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Cellular Stress Amplifies TLR3/4-Induced CXCL1/2 Gene Transcription in Mononuclear Phagocytes via RIPK1

Chenyang Zhao, Paul G. Pavicic, Jr., Shyamasree Datta, Dongxu Sun, Michael Novotny, and Thomas A. Hamilton

The impact of environmental stressors on the magnitude of specific chemokine gene expression was examined in mouse bone marrow–derived macrophages stimulated through various TLRs. Levels of TLR-stimulated CXCL1 and CXCL2 but not CXCL10 or CCL5 mRNAs were selectively enhanced (>10-fold) in stressed macrophages. The amplification was also manifested for other proinflammatory cytokines, including TNF-α, IL-1α, and IL-6. Responses through TLR3 and TLR4 exhibited the greatest sensitivity, reflecting a requirement for Toll/IL-1R domain–containing adaptor-inducing IFN-β (TRIF), the adaptor protein selectively associated with these TLRs. IFN regulatory factor 3, a transcription factor that is downstream of TLR4/TRIF signaling, was not required for sensitivity to stress-induced chemokine amplification. Rather, receptor-interacting protein kinase 1, a kinase also linked with TLR3/4/TRIF signaling, is required and involves a stress-dependent increase in its abundance and ubiquitination. Whereas NF-κB activation is necessary for TLR-induced chemokine gene transcription, this factor does not appear to be the primary mechanistic target of environmental stress. The application of stress also enhanced chemokine expression in macrophages infiltrating the peritoneal cavity but was not observed in the resident peritoneal cells or in the liver. These findings identify novel mechanisms for modulating the magnitude and duration of selective TLR-induced chemokine and cytokine gene expression and further establish the importance of cell stress pathways in coordinating the outcomes of cellular and tissue injury. The Journal of Immunology, 2014, 193: 879–888.
peritoneal macrophages were prepared as described previously (32). RAW264.7 mouse macrophage cells were maintained in MEM contain- ing penicillin, streptomycin, and 10% FBS in humidified 5% CO2. Transient transfections of siRNA oligonucleotides in BMDMs and RAW264.7 cells were done using an Amaxa Nucleofector Kit V. Cells (5 × 10^6) were transfected with 15 µg siRNA oligonucleotide duplex and cultures were rested for 48–96 h following transfection prior to further experimental treatments. For amino acid restriction (AAR) experiments, cells were washed three times with PBS, cultured with lysine-, leucine-, and methionine-deficient MEM supplemented with 10% dialyzed FBS, penicillin, and streptomycin (AAR medium) for 1 h, washed again, and fresh AAR medium was added for 16 h prior to further treatments. Promoter reporter plasmids were constructed by linking either five copies of the mouse CXCL10 xB1 sequence element (33, 34) or 290 nucleotides from the 5′ flanking region of the mouse CXCL1 gene (35, 36) into the cell cloning site of the pGL2 Basic plasmid (Promega). Plasmids were transfected in RAW264.7 cells using SuperFect (Qiagen) and rested for 24 h prior to further experimental treatment. Luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer’s instructions.

Preparation of liver nonparenchymal cells
Nonparenchymal cells were isolated from the mouse liver as described (37). Briefly, the liver was perfused with collagenase IV (Sigma-Aldrich, St. Louis, MO) solution (1.0 mg/mL) via the portal vein, minced, and digested with collagenase for 30 min at 37°C with agitation. The digests were filtered through 200-µm nylon mesh, and the nonparenchymal cells were enriched by Percoll density gradient centrifugation, collected, and resuspended in culture medium in polypropylene tubes for treatment as described in the text. Myeloid cell content was assessed by flow cytometry following immunostaining with anti-CD11b.

