to up-regulate the mRNA expression and protein levels of cytokines (IL-6 and IL-12) and costimulatory molecules, such as CD86, CD80, and ICAM-1 (Fig. 4). We suggest that the combination of direct activation of DC in an environment that contains large amounts of necrotic tumor debris (potential tumor antigens) and a less immunosuppressive tumor microenvironment (Table 1) likely explains the ability of DMXAA to induce such effective antitumor immune responses capable of curing even large, established tumors. The signals that can activate DC to effectively present tumor antigens has been an area of active interest (10, 17, 31, 32). There are several distinct mechanisms leading to DC activation (17, 33). One group of activators include non–self-pathogen–associated molecules such as LPS, flagellin, and unmethylated DNA rich in CG motifs (CpG DNA) that are recognized by germ line–encoded pattern-recognition receptors (18). The best studied of these are the TLRs. TLRs act as sensors that, when stimulated, result in the rapid signaling of proinflammatory and costimulatory pathways (34). An increasing number of endogenous signals released from dying cells that also signal danger to DC are being recognized, including uric acid crystals, heat shock proteins, extracellular matrix components, guanine derivatives, and self DNA (10, 33, 35, 36). Some, but not all, of these endogenous stimulators are also recognized by TLRs.

It is currently unclear how DMXAA activates DC. Although it has been shown that DMXAA can activate NFκB in a number of cell types, despite more than 15 years of research on DMXAA and FAA, no DMXAA receptor has been identified, and the molecular mechanisms responsible for this activation remain unknown (2, 14–16). As discussed above, one obvious set of candidate receptors in DC are the TLRs, of which there are now more than 10 recognized members. For example, the immunostimulatory activity of small organic molecules like guanine nucleoside analogues are mediated through TLR-7 (35). As a way to study this question, we exposed DC derived from mice lacking the adapter protein MyD88 to DMXAA. All TLRs, except TLR3, have been shown to use the MyD88-dependent pathway, which leads to NFκB activation and proinflammatory cytokine production (34). Whereas signaling from our positive control (CpG DNA using TLR9) was completely abolished, DMXAA-induced IL-12 production and CD86 and CD40 up-regulation were independent of MyD88 (Fig. 4E and F). We also found the effects of DMXAA on DC to be independent of TLR3 expression. Thus, although we do not know whether DMXAA binds to a surface receptor on DC and initiates a signaling cascade or whether it diffuses into the cell and interacts directly with a cytoplasmic signaling molecule, such as a member of the NOD (nucleotide-binding oligomerization domain) protein family (37), we provide strong evidence that TLRs are not involved in this process. This idea is supported by (a) the different kinetics of mRNA expression seen after DMXAA compared with LPS and CpG, (b) the observation that costimulatory molecules (versus cytokines) are more affected by DMXAA than by LPS and CpG (Table 2), and (c) preliminary data looking at the interaction of DMXAA and TLR-dependent activation showing additive effects (data not shown). We also have data to suggest that DMXAA-induced macrophage activation is also MyD88 independent (38). Further work will be required to understand the mechanism by which DMXAA activates DC. Determining exactly where and how DMXAA interacts with the NFκB signaling pathway is an area of ongoing investigation.
There are a number of implications (and further questions) raised by this study. First, these studies add further support to the idea that DMXAA may be useful when combined with other types of immunotherapy such as vaccines or adoptive transfer. For example, Kanwar et al. (39) found that DMXAA combined with B7.1 (CD80)–mediated immunotherapy overcomes immune resistance and leads to the eradication of large tumors and multiple tumor foci. We have some preliminary data to support this hypothesis in vaccine trials and in adoptive transfer studies using transgenic OT-1 T cells (that have a transgenic T-cell receptor recognizing the OVA peptide SIINFEKL) in combination with DMXAA to treat EG7 tumors. Second, our observations suggest that DMXAA may be similar to an endogenous or microbial counterpart that may define a new pathway in innate immunity. Third, although DMXAA is currently in clinical trials (3–5), counterexamples suggest that DMXAA may be similar to an endogenous or microbial counterpart that may define a new pathway in innate immunity. That DMXAA may be similar to an endogenous or microbial counterpart that may define a new pathway in innate immunity. That DMXAA may be similar to an endogenous or microbial counterpart that may define a new pathway in innate immunity. That DMXAA may be similar to an endogenous or microbial counterpart that may define a new pathway in innate immunity.

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The Vascular Disrupting Agent, DMXAA, Directly Activates Dendritic Cells through a MyD88-Independent Mechanism and Generates Antitumor Cytotoxic T Lymphocytes


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