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Activation of mitogen-activated protein kinases by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) plays an important role in macrophage stimulation

Jing Sun [a], Liang-Chuan S. Wang [a], Zvi G. Fridlender [a], Veena Kapoor [a], Guanjun Cheng [a], Lai-Ming Ching [b], Steven M. Albelda [a,*]

[a] Thoracic Oncology Research Laboratory, University of Pennsylvania School of Medicine, 1016B ARC, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318, United States
[b] Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

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ABSTRACT

The small molecule anti-tumor agent, 5,6-dimethylxanthenone-4-acetic acid (DMXAA, now called Vadimezan) is a potent macrophage and dendritic cell activating agent that, in the murine system, results in the release of large amounts of cytokines and chemokines. The mechanisms by which this release is mediated have not been fully elucidated. The mitogen-activated protein kinase (MAPK) pathways play an important role in the regulation of proinflammatory cytokines, such as TNF-α, IL-1β, as well as the responses to extracellular stimuli, such as lipopolysaccharide (LPS). The results of this study demonstrate that DMXAA activates three members of mitogen-activated protein kinase (MAPK) superfamily, namely p38 MAPK, extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), and c-Jun N-terminal kinases (JNKs) via a RIP2-independent mechanism in murine macrophages. By using selective inhibitors of MAPKs, this study confirms that both activated p38/MK2 pathways and ERK1/2 MAPK play a significant role in regulation of both TNF-α and IL-6 protein production induced by DMXAA at the post-transcriptional level. Our findings also show that interferon-γ priming can dramatically augment TNF-α protein secretion induced by DMXAA through enhancing activation of multiple MAPK pathways at the post-transcriptional level. This study expands current knowledge on mechanisms of how DMXAA acts as a potent anti-tumor agent in murine system and also provides useful information for further study on the mechanism of action of this potential anti-tumor compound in human macrophages.

1. Introduction

5,6-Dimethylxanthenone-4-acetic acid (DMXAA, now named Vadimezan) is a xanthone derivative with potent anti-tumor effects in multiple mouse models [1–4]. Work from our group and others have shown that DMXAA reduces tumor growth in several mouse tumor models through two phases. The early phase effect is mediated by macrophages that release significant amounts of TNF-α and nitric oxide (NO), causing hemorrhagic necrosis in tumors. The late phase response is mediated by migration of activated tumor-specific $CD8^+$ T-cells partly due to secretion of chemokines, such as CCL2 (MCP-1), CXCL10 (IP-10), and CCL5 (RANTES) induced by DMXAA in mouse macrophages and dendritic cells [1–4].

The wide range of cytokines and chemokines induced by DMXAA in mouse models raises interesting questions about the cell signaling pathways activated. It is known that DMXAA can activate the NF-κB, TBK1–IRF-3 and NOD pathways [5–7]. Given the broad importance of the mitogen-activated protein kinase (MAPK) pathways in the regulation of cytokines (see below), and one report suggesting the involvement of p38 MAP kinase in the action of DMXAA in endothelial cells [8], we hypothesized that DMXAA would be able to activate p38 MAPK along with other members of this family, the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), and c-Jun N-terminal kinases (JNKs) in macrophages.

The MAPKs are serine–threonine protein kinases that are activated in response to a variety of extracellular stimuli and

Abbreviations: ARE, adenylate/uridylate-rich elements; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; IFNAR, interferon alpha receptor; IL-6, interleukin 6; IP-10, interferon gamma-induced protein 10 kDa; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MK-2, MAP kinase activated kinase-2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NOD, nucleotide oligomerization domain; PCR, polymerase chain reaction; RANTES, regulated upon Activation, Normal T-cell Expressed, and Secreted or CCL5; RIP2, receptor-interacting protein 2 or RICK; TBK–IRF3, TANK-binding kinase-interferon regulatory factor 3; TBP, TATA-binding protein; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; TTP, tristetraprolin; 3′-UTR, 3′-untranslated region.

* Corresponding author. Tel.: +1 215 573 9933; fax: +1 215 573 4469.
E-mail address: albelda@mail.med.upenn.edu (S.M. Albelda).

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mediate signal transduction from the cell surface to the nucleus. They are widely expressed in multiple cell types and the MAPK signaling cascade controls such fundamental cellular processes as proliferation, differentiation, survival and apoptosis [9,10].

The MAPK superfamily is composed of three distinct subgroups, namely p38MAPK, ERK1 and ERK2, and JNKs. These kinases share 60–70% amino-acid sequence identity but, differ in the sequence and size of their activation loops, as well as in their activation in response to different stimuli. In general, activation of the ERKs is induced by growth-promoting mitogenic stimuli, whereas the JNKs and p38 MAPKs respond to environmental stresses, such as osmotic shock, hypoxia, heat shock, ultraviolet radiation and inflammatory cytokines [9,11].

p38 MAPKs are expressed in four distinct isoforms; two ubiquitously expressed isoforms, p38α and p38β, and two tissue-specific isoforms, p38γ and p38δ. p38 MAPKs have a central role in the regulation of proinflammatory cytokine production through both transcriptional and post-transcriptional mechanisms [12,13]. The p38/MK2 signal axis plays a very important role in upregulation of the proinflammatory cytokine production, such as TNF-α and IL-6 biosynthesis induced by LPS through stabilization and enhanced translation of mRNAs containing the adenylate/uridylate-rich elements (AREs) present in their 3′-untranslated region (3′-UTR) by phosphorylation of ARE-binding proteins [14–17].

In addition, many studies have shown that both ERK1/2 and JNKs MAPKs also play an important role in the regulation of proinflammatory cytokine production. JNK MAPKs regulate proinflammatory cytokine production mainly through transcriptional regulation mechanisms [18,19]. As for ERK1/2 MAPKs, studies have shown that the ERK pathway is involved in upregulation of TNF-α production by increasing TNF-α promoter activity via increased DNA binding activity of Egr-1 and NF-κB to the TNF-α promoter [20,21]. In addition to transcriptional regulation, ERK signaling pathway also plays an important role in post-transcriptional regulation of TNF-α production through regulating nucleocytoplasmic mRNA transport via a mechanism that targets the ARE in the 3′UTR of the TNF-α RNA through a Tpl2, also known as Cot, dependent mechanism [22].

Given that the cytokines secreted from activated macrophage are central effectors in DMXAA-mediated anti-tumor effects, we used both a murine macrophage cell line (MHS) and primary thioglycollate-induced peritoneal macrophages to determine if DMXAA could induce phosphorylation of MAPKs. In addition, we also used specific pharmacologic inhibitors of MAPKs to evaluate the role of MAPKs in DMXAA-induced cytokines secretion and the priming effect of IFN-γ on DMXAA.

2. Materials and methods

2.1. Mice

Pathogen-free BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA), and heterozygous RICK$^{+/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The heterozygous RICK$^{+/-}$ mice were bred to obtain the homozygous RICK$^{-/-}$ and wild type littermates. Animals were housed in the animal facility at the Wistar Institute (Philadelphia, PA). The animal use committees of the Wistar Institute and University of Pennsylvania approved all protocols in compliance with the care and the use of animals.

2.2. Cell culture and reagents

The murine macrophage cell line, MHS, was purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc, Lawrenceville, GA), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 0.45% glucose, 20 mM sodium bicarbonate, 10 mM HEPES. Thioglycollate-elicited mouse peritoneal macrophages were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. The cultures were maintained at 37 °C in an atmosphere containing 5% CO_2. All cell lines were regularly tested and maintained negative for Mycoplasma contamination.

DMXAA was purchased from Sigma–Aldrich (St Louis, MO), and was dissolved in deionized water. Muramyl dipeptide (MDP) was purchased from InvivoGen (San Diego, CA). Anti-mouse type I IFN receptor antibody (anti-mouse IFNAR-1) was purchased from BioLegend (San Diego, CA). Recombinant mouse interferon beta was purchased from PBL Biomedical Laboratories (Piscataway, NJ). BIRB796, a p38 inhibitor, was purchased from Axon Medchem BV (Groningen, The Netherlands). Mouse recombinant IFN-γ protein, FR180204 (ERK1/2 inhibitor) and 420135 (JNK1, 2/3 inhibitor) were purchased from Calbiochem (La Jolla, CA). All MAPK inhibitors were dissolved in DMSO first, and diluted to test concentration with culture medium and the final amount of DMSO in all the cultures was less than 0.5%.

