

Curcumin: A Double Hit on Malignant Mesothelioma

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Abstract

Inflammation is a key mediator in the development of malignant mesothelioma, which has a dismal prognosis and poor therapeutic strategies. Curcumin, a naturally occurring polyphenol in turmeric, has been shown to possess anticarcinogenic properties through its anti-inflammatory effects. Inflammasomes, a component of inflammation, control the activation of caspase-1 leading to pyroptosis and processing of proinflammatory cytokines, interleukin (IL)-1 β and IL-18. In the present study, we investigate the role of curcumin in pyroptotic cell death of malignant mesothelioma cells. Using *in vitro* models with mouse and human malignant mesothelioma cells, curcumin is shown to induce pyroptosis through activation of caspase-1 and increased release of high-mobility group box 1 (HMGB1) without processing of IL-1 β and IL-18. Absence of IL-1 β processing in response to curcumin-mediated caspase-1 activation is attributed to blockade of pro-IL-1 β priming through inhibition of the NF- κ B pathway. Furthermore, curcumin's cytotoxicity in malignant mesothelioma cells is demonstrated to be dependent on pyroptosis as inhibition of caspase-1 resulted in protection against curcumin-induced cell death. We also demonstrate that curcumin-mediated caspase-1 activation is oxidant dependent by using N-acetyl-L-cysteine (NAC) to inhibit pyroptosis. PCR array analysis using the human inflammasome template revealed that curcumin significantly downregulated levels of inflammasome-related gene expression involved in inflammation, e.g., NF- κ B, toll-like receptors (TLR), and IL-1 β . Our data indicate that curcumin has a double effect on malignant mesothelioma cells through induction of pyroptosis while subsequently protecting against inflammation. *Cancer Prev Res*; 7(3); 330–40. ©2014 AACR.

Introduction

Malignant mesothelioma is an asbestos-related malignancy with a dismal prognosis and poor therapeutic strategies. Malignant mesothelioma arises from the mesothelial cells that line the pleural cavity, pericardium, and peritoneum. Therapeutic interventions for malignant mesothelioma include chemotherapy, surgery, radiation, immunotherapy, targeted molecular therapy, and gene therapy; however, the median survival remains poor at 9 to 12 months from the time of diagnosis (1). Curcumin is a naturally occurring polyphenol in the spice turmeric, which comes from the rhizomes of the herb *Curcuma longa*. Prior research has identified a broad range of anticarcinogenic potential of curcumin in various cancer types through its anti-inflammation, antiproliferative, antiangiogenic, proapoptotic, and enhancing chemoradiation properties (2–7). However, little research has explored the therapeutic role of

curcumin in malignant mesothelioma. If proven to be effective in malignant mesothelioma treatment, curcumin would serve as an excellent addition to available therapeutic options as this compound has a relatively low cost and low cytotoxic profile compared with its chemotherapy counterparts (8). Furthermore, effective compounds will be urgently needed because of the incidence and mortality of malignant mesothelioma in less-developed countries with economically disadvantaged populations (9).

Inflammation plays an important role in the development of multiple cancers, including malignant mesothelioma (10, 11). Caspase-1 mediates the activation of interleukin (IL)-1 β and IL-18 and an inflammatory cell death process termed pyroptosis that is distinct from necrosis and apoptosis (12). Initially synthesized as an inactive protein (pro-caspase-1), caspase-1 becomes biologically functional through autoprocessing into active caspase-1, which consists of two small (p10) and two large subunits (p20; ref. 13). Autoprocessing of caspase-1 typically occurs through classic inflammasomes composed of nod-like receptors (NLR) and an adaptor protein, apoptosis-associated speck like protein containing a caspase-1 recruitment domain known as PYCARD or ASC (14). Additional modes of caspase-1 activation may occur through inflammasomes with direct caspase-1 recruitment domains (ASC independent) such as NLRC4 and NLRP1 (15) or through an assembly of ASC dimers

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known as the pyroptosome (16). One of the best-characterized inflammasome is that consisting of NLRP3, ASC, and caspase-1, which has previously been shown to be activated by asbestos in macrophages (17) and mesothelial cells (unpublished data). Here, we are the first to report the ability of curcumin to enhance malignant mesothelioma cell killing via induction of pyroptosis without activation of classic inflammasome-related cytokines, IL-1 β and IL-18, due to inhibition of toll-like receptor (TLR) and NF- κ B pathways. In addition, we demonstrate that curcumin-initiated pyroptosis is dependent on reactive oxygen species (ROS) production and independent of the NLRP3 inflammasome.

Materials and Methods

Reagents

Curcumin (77.5% curcumin, 18.27% demethoxycurcumin, and 4.21% bisdemethoxycurcumin; also called C3 complex) was kindly supplied by Dr. Bharat Aggarwal (MD Anderson Cancer Center, Houston, TX) and curcumin ($\geq 94\%$ curcuminoid content, $\geq 80\%$ curcumin) was purchased from Sigma-Aldrich. A 10-mmol/L solution of curcumin was prepared in dimethyl sulfoxide (DMSO) and stored in 100- μ L aliquots at -20°C and diluted as needed in cell culture medium. Crocidolite asbestos was obtained from the National Institute of Environmental Health Sciences (NIEHS) and physical and chemical characterization has been previously reported (18). N-acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich. DMSO was used as a control treatment in all *in vitro* experiments.

Cell culture and exposure to reagents

Mouse malignant mesothelioma cells (#40) were obtained from Dr. Agnes Kane (Brown University, Providence RI) and maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and supplemented with penicillin (50 U/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$). Human mesothelial LP9/TERT-1 (LP9), cell line phenotypically and functionally resembling normal human mesothelial cells (19), were obtained from Dr. James Rheinwald (Brigham and Women's Hospital, Boston, MA). HMESO cells have been previously characterized by Reale and colleagues (20). H2595 and H2461 were contributed by Dr. Harvey Pass (New York University, New York, NY; ref. 21). Cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the Promega CELL ID System (Promega). All cells were maintained in appropriate cell culture medium as described before (22). Crocidolite asbestos fibers were prepared and added to cell culture medium as previously described (23). For NAC treatments, HMESO cells were grown to 80% to 90% confluency and treated with NAC (Sigma) 10 mmol/L for 18 hours after pH adjustment (24) before curcumin treatments. In experiments involving actinomycin D (Sigma), cells were treated with 10 $\mu\text{g}/\text{mL}$ of actinomycin D for 30 minutes before curcumin treatments.

MTS assay

Malignant mesothelioma cells were treated with different concentrations of curcumin (0–50 $\mu\text{mol}/\text{L}$) for 24 to 72 hours, and cell viability was measured using the colorimetric MTS assay, CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) as per the manufacturer's recommendations (22).

Quantitative real-time PCR

Total RNA (1 μg) from different cell types was reverse-transcribed as described previously (23) with random primers using the Promega AMV Reverse Transcription System (Promega). In LP9 cells with various exposure times to asbestos and curcumin, the *NLRP3* and *pro-IL-1 β* genes were evaluated using the $\Delta\Delta C_t$ method. Hypoxanthine phosphoribosyl transferase (*hprt*) and 18s ribosomal RNA controls were used as normalization controls.

PCR array

HMESO cells were grown to 90% confluency and treated with curcumin (40 $\mu\text{mol}/\text{L}$ for 48 hours). RNA was isolated and purified using a Qiagen RNeasy Plus Kit (Qiagen) as previously described (22). After quality assessment, 500 ng of RNA was used for cDNA synthesis using the RT² First Strand Kit (SABiosciences). Quantitative Real-Time PCR (qRT-PCR) was performed by the Vermont Cancer Center DNA Analysis Facility using RT² Real-Time SYBR Green PCR Master Mix and Human Inflammasomes RT² Profiler PCR Arrays (Qiagen; 7900HT Sequence Detection System, Applied Biosystems). Data were analyzed using an online spreadsheet-based data analysis template (SABiosciences). qRT-PCR (TaqMan) was used to validate selected genes using Assays-on-Demand (AOD) primers and probes from Applied Biosystems.

Cell viability determination

Viability of HMESO cells after caspase-1 inhibition and curcumin treatment was studied by growing HMESO cells to 90% confluency per well in a 12-well plate ($n = 3$). At 90% confluency, HMESO cells were maintained in low serum-containing medium (0.5% FBS) for 24 hours before treatment with caspase-1 inhibitor (EMD Biosciences; 40 $\mu\text{mol}/\text{L}$ for 1 hour) followed by curcumin (40 $\mu\text{mol}/\text{L}$ for 48 hours). Cells were trypsinized and counted using a hemocytometer.

Caspase-1 activity assay

Caspase-1 activity was measured using the Caspase-1 Colorimetric Assay (R&D Systems) according to the manufacturer's directions and normalized to total protein.

Detection of HMGB1, IL-1 β , and caspase-1 (p20) in medium

The media from treated cells were collected before cell lysis and 500 to 1,000 μL was concentrated in Amicon Ultra Centrifugal Filters with a 10K membrane (Millipore) by spinning at 14,000 $\times g$ for 30 minutes. Western blot analyses were performed on concentrated supernatants. A rabbit

polyclonal HMGB1 antibody (Abcam) was used at a dilution of 1:500. A rabbit polyclonal caspase-1 (p20) antibody (Cell Signaling Technology) was used at a dilution of 1:500. A rabbit polyclonal IL-1 β antibody (Cell Signaling Technology) was used at a dilution of 1:500.

Western blot analysis

Cells were exposed to agents as described above, and lysates were prepared as previously described (23). The amount of protein was determined using the RC DC Protein Assay (Bio-Rad). Western blots were performed as described previously (23), using monoclonal rabbit NLRP3 antibodies (Novus Biologicals) at a dilution of 1:500 and a mouse β -Actin antibody (Abcam) at a dilution of 1:2,000. Quantity One was used to quantify band density. Blots are representative of at least two or more different experiments.

ELISA for IL-1 β and IL-18

All source material for IL-1 β and IL-18 ELISAs came from cell culture medium that was concentrated as described above. Human and mouse IL-1 β kits (BioLegend) and human and mouse IL-18 ELISA kits (Medical & Biological Laboratories) were used. All ELISA assays were performed according to the manufacturer's instructions. The entire concentrate from 500 to 1,000 μ L of cell supernatant was used from each dish.

Malignant mesothelioma cell transfection with siNLRP3

ON-TARGETplus Non-Targeting siRNA #1 (scrambled control) or ON-TARGETplus SMARTpool mouse NLRP3 and ASC siRNA (5 nmol/L; Dharmacon) were transfected into mouse malignant mesothelioma cells at 80% confluency using Lipofectamine 2000 (Invitrogen) as previously described (25). The efficiency of NLRP3 or ASC knock-down was determined by qRT-PCR after 30 hours. After 30 hours post transfection, mouse malignant mesothelioma cells were treated with 40 μ mol/L of curcumin for 24 hours and caspase-1 activity was measured as described above.

Nitro blue tetrazolium assay for ROS detection

HMESO cells were grown to 90% confluency in 12-well plates and treated with curcumin (40 μ mol/L for 6 hours). Cells were washed with Hanks Balanced Salt Solution (HBSS) without phenol red and incubated with Nitro blue tetrazolium (NBT), purchased from Sigma-Aldrich for 45 minutes. One set of dishes was fixed in 100% methanol for 5 minutes and the other was trypsinized and counted using a hemocytometer. Fixed cells were then washed twice with HBSS without phenol red. NBT was solubilized in 560 μ L of 2 mol/L KOH and 480 μ L DMSO and 100 μ L was transferred to a 96-well plate and read at wavelength 630 nm.

In vivo malignant mesothelioma tumor model

For allograft model, mouse malignant mesothelioma cells #40 (2×10^6 cells in 50 μ L 0.9% NaCl, pH 7.4) were injected into the lower left quadrant of the peritoneal cavity of 8-week-old male C57/BL6 mice. For the xenograft model,

HMESO cells (2×10^6 cells in 50 μ L 0.9% NaCl, pH 7.4) were injected into the lower left quadrant of the peritoneal cavity of 6- to 8-week-old male Fox Chase SCID (severe combined immunodeficient) mice. Each treatment group ranged from 4 to 8 mice. Curcumin treatments were initiated 24 hours to 1 week post malignant mesothelioma cell injections. Mice were treated daily with oral curcumin via gavage or three times per week intraperitoneal injections of curcumin with a vehicle of corn oil or DMSO. Cisplatin 2 mg/kg intraperitoneal injections were performed at week 1 and week 2 post-malignant mesothelioma inoculations alone and in combination with curcumin. At 4 weeks post-malignant mesothelioma cell injection, mice were euthanized by intraperitoneal injection of sodium pentobarbital. Following euthanization, malignant mesothelioma tumors were collected, weighed, and measured using calipers. All experiments using mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Vermont College of Medicine (Burlington, VT).

Statistical analyses

Statistical significance was determined using a one way ANOVA followed by a Newman-Keuls multiple comparisons test or a Student *t* test. Comparisons yielding *P* values below 0.05 were determined to be statistically significant from each other.

Results

Curcumin induced NLRP3 inflammasome priming and caspase-1 activation, but not cytokine maturation in mouse malignant mesothelioma cells

Cytotoxic doses of curcumin in mouse malignant mesothelioma cells were established using MTS assays by treating cells with escalating doses of curcumin (0–50 μ mol/L) for 24 to 72 hours. As shown in Fig. 1A, all curcumin doses of 40 and 50 μ mol/L for 48 and 72 hours significantly inhibited mouse malignant mesothelioma cell growth compared with control. For this reason, the concentration of curcumin 40 μ mol/L was selected for subsequent experiments.

Inflammasome activation is a two-step process that requires priming followed by activation (26). To investigate the priming step of NLRP3 activation, mRNA levels of NLRP3 were analyzed, following curcumin treatment. Treatment with curcumin 40 μ mol/L for 48 hours in mouse malignant mesothelioma cells resulted in significantly increased NLRP3 mRNA levels (Fig. 1B), which was a result of increased transcription as demonstrated through amelioration of this effect by actinomycin D pretreatment (Fig. 1C). Control (DMSO) and curcumin-treated mouse malignant mesothelioma cells did not show a significant difference in pro-IL-1 β mRNA levels (Fig. 1D). In addition, curcumin downregulated mRNA levels of ASC (Fig. 1E). In support of pyroptosis, caspase-1 activation was significantly increased in response to curcumin treatment (Fig. 1F). HMGB1 is marker of cell death (27) and was analyzed as a parameter to support the occurrence of pyroptosis.

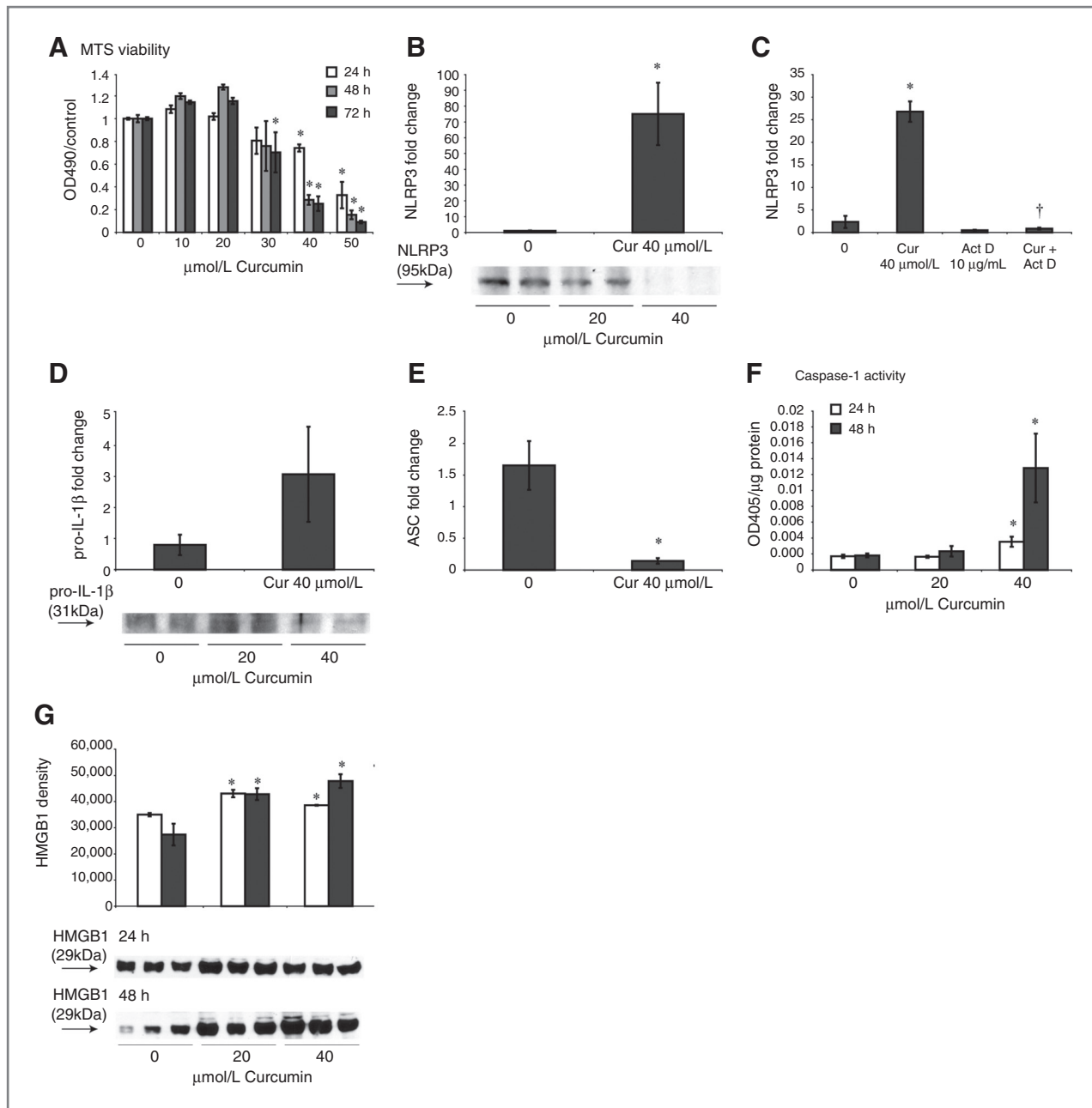


Figure 1. Curcumin induced NLRP3 priming and caspase-1 activation in mouse malignant mesothelioma cells without proinflammatory cytokine processing. A, MTS assays revealed that curcumin inhibited mouse malignant mesothelioma cell growth in a manner that was dose and time dependent. Mouse malignant mesothelioma cells treated with curcumin (40 μmol/L for 48 hours) resulted in increased mRNA levels of NLRP3 (B) compared with control malignant mesothelioma cells, which was inhibited by actinomycin D pretreatment for 1 hour (C). Pro-IL-1β mRNA levels were not significantly altered by curcumin (D), whereas ASC mRNA levels were significantly decreased (E). Caspase-1 activation (F) and HMGB-1 release (G) significantly increased in mouse malignant mesothelioma cells in response to curcumin (20 and 40 μmol/L for 48 hours). *, $P \leq 0.05$ when compared with control; †, $P \leq 0.05$ when compared with curcumin-only treatment group.

Curcumin treatment resulted in significantly increased extracellular (secreted) levels of HMGB1 in the medium of mouse malignant mesothelioma cells (Fig. 1G).

As previously described, active caspase-1 is responsible for activation of the proinflammatory cytokines, IL-1β and IL-18. Processing of these cytokines was investigated by

ELISA, which demonstrated undetectable levels of IL-1β and IL-18 in control and curcumin-treated mouse malignant mesothelioma cells (data not shown). In addition, IL-1β Western blot analysis was performed and also revealed no detectable levels of IL-1β in curcumin-treated mouse malignant mesothelioma cells (data not shown), thus

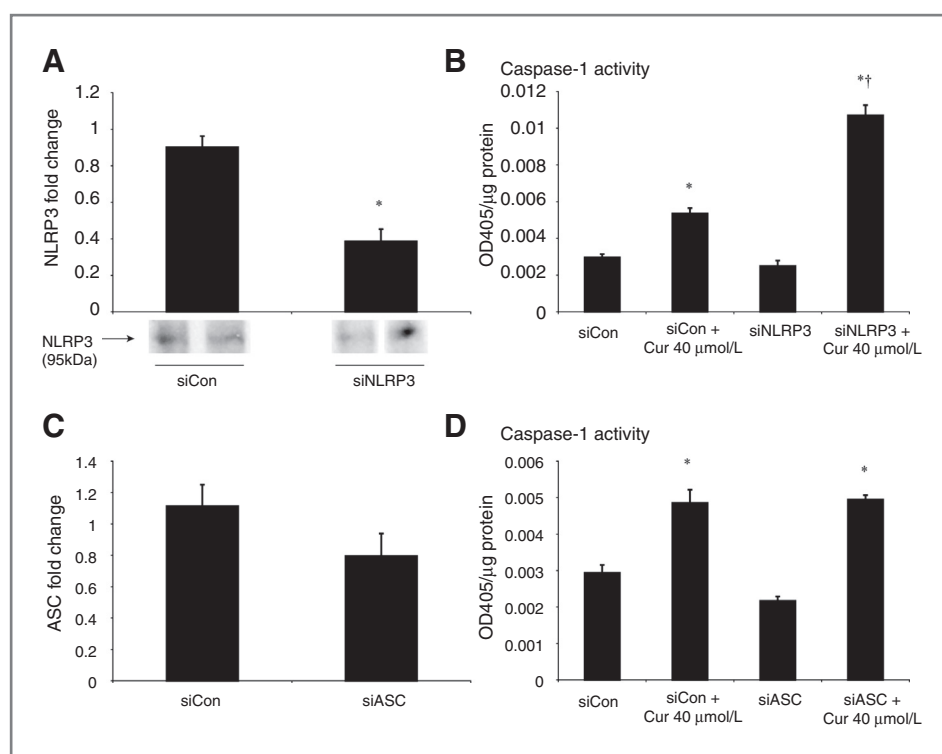


Figure 2. NLRP3 and ASC inhibition did not attenuate curcumin-induced caspase-1 activation. Mouse malignant mesothelioma cells transfected with siNLRP3 or siASC suppressed NLRP3 (A) and ASC (C) mRNA levels, however did not inhibit caspase-1 activity (B and D) when treated with curcumin (40 $\mu\text{mol/L}$ for 24 hours). *, $P \leq 0.05$ when compared with control; †, $P \leq 0.05$ when compared with siCon + curcumin.

confirming that IL-1 β was not processed as a result of caspase-1 activation by curcumin.

Silencing of NLRP3 or ASC by siRNA did not inhibit curcumin-induced caspase-1 activation in mouse malignant mesothelioma cells

As an attempt to link our findings of NLRP3 priming with caspase-1 activation in curcumin-treated mouse malignant mesothelioma cells, we evaluated whether curcumin-induced caspase-1 activation was NLRP3 dependent. NLRP3 mRNA was significantly reduced by siRNA in mouse malignant mesothelioma cells (Fig. 2A); however, caspase-1 activation by curcumin was not affected (Fig. 2B). Next, we knocked down expression of ASC in mouse malignant mesothelioma cell by siRNA (Fig. 2C), which again had no effect on curcumin's ability to activate caspase-1 (Fig. 2D).

Curcumin treatment in human malignant mesothelioma cells resulted in NLRP3 priming and caspase-1 activation without proinflammatory cytokine secretion

MTS assays were performed to confirm cytotoxic curcumin doses in HMESO, H2595, and H2461 cells. Figure 3A–C reveals that curcumin had a dose- and time-dependent inhibitory effect on cell growth of human malignant mesothelioma cells. Similar to mouse malignant mesothelioma cells, curcumin doses of 30 to 50 $\mu\text{mol/L}$ at all time points resulted in significant cytotoxicity of human malignant mesothelioma cells. Curcumin-induced priming of NLRP3 in HMESO cells was demonstrated by significantly

increased mRNA (Fig. 3D) levels of NLRP3 compared with control cells. Actinomycin D pretreatment diminished the curcumin-induced increase in NLRP3 mRNA (Fig. 3E), thus indicating that this effect occurred by transcriptional upregulation of NLRP3 and not stabilization of mRNA. Caspase-1 activity significantly increased in HMESO cells in response to curcumin treatment as shown by increased caspase-1 activity measured by caspase-1 assay (Fig. 3F). Consistent with the cytokine analysis in mouse malignant mesothelioma cells, curcumin did not induce significant changes in processed IL-1 β and IL-18 levels in HMESO cells (Fig. 3G and H). NLRP3 protein (Fig. 3I) and caspase-1 p20 protein was also increased by curcumin (Fig. 3J). In addition, HMGB1 was increased in the medium of HMESO cells treated with curcumin (Fig. 3K).

Curcumin kills HMESO cells through pyroptosis

Pyroptotic cell death requires active caspase-1. To prove that curcumin induces cell death through pyroptosis, HMESO cells were pretreated with a caspase-1 inhibitor and then treated with curcumin (40 $\mu\text{mol/L}$ for 48 hours). Figure 4 demonstrates that HMESO cells were significantly less susceptible to curcumin-induced cytotoxicity in the presence of caspase-1 inhibition.

Curcumin-induced priming of NLRP3 and pyroptosis in HMESO cells is ROS dependent

ROS is a known activator of the NLRP3 inflammasome in macrophages (17). Curcumin has been shown to have both antioxidant and prooxidant effects in different cell types and experimental settings (28–30), although no data exist

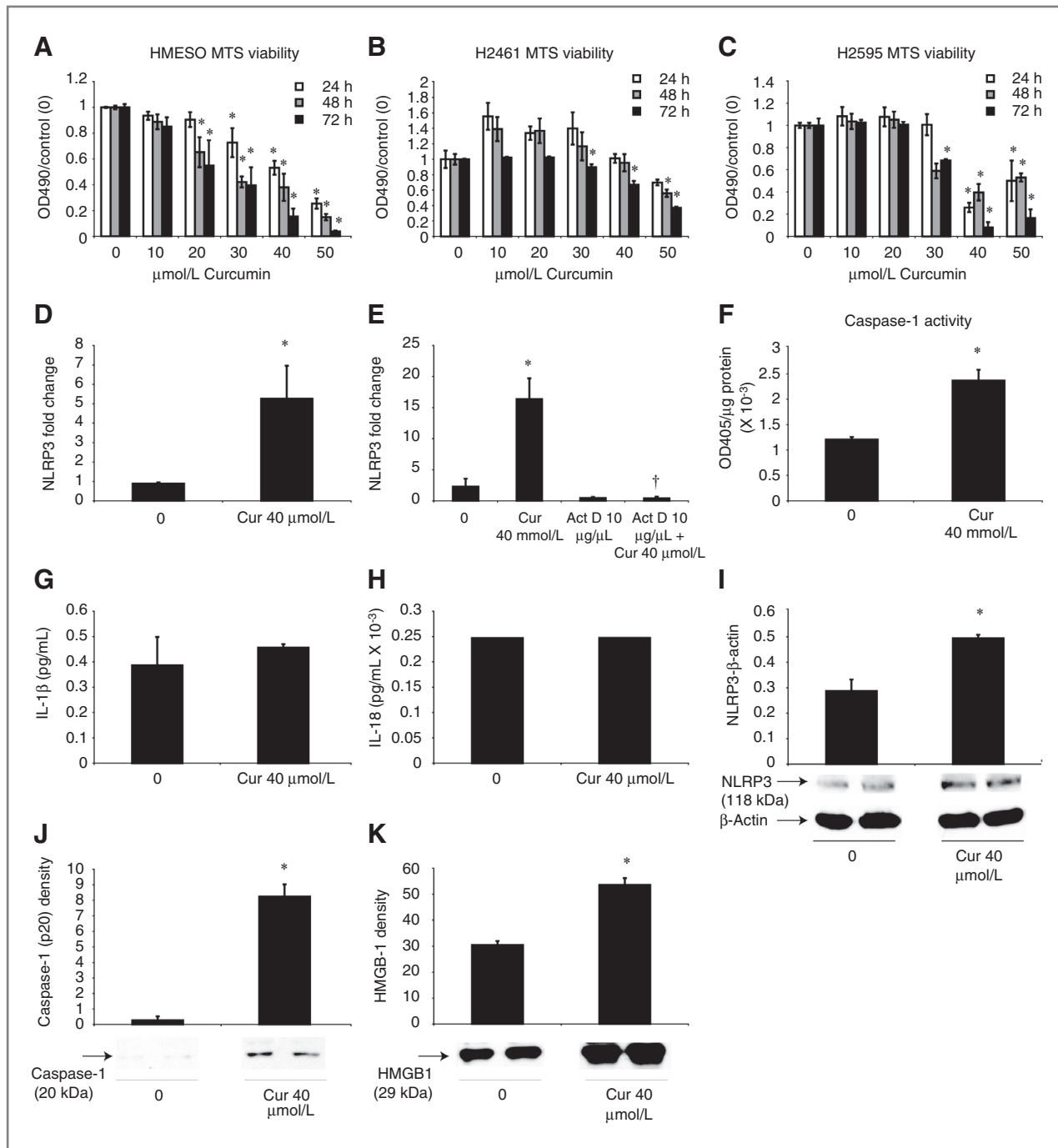


Figure 3. Curcumin mediates NLRP3 priming and caspase-1 activation without proinflammatory cytokine secretion in human malignant mesothelioma cells. MTS assays revealed that curcumin inhibited HMESO (A), H2461 (B), and H2595 (C) cell growth in a manner that was dose and time dependent. HMESO cells treated with curcumin (40 $\mu\text{mol/L}$ for 48 hours) resulted in increased mRNA levels (D) and protein levels (I) of NLRP3 compared with control malignant mesothelioma cells. Pretreatment with actinomycin D 1 hour before curcumin treatment inhibited curcumin-induced increased mRNA levels of NLRP3 (E). Caspase-1 activation was significantly increased in response to curcumin (40 $\mu\text{mol/L}$ for 48 hours) when measured by caspase-1 activity assay (F) and Western blot analysis of p20 (J) without significant change in secretion of IL-1 β (G) and IL-18 (H). Curcumin treatment (40 $\mu\text{mol/L}$ for 48 hours) resulted in increased HMGB1 release in HMESO cells compared with control cells (K). *, P value ≤ 0.05 when compared with control. †, $P < 0.05$ when compared with curcumin-only treatment group.

regarding curcumin regulated ROS in malignant mesotheliomas. For this reason, NBT assays were performed to determine whether curcumin alters ROS production in HMESO cells. As shown in Fig. 5A, curcumin treatment

(40 $\mu\text{mol/L}$ for 6 hours) of HMESO cells increased ROS production compared with control cells, which was inhibited by NAC pretreatment (Fig. 5B). In addition, pretreatment with NAC decreased curcumin-induced increases in

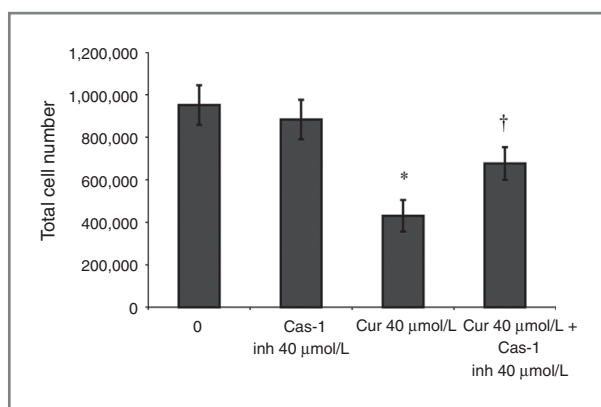


Figure 4. Curcumin kills human malignant mesothelioma cells through pyroptosis. Curcumin (40 µmol/L for 48 hours) significantly decreased HMESO cells compared with control. Pretreatment with caspase-1 inhibitor for 1 hour significantly reduced the inhibitory effect of curcumin. *, $P \leq 0.05$ when compared with control; †, P value ≤ 0.05 when compared with curcumin-only treatment group.

NLRP3 mRNA (Fig. 5C), caspase-1 activity (Fig. 5D), cleavage of p20 (Fig. 5E), and HMGB-1 release (Fig. 5F) in HMESO cells; however, no significant effect of NAC was observed on NLRP3 protein expression (Fig. 5G).

Curcumin alters inflammasome-related gene expression in HMESO cells

A PCR array using a "Human Inflammasomes" template on HMESO cells treated with curcumin (40 µmol/L for 48 hours) compared with control cells showed that curcumin treatment resulted in significantly ($P \leq 0.05$) reduced levels of inflammasome-related gene expression involved in inflammation, NF-κB, TLRs, and IL-1 pathways (Table 1). In addition, MYD88, *NLRC4*, and *TXNIP* were downregulated by curcumin. Genes that were significantly upregulated by curcumin in HMESO cells included *heat shock protein 90 kDa alpha class A member 1 (HSP90AA1)*, *IL-12*, *IL-6*, and *Mediterranean fever (MEFV)*.

Curcumin in combination with asbestos resulted in increased NLRP3 and pro-IL-1β priming in mesothelial cells

To determine curcumin's effect on asbestos-initiated inflammasome processes in human mesothelial cells, LP9 cells were pretreated with curcumin followed by asbestos exposure. The combination of curcumin pretreatment followed by asbestos exposure resulted in increased steady state mRNA levels of NLRP3 (Supplementary Fig. S1A) and pro-IL-1β (Supplementary Fig. S1B) compared with control and asbestos-only-treated LP9 cells. No accumulative effects of curcumin pretreatment in combination with asbestos were present in caspase-1 activation (Supplementary Fig. S1C) as asbestos only and curcumin pretreatment with asbestos treatment groups were not significantly different. Curcumin treatment alone had no effect on LP9 cells when analyzed for the above parameters.

Curcumin treatment *in vivo* did not reduce malignant mesothelioma tumor burden

Various doses of curcumin administered through different routes in murine allograft and xenograft models of malignant mesothelioma did not reduce tumor burden (Supplementary Fig. S2A–S2C). In addition, curcumin did not have synergistic effects with cisplatin treatment in the allograft model (Supplementary Fig. S2B).

Discussion

Prior studies have shown that curcumin causes cell death by apoptosis (31) and autophagy (32); however, we are the first to demonstrate that curcumin induces cell death by pyroptosis in mouse and human malignant mesothelioma cells. Pyroptosis is a caspase-1-mediated, programmed, proinflammatory cell death that results in loss of plasma membrane integrity and release of cytoplasmic contents (12). Impaired pyroptosis has been reported to enhance inflammation-induced colon cancer (33). Consistent with the above report, induction of caspase-1 has been shown to be beneficial in other cancers such as prostate and renal cancer (34, 35). Here, we use two different techniques (caspase-1 activity assays and Western blot analysis of secreted p20) to clearly demonstrate that curcumin mediates caspase-1 activation in malignant mesothelioma cells. Furthermore, we show that caspase-1 was necessary to exert the cytotoxic effects of curcumin as caspase-1 inhibition before curcumin treatment resulted in protection against curcumin-induced cell death. Even more intriguing is the finding that curcumin can induce pyroptosis while preventing processing of proinflammatory cytokines, IL-1β and IL-18. This finding is consistent with research performed in dendritic cells that reports inflammasome-independent regulation of pyroptosis and cytokine processing in response to *Listeria monocytogenes* p60 protein (36). In addition, research performed in pathogen-infected macrophages suggests that two distinct inflammasome complexes may form that differentially mediate cytokine secretion and/or pyroptosis (37).

The ability of curcumin to provide protection against processing of classic inflammasome cytokines is an important finding because of the detrimental effects these cytokine may convey. IL-1β has been linked to inflammasome-dependent carcinogenic inflammation through its role in tumor progression and chemoresistance in multiple cancer types (14). In addition, IL-1β has been shown to promote mesothelial cell proliferation and transformation, leading to malignant mesothelioma development (38). IL-18 has also been linked to tumor progression as this cytokine was shown to be immunosuppressive and support metastatic function in melanoma and colon carcinoma (39). In our *in vitro* models, curcumin may be preventing cytokine processing by blocking priming of cytokine precursors, pro-IL-1β and pro-IL-18, which is a necessary step required before cytokine activation (40). We show that pro-IL-1β was not primed by curcumin in mouse malignant mesothelioma cells and found to be significantly downregulated in human

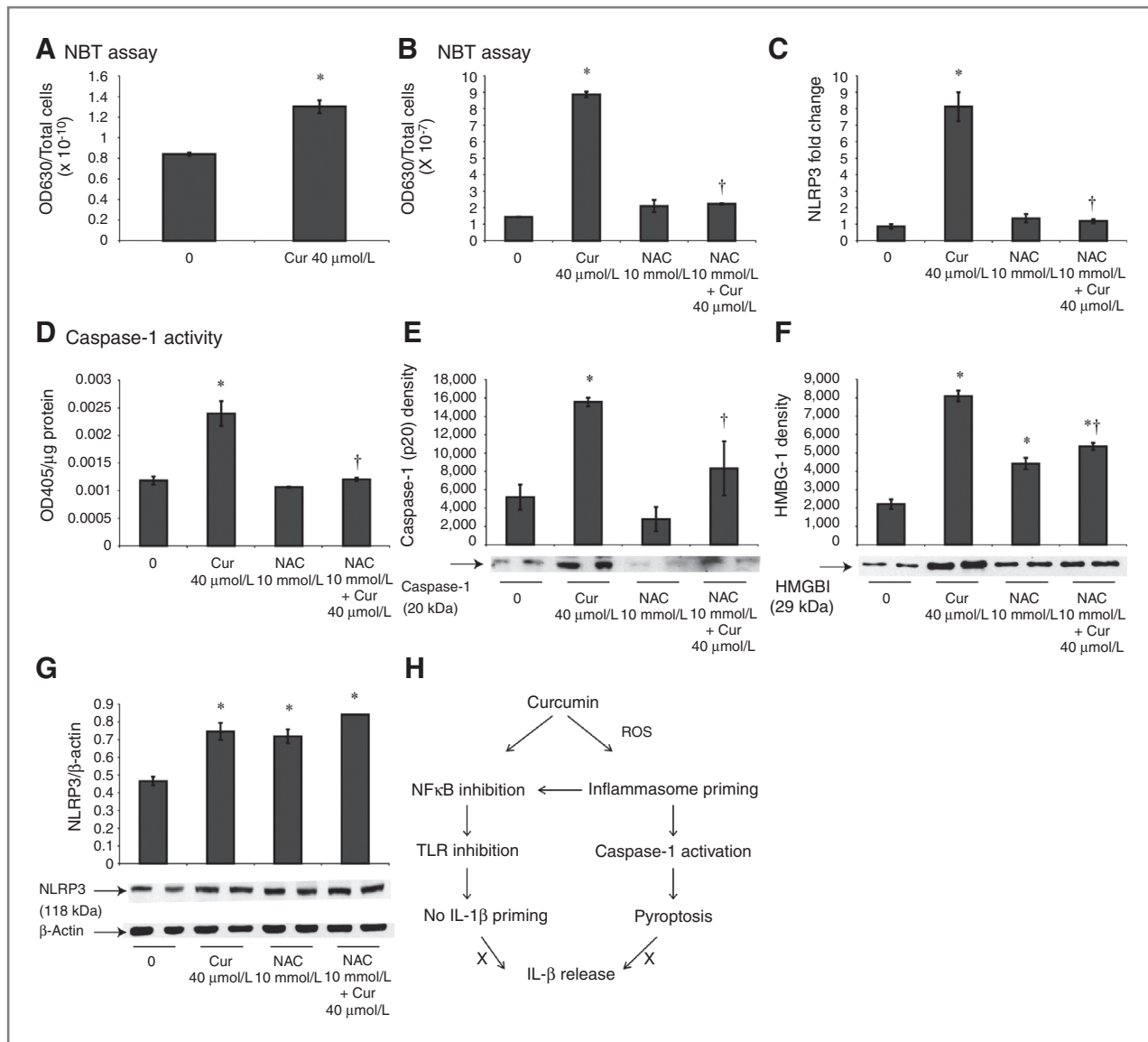


Figure 5. Curcumin-induced pyroptosis is ROS dependent. Curcumin treatment (40 μmol/L for 6 hours) resulted in significantly increased ROS in HMESO cells (A) compared with control cells, which was attenuated by NAC pretreatment (40 μmol/L for 18 hours; B). Pretreatment with NAC (40 μmol/L for 18 hours) blocked increased NLRP3 mRNA levels (C), caspase-1 activity (D), cleavage of p20 (E), and HMGB1 release (F) by curcumin (40 μmol/L for 48 hours) in HMESO cells. Expression of NLRP3 protein (G) was not altered with NAC pretreatment. H, schema showing regulation of pyroptosis by curcumin. *, $P \leq 0.05$ when compared with control; †, $P < 0.05$ when compared with curcumin-only treatment group.

malignant mesothelioma cells. Cytokine priming typically involves TLR stimulation with downstream involvement of NF-κB (41, 42). In our studies, we found that curcumin treatment of malignant mesothelioma cells resulted in downregulation of inflammasome-related genes belonging to the TLR, IL-1, and NF-κB pathways, thus providing a link to curcumin-induced inhibition of procytokine expression and subsequent cytokine processing (maturation; Fig. 5H).

Another parameter to support the occurrence of pyroptosis is HMGB1, which is passively released into the extracellular space upon loss of membrane integrity (43). HMGB1 is a damage-associated molecular pattern, and has been studied as a positive prognostic biomarker for

response to chemotherapy in patients with breast cancer (27). This study concluded that HMGB1 was increased in the plasma of patients who had a positive response to chemotherapy (epirubicin/docetaxel) compared with patients who were nonresponders. Other recent studies suggest that HMGB1 requires cytokines, such as IL-1β, to induce strong inflammatory responses, which involves signaling through TLRs and RAGE receptors (44). Our data showing increased HMGB1 release by curcumin suggest that HMGB1 is not inducing proinflammatory effects in this setting as curcumin blocks IL-1β processing and promotes downregulation of TLR signaling pathway genes (*MYD88*, *IRAK1*, and *TIRAP*).

Table 1. PCR array analysis showing significant altered expression of important tumorigenesis-related genes by curcumin in HMESO cells as compared with control

Gene name	Increase/decrease (fold)	Function	Validation (qRT-PCR)
Caspase-8 (CASP8)	-1.76	Apoptosis	
CASP8 and FADD-like apoptosis regulator (CFLAR)	-1.66	Apoptosis	
Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta (IKKB)	-1.89	NF- κ B pathway promoter	Yes
IL-1 β	-3.11	Inflammation, drug resistance	Yes
Interleukin-1 receptor associated kinase (IRAK1)	-2.28	IL-1 pathway promoter	
Myeloid differentiation primary response gene 88 (MYD88)	-2.3	IL-1 and TLR signal transducer	Yes
NFKB1	-3.13	Inflammation, proliferation	
NLR family, CARD domain containing 4 (NLRC4)	-1.76	Inflammasome	
PYD and CARD containing (PYCARD)	-2.4	Caspase-1 adaptor protein	Yes
TGF- β activated kinase 1/MAP3K7 binding protein 1 (TAB1)	-2.66	Signal transduction TGF- β , IL-1	
TGF- β activated kinase 1/MAP3K7 binding protein 2 (TAB2)	-1.97	Signal transduction IL-1, NF- κ B	
Toll-IL 1 receptor domain containing adaptor protein (TIRAP)	-2.09	TLR adapter protein, NF- κ B activation	
Thioredoxin interacting protein (TXNIP)	-4.89	Thioredoxin inhibitor	Yes
HSP90AA1	10.15	ROS production	
IL-12A	18.79	Th1 response	Yes
IL-6	3.73	Inflammation, maturation of B cells	
MEFV	1.57	Inflammasome adapter protein	

The molecular pathway by which curcumin activates caspase-1 leading to pyroptosis remains unclear. Here, we show for the first time that curcumin upregulates NLRP3 mRNA as well as protein levels in human malignant mesothelioma cells. Despite these findings, our data indicate that activation of the NLRP3 inflammasome is not required for curcumin-induced pyroptosis as reduction in NLRP3 or ASC mRNA levels via siRNA did not attenuate caspase-1 activation by curcumin. In support of this finding, we demonstrated that curcumin downregulates the gene expression of *TXNIP* in malignant mesothelioma cells, which is a direct ligand for activation of the NLRP3 inflammasome (45). Another possibility could be that reduction in NLRP3 expression leads to a compensatory upregulation of other inflammasomes that are capable of caspase-1 activation. In addition, our data indicate that caspase-1 is not activated by curcumin through a pyroptosome or other inflammasomes that require ASC because siASC failed to suppress curcumin-induced caspase-1 activation. Furthermore, curcumin treatment in mouse and human malignant mesothelioma cells significantly downregulated ASC. It is possible that curcumin could activate caspase-1 through ASC-independent inflammasomes, although our PCR array data indicate that curcumin downregulates *NLRC4* gene expression. In addition, we performed analysis of NLRP1 via PCR array and qRT-PCR in malignant mesothelioma cells treated with and without curcumin (data not shown).

PCR array data initially revealed an upregulation of *NLRP1* gene expression by curcumin. However, the values generated from the analysis were very close to the threshold cutoff values. For this reason, we attempted validation with qRT-PCR using AOD primers and probes for NLRP1, which repeatedly showed undetectable levels of mRNA expression of NLRP1 in control and curcumin-treated malignant mesothelioma cells. These results suggest that curcumin is not signaling through NLRC4 or NLRP1 inflammasomes either; however, confirming these findings would require siNLRC4 and siNLRP1 experiments.

Regardless of the exact mechanisms involved in curcumin-induced caspase-1 activation in malignant mesothelioma cells, our findings implicate ROS in this process. Prior studies have demonstrated that curcumin exerts its biologic effects through prooxidant mechanisms (28, 29). Here, we show that curcumin increases ROS production in malignant mesothelioma cells and that ROS production is required, in part, for NLRP3 priming, HMBG1 release, and caspase-1 activation by curcumin. In contrast, NLRP3 protein expression was not altered by reduction in curcumin-induced ROS, which further suggest that NLRP3 is not mediating pyroptosis. Taken together, our data imply that curcumin generates ROS, which may then activate inflammasomes, leading to caspase-1 activation or may directly activate caspase-1, resulting in pyroptosis (Fig. 5H). The source of ROS generated by curcumin remains unclear. One possible

link may be HSP90, which was upregulated in malignant mesothelioma cells by curcumin. HSP90 has been shown to regulate nicotinamide adenine dinucleotide phosphate oxidases and increase ROS production (46, 47).

Although curcumin-upregulated NLRP3 may not be necessary for pyroptosis, this protein may serve as a mediator for regulation of the NF- κ B pathway by curcumin in malignant mesothelioma cells. Numerous reports have highlighted curcumin as an inhibitor of NF- κ B in multiple cancers (48). The NF- κ B pathway is implicated in mesothelial cell transformation and malignant mesothelioma tumor progression (49). Our PCR array data revealed that curcumin downregulated the gene expression of *NFKB1* in addition to other genes involved in promotion of the NF- κ B pathway (*IKBKB*, *TIRAP*, and *TAB2*) in malignant mesothelioma cells. Interestingly, it has been reported that increased levels of NLRP3 alone (42) and ASC alone block NF- κ B activity through inhibition of p65 nuclear translocation. However, coexpression of NLRP3 and ASC resulted in NF- κ B activation (50). These reports indicate that the balance of NLRP3 and ASC expression influence regulation of NF- κ B signaling and provide a context to interpret our findings that curcumin upregulates NLRP3 mRNA and protein levels and downregulates gene and mRNA expression of ASC in malignant mesothelioma cells. Hence, curcumin may be downregulating NF- κ B by upregulation of NLRP3 levels with subsequent downregulation of ASC.

We also investigated the role of curcumin in asbestos-initiated inflammasome and caspase-1 processes in mesothelial cells. To our surprise, we found that curcumin significantly enhances asbestos-induced increases of NLRP3 and pro-IL-1 β mRNA levels in mesothelial cells. As described previously, increased levels of NLRP3 may have biologic signaling importance; however, it remains unclear how curcumin modulates the detrimental effects of asbestos in mesothelial cells.

Although our *in vitro* data strongly showed that curcumin is cytotoxic to malignant mesothelioma cells through induction of pyroptosis, our *in vivo* experiments failed to show corresponding results. These findings are likely a consequence of the low solubility and poor bioavailability of curcumin, which has limited its role as a cancer therapy in

clinical trials (8). Our future studies involve use of synthetic curcumin analog to increase the cellular uptake.

In conclusion, we demonstrate that curcumin induces cytotoxic effects on malignant mesothelioma cells through pyroptosis in a process involving ROS production that is independent of the NLRP3 inflammasome. In addition, curcumin has anti-inflammatory effects by blocking cytokine processing of IL-1 β and IL-18 and genes involved in the NF- κ B pathway. These results provide evidence that curcumin warrants further investigation as a therapeutic agent in malignant mesothelioma, although future studies must include improved curcumin analogs or enhanced modes of delivery to overcome curcumin's most challenging feature, which is limited bioavailability.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.M. Miller, A. Shukla

Development of methodology: J.M. Miller

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.K. Thompson, C.M. Westbom, M. Sayan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Miller, M.B. MacPherson, A. Shukla

Writing, review, and/or revision of the manuscript: J.M. Miller, M.B. MacPherson, M. Sayan, A. Shukla

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Miller, M.B. MacPherson, S.L. Beuschel, M. Sayan

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