mRNA determination
Total RNA was prepared using TRI Reagent and analyzed either by Northern blot hybridization as described previously (38) or by reverse transcription and real-time PCR using 0.5-1 µg total RNA as previously described (39). Autoradiographs were quantified by image analysis using ImageJ software. CXCL1 mRNA levels were normalized to levels of GAPDH mRNA measured in the same RNA sample. Specific primers used for PCR as described below were obtained from Eurofins MWG Operon: CXCL1 (40), forward, 5′-CACAGGGGCGCCTATCCGCAA-3′; reverse, 5′-CAAAGGCAGACCCCTGGAC-3′. RNase inhibitor was from Roche and Moloney murine leukemia virus reverse transcriptase was from Promega. SYBR Green PCR Master Mix (2×) was purchased from Applied Biosystems. Brewer’s thiglycollate broth was obtained from BD Biosciences. PerkinElmer Life Sciences was the source of [α–32P]CTP. A CXCL1 ELISA kit was purchased from R&D Systems. A Nuclear Extract Kit and a TransAM NF-κB p65 transcrip- tion factor assay kit were purchased from Active Motif. Mouse anti-RIPK1 Ab (catalog no. 610458) was purchased from BD Transduction Laboratories; rabbit anti-p65 mAb (catalog no. 8242) was purchased from Cell Signaling Technology; anti-ubiquitin Ab (catalog no. SC 8017) was obtained from Santa Cruz Biotechnology; mAb specific for GAPDH was purchased from Millipore (catalog no. MAB374); Ab specific for CD95 (CD178) Ab was purchased from BD Biosciences (catalog no. 554255). The small interfering RNAs (siRNAs)—5′-CCACUGUACUGAUCAGUGAA-3′ for RIPK1 (30) and 5′-GGGAUAUCAUGGUAAGGGCUUU-3′ for XBP1 (31)—were purchased from Thermo Scientific Dharmacon RNAi Technologies.

Mice, cell culture, and siRNA transfection
C57BL/6, CHOP+/-, TRIF-/-, and GCN2-/- mice on a C57BL/6 background were obtained from The Jackson Laboratory. TRIF-/-defi- cient mice were provided by Dr. Perry Blackshear (National Institutes of Health). IFN regulatory factor 3 (IRF3)-/- mice (C57BL/6 background) were obtained from RIKEN Bio Resource Center (Tsukuba, Japan). Mouse BMDCs were prepared from bone marrow cells that were collected from femurs by flushing with cold HBSS. The cell suspensions were then passed through a 100-µm nylon cell strainer (BD Falcon), collected by centrifugation, and resuspended in DMEM containing 10% FBS, 100 U/ml pen- icillin, streptomycin, and 50 µg/ml M-CSF (R&D Systems). Splenocytes (5 × 10^6) were seeded in 100-mm dishes and cultured at 37°C with 5% CO2 for 7 d. Nonadherent cells were removed by washing, and the BMDCs were treated as indicated in the text. Thioglycollate-elicited materials and Methods
Reagents
DMEM, RPMI 1640, lysine-leucine-methionine–deficient DMEM, Dul- becco’s PBS, and antibiotics were obtained from Central Cell Services of the Lerner Research Institute. FBS was purchased from Atlas Biologicals. Dialyzed FBS and random primers were obtained from Invitrogen. The Amaxa Nucleofector Kit V was obtained from Lonza. TRI Reagent was purchased from Molecular Research Center, and nylon transfer mem- brane was purchased from GE Healthcare Life Sciences. Tunicamycin (Tm), thapsigargin (Tg), formamide, MOPS, salmon sperm DNA, and diethyl pyrocarbonate were obtained from Sigma-Aldrich. TRIF agonists (Pam3CSK4, LPS, and polyinosinic-polycytidylic acid [poly(lC)]) were purchased from Genzyme. RT-PCR system (PerkinElmer). For determination of RIPK1 ubiquitination, treated samples, and Abs, and samples were visualized with a Western Lightning Plus-ECL manufacturer’s instructions. Northern blot hybridization as described previously (38) or by reverse transcription and real-time PCR using 0.5-1 µg total RNA as previously described (39). Autoradiographs were quantified by image analysis using ImageJ software. CXCL1 mRNA levels were normalized to levels of GAPDH mRNA measured in the same RNA sample. Specific primers used for PCR as described below were obtained from Eurofins MWG Operon: CXCL1 (40), forward, 5′-CACAGGGGCGCCTATCCGCAA-3′; reverse, 5′-CAAAGGCAGACCCCTGGAC-3′. RNase inhibitor was from Roche and Moloney murine leukemia virus reverse transcriptase was from Promega. SYBR Green PCR Master Mix (2×) was acquired from Applied Biosystems. Brewer’s thiglycollate broth was obtained from BD Biosciences. PerkinElmer Life Sciences was the source of [α–32P]CTP. A CXCL1 ELISA kit was purchased from R&D Systems. A Nuclear Extract Kit and a TransAM NF-κB p65 transcrip- tion factor assay kit were purchased from Active Motif. Mouse anti- RIPK1 Ab (catalog no. 610458) was purchased from BD Transduction Laboratories; rabbit anti-p65 mAb (catalog no. 8242) was purchased from Cell Signaling Technology; anti-ubiquitin Ab (catalog no. SC 8017) was obtained from Santa Cruz Biotechnology; mAb specific for GAPDH was purchased from Millipore (catalog no. MAB374); Ab specific for CD95 (CD178) Ab was purchased from BD Biosciences (catalog no. 554255). The small interfering RNAs (siRNAs)—5′-CCACUGUACUGAUCAGUGAA-3′ for RIPK1 (30) and 5′-GGGAUAUCAUGGUAAGGGCUUU-3′ for XBP1 (31)—were purchased from Thermo Scientific Dharmacon RNAi Technologies.
P-40, 20 mM 2-ME, 250 mM NaCl, 2 mM DTT, 1 mM NEM and protease inhibitor mixture (Sigma-Aldrich) for 30 min. Cell debris was removed by centrifugation and the supernatant was supplemented with 0.1 volume of 10% SDS to give a final concentration of 1% SDS. Then lysates were boiled for 5 min, placed on ice, and lysis buffer was added to achieve a final SDS concentration of 0.1% and incubated with beads and Abs overnight at 4°C prior to analysis as described above.