2.3. Isolation of thioglycollate-elicited mouse peritoneal macrophages

BALB/c mice were injected intra-peritoneally with 2 ml of thioglycollate (1 mg in total; Becton, Dickinson and company, Sparks, MD). Four days later, peritoneal cavities were lavaged with 10 ml of culture medium and lavage fluid was spun at 1500 rpm for 5 min to collect the cells. Cells were then resuspended with 10 ml RBC lysing reagent (BD Biosciences Pharmingen, San Diego, CA) for 10 min to lyse erythrocytes. Peritoneal macrophages were then enriched by plating the peritoneal exudates in 6-well plates, and washing off all the non-adherent cells 3 h later. At least 85% of adherent cells isolated were macrophages, confirmed by cytospins stained with hematoxylin and eosin.

2.4. Blockade of MAPK pathways with pharmacological inhibitors

Mouse MHS cells or thioglycollate-elicited peritoneal macrophages were seeded in 24-well plates at 300,000 cells/well overnight. On the following day, cells were then treated with different MAPK pathway inhibitors, BIRB796 (0.1 μM), FR180204 (5 μM), or 420135 (10 μM). An hour later (or 2 h for BIRB796), the culture supernatants were removed and fresh culture medium containing DMXAA at 20 μg/ml was added to the culture for an additional 5 h. Fresh FR180204 and 420136 inhibitors were also added to the culture at this point to ensure continued ERK and JNK enzyme inhibition. When the 5 h incubation was over, total RNA was extracted (see below) from the cells and analyzed with real-time PCR. The culture supernatants were also collected for cytokine assays.

2.5. Cytokine and chemokine analysis

Cytokines/chemokines in the culture supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) kits to detect murine IFNβ (VeriKine Mouse IFN Beta ELISA Kit, PBL Biomedical Laboratories), IP-10 (DuoSet Mouse CXCL10/IP10/CRG-2, R&D Systems, Inc., Minneapolis, MN), IL-6 (BD Biosciences Pharmingen, San Diego, CA), TNF-α (BD Biosciences Pharmingen), and MCP-1 (BD Biosciences Pharmingen). The ELISAs were carried out according to the instructions of the manufacturers.

2.6. Immunoblots

MHS cells or thioglycollate-elicited mouse peritoneal macrophages were seeded in 60-mm tissue culture plates overnight and stimulated with either medium or DMXAA at 20 μg/ml in presence of 0.5 mM sodium orthovanadate for 2 h. The cells were then lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN)) with 1 mM PMSF and 2 mM sodium orthovanadate. Protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Equal amounts of whole cell lysates from each sample were electrophoresed on 4–12% NuPAGE Novex Tris Acetate Mini Gels (Invitrogen) and transferred onto nitrocellulose membranes. Monoclonal anti-β-actin antibody was used to verify equal loading. Monoclonal anti-β-actin antibody was purchased from Sigma. Rabbit anti-mouse phospho-p38, phospho-MAPKAPK-2, phospho-ERK1/2, p38 MAPK, ERK1/2, SAPK/JNK mAbs and mouse monoclonal anti-phospho-SAPK/JNK antibody were purchased from Cell Signaling Technology (Danvers, MA).

2.7. RNA isolation and quantitative real-time PCR

Quantitation of mRNA levels was done as previously described [7]. Cells from control (Medium) and DMXAA-treated cultures were harvested at various time points and homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA). Three microgram of RNA from each condition was reverse transcribed using 0.5 μg oligo(dT) (Promega, Madison, WI), 1 mmol/l deoxynucleotide triphosphates, and 1 unit SuperScript III reverse transcriptase in 1× First-Strand Buffer and 10 mmol/l DTT (Clontech, Palo Alto, CA) for 60 min at 50 °C. Equal amounts of cDNA from each condition were pooled. Primers were obtained from the literature or designed using standard protocols. Primer sequences can be obtained from the authors on request. Semi-quantitative analysis of gene expression was done using a Smart Cycler System (Cepheid, Sunnyvale, CA) following the manufacturer's protocol for SYBR Green kit supplied by Roche Applied Science. cDNA concentrations from each pool were normalized using β-actin or GAPDH as a control gene. Relative levels of expression of each of the selected genes (fold change versus medium control) were determined. Each sample was run in triplicate or quadruplicate and the experiment was repeated at least three times. PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide.

2.8. Statistical analysis

Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student's t test. Comparisons with more than two groups were done using ANOVA with appropriate post hoc testing. Differences were considered significant when p was <0.05. Data are presented as mean ± SEM. Results are representative of two to four independent experiments.

3. Results

3.1. DMXAA activates the p38–MK2 signal axis via a type I interferon-independent mechanism

To determine if DMXAA could induce phosphorylation of p38 MAP kinase, MHS cells were stimulated with either medium alone or DMXAA at 20 μg/ml at different time points and whole cell lysates were analyzed by immunoblotting using an anti-phospho-p38 antibody. As shown in Fig. 1A, DMXAA induced clear phosphorylation of p38 MAP kinase in as early as 30 min, with a peak at 2 h.

DMXAA can induce large amount of type I interferon secretion from macrophages [6] and type I IFNs can activate MAPK pathways [23–25]. To rule out a possible autocrine effect of type I interferons induced by DMXAA, we thus quantified the time course of IFNβ secretion level in murine macrophages treated with DMXAA via ELISA. MHS cells secreted no IFNβ at 2 h, but made large amounts (>4500 pg/ml) at 5 h (Fig. 1B). Peritoneal Macs secreted only small amounts of IFNβ at baseline (<10 pg/ml), that was virtually unchanged at 2 h, but increased 7–10 fold at 5 h (Fig. 1C). To further rule out autocrine IFNβ secretion, we stimulated MHS cells with medium alone or DMXAA for 2 h, with or without pre-treatment of the cells with a blocking antibody against type I interferon receptor (IFNAR-1). Immunoblots on whole cell lysates showed that DMXAA clearly induced phosphorylation of p38 in the presence of IFNAR-1 blockade (Fig. 1D). To prove the IFNAR-1 blocking efficiency, MHS cells were stimulated with medium alone or IFNβ at 5 ng/ml for 5 h, with or without pre-treatment of the cells with a blocking antibody against type I interferon receptor (IFNAR-1) at 10 μg/ml for 1 h. The supernatants were collected for detection of IP-10 secretion by ELISA. The result showed that IP-10 secretion induced by IFNβ was completely blocked in the presence of blocking antibody against type I interferon receptor (IFNAR-1) (Supplemental Fig. S1). These data, plus the kinetics, show that the activation of p38 by DMXAA is through an IFN-independent pathway.

To determine if DMXAA can also activate a downstream target of p38, such as MAP kinase activated kinase-2 (MK2), both MHS cells and thioglycollate-induced macrophages were treated with either medium alone or DMXAA at 20 μg/ml for 2 h. The whole cell lysates were analyzed by immunoblotting using anti-phospho-p38 and anti-phospho-MK2 antibodies. Both p38 and β-actin were used as loading controls. Fig. 1E shows that DMXAA clearly activates p38 MAPK and its downstream target, MK2 in both cell types.

3.2. Activated p38–MK2 pathways play a role in regulation of both TNF-α and IL-6 protein production induced by DMXAA at the post-transcriptional level

To determine the potential biological role of the activated p38/MK2 pathway in the regulation of cytokine production induced by DMXAA, a specific p38 MAPK inhibitor, BIRB796 (which is much more specific than the more commonly used SB203580) was used [26,27]. First, both MHS cells and peritoneal macrophages were pre-treated with BIRB796 at 0.1 μM for 2 h followed by DMXAA stimulation at 20 μg/ml for 2 h. The whole cell lysates were analyzed by immunoblot using the anti-phospho-p38 antibody with both p38 and β-actin as loading controls. BIRB796 potently inhibited the phosphorylation of p38 MAPK induced by DMXAA in both cell types (Fig. 2A).

Based on these inhibitory effects of BIRB796 on phosphorylation of p38 MAPK induced by DMXAA, both MHS cells and peritoneal macrophages were pre-treated with BIRB796 at 0.1 μM for 2 h followed by DMXAA stimulation at 20 μg/ml for 5 h. The supernatants were collected and cytokine and chemokine protein secretion was measured by ELISA. BIRB796 significantly ($p < 0.05$) decreased DMXAA-induced TNF-α and IL-6 protein secretion by about 40–70% in both MHS cells (Fig. 2B) and thioglycollate-induced macrophages (Fig. 2C). In addition, we also treated the MHS cells with BIRB796 at 0.5 μM and showed even higher decreased TNF-α protein secretion (Supplemental Fig. S2).

Next, we wanted to see whether or not inhibition of MAPK affects DMXAA-induced TNF-α and IL-6 at transcriptional level. Application of DMXAA strongly induced the mRNA levels of TNF-α and IL-6 in both cell types (Fig. 2D and E), measured by real-time

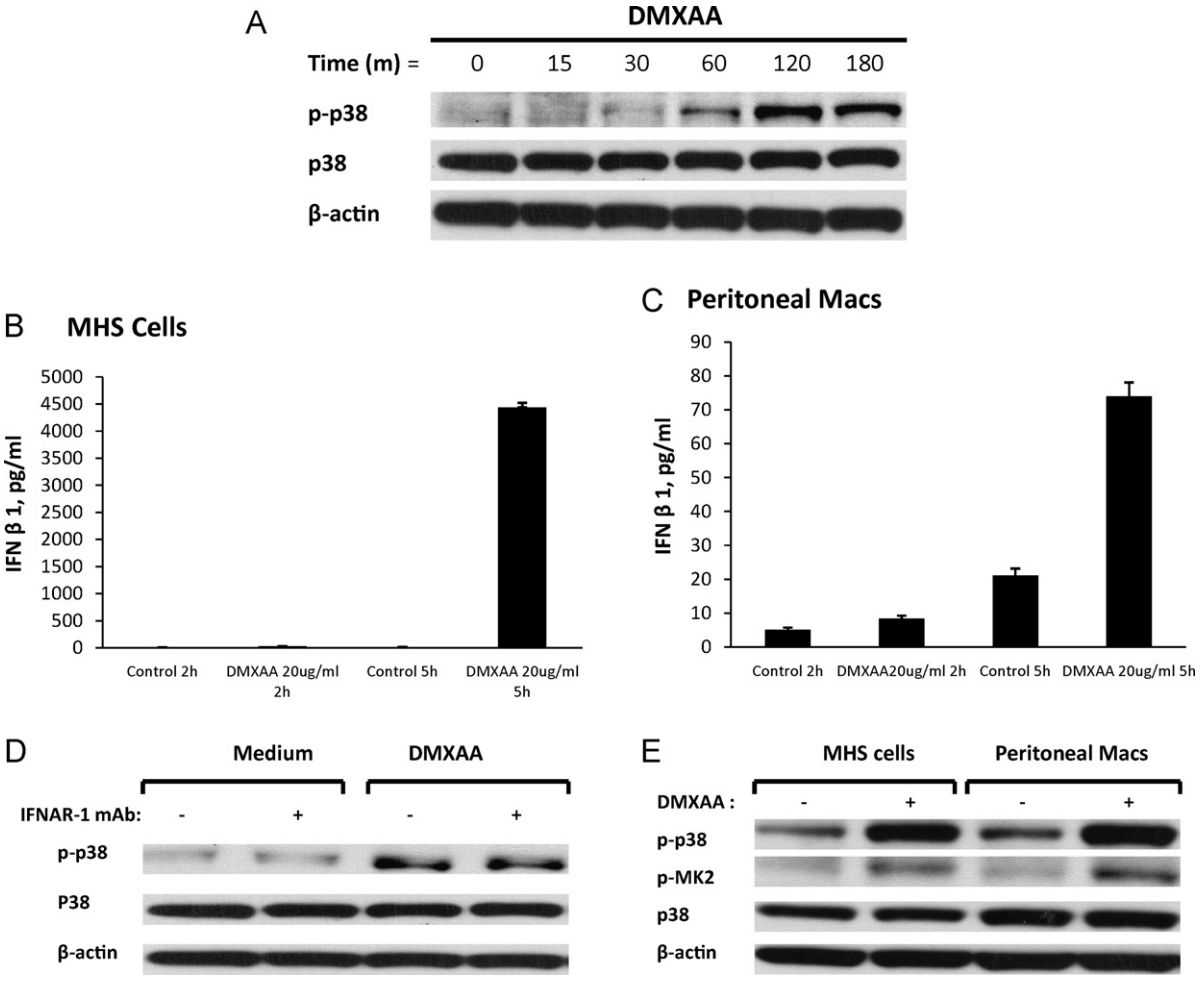

Fig. 1. DMXAA activates p38 pathway in mouse macrophages via an interferon-independent mechanism. Mouse macrophages, MHS (A, B, and D) and thioglycollate-elicited peritoneal macrophages (C and E), were stimulated with 20 μg/ml DMXAA in culture. At various time points, the culture supernatants and the cells were collected and assayed for IFNβ1 protein secretion (B and C) and amount of p38 and MK2 phosphorylation (A, D, and E) with ELISA and immunoblots respectively. The values for IFNβ1 protein concentrations were expressed as the mean ± SEM. Both p38 and β-actin expression were used to show equal loading of lanes.

PCR. However, in contrast to the protein results above, administration of BIRB796 had no significant effects on the changes of DMXAA-induced mRNA expression levels in TNF-α or IL-6 in MHS cells (Fig. 2D) or macrophages (Fig. 2E). BIRB796 actually slightly increased mRNA levels in most cases.

Taken together, these data suggest that the activated p38/MK2 pathway plays an important role in the regulation of DMXAA-induced TNF-α and IL-6 protein secretion in macrophages, but does so at the post-transcriptional level.

3.3. DMXAA also activates the ERK1/2 and SAPK/JNK1, 2/3 MAPKs

The ERK1/2 and SAPK/JNK1, 2/3 pathways are also involved in the regulation of proinflammatory cytokine production [18–22]. We thus determined if DMXAA might also activate ERK1/2 and SAPK/JNK1, 2/3 MAPKs. Accordingly, both MHS cells and thioglycollate-induced macrophages were treated with either medium alone or DMXAA at 20 μg/ml for 2 h. The whole cell lysates were analyzed by immunoblotting using anti-phospho-ERK1/2 or anti-phospho-SAPK/JNK1, 2/3 antibodies using both ERK or SAPK/JNK and β-actin as a loading controls.

Fig. 3A shows that DMXAA clearly activates ERK1/2 and SAPK/JNK1, 2/3 MAPKs through induction of phosphorylation of ERK and SAPK/JNK without changing total protein level in both MHS cells and thioglycollate-induced macrophages.

To examine the functional consequences in MHS cells, we used an ERK-selective inhibitor, FR180204, which inhibits the kinase activity of ERK1 and ERK2 with 30-fold greater selectivity against p38α and more than 100-fold greater selectivity against other kinases [28,29]. We also used a SAPK/JNK1, 2/3-selective inhibitor, 420135, with 1000-fold selective for JNKs over other MAP kinases including ERK, p38 and little inhibitory activity against other 74 kinases [30].

MHS cells were pre-treated with either medium alone or FR180204 at 5 μM or 420135 at 10 μM for 1 h followed by DMXAA stimulation at 20 μg/ml with the continuing presence of inhibitors at the same concentration for 5 h. The supernatants and mRNA were collected as above. The ERK1/2 inhibitor, FR180204, has a significant inhibitory effect (30–50%) on DMXAA-induced TNF-α and IL-6 protein secretion (Fig. 3B and Supplemental Fig. S3). In contrast, the SAPK/JNK1, 2/3 inhibitor, 420135, showed no significant inhibitory effects on DMXAA-induced TNF-α and IL-6 protein secretion (Fig. 3C). Moreover, we showed that DMXAA-induced TNF-α and IL-6 production were completely abolished in the presence of all three MAPK inhibitors (Supplemental Fig. S4).

Neither FR180204 nor 420135 showed any significant effects on DMXAA-induced TNF-α and IL-6 gene expression (Fig. 3D and E). These data indicate that activated ERK1/2 MAPK also plays an important role in regulation of both TNF-α and IL-6 protein production induced by DMXAA at the post-transcriptional level, while the SAPK/JNK1, 2/3 pathway is not involved.

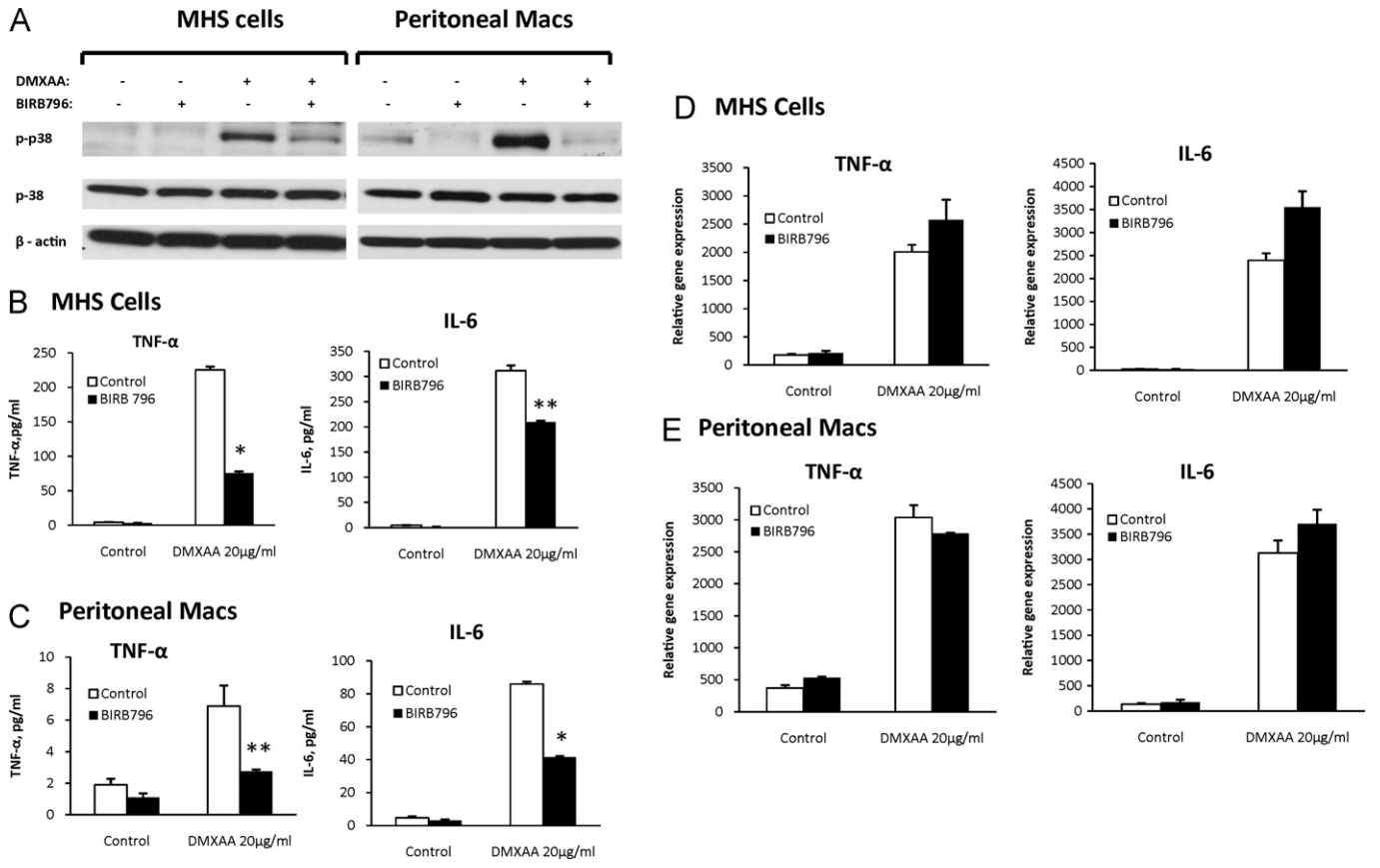

Fig. 2. Blockade of p38-mediated pathway activation attenuated DMXAA-induced TNF-α and IL-6 production at the post-transcriptional level. Both MHS cells and thioglycollate-elicited macrophages were pre-treated with BIRB796 at 0.1 μM for 2 h, followed by DMXAA stimulation at 20 μg/ml. The cells were harvested 2 h post-DMXAA treatment to look at p38 pathway activation by western blots (A). The culture supernatant and cell lysates were collected 5 h after DMXAA treatment to look at TNF-α and IL-6 protein level by ELISAs (B and C) or mRNA level by real-time PCR (D and E). Results represent the mean ± SEM for at least three independent experiments. $^{*}p < 0.01$ and $^{**}p < 0.05$, determined with Student's *t*-test.

3.4. MAPK pathways do not play a role in the production of DMXAA-induced chemokines such as IP-10 (CXCL10) or MCP-1 (CCL2)

Another striking feature of macrophages activated by DMXAA is the production of large amount of chemokines such as IP-10, MCP-1 and RANTES (CCL5) [3]. To determine if activated MAPK pathways play a role in regulation of these chemokines, MHS cells were pre-treated either with medium alone or with BIRB796 at 0.1 μM, FR180204 at 5 μM, or 420135 at 10 μM for 1 h followed by DMXAA stimulation at 20 μg/ml with the continuing presence of the inhibitors at the same concentration for 5 h. Chemokine levels in the supernatants were measured by ELISA and the cells were lysed for collection of mRNA and RT-PCR.

As expected, DMXAA stimulated the MHS cells to produce large amounts (>5000 pg/ml) of both IP-10 and MCP-1 (Fig. 4) and increased the mRNA expression levels of these chemokines, as well as that of RANTES (Table 1). However, unlike our data with cytokines, none of the inhibitors lowered DMXAA-induced chemokine protein secretion (Fig. 4) or mRNA expression levels (Table 1). Our results thus show that none of the activated MAPK pathways seem to play a role in regulation of DMXAA-induced chemokine production at either the transcriptional or post-transcriptional level.

3.5. Interferon-γ priming augments TNF-α protein secretion induced by DMXAA through enhancing activation of MAPK pathways

IFN-γ is known to "prime" or enhance TNF-α, or nitric oxide production by macrophages in response to either LPS or micro-particulate β-glucan. We therefore explored the role of IFN-γ priming in the production of proinflammatory cytokine induced by DMXAA in macrophages. MHS cells were thus treated with either medium alone or DMXAA at 20 μg/ml for 5 h with or without IFN-γ priming for 2 h. The supernatants were collected for detection of cytokines. We found that TNF-α protein production was augmented by 5- to 6-fold by IFN-γ priming (Fig. 5A). Interestingly, the marked increase we saw in TNF-α protein secretion after priming was not accompanied by any increase in the amount of TNF-α mRNA expression above that seen induced by DMXAA (Fig. 5B), indicating that the priming effect was due to post-transcriptional regulation.

To explore the role of the MAPK pathways in this effect, MHS cells were pre-treated with IFN-γ at 5 ng/ml for 2 h and then stimulated with DMXAA at 20 μg/ml for 2 h. The whole cell lysates were analyzed by immunoblotting with anti-phospho-p38, anti-phospho-MK2, anti-phospho-ERK1/2 and anti-phospho-SAPK/JNK1, 2/3 antibodies using both p38 and β-actin as loading controls. As shown in Fig. 5C, we found that IFN-γ alone had very small effects on MAPK activation, whereas, IFN-γ priming significantly enhanced the activation of MAPKs induced by DMXAA, with an especially strong effect seen in ERK1/2 MAPK activation.

To determine the functional effects of these changes in MAPK activation, MHS cells were pre-treated with MAPK-specific inhibitors for 1 h, followed by IFN-γ priming at 5 ng/ml for 2 h and then stimulation with DMXAA at 20 μg/ml for 5 h. The supernatants were collected for detection of TNF-α protein by ELISA. As mentioned above, in the absence of inhibitors, we saw

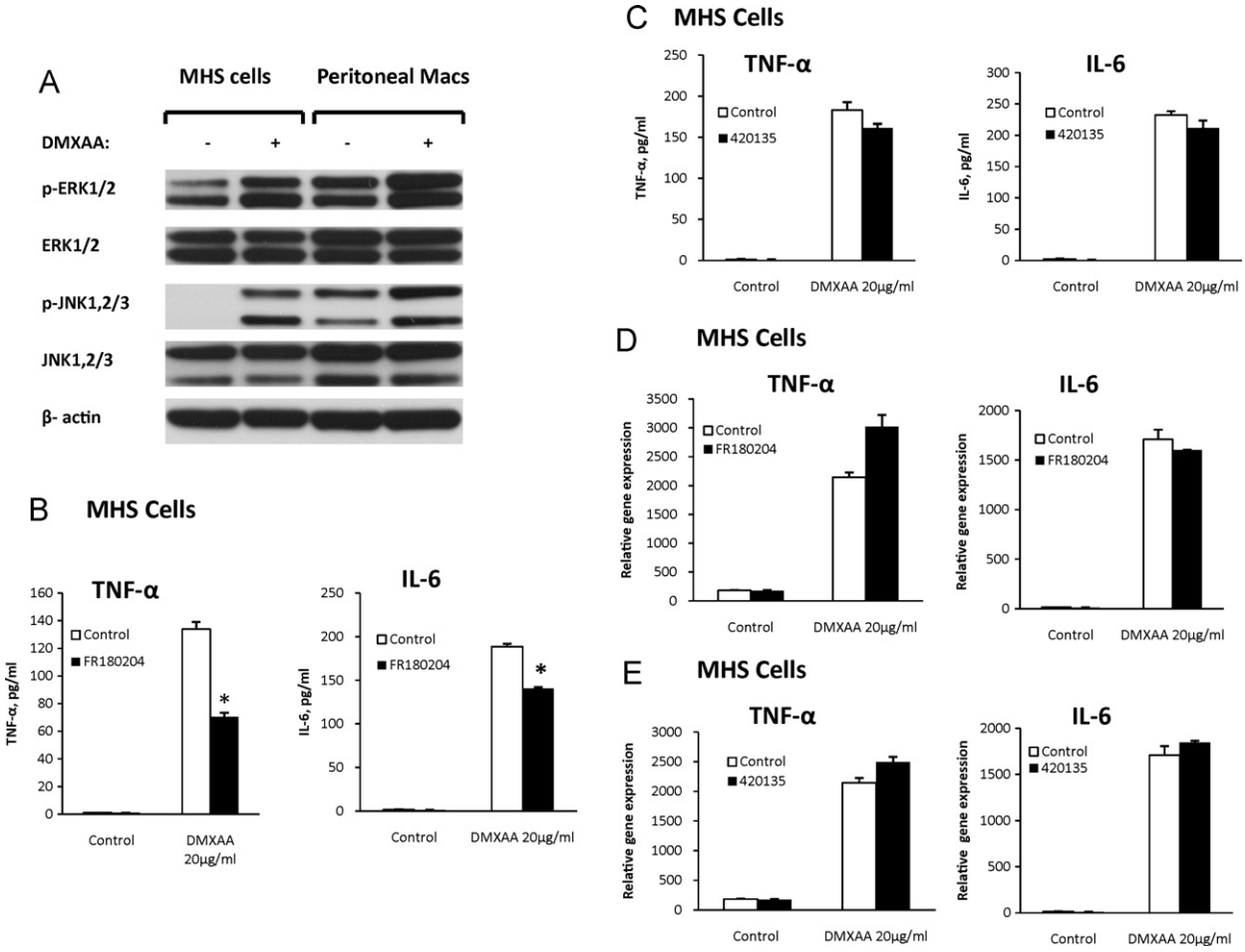

Fig. 3. DMXAA also activates ERK1/2 and SAPK/JNK1, 2/3 in macrophages, but only ERK1/2 signaling pathway plays a role in DMXAA-stimulated TNF-α and IL-6 production. Mouse macrophages, MHS and thioglycollate-elicited peritoneal macrophages were stimulated with 20 μg/ml DMXAA in culture for 2 h. Cells were then lysed, and amount of ERK1/2 and JNKs phosphorylation were analyzed with immunoblots (A). To investigate the role of ERK1/2 and JNKs in DMXAA-induced TNF-α and IL-6 production, FR180204 (B and D) and 420135 (C and E), the pharmacologic inhibitors for ERK1/2 and JNKs, respectively, were used. MHS cells were pre-treated with the inhibitors for 1 h, and DMXAA was then added to the culture and incubate for an additional 5 h. The culture supernatants were collected and assay for secreted TNF-α and IL-6 protein level, and the RNA were extracted from the cells and analyzed with real-time PCR to look at the mRNA expression. The data are expressed as the mean ± SEM. $^{*}p < 0.01$.

that IFN-γ priming dramatically augmented DMXAA-induced TNF-α protein secretion (Fig. 5D–F). This IFN-γ-dependent augmentation was diminished by applying the p38 or ERK1/2 specific inhibitors, BIRB796 or FR180204, but not by the JNKs inhibitor 420135 (Fig. 5D–F). These findings suggest that interferon-γ priming augments TNF-α protein secretion induced by DMXAA through enhancing activation of p38/MK2 signal axis and ERK1/2 MAPK pathways.

3.6. The activation of MAPK pathways by DMXAA is via RIP2 independent mechanism

Our group has recently shown that DMXAA has the ability to activate the nucleotide oligomerization domain 1 and 2 (NOD1 and NOD2) cytosolic signaling pathway [7]. It is known that NOD1 and NOD2 can activate MAPK pathway members, primarily by interacting with a protein kinase named RIP2 (receptor-interacting protein 2; also called RICK, RIPK2 and CARDIAK) [27]. To determine if DMXAA-induced activation of MAPK pathways was occurring through this signaling pathway, we took advantage of mice in which RIP2 was deleted, thus attenuating NOD1 or NOD2 signaling. Accordingly, wild type and RIP2$^{-/-}$ mice were injected intraperitonally with 2 ml of thioglycollate for 4 days. Purified peritoneal macrophages were seeded in 6 cm plates overnight and were stimulated with either medium alone, DMXAA at 20 μg/ml or muramyl dipeptide (MDP – a known ligand for NOD2) at 10 μg/ml for 2 h. The whole cell lysates were then analyzed by immunoblotting to assess phosphorylation of p38, ERK1/2 or JNKs.

As shown in Fig. 6, as expected, in wild-type (RIP2$^{+/+}$) mice (left three lanes), DMXAA increased phosphorylation of p38, ERK1/2 and JNKs. The NOD2 ligand, MDP, had an even stronger effect on all three proteins. In the RIP2$^{-/-}$ mice (right three lanes), the MDP-induced phosphorylation of p38, ERK1/2 and JNKs was clearly decreased. In contrast, there was no change in the DMXAA-induced MAPKs activation. The study shows that the activation of MAPKs by DMXAA is via a RIP2-independent mechanism.

4. Discussion

5,6-Dimethylxanthenone-4-acetic acid (DMXAA, now named Vadimezan) is a cell permeable, small molecule in the xanthone class that shows potent antitumor activity in several mouse models. In human, the clinical benefits of this drug have also been demonstrated in both phase I and phase II clinical trials [31–35]. The mechanism by which DMXAA exerts its anti-tumor actions has been of significant interest ever since its initial discovery in the 1990s. In the murine system, actions on both endothelial cells and leukocytes, in particular macrophages and dendritic cells, have been demonstrated [1–4]. With regard to macrophages, it is known that DMXAA can induce the secretion of large amounts of a number

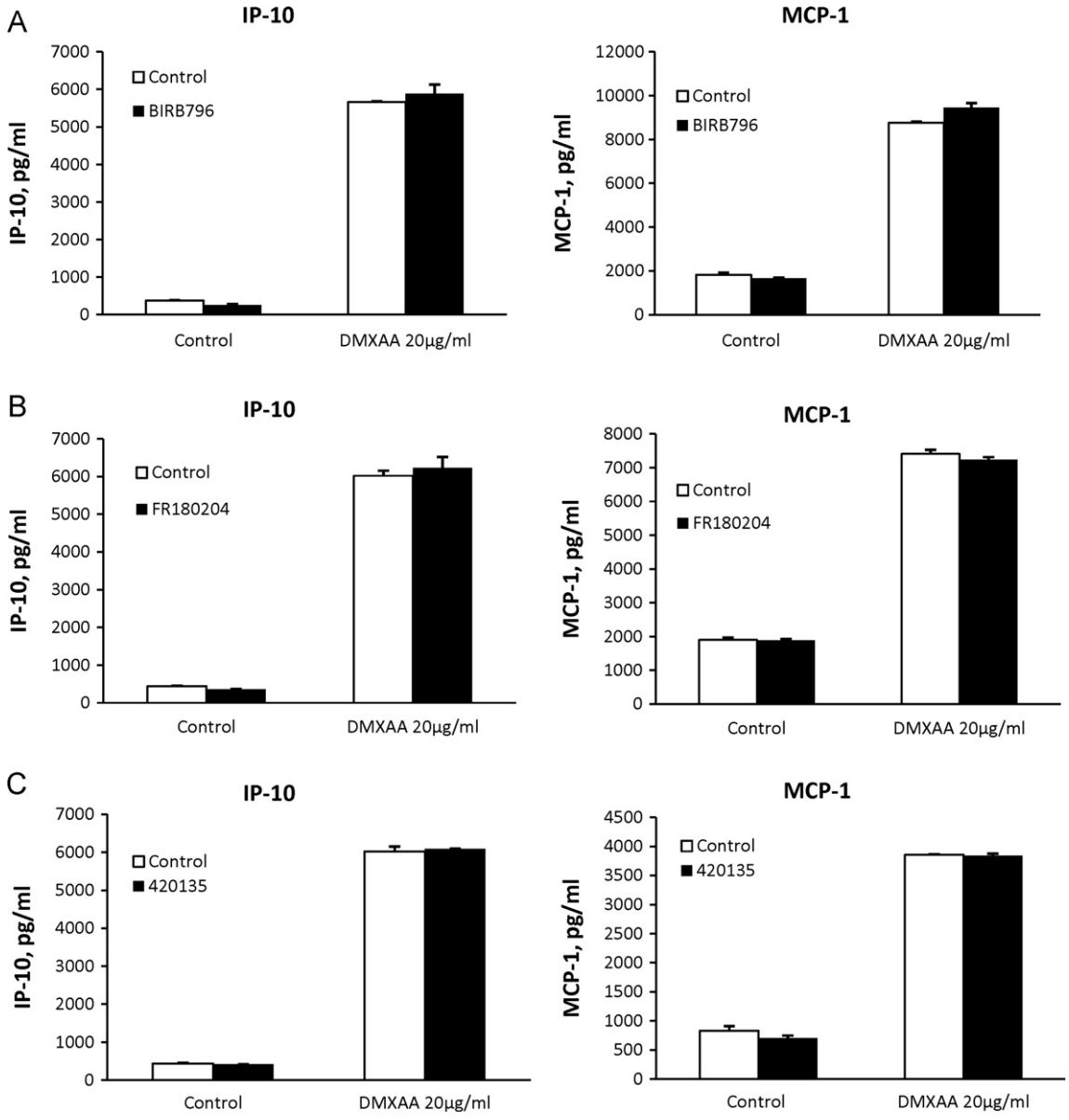

Fig. 4. DMXAA-stimulated IP-10 and MCP-1 productions are MAPK pathway independent. MHS cells were pre-treated with different MAPK pathway inhibitors, BIRB796 (A), FR180204 (B) or 420135 (C) for 1 h, prior to DMXAA stimulation. After 5 h incubation time, culture supernatants were collected and assayed for IP-10 (left panel) and MCP-1 (right panel) with ELISA. The data are expressed as the mean ± SEM.

of proinflammatory cytokines and chemokines (i.e. TNF-α, IL-6, IP-10, and interferon-β). One particularly interesting aspect of DMXAA is that this macrophage activation appears to involve multiple signaling pathways. There are data to implicate the NF-κB pathway [5], the TBK1–IRF3 signaling axis [6], and the NOD pathways [7]. To date, however, to our knowledge, there is only one previous study that indirectly implicated a role for p38 MAP kinase in the actions of DMXAA [8]. In this study, the formation of DMXAA-induced tumor cell networks on matrigel was blocked by addition of the p38 inhibitor SB203580. Given the biological significance of MAPK pathways in inflammation and the multi-faceted activation events induced by DMXAA, we explored the role of the MAPKs signaling family in DMXAA-induced proinflammatory cytokine production.

Our results clearly show that DMXAA can induce rapid phosphorylation (beginning at 30 min) of all three MAPK pathways in murine macrophages (Figs. 1A and 3A). Given the fact that DMXAA can stimulate large amounts of type I interferon secretion from macrophages [6] and type I interferons can activate MAPK pathway [23–25], it was necessary to determine if the phosphorylation of MAPKs by DMXAA was due to a direct effect or due to secondary autocrine signaling via the type I interferons induced by DMXAA. To examine this issue, we used two approaches. First, we quantified the kinetics of IFNβ production (Fig. 1B and C). In MHS cells, we were unable to detect any IFNβ in the cell supernatants at 2 h after DMXAA stimulation, as compared to large amounts at the 5 h time point (4.5 ng/ml). In peritoneal macrophages, we detected only minute amounts (<10 pg/ml) of IFNβ at 2 h. We believe that the delayed appearance of IFNβ protein makes IFN-induced MAPKs phosphorylation highly unlikely. Second, we stimulated the MHS cells with DMXAA in the presence or absence of a blocking antibody against the type I interferon receptor (IFNAR-1) to inhibit any potential secondary autocrine effects of type I interferons. Robust phosphorylation of p38 induced by DMXAA was seen in a concentration of the IFNAR-1 antibody that blocked IFN-mediated induction of IP-10 (Fig. 1D and Supplemental Fig. S1). Taken

Table 1
Chemokine gene expression in MHS cells treated with DMXAA with or without the presence of various MAPK inhibitors.

Genes	Treatments	mRNA expression
IP-10	DMXAA	52.9 ± 4.98
	DMXAA/BIRB796	56.1 ± 1.38
	DMXAA/FR180204	51.55 ± 1.65
	DMXAA/420135	70.59 ± 3.29
MCP-1	DMXAA	5.96 ± 0.61
	DMXAA/BIRB796	8.68 ± 0.08
	DMXAA/FR180204	6.12 ± 0.02
	DMXAA/420135	8.4 ± 0.33
RANTES	DMXAA	17.08 ± 0.53
	DMXAA/BIRB796	15.98 ± 0.79
	DMXAA/FR180204	21.59 ± 1.53
	DMXAA/420135	22.88 ± 1.68
IFNβ1	DMXAA	84.22 ± 3.0
	DMXAA/BIRB796	99.11 ± 2.30
	DMXAA/FR180204	84.19 ± 18.33
	DMXAA/420135	82.09 ± 33.67

mRNA expression by RT-PCR: fold change over control.
The mean fold increase in message level (from quadruplicate samples) is tabulated along with the SE.

together, these results indicate that phosphorylation of MAPK induced by DMXAA is a primary effect that is independent of the interferon/IRF-3 pathway.

Our group has recently shown that DMXAA can also activate the intracellular NOD signaling pathway. Since it is known that NOD1 and NOD2 can activate MAPKs through interacting with a protein kinase named RIP2 (receptor-interacting protein 2; also called RICK, RIPK2 and CARDIAK) [27], we wanted to determine if DMXAA-induced MAPK activation worked through this signaling pathway. To evaluate this, we exposed macrophages derived from RIP2 knock-out mice to DMXAA. Our data (Fig. 6) showed that DMXAA was still able to induce phosphorylation of all three MAPKs independently of the NOD/RIP2 pathway.

Studies showing the induction of phosphorylation of key members of each of the three MAPK pathways by DMXAA were followed by experiments evaluating the functional significance of each pathway using specific pharmacologic inhibitors. We first tested a commonly used p38 MAPK inhibitor, SB203580, and were able to detect inhibition of proinflammatory cytokine secretion induced by DMXAA (data not shown). However, recent reports showed that this inhibitor was found to also inhibit RIP2 kinase

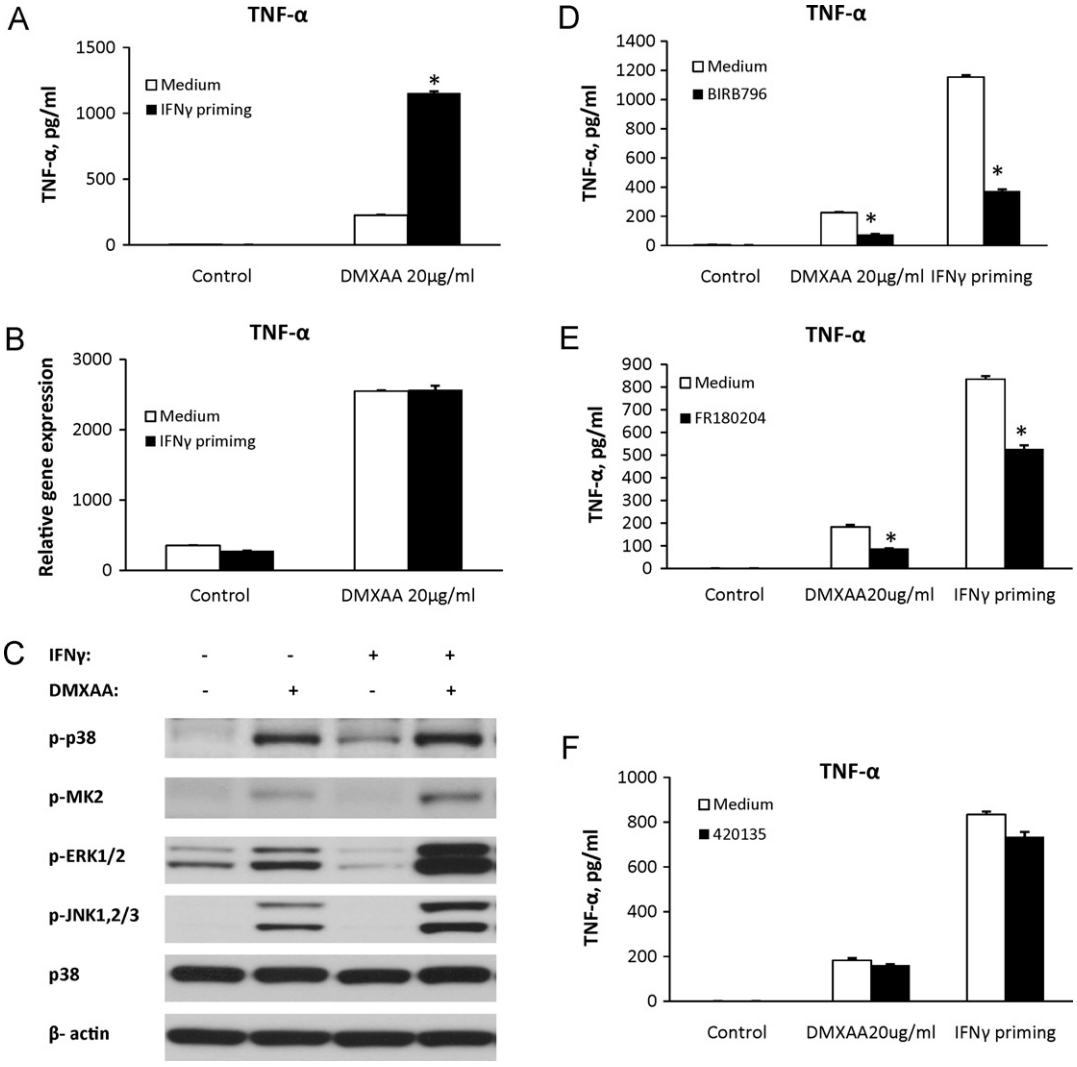

Fig. 5. Effect of IFN-γ priming on DMXAA-stimulated MAPK pathways activation and TNF-α production. MHS cells were pre-exposed to IFN-γ, followed by DMXAA treatment at 20 μg/ml. The culture supernatants were collected to measure TNF-α production by ELISA (A), or the RNA was extracted to measure the TNF-α mRNA level with real-time PCR (B). The cells were also extracted to determine the effect of IFN-γ priming on DMXAA-induced MAPK pathways activation (C). To study the role of various MAPK pathways in the IFN-γ priming effect, MHS cells were primed with IFN-γ for 2 h with or without the inhibitors, BIRB796 (D), FR180204 (E), or 420135 (F) pretreatment for 1 h, followed by treating with DMXAA for an additional 5 h. The culture supernatants were then collected for TNF-α ELISA. The data are presented as the mean ± SEM, and *a statistically significant reduction of TNF-α production ($p < 0.01$).

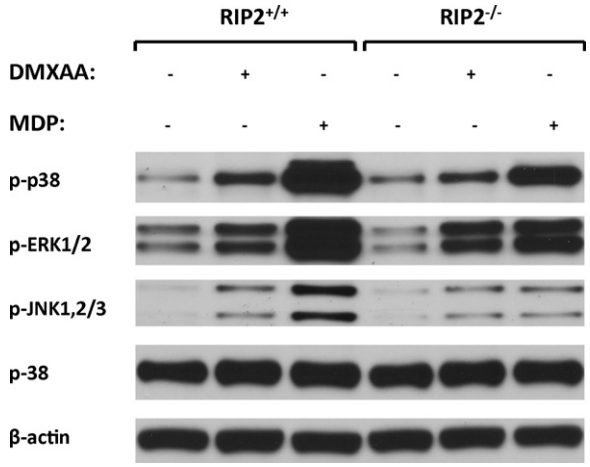

Fig. 6. The activation of MAPK pathways by DMXAA is RIP2 independent. Thioglycollate-elicited peritoneal macrophages from the wild type and RIP2 knockout mice were treated with DMXAA at 20 μg/ml or MDP at 10 μg/ml for 2 h, and the whole cell lysates were extracted and analyzed by immunoblot for phosphorylated p38, ERK1/2, and JNKs. Both p38 and β-actin expression were used to show equal loading of lanes.

with even greater potency than p38 MAPK [27,36]. Although we had evidence to suggest that RIP2 was not involved (Fig. 6), we repeated the inhibitory experiments using a much more specific p38 MAPK inhibitor, BIRB796, at 0.1 μM, a concentration at which the drug is reported to inhibit p38 MAPK specifically, without blocking RIP2, ERK1/2, JNKs or many other protein kinases [29]. Our results demonstrated that both TNF-α and IL-6 protein secretion stimulated by DMXAA was reduced by pre-treatment of macrophages with BIRB796 (Fig. 2B, C and Supplemental Fig. S2). We also applied an ERK-selective inhibitor, FR180204 at 5 μM. At this concentration, the inhibitor is reported to show much higher specific inhibitory effect on the kinase activity of ERK1 and ERK2 over p38 and other kinases [28,29]. Again, we saw reduction of DMXAA-stimulated cytokine secretion (Fig. 3B and Supplemental Fig. S3). Finally, we used a SAPK/JNK1,2/3-selective inhibitor, 420135, which has 1000-fold selectivity for JNKs over other MAP kinases including ERK, p38 and little inhibitory activity against another 74 kinases [30]. In this case, the inhibitor had no effect on DMXAA-induced cytokine production (Fig. 3C). Our data thus show that the p38 and ERK1/2 MAPKs play a functional role in the regulation of proinflammatory cytokine production induced by DMXAA, but interestingly, MAPK pathways seem to play little role in the induction of chemokines by DMXAA (Fig. 4).

We also explored the mechanism by which MAPK signaling affected the production of cytokines after DMXAA stimulation. There are a number of reports showing effects of p38 due to transcriptional regulation. p38 MAPK is known to regulate various transcription factors, such as CHOP [37], and ATF-2, Elk-1 [38] by phosphorylation. Studies have shown that p38 MAPK regulates NF-κB-dependent gene transcription via regulating DNA binding of the TATA-binding protein (TBP) to the TATA box [39,40]. ERK pathway has been reported to be involved in upregulation of TNF-α production by increasing TNF-α promoter activity via increased DNA binding activity of Egr-1 and NF-κB to TNF-α promoter [20,21]. However, there are also previous studies demonstrating that the p38–MK2 signaling pathway plays an important role in the post-transcriptional regulation of both TNF-α and IL-6 protein secretion by regulating either translation or mRNA stability. The mechanism appears to be dependent on the binding status of phosphorylated adenine/uridine-rich element (ARE)-binding proteins, such as Tristetraprolin (TTP), to the ARE in the 3′ untranslated region of TNF-α and IL-6 mRNA [15–17,41]. Similarly, in addition to transcriptional regulation, Tpl2/ERK signaling pathway also play an important role in post-transcriptional regulation of TNF-α production through regulating nucleocytoplasmic mRNA transport via a mechanism that targets the ARE in the 3′UTR of the TNF-α mRNA [22]. Another possibility is that MNK/eIF4E pathway may be involved in the regulation of TNF-α production. MNK kinases are downstream targets of both the p38 and ERKs MAP kinases [42,43], and have been shown to be involved in the regulation of TNF and other pro-inflammatory cytokines in response to various TLR agonists [44,45]. We have no direct evidence for this pathway yet, however, we plan to study the MAPK-MNK/eIF4E signaling axis in the future.

It was therefore of interest to analyze changes in both protein production and mRNA expression. We found that DMXAA stimulated large increases in both mRNA and protein secretion of macrophage TNF-α and IL-6 (Figs. 2 and 3). However, although the blockade of either p38 MAPK or ERK1/ERK2 significantly inhibited the production of TNF-α and IL-6 protein secretion, the inhibitors had no effect on the mRNA expression levels (Figs. 2 and 3). These data suggest that the activated p38 and ERK1/ERK2 signaling pathways induced by DMXAA affect the secretion of TNF-α and IL-6 primarily through regulating either translation or mRNA stability, not cytokine transcription.

Finally, we explored the role of the MAPKs in the phenomenon called "macrophage priming". This is a well-known process by which low levels of IFN-γ, that has very little effect on their own, but can synergize with LPS or microparticulate β-glucan to enhance the production of TNF-α, IL-6 or nitric oxide [46–48]. The mechanisms underlying the IFN-γ priming action are complicated and still not known for certain, however, some studies have suggested IFN-γ priming can augment DNA binding of NF-κB in response to LPS [46] or can cause up-regulation of LPS uptake and expression of the intracellular TLR4–MD-2 complex [49]. Other studies have demonstrated that the priming of macrophages by IFN-γ is highly dependent on glycogen synthase kinase-3 [47] or is dependent on the presence of functional NOD2 protein [50]. The MAPKs may also be involved. At least one study suggested that IFN-γ can rapidly induce ERK1/2 phosphorylation which then plays a vital role in IFN-γ-inducible macrophage nitric oxide generation besides the involvement of JAK2-STAT1 pathway activated by IFN-γ [51]. Another study suggested that activation of p38 MAPK by IFN-γ participates in the regulation of cytokines such as TNF-α, and iNOS gene expression [52].

Priming of the DMXAA effect by LPS in leukocytes has been previously described [53]. We found that exposure of macrophages to a low dose of IFN-γ alone for 5 h did not increase TNF-α protein secretion, but a clear priming effect could be seen by pretreatment with IFN-γ (Fig. 5A). When we explored the involvement of MAPKs in this process, we saw a small increase of phosphorylation of p38 and ERK1/2 after IFN-γ treatment alone, but increased phosphorylation of all three MAPKs, especially ERK1/2, after DMXAA exposure in IFN-γ primed cells. We also saw clear blunting of TNF-α production in the primed cells after blocking p38 and ERK1/2. Thus, our data show that one of the mechanisms of synergistic effect between IFN-γ and DMXAA in augmenting TNF-α production in mouse macrophages is via post-transcriptional regulation due to enhancement of DMXAA-induced p38 and ERK1/2 MAPK activation through IFN-γ priming.

One important caveat to consider in our studies was that we used pharmacologic inhibitors of MAPKs. Since the three MAP kinases share 60–70% amino-acid sequence identity, we took care to use the most specific inhibitors available for each MAPK. In our p38 MAPK inhibitory experiments, we used a p38 MAPK inhibitor, BIRB796, which is much more specific than the most commonly used p38 MAPK inhibitor, SB203580, which also inhibits RIP2. In our hands, BIRB796 showed very strong inhibitory effects on the

DMXAA-induced phosphorylation and activation of p38 MAPK at 0.1 μM with no inhibitory effects on the phosphorylation of ERK1/2, JNKs MAPKs (data not shown). This supports the conclusion that the effects of BIRB796 are primarily due to p38 MAPK inhibition. For the ERK1/2 and JNKs MAPK blocking studies, we used recently developed inhibitors that directly inhibit either ERK1/2 or JNKs MAPK activity instead of the more commonly used MEK1/2 or MKK4/7 inhibitors. The two inhibitors used in this study, FR180204 and 420135 are reported to have much more specific inhibitory effects on the kinase activity of ERK1/2 or JNKs (respectively) than of p38 MAPK and other kinases [28–30]. It is recognized, however, that no inhibitor is completely specific. Although we would have liked to use genetic approaches to study these pathways (as we did with the RIP2 KO mice), attempts to achieve complete knockdown p38 or ERK1/2 MAPK using knockout mouse technology has been limited by either the critical role of p38α or ERK2 MAPK in mouse development, causing embryonic lethality or compensation by different isoforms of MAPKs [10,54].

A major unanswered, but important question in these experiments is how a small, cell permeable molecule like DMXAA is able to activate all three MAPKs, in addition to the NF-κB, TBK1/IRF3, and NOD/RIP2 signaling networks. This question has been pursued for more than a decade by our labs and others without a clear positive answer. It has been shown, however, using knockout mice and other approaches, that DMXAA does not function through all known TLRs, cytosolic helicase receptors, or MyD88 [4,6]. Our studies have supported these findings. Finding the "DMXAA receptor" remains a goal that may be especially valuable in correlating findings in murine versus human cells.

In conclusion, this paper provides strong evidence that DMXAA is able to induce phosphorylation of all three MAPK pathways in murine macrophages and that at least two of these subfamilies (p38 and ERK1/2) play a role in the increased production of proinflammatory cytokines, such as TNF-α and IL-6 through a post-transcriptional mechanism. These data further expand our knowledge on mechanism how DMXAA acts as a potent anti-tumor agent and will hopefully be helpful in understanding its potential efficacy in human cancer therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.07.086.

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