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

Africa Wallace, David F. LaRosa, Veena Kapoor, Jing Sun, Guanjun Cheng, Arminder Jassar, Aaron Blouin, Lai-Ming Ching, and Steven M. Albelda

1Thoracic Oncology Research Laboratory, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania and 2Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

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 (CA4P), did not induce DC activation in the TDLN, did not increase thenumber of tetramer-positive cells, and was not an antitumor 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 antitumor effect. 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.

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 antitumor effect. 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.

Note: A. Wallace and D.F. LaRosa contributed equally to this work.

Requests for reprints: Steven Albelda, Thoracic Oncology Research Laboratory, BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6166. Phone: 215-573-9933; Fax: 215-573-8469. E-mail: Albelda@mail.med.upenn.edu.

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Institute. MyD88 combined immunodeficiency (SCID) mice were purchased from the Wistar or the Jackson Laboratory. Severe mice were purchased from Taconic Laboratories. The sodium salt of DMXAA was synthesized at the Auckland Cancer Society Research Center. DMXAA was formulated in normal saline and used as described (10). In 24-well plates, 1 × 10^6 cells per well were seeded in 1 mL of Iscove’s modified Dulbecco’s medium (IMDM) with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL 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 inoculated with Tumor-bearing EG7 flank tumors (1 × 10^6 single cell suspensions in 100 µL PBS. Tumors were measured twice weekly, and volumes were estimated using the formula A x B x C/2.[23] Tumor volume doubling time was calculated from the mean of volume measurements for each group. Tumor necrosis was quantified using image analysis software (ImageJ, NIH) for 15 × 10^6 cells were added to cells suspended in at least 500 µl buffer comprised of PBS with 2 mmol/L EDTA and 0.5% bovine serum albumin.

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 GMEM media containing methionine 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−/− 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 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 used as described (10). In 24-well plates, 1 × 10^6 cells per well were seeded in 1 mL of 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+ cells. Briefly, 1 mL 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 Biotec) per manufacturer’s instructions. Cells were then positively selected using MACS LS columns (Miltenyi Biotec) and washed before use in experiments.

Protein studies for cytokine and chemokine levels. To compare changes in cytokine and chemokine protein levels within tumors, mice bearing EG7 flank tumors (n = 5 in each group) that were 200 to 300 mm³ 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 3,000 rpm for 10 min and filtered through a 1.2-µm filter. Total protein in each sample was determined. Mouse cytokine expression was measured using a mouse cytokine bead Lincoplex kit using 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 (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 cytokine concentrations in supernatants were determined by commercial ELISA kits (OptEIA, BD Pharmingen) according to the manufacturer’s instructions. Cells were washed, Fcγ/I/II 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/QiAasher spin homogenizer (Qiagen Inc.). RNA (3 µg) from each condition was reverse transcribed using 4 µL oligo(dT) (Promega) and 1 µL random hexamers. Final reaction volumes (20 µL) contained 1 unit of SuperScript II reverse transcriptase (Invitrogen). The reaction was incubated at 42°C for 60 min. Equal amounts of cDNA from each condition was pooled. Primers were designed from the literature or designed using standard protocols. Primer sequences can be obtained from the authors upon request. Semiquantitative 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 x 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
Table 1. Effect of DMXAA on cytokine concentrations within EG7 tumors

<table>
<thead>
<tr>
<th></th>
<th>MCP-1 (pg/mL)</th>
<th>TNF-1α</th>
<th>RANTES</th>
<th>MIP-1α</th>
<th>KC</th>
<th>IP-10</th>
<th>IFN-γ</th>
<th>IL-12</th>
<th>IL-10</th>
<th>IL-6</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>106</td>
<td>7.6</td>
<td>2</td>
<td>39</td>
<td>16</td>
<td>75</td>
<td>0.3</td>
<td>1.5</td>
<td>13</td>
<td>44</td>
</tr>
<tr>
<td>DMXAA</td>
<td>474</td>
<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>
</tr>
<tr>
<td>Fold change</td>
<td>4.5*</td>
<td>6.1*</td>
<td>6.5†</td>
<td>4.5†</td>
<td>5.9†</td>
<td>6.4†</td>
<td>3.0†</td>
<td>1.5</td>
<td>1.5†</td>
<td>10.8†</td>
</tr>
</tbody>
</table>

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.
CD80 (mean fluorescent intensity of 39.5 versus 61; Fig. 3C) were also up-regulated, indicating DC maturation/activation. The experiment was repeated, however, this time also examining the non-draining lymph node. As shown in Table 2A, FACS showed similar increases in the expression levels of CD68, CD80, and ICAM-1 on the DCs from the TDLNs and non-TDLNs. We did not see increases in CD40 at this 24-h time point.

We also did FACS to determine if DMXAA affected the numbers of plasmacytoid DCs in the TDLNs. Using a definition of pDCs as the CD11c{	extsuperscript{+}}/GR-1{	extsuperscript{−}}/B220{	extsuperscript{+}} cell population (11), we found similar levels of pDCs in control (9.4%) and DMXAA-treated (14%) animals.

**Tumor necrosis is not sufficient for DC activation.** One mechanism by which DMXAA might be activating DCs could be by 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{	extsuperscript{+}} 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 *vivo*.

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, 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.

**Activation of DC by DMXAA is MyD88 independent.** Although it has been established that DMXAA activates macrophages through a pathway dependent on the transcription factor nuclear factor κB (NFκB; ref. 14–16), other upstream signals involved in this pathway remain unknown. DCs use germ line-encoded sensors that recognize specific danger signals, which include TLRs (17, 18). Upon stimulation, all TLRs (except TLR3) use the intracellular adaptor molecule MyD88 for signaling. To determine if DMXAA activation of DCs required MyD88, we examined the effect of DMXAA on BMDC derived from MyD88−/− mice. As shown in Fig. 4E, whereas IL-12 production induced by CpG was completely inhibited in the DC from MyD88−/− mice, the effects of DMXAA on mouse DC IL-12 production was independent of MyD88. 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

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

A. Expression levels of activation markers (mean fluorescence intensity using FACS) on DC from TDLNs and non-TDLNs

<table>
<thead>
<tr>
<th></th>
<th>TDLNs</th>
<th>Non-TDLN</th>
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<tbody>
<tr>
<td></td>
<td>CD86</td>
<td>CD40</td>
</tr>
<tr>
<td>Control</td>
<td>76</td>
<td>12.7</td>
</tr>
<tr>
<td>DMXAA</td>
<td>352</td>
<td>15.3</td>
</tr>
<tr>
<td>Combrestatin</td>
<td>77</td>
<td>13.6</td>
</tr>
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</table>

B. Expression levels of mRNA for DC activation markers in vitro

<table>
<thead>
<tr>
<th>Activation marker</th>
<th>Treatment</th>
<th>mRNA expression by real-time RT-PCR: fold change over untreated</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>IL-6</td>
<td>LPS</td>
<td>308,600 ± 71,000</td>
</tr>
<tr>
<td></td>
<td>CpG</td>
<td>39,716 ± 4870</td>
</tr>
<tr>
<td></td>
<td>DMXAA</td>
<td>71 ± 13</td>
</tr>
<tr>
<td>IL-12</td>
<td>LPS</td>
<td>34 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>CpG</td>
<td>34 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>DMXAA</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>CD86</td>
<td>LPS</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>CpG</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>DMXAA</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>CD40</td>
<td>LPS</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CpG</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>DMXAA</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>LPS</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CpG</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>DMXAA</td>
<td>1 ± 0.1</td>
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</tbody>
</table>

**NOTE:** (A) Tumor-bearing animals were treated with saline (control), DMXAA, or combrestatin (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.
tumor vessel occlusion, and ischemic necrosis; (i.e., transforming growth factor-β and IL-10), as well as other T-cell–suppressive substances, such as prostaglandin E2 or arginine (26–28).

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 efficient 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), that DMXAA may be similar to an endogenous or microbial flavanoid molecule DMXAA effectively induces strong and antigen-specific antitumor CTL responses by interacting with both the innate and acquired immune system at multiple levels. An important new finding in this paper is that DMXAA can be added to the list of agents that have the ability to directly activate DCs.

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### References


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