NF-κB DNA binding ELISA

BMDMs were fractionated using a Nuclear Extract Kit according to the manufacturer’s instructions. The nuclear fractions were used to analyze the DNA binding activity of p65 using the TransAM NF-κB p65 kit.

Results

Cellular stress amplifies expression of a subset of TLR-induced chemokine and cytokine genes

Cellular stress has been shown to promote elevated expression of a limited selection of TLR-induced inflammatory cytokines in macrophages, including IL-6, the p19 subunit of IL-23, and IFN-β (23–26). Additionally, although more modest effects on other TLR target genes have been reported, early chemokine expression has not been well characterized (24, 27). To examine this in more detail BMDMs were exposed to Tm, a protein glycosylation inhibitor known to promote the UPR, for the indicated times followed by stimulation with LPS for 6 h. The level of mRNA encoding the chemokine CXCL1 was markedly elevated (>20-fold) in cultures exposed to Tm for 4 h prior to LPS (Fig. 1A) whereas only modest change was observed in cells undergoing simultaneous exposure to Tm and LPS (Tm treatment time of 0). Moreover, CXCL1 mRNA expression was also prolonged in cultures exposed to Tm for 6 h prior to LPS treatment as compared with unstressed macrophages where the response was transient and levels of CXCL1 mRNA returned to baseline by 6 h after LPS (Fig. 1B). A similar pattern of enhanced expression was also observed with CXCL2 mRNA whereas levels of mRNAs encoding CXCL10 and CCL5, although induced by LPS, were not enhanced (Fig. 1C). These findings suggest a selective effect of stress on the expression of chemokines with the signature ELR-CXC motif known to target neutrophils. The effects of stress engagement on TLR response were not limited to chemokines, however, as comparable alterations in levels of TNF-α, IL-1α, and IL-6 were also observed (Fig. 1D).

Characteristics of stress-amplified chemokine expression

We compared several routes of inducing cell stress, including Tm, an inhibitor of protein glycosyltransferase (48), Tg, an inhibitor of the ER Ca2+ ATPase (49), or essential AAR, for their ability to amplify CXCL1 expression in BMDMs treated with three different TLR-specific ligands (Pam3CSK4 for TLR2, poly(I:C) for TLR3, LPS for TLR4). Cultures were exposed to Tm or Tg for 6 h or deprived of essential amino acids (Leu, Met, Lys) for 16 h followed by stimulation for 6 h with the individual TLR-specific ligands (Fig. 2A). A similar experimental design was used to examine chemokine expression in the RAW264.7 cell line (Fig. 2B). All three routes of inducing cell stress resulted in elevated expression of LPS-stimulated CXCL1 mRNA, although the magnitude of response varied with the stress inducer and TLR ligand employed. In both cell types treatment with Tm elevated CXCL1 mRNA when stimulated through TLR3 or TLR4 but not TLR2, whereas cells exposed to Tg or AAR exhibited amplification for responses to all three TLRs. Although the magnitude of response to TLR4 was generally greater than for other TLRs, particularly in BMDMs, this does not reflect greater total chemokine production but rather greater sensitivity to the stress mechanism. CXCL2 expression was modulated comparably to CXCL1 and revealed a similar distinction between TLR2 and TLR3/TLR4 in Tm-stressed cells (Fig. 2C). Consistent with the findings in Fig. 1, levels of CXCL10 (Fig. 2C) and CCL5 (not shown) mRNAs exhibited only modest differences between stressed and nonstressed macrophages. CXCL10 is only poorly induced by TLR2, as it depends on the IRF3 component of the TRIF pathway that is specifically engaged by TLR3 and TLR4 (50, 51). The levels of secreted CXCL1 protein are also selectively increased in LPS-stimulated

FIGURE 1. Cellular stress amplifies expression of a subset of TLR-induced chemokine and cytokine genes. (A) BMDMs treated with DMSO or Tm (1 μg/ml) for the indicated times were subsequently stimulated with LPS (100 ng/ml) for 6 h. Levels of CXCL1 and GAPDH mRNA were determined by Northern hybridization and autoradiographs were quantified using ImageJ software. CXCL1 levels were normalized to GAPDH levels in the same samples and are presented as fold change relative to samples treated with DMSO and LPS for 6 h. Results are representative of more than four separate experiments. (B) BMDMs were treated with DMSO or Tm for 6 h followed by LPS for the indicated times prior to determination and quantification of CXCL1 mRNA levels as in (A). Data are presented as the fold change relative to samples treated with DMSO but without LPS. (C) BMDMs were treated for 6 h with DMSO or Tm prior to addition of LPS for 6 h. Levels of CXCL1, CXCL2, CCL5, and CXCL10 mRNA were measured by real-time PCR as described in Materials and Methods and normalized to levels of GAPDH mRNA. Values presented are the fold induction relative to cultures treated with DMSO alone for 12 h and are the means of duplicate determinations ± half of the range. (D) RNA from the experiment described in (C) was employed to determine levels of TNF-α, IL-6, and IL-1α mRNAs.
levels of TLR2 and TLR4 mRNAs. (A) RAW264.7 cells were treated and analyzed as in (A). (C) RNA from the experiments described in (A) was employed to determine levels of CXCL2 mRNA in cultures stimulated through TLR2 or TLR4 and CXCL10 mRNA in cultures stimulated through TLR4. (B) RAW264.7 cells were treated and analyzed as in (A). (C) RNA from the experiments described in (A) was employed to determine levels of CXCL2 mRNA in cultures stimulated through TLR2 or TLR4 and CXCL10 mRNA in cultures stimulated through TLR4. (D) RAW264.7 cells were treated and analyzed as in (B). (E) RAW264.7 cells were treated and analyzed as in (A). (F) RNA from the experiments described in (A) was employed to determine levels of TLR2 and TLR4 mRNAs.

**FIGURE 2.** Characteristics of stress-amplified chemokine expression. (A) BMDMs were cultured with Tm (1 μg/ml), Tg (50 nM), or under conditions of AAR (Leu, Met, Lys) for 6 (Tm, Tg) or 16 h (AAR) prior to stimulation with ligands for TLR2 (Pam3Cys), TLR3 (poly(I:C)), or TLR4 (LPS) for an additional 6 h. Levels of CXCL1 and GAPDH mRNA were determined by real-time PCR as in Fig. 1C. Values presented are the fold induction relative to cultures treated with DMSO alone for 12 h and are the means of duplicate determinations ± half of the range. (B) RAW264.7 cells were treated and analyzed as in (A). (C) RNA from the experiments described in (A) was employed to determine levels of CXCL2 mRNA in cultures stimulated through TLR2 or TLR4 and CXCL10 mRNA in cultures stimulated through TLR4. (D) BMDMs were treated with DMSO or Tg for 6 h prior to stimulation with LPS for 6 or 24 h. Supernatants were collected and used to determine protein levels of CXCL1 by ELISA. Results are presented as the means of duplicate determinations ± half of the range. (E) BMDMs from WT, TRIF−/−, or IRF3−/− mice were treated with DMSO or Tm for 6 h followed by LPS for 6 h. Levels of CXCL1 and GAPDH mRNAs were determined and quantified as in (A). (F) RNA from the experiments described in A was employed to determine levels of TLR2 and TLR4 mRNAs.

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**UPR pathways are not required for stress-enhanced CXCL1 expression**

Prior studies have reported important roles for several components of the UPR in the selective enhancement of cytokine expression in inflammatory macrophages (23, 24, 26, 27). For example, the enhanced expression of both IL-6 and the p19 component of IL-23 have been shown by several laboratories to be dependent on CHOP, a transcription factor activated via the UPR (24–26, 53). We used several experimental strategies to test the possible role for these factors in the Tm-mediated amplification of LPS-stimulated CXCL1 expression. Transfection of BMDMs with siRNA targeting XBP1 reduced the levels of spliced XBP1 mRNA by >70% as compared with control siRNA-treated cultures but did not alter the effects of stress on LPS-induced CXCL1 mRNA levels (Fig. 3A). To evaluate the possible participation of CHOP, we measured CXCL1 mRNA levels in unstressed or Tm-treated, LPS-stimulated BMDMs from WT and CHOP−/− mice. Exposure to Tm enhanced chemokine expression comparably in both cell populations (Fig. 3B). In a third approach we compared the response of BMDMs from WT or GCN2−/− mice to AAR (GCN2 is an eIF2α kinase that is activated during AAR and initiates cell stress by inducing ATF4 and CHOP) (54). Whereas CHOP mRNA is strongly induced during AAR in WT macrophages, this is, as expected, abrogated using BMDMs from GCN2−/− mice (Fig. 3C). Also noteworthy is the finding that AAR did not activate other UPR pathways (e.g., XBP1 splicing or BIP expression) (Fig. 3C). Importantly, the effects of AAR on elevation of CXCL1 mRNA expression were similar in WT and GCN2−/− macrophages (Fig. 3C). Hence, deficiency of CHOP or XBP1 does not compromise stress-amplified chemokine expression. Moreover, other components of the UPR are not induced during AAR-mediated stress and thus are not required, at least in this context. These findings are consistent with a recent report showing that cell stress can amplify inflammasome activity independently of the UPR (55).

**Tm-induced stress enhances TLR4 signaling via RIPK1 kinase**

Our finding that Tm-driven amplification of chemokine expression is independent of CHOP and XBP1 prompted us to consider other stress-sensitive mediators. Because of the requirement for TRIF but not IRF3 in stress-mediated amplification of LPS-induced chemokine expression, we examined RIPK1, a kinase that participates in TNFR, TLR3, and TLR4 signaling to NF-κB (28, 29, 56, 57). Importantly, RIPK1 is also required for NF-κB activation during the DNA damage response and is an essential component of the necroptosis pathway (58–60). siRNA-mediated depletion of RIPK1 almost fully abrogated the amplification of LPS-induced CXCL1 levels in Tm-treated BMDMs as measured by Northern hybridization (Fig. 4A). Interestingly, the depletion of RIPK1 did not compromise the basal TLR4-induced CXCL1 and CXCL2 mRNA levels and indeed resulted in a modest elevation that cannot be appreciably increased by either Tm or Tg (Fig. 4B).
however, that the effects of RIPK1 depletion vary with the specific target gene as levels of IL-1α in response to LPS were markedly reduced in both resting and stressed BMDMs (Fig. 4C). It has been shown that the kinase activity of RIPK1 is not essential for NF-κB activation (57, 61, 62). Rather, NF-κB activation depends on ubiquitin modification of RIPK1 on Lys377 (56, 57, 62, 63). Tm treatment of BMDMs for 6 h markedly enhanced the abundance of cellular RIPK1 protein as measured by Western blot. Importantly, stress treatment resulted in ubiquitination of RIPK1 as well (Fig. 4D). Treatment with Tg also induced increased levels of RIPK1 protein and ubiquitination (Fig. 4E and not shown). Stress-mediated enhancement of TLR4-stimulated CXCL1 expression is not compromised in BMDMs treated with the specific RIPK1 kinase inhibitor NEC1, suggesting that the kinase activity is not essential for the role of RIPK1 in mediating the effects of cell stress on CXCL1 expression (Fig. 4F).

A recent report demonstrated that apoptosis induced via FAS/CD95 interaction can promote RIPK1-dependent chemokine production as part of a program to help clear apoptotic cells by soliciting phagocytes (64). In our experiments, we did not find evidence for significant increases in apoptosis during treatment of stressed macrophages with LPS (not shown), suggesting that apoptosis was not the basis for enhanced chemokine expression. Cullen et al. (64) also identified a requirement for cellular cIAPs in the FAS-mediated chemokine response. To determine whether cIAPs were required for stress-enhanced chemokine expression, we treated BMDMs with the cIAP inhibitor AT 406, a smac mimetic that results in the rapid degradation of cIAP1/2 (65) along with Tg for 6 h prior to stimulation with LPS. Whereas AT 406 resulted in significant reduction in levels of cIAP, there was no effect on the pattern of CXCL1 mRNA expression in either resting or stressed macrophages (Supplemental Fig. 1A). Furthermore, macrophages treated with a monoclonal anti-CD95 Ab that can trigger apoptosis did not induce CXCL1 mRNA expression in either resting or stressed BMDMs (Supplemental Fig. 1B). Finally, we considered the possibility that LPS-induced TNF-α, the expression of which is also elevated in stressed cells, might be responsible for the enhanced chemokine production. The addition of TNF-α (20 ng/ml) to BMDMs alone or along with LPS and/or stress inducers did not alter the pattern of CXCL1 mRNA expression nor did the inclusion of a neutralizing Ab against TNF-α (Supplemental Fig. 1C). Hence we concluded that the mechanism described by Cullen et al. (64) was not responsible for the elevated chemokine expression observed in LPS-stimulated stressed macrophages.

Stress enhances TLR4-induced CXCL1 gene transcription

The magnitude and duration of chemokine gene expression is known to be controlled both by transcription and mRNA half-life (66, 67). To assess CXCL1 transcription in TLR4-stimulated macrophages, a set of primers amplifying a fragment that contains both intronic and exonic sequences was employed as a measure of primary transcript abundance (see schematic in Fig. 5A). Using total RNA prepared from stressed and unstressed macrophages treated for various times with LPS, the levels of primary transcript predicted the temporal pattern of mature mRNA accumulation (compare Fig. 5A and Fig. 1B), indicating that gene transcription is a target for the action of cell stress. Furthermore, depletion of RIPK1 protein also compromised the stress-mediated increase in CXCL1 primary transcript abundance (Fig. 5B). Whereas mRNA half-life is well documented to control CXCL1 expression (67), the half-life of CXCL1 mRNA in BMDMs does not appear to be regulated by cell stress. CXCL1 mRNA decay was not significantly changed by cell stress (Fig. 5C), and the abundance of CXCL1 mRNA was comparably amplified by Tm in BMDMs prepared from WT mice or mice deficient in tristetraprolin, a protein known to be the predominant regulator of CXCL1 half-life (47) (Fig. 5D). The transcription factor NF-κB is an important determinant of LPS-induced CXCL1 gene transcription and is a major downstream mediator in RIPK1-dependent signaling (36, 57, 62). The activation and function of this factor does not, however, appear to be the major target for the action of cell stress in enhancing CXCL1 expression. This is based on several observations. First, the magnitude and kinetics of nuclear localization and DNA binding activity of p65 are similar in untreated or Tm-pre-treated BMDMs following TLR4 stimulation (Fig. 5E, 5F). Second, we evaluated RAW264.7 cells transiently transfected with reporter plasmids in which luciferase expression is driven by either five tandemly arranged EB sites or a 290-bp fragment from the CXCL1 promoter (36) that contains two EB sites and observed that EB-driven transcripts exhibit sensitivity to TLR4 but not cell stress (Fig. 5G). Hence, the enhanced transcription apparently depends on sequences not contained within the 290-nucleotide fragment from the CXCL1 promoter used in this...
seems not to be achieved by enhancing the activity of NF-κB expression via modulation of gene transcription, although the effect amplifies both the magnitude and duration of CXCL1 mRNA expression. Taken together, these data suggest that cell stress from resident macrophages, consistent with the results of in vitro experiments. Stress did not alter LPS-induced chemokine expression in both cell populations by treatment with LPS, particularly those infiltrating damaged tissues. To test this hypothesis we initially determined the sensitivity to stress in resident myeloid cell populations prepared from the liver and the peritoneal cavity following injection of thioglycollate broth show substantial amplification of CXCL1 mRNA and protein levels when treated with Tg followed by LPS as compared with cells treated with LPS alone (Fig. 6C). Similar outcomes were obtained in vivo when mice were injected i.p. with the stress inducer Tg followed 6 h later by LPS. Stress did not alter LPS-induced CXCL1 mRNA expression in the liver and did not appreciably alter levels of circulating chemokine in the serum (Fig. 6D). The sequential exposure of macrophages to Tg followed by LPS within the peritoneal cavity resulted in a dramatic increase in levels of CXCL1 mRNA in the elicited cell population and in secreted protein in the peritoneal wash as compared with those receiving LPS alone (Fig. 6E). Similar treatment of unmanipulated mice did not show stress-mediated enhancement of chemokine secretion from resident macrophages, consistent with the results of in vitro treatments (Fig. 6E). These findings support the hypothesis that stress-dependent modulation of myeloid cell cytokine production occurs selectively within the population of macrophages infiltrating tissues in response to inflammatory conditions. In contrast, resident myeloid cell populations do not exhibit this sensitivity.

**Discussion**

Several recent studies have demonstrated that expression of specific inflammatory cytokine genes can be markedly amplified in TLR-stimulated macrophages undergoing cellular stress responses, but the spectrum of cytokine loci that are sensitive and the mechanistic appreciation of how cellular stress inducers affect TLR signaling remain poorly defined (23, 24, 26, 27). In particular, the effects of stress on expression of chemokines that recruit neutrophils, among the earliest events following injury or infectious challenge, have not been explored. In the present study we examined how different environmental stress conditions affect TLR-induced chemokine expression. The findings support the following conclusions. First, macrophages engaged in cell stress exhibit a dramatic increase in the transcription of a subset of chemokine and cytokine genes that include CXCL1 and CXCL2 but not CXCL10 or CCL5. Second, although multiple stress inducers can amplify chemokine and cytokine gene expression in macrophages following stimulation through TLRs, responses from the TLR3/TLR4/TRIF pathway exhibit the greatest sensitivity. Third, whereas XBP1 and CHOP, components of the UPR, are implicated in stress-mediated increases in TLR-induced IFN-β, IL-23p19, and IL-6 expression, the effects of stress on CXCL1 expression are independent of both factors. Fourth, RIPK1, a stress-sensitive signaling protein in the TLR3 and TLR4 pathways, is necessary for the amplified magnitude and duration of chemokine gene transcription in stressed macrophages. Fifth, surprisingly, whereas the effects involve amplification of transcription, neither IRF3 nor NF-κB appears to be a primary determinant of stress-mediated amplification of chemokine production. Finally,
the sensitivity of myeloid cells for stress enhancement of chemokine expression appears to be restricted to inflammatory macrophages whereas resident myeloid cells from the liver or peritoneal cavity do not exhibit sensitivity. Note that the three mechanistically distinct treatments used to induce cellular stress responses all had similar effects on chemokine and cytokine expression induced in response to TLR4. We did, however, observe TLR selectivity in responses obtained using different stress mechanisms (e.g., TLR3/4, but not TLR2, are sensitive to Tm-induced stress). Hence, there appears to be substantial mechanistic complexity and diversity in the interface between cellular stress and inflammatory TLR signaling pathways. This mechanistic diversity makes it particularly challenging to identify common molecular events and this represents an area for further exploration.

Because multiple laboratories have identified a relationship between responses to environmental stress and the magnitude and duration of inflammatory cytokine expression, particularly in myeloid cell populations, it is important to consider the present findings in the context of these earlier studies (23–27, 52, 68). Stress responses that enhance expression of IFN-β, IL-6, and IL23p19 are well documented (23–26), but the mechanisms involved in amplification of chemokine transcription observed in the present study are readily distinguished from these based on differential dependency on IRF3 and CHOP; whereas IFN-β requires IRF3 and both IL-6 and IL-23p19 require CHOP, CXCL1 expression is comparably amplified in macrophages deficient in either gene. Effects on a broader spectrum of inflammatory cytokine genes have been linked to endoplasmic reticulum stress responses involving the inositol requiring kinase and endonuclease 1/XBP1 pathway (24, 27). Our findings, however, demonstrate that the modulation of chemokine gene expression is not dependent on XBP1 based on siRNA-mediated depletion. Furthermore, under conditions of AAR in GCN2-deficient macrophages, the XBP1 pathway is not activated and yet the amplification of gene expression remains intact (see Fig. 3C). Although the UPR-mediated activation of ATF6 has been reported to contribute to acute phase gene expression (69), we have not explicitly examined this pathway in the effect of cell stress on chemokine expression. Thus, a mechanistic connection with UPR stress remains a possibility. Nevertheless, three mechanistically distinct routes of inducing cell stress in mouse macrophages result in markedly elevated and prolonged transcription of CXCL1 that does not appear to be linked with the specific UPR pathways reported for other stress-sensitive inflammatory cytokine genes.

The absence of a role for the UPR prompted a search for additional routes/mechanisms through which stress and inflammatory signaling might be linked. RIPK1 is known to contribute to inflammatory cytokine expression downstream of TNFR, TLR3, and TLR4, and also as a sensor of cellular stress response to DNA damage (57, 62, 70, 71). Moreover, the requirement of TRIF for activation of NF-κB p65 DNA binding activity as described in Materials and Methods. Results are presented as the means of duplicate determinations ± half of the range. (C) RAW264.7 cells transiently transfected with the indicated luciferase reporter plasmids were treated with DMSO or Tm for 6 h followed by LPS for 6 h. Total RNA was prepared and used to determine luciferase mRNA by Northern hybridization as in Fig. 1. Results are representative of three separate experiments.

FIGURE 5. Stress enhances TLR4-induced CXCL1 gene transcription. (A) BMDMs were treated with DMSO or Tm for 6 h followed by LPS for the indicated times, and total RNA was used to determine levels of CXCL1 primary transcripts by real-time PCR as described in Materials and Methods. Values presented are the fold induction relative to cultures treated with DMSO alone for 12 h and are the means of duplicate determinations ± half of the range. