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Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin

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Abstract

Stressed cells undergoing necrosis release molecules that acts as endogenous danger signals to alert and activate innate immune cells. Both HMGB1 and HSP70 are induced in activated monocytes/macrophages and also are released from stressed or injured cells. We investigated whether HMGB1 and HSP70 released from necrotic monocytes/macrophages, can act as danger signals to mediate proinflammatory cytokine responses to bacterial endotoxin or lipopolysaccharide (LPS). We show that cell lysate, obtained from necrotic cells directly stimulates the proinflammatory cytokine and chemokine responses in human monocyte/macrophage cell line, THP-1, as revealed by the induction of TNF- α , IL-6 and IL-8 mRNA expression and protein production. In the presence of LPS, necrotic cell lysate induced a more robust increase in all three proteins. We found that HMGB1 and HSP70 were indeed present in the necrotic cell lysate and were responsible for the significant induction of the proinflammatory cytokine expression, as neutralization with antibodies against both proteins blocked the increase in the cytokine production seen after incubating LPS-stimulated cells with the necrotic cell lysate. We also found that the newly identified triggering receptor expressed on myeloid cells-1 (TREM-1) was involved in mediating the HMGB1- and HSP70-induced cytokine production. Blocking TREM-1 on THP-1 cells with a recombinant chimera prevented the increase in cytokine production, while simultaneous blocking of TLR4 and TREM-1 completely abolished the proinflammatory response, suggesting that TREM-1 synergizes with TLR4 to mediate the effects of such signals from necrotic cells. In addition, blocking HMGB1 or HSP70 simultaneously with TREM-1 did not decrease the cytokine level further, confirming the involvement of TREM-1 in mediating the effect of HMGB1 and HSP70. Although the interaction of HMGB1 and HSP70 with TREM-1 induced I κ B α and p38 expression, both of which are required for the inflammatory cytokine expression, blockade of TREM-1 did not affect I κ B α expression but markedly reduced p38 activation, as revealed by Western blot analysis. Together, these results demonstrate that HMGB1 and HSP70 released from necrotic cells function as endogenous danger signals to augment the proinflammatory responses in monocytes/macrophage and that TREM-1 relays such signals to the cytokine expression cascade. This mechanism may contribute to the amplification and persistence of the inflammatory response to bacterial infection.

Keywords

Endogenous signal; HMGB1; HSP70; Cytokines; Inflammation; Macrophages

1. Introduction

It is well known that the optimal immune response develops in the presence of adjuvant [1,2]. In infection, microbial components such as bacterial lipopolysaccharide, provide such adjuvant, which augments the stimulation of innate immune cells such as dendritic cells (DCs) and macrophages [1,3] to produce cytokines and inflammatory mediators. However, there are situations in which an immune response can develop in the absence of exogenous adjuvants, such as in autoimmunity and in responses to tumor. This led to the hypothesis that cell stress or death may result in the release of endogenous danger signals that act as adjuvants to stimulate an immune response. Previous studies have proposed that DCs, for example, respond to endogenous danger signals released by cells undergoing stress or necrotic death [4]. DCs were activated when co-cultured with necrotic, but not normal, cells [5]. Recently, it has been demonstrated that dying cells co-injected with antigen into animals provided an adjuvant effect for activating T-cell responses [6,7].

Previous studies showed that HMGB1 protein acts as a late mediator of inflammation and endotoxin lethality [8,9]. HMGB1 is released from monocyte/macrophage stimulated with LPS [8]. It can also be released from injured or necrotic cells, where it stimulates necrosis-induced inflammation [9]. Recombinant HMGB1 activates monocytes in vitro and induces the release of proinflammatory mediators, including TNF- α , IL-6, and IL-8 [10] and antibodies against HMGB1 protect against endotoxin-induced lethality [8]. HSP70 belongs to a family of proteins known as heat shock proteins (HSPs), which are expressed at low level under normal physiological conditions [11], but are significantly induced by stressful stimuli such as bacterial endotoxin and other proinflammatory triggers [12]. Although the primary function of HSPs, including HSP70, is to stabilize protein folding, there is a growing interest in their immune function. HSP70 levels, for example, are increased in traumatized patients and children with septic shock [13]. In addition, exogenously expressed HSP70 stimulates proinflammatory responses in human monocytes and up-regulates IL-6, IL-1 β , and TNF- α expression [14].

Most studies addressing the proinflammatory role of HMGB1 and HSP70 used either the purified recombinant proteins or those ectopically expressed in transfected cells. We hypothesized that HMGB1 and HSP70 released from necrotic cells may act as endogenous danger signals to directly modulate the proinflammatory cytokine cascade. Using the human monocyte/macrophage-like cells, THP-1, we identified HMGB1 and HSP70 as two such signals and determined their contribution toward a TREM-1-mediated monocyte/macrophage response to bacterial endotoxin responsible for chronic inflammation.

2. Materials and methods

2.1. Cell culture and treatment

The human monocytic cell lines, THP-1 and U-937, were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C. For experiments, cells were stimulated for 6 h with 0.5 μ g/ml of gram-negative bacterial lipopolysaccharide (LPS, *E. coli* 055:B5; Sigma, St. Louis, MO).

2.2. Induction of necrosis

Cells were cultured in complete medium at 1×10^6 cells/ml and stimulated with $0.5 \mu\text{g/ml}$ of LPS, in order to drive HMGB1 and HSP70 expression. After 12 h, cells were harvested washed twice with sterile PBS and then resuspended in complete culture medium at 1×10^6 cells/ml. At this point, cell viability was more than 95% as determined by trypan blue exclusion. These cells were immediately used for necrosis induction by repetitive (6 cycles) freezing with liquid nitrogen and thawing at 37°C . Necrotic cell lysate (NCL) was obtained by centrifugation at 15,000 rpm for 20 min.

NCL was added to the freshly cultured cells at 50% (v/v), i.e., 50% fresh medium and 50% NCL and cell density was adjusted to 1×10^6 cells/ml.

2.3. Blocking experiments

To investigate the role of TLR4, HSP70 and HMGB1 in the proinflammatory cytokine induction by NCL, cultures were treated with $8 \mu\text{g/ml}$ anti-TLR4 (eBioscience, San Diego, CA), anti-HSP70 (R&D Systems, Minneapolis, MN), or anti-HMGB1 (Abcam, Cambridge, MA) antibodies, as well as isotype controls. To investigate the involvement of RAGE and TREM-1 receptors in the inflammatory cytokine response, anti-RAGE antibody or recombinant TREM-1 fusion chimera (rTREM-1/Fc) (R&D Systems) was added to the culture at 8 and $2 \mu\text{g/ml}$, respectively. All antibodies, including isotype controls, and the recombinant protein were added 1 h before reconstituting the culture with NCL.

2.4. Cytokine and chemokine assay

TNF- α , IL-6 and IL-8 protein concentrations in the culture supernatant were determined using ELISA kits following the manufacturer's protocol (R&D Systems, Minneapolis, MN). The samples, along with known standards, were assayed in duplicate. The lower detection limit was 5.1 pg/ml for TNF- α , 0.7 pg/ml for IL-6 and 7.5 pg/ml for IL-8.

2.5. Quantitative real-time PCR

Total RNA was extracted from THP-1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). The RT reactions were performed for 1 h at 42°C in a total volume of $50 \mu\text{l}$ containing $2 \mu\text{g}$ RNA and Superscript reverse transcriptase II (Invitrogen). Real-time PCR was performed with specific primer and probe sets corresponding to human TNF- α , IL-6, and IL-8 genes (Applied Biosystems, Foster City, CA). Each set contained two primers and an internal fluorogenic probe that was labeled with the fluorescent dyes carboxyfluorescein (FAM) as a reporter dye and *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (TAMRA) as a quencher dye, as follows: TNF- α forward 5'-TCTTCTCGAACCCCGAGTGA-3', reverse 5'-GGAGCTGCCCCCTCAGCTT-3', and internal probe 5'-FAM-AGCCTGTAGCCCATGTTGTAGCAAACCCT-TAMRA-3'; IL-6 forward 5'-CGACGTCCTGTACTGTTGAGT-3', reverse 5'-ACTCCACGGGTACGATGTAAA-3', and internal probe 5'-FAM-TAACCCGTGCAGCCGCCCTGGCGTCAG-TAMRA-3'; IL-8 forward 5'-CTGGCCGTGGCTCTCTTG-3', reverse 5'-CCTTGGCAAACACTGCACCTT-3', and internal probe 5'-FAM-CAGCCTTCCTGATTTCTGCAGCTCTGTGT-TAMRA-3'. The PCR reactions were performed in duplicate. The reaction mixture ($25 \mu\text{l}$) contained $5 \mu\text{l}$ cDNA, $12.5 \mu\text{l}$ of $2\times$ TaqMan universal master mix, 400 nM of each primer and 200 nM of internal probe. The reaction conditions were 2 min at 50°C , 10 min at 95°C , and 40 cycles with 15 s at 95°C and 1 min at 60°C (combined annealing and extension), using ABI prism 7700 Sequence Detection System, which monitors the amplified product in real-time by the analysis of emitted fluorescence. GAPDH mRNA was also amplified (using GAPDH TaqMan kit; Applied Biosystems) as an internal control. Sample data were normalized to GAPDH mRNA and are presented as fold change relative to medium control.

2.6. Western blot analysis

Treated or untreated cells were harvested, washed once in cold phosphate-buffered saline (PBS) and then lysed in buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, and 100 µg/ml PMSF. Cell lysates were cleared by centrifugation and protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). Equal amounts (50 µg) were boiled for 5 min in equal volumes of sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 15% glycerol and 10% 2-mercaptoethanol). Samples were chilled on ice and separated on an 8–16% gel (Pierce, Rockford, IL) and then electroblotted onto PVDF membranes (Pierce Biotechnology, Rockford, IL) using the Novex Xcell MiniGel System. Membranes were probed overnight with appropriate dilutions of primary Abs against IκB, p38 MAPK and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). After washing twice with TBS/0.05% Tween 20, appropriate peroxidase-conjugated secondary antibodies were applied. Blots were washed twice and incubated in enhanced chemiluminescence detection reagent (Amersham Bioscience, Piscataway, NJ). Specific bands were visualized by exposure to X-ray film. Membranes were stripped and reprobed with antibodies specific to phospho IκBα and p38 (Santa Cruz).

2.7. Data analysis

Differences between treatment groups were analysed by Student's *t*-test. Data are presented as mean ± standard error of the mean (S.E.M.) of at least three independent experiments. *p* < 0.05 was considered significant.

3. Results

3.1. Production of proinflammatory cytokines and chemokine by activated THP-1 cells

Bacterial endotoxin induces proinflammatory cytokine production by monocytes/macrophages [15,16] and LPS has been shown to stimulate the production of the cytokines TNF-α, IL-6 and the chemokine IL-8 in monocytes/macrophages [17,18,14]. To demonstrate the ability of LPS to induce proinflammatory cytokines in THP-1, cells were stimulated for 6 h with 0.5 µg/ml of LPS and TNF-α, IL-6, and IL-8 secretions were measured in the culture supernatant by ELISA. Under these conditions, LPS induced secretion of significant (*p* < 0.05) amounts of TNF-α, IL-6, and IL-8 (Fig. 1).

3.2. Necrotic cell lysate augments proinflammatory cytokine expression in the presence of LPS

Necrotic cells have been shown to activate the immune functions of DCs and T-cells. We investigated whether necrotic cell products (NCL) can affect the proinflammatory cytokine expression in THP-1 cells. Necrosis was induced in THP-1 cells that had been stimulated with LPS for 12 h and the NCL was used to condition the freshly cultured THP-1 cells. As shown in Fig. 2A, NCL added at 50% (v/v) to THP-1 culture for 6 h significantly increased the cytokine production as compared with medium control. However, such increase was still far less than that induced by LPS alone. When cells were incubated with NCL in the presence of LPS, we measured a more robust increase in the levels of TNF-α, IL-6, and IL-8. Such an increase was more than twice the levels seen after stimulation with LPS alone.

We wished to identify molecule(s) in the NCL that may be responsible for augmenting the LPS-induced cytokine production. Both HMGB1 and HSP70 have been reported to be released by activated as well as stressed or necrotic cells and their recombinant proteins have been shown to activate innate immune cells. We used specific antibodies to neutralize HMGB1 or HSP70 activity that may be present in the NCL. NCL was treated with the antibodies for 1 h before adding it to the THP-1 culture. The results (Fig. 2A) show that anti-

HMGB1 or anti-HSP70 significantly reduced the increase in the NCL-induced production of all three proteins, as compared with cells treated with LPS plus NCL. In addition, the simultaneous blockade of HMGB1 with HSP70 did not show any significant changes in cytokine production over that seen after the blockade of either HMGB1 or HSP70 alone, indicating there was no additive or synergistic effect. Treatment with isotype control antibody did not exhibit any significant changes compared to LPS plus NCL (data not shown). These data show that HMGB1 and HSP70 of the necrotic cell product played a role, at least in part, in the up-regulation of LPS-induced TNF- α , IL-6, and IL-8 secretion from THP-1 cells. Similar cytokine levels were obtained with U-937 cells (Fig. 3).

We further examined whether the cytokine induction by NCL was mediated at the level of gene expression by analyzing mRNA expression by real-time PCR. NCL markedly enhanced the cytokine mRNA expression in the presence of LPS (Fig. 2B). The presence of HMGB1 and HSP70 antibodies in the culture significantly ($p < 0.05$) reduced the increase in mRNA expression observed upon incubation with NCL. Together, these results suggest that HMGB1 and HSP70 were present in the NCL and acted as co-signals to up-regulate the LPS-induced TNF- α , IL-6, and IL-8 gene expression and protein production.

3.3. Up-regulation of LPS-induced cytokine production by HMGB1 and HSP70 is mediated by TREM-1

Toll-like receptor 4 (TLR4) is involved in the activation of innate immune pathways such as inflammation and cytokine production [19,20] and this is attributed to TLR4-mediated recognition of LPS [21]. Since our data showed that HMGB1 and HSP70 were involved in the enhancement of cytokine production in the presence of LPS, we investigated the contribution of TLR4 to the increase in the cytokine production from cells treated with LPS plus NCL. Blocking TLR4 with anti-TLR4 antibody significantly reduced, but did not completely prevent, the cytokine induction in cells treated with LPS plus NCL (Fig. 4), suggesting that an additional pathway may be involved in signaling the cytokine production in the presence of NCL. Treatment with control antibody did not show significant difference compared with the positive control (data not shown).

HMGB1 has been shown to bind to the receptor for advanced glycation end products (RAGE) and triggers inflammation [22]. However, treatment of THP-1 cells with anti-RAGE antibody did not exhibit changes in the cytokine induction by LPS plus NCL (data not shown). On the other hand, Triggering receptor on myeloid cells-1 (TREM-1) is a newly identified receptor expressed on neutrophils and monocytes/macrophages [23]. TREM-1 has been shown to synergize with TLRs to amplify the inflammatory response initiated by LPS [24] and induces TNF- α and IL-8 production [23]. We investigated the effect of blocking TREM-1 on the cytokine production in response to LPS and NCL. As shown in Fig. 4, blocking TREM-1 with TREM-1 fusion protein (rTREM-1/Fc) did not affect the LPS-mediated cytokine production. Blocking TREM-1 simultaneously with blocking HMGB1 or HSP70 did not affect the cytokine production either. In contrast, blocking TREM-1 abolished the NCL-induced cytokine production while blocking TREM-1 simultaneously with blocking HMGB1 or HSP70 activity had no additional effects. These results suggest that TREM-1 is not required for LPS-induced cytokine production but is essential for mediating the effect of NCL. We also examined whether blocking TREM-1 will influence the increase in cytokine production seen after adding NCL to LPS-treated cells. Treatment of THP-1 cells with rTREM-1 markedly decreased all three proteins while treatment with rTREM-1 in the presence of anti-HMGB1 or anti-HSP70 did not show any significant changes compared with rTREM-1 alone (Fig. 4). Blocking TREM-1 simultaneously with TLR4 completely inhibited the cytokine production. Together, the data presented in Fig. 4 suggest that TREM-1, although not required for LPS-induced cytokine production, is

involved in mediating the proinflammatory effects of HMGB1 and HSP70 and in enhancing the LPS-induced cytokine production by NCL.

3.4. Blocking TREM-1 attenuates p38 MAPK expression but not NF- κ B activation

Previous studies reported that MAP kinase and NF- κ B pathways are critical for monocyte/macrophage activation and proinflammatory cytokine production in response to a variety of stimuli [25,26]. NF- κ B is regulated by its cytoplasmic inhibitor, I κ B α , which, when activated, releases NF- κ B to migrate to the nucleus and activate target genes [27]. Indeed, TNF- α , IL-6, and IL-8 expression are regulated transcriptionally by NF- κ B and post-transcriptionally by p38 MAPK through stabilizing mRNA [28–32,17]. In addition, recombinant HMGB1 and HSP70 have been shown to induce proinflammatory cytokine by mechanisms involving NF- κ B and p38 [14,33]. Since TREM-1 was involved in the up-regulation of cytokine production by NCL in the presence of LPS stimulation, we examined whether these two pathways were involved in the NCL-enhanced cytokine production and whether blocking TREM-1 had an effect on the NCL-enhanced cytokine production through NF- κ B or p38 pathway. Western blot analysis showed that NCL induced I κ B α expression and phosphorylation similar to LPS. However, blocking TREM-1 did not notably affect I κ B α pathway (data not shown). In addition, while NCL alone induced p38 expression and increased the LPS-induced expression of p38, it did markedly phosphorylate and activate p38 in the presence of LPS (Fig. 5). NCL alone slightly activated p38. These results suggest that HMGB1 and HSP70 released from necrotic cells function as signals to enhance the LPS-induced p38 activation and cytokine production.

4. Discussion

Necrotic cells activate dendritic cells by inducing cytokine expression [34]. This study suggests that cell injury or death during the course of inflammation make available endogenous signals that amplify and prolong the inflammatory response. We have examined the ability of necrotic cell products to induce proinflammatory cytokines and chemokine at the mRNA and protein level in THP-1 cells. In this experimental model, necrotic cell lysate (NCL) induced comparable levels of TNF- α , IL-6 and IL-8 at 6 h when compared with medium control. The induction of these inflammatory mediators was significantly enhanced in the presence of bacterial LPS.

Previous studies have shown that both HMGB1 and HSP70 function as inflammatory mediators [8,14]. Because their levels are increased under certain stressful conditions such as cell injury and inflammation [8,9,12,13], they may act as endogenous signals to promote and exacerbate the inflammatory responses. Our blocking experiments demonstrated that both HMGB1 and HSP70 were part of the necrotic cell products, because blocking both proteins with specific antibodies significantly decreased the cytokine expression induced by LPS plus NCL. Our results support a previous study showing that HMGB1 $^{-/-}$ necrotic cells have greatly reduced ability to promote inflammation, as demonstrated by the reduced expression of TNF- α [9]. In addition, exogenously expressed HMGB1 and HSP70 have been shown to activate human monocytes and induce TNF- α , IL-6 and IL-8 expression [12,10,14]. The role of HSP70 in inflammation has been controversial. While some studies showed that it acts as endogenous signal to promote inflammation [28,35,36], other studies have suggested that it may play a protective role in response to tissue injury or inflammatory insults [37,38]. One explanation is that HSPs may have different effects depending on the context in which they are presented to the immune system. For example, intracellular HSP72 has been shown to decrease proinflammatory cytokine expression while extracellular HSP72 increase their expression [39,40]. In THP-1 cells, our data showed that HSP70 in the NCL was proinflammatory, as it enhanced the cytokine and chemokine expression. The finding that simultaneous blockade of HMGB1 and HSP70 did not produce an additive effect

suggests that either protein can induce similar effects, and does not require interactive effects with the other protein.

We performed blocking experiments to identify the receptor(s) involved in the induction of cytokine expression by NCL. HMGB1 has been shown to bind to RAGE and trigger inflammation [23]. Our data (not shown) indicated that blocking RAGE had no effects on the up-regulation of cytokine production by NCL in LPS-treated cells. With this regard, a previous study has shown that RAGE-blocking antibodies did not completely block cellular activation by HMGB1, and suggested that there may be other HMGB1-binding receptors than RAGE [41]. In addition, TLR4 may influence HSP70 signaling and activation of macrophages [36]. Blocking TLR4 did not abolish the cytokine expression, suggesting that other cell surface molecules may mediate the effect of HMGB1 and HSP70 in the NCL on the cytokine induction.

While its ligands are unknown, TREM-1 stimulates secretion of proinflammatory cytokines and chemokines and amplifies the inflammatory responses initiated by TLRs (reviewed in [23]) [24]. In addition, using a model of septic shock, blocking TREM-1 protected mice from death [24]. Engagement of TREM-1 in combination with microbial ligands that activate TLRs synergistically increased production of TNF- α and IL-8 [42,43]. Our results demonstrated that blocking TREM-1 significantly reduced the up-regulation of LPS-induced cytokine expression by NCL, while blocking TREM-1 and TLR4 completely abolished the cytokine production, suggesting that TREM-1 was involved in the augmentation of cytokine expression in THP-1 cells. A previous study has shown that TREM-1 fusion protein decreased the ability of sera from patients with sepsis to activate monocytes, and suggested that TREM-1 unidentified ligands may be present in the sera of septic patients [44]. Our study supports this previous observation and suggests that HMGB1 and HSP70 proteins in the NCL may activate TREM-1 and thus up-regulate the proinflammatory response in THP-1 cells. We found that simultaneous blockade of TREM-1 with HMGB1 or HSP70 on the LPS plus NCL-induced cytokine production was similar to the effect of blockade of TREM-1 alone, suggesting that HMGB1 and HSP70 utilize the same (TREM-1) pathway to enhance LPS-induced cytokine production. In addition, pathways other than TREM-1, or other endogenous signals in the NCL may contribute to enhancing LPS-induced cytokine production, because blocking TREM-1 alone or simultaneously with HMGB1 or HSP70 did not decrease the cytokine induced by LPS plus NCL to the level seen upon blocking TLR4 alone.

Activation of p38 MAPK contributes to IL-6, IL-8 and TNF- α production [45,33], while attenuation of p38 and NF- κ B phosphorylation down-regulates IL-8 and TNF- α production by THP-1 cells [33]. Our results indicated that NF- κ B was induced by NCL and also by LPS but was not inhibited by the blockade of TREM-1 in the presence of LPS plus NCL, suggesting that NCL activates NF- κ B and that NF- κ B activation occurs in the absence of TREM-1. Such dual activation may be sufficient to induce cytokine expression without TREM-1 co-signal. On the other hand, p38 was slightly activated by NCL. Its activation was markedly enhanced in the presence of LPS and was reduced by blocking TREM-1, suggesting that TREM-1 was involved in the enhancement, by NCL, of LPS-induced cytokine production. This is very significant, because it suggests that NCL with its HMGB1 and HSP70 components may indeed act as ligands for TREM-1. It has previously been reported that TREM-1 and TLRs cooperate to promote inflammation [25] through NF- κ B and MAP kinase activation [23].

This study has shown that necrotic cells contain endogenous signals that promote inflammation. Two such signals, HMGB1 and HSP70 directly up-regulate the endotoxin-induced proinflammatory cytokines and chemokine through activation of NF- κ B and p38

signaling pathways. This study identifies TREM-1 as part of a receptor assembly pathway that transduces the proinflammatory endogenous signal to the cytokine gene expression cascade.

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Fig. 1. LPS induces proinflammatory cytokine and chemokine expression in THP-1 cells. Cells were stimulated with 0.5 μ g/ml of LPS for 6 h. The TNF- α , IL-6 and IL-8 protein production was measured in the culture supernatant by ELISA. * $p < 0.05$ vs. medium control.

Fig. 2.

Necrotic cell lysate (NCL) contains HMGB1 and HSP70 that up-regulate the LPS-induced proinflammatory cytokine and chemokine production. Cells were incubated for 6 h with NCL, or NCL plus LPS in the presence or absence of anti-HMGB1 or anti-HSP70 or both. (A) Protein production as measured by ELISA. (B) mRNA expression as determined by real-time PCR. Data were normalized to GAPDH mRNA and are presented as fold change relative to medium control (assigned as one fold). * $p < 0.05$ vs. LPS alone; ** $p < 0.05$ vs. positive control (LPS plus NCL).

Fig. 4.

Blocking TREM-1 reduces the NCL-mediated increase in the LPS-induced cytokine production. Cells were treated for 6 h with LPS, NCL, or LPS plus NCL in the presence or absence of rTREM-1 and anti-HMGB1, anti-HSP70, or anti-TLR4. Protein levels were assayed in the culture supernatant after 6 h. * $p < 0.05$ vs. NCL alone; ** $p < 0.05$ vs. LPS plus NCL.

Fig. 5. NCL activates p38 MAPK pathway in THP-1 cells. Cells were treated for 6 h with NCL, LPS, or both in the presence or absence of rTREM-1. Proteins were fractionated, blotted, and then probed with specific antibodies to determine the expression of total and phosphorylated p38 MAPK. P-p38, phosphorylated p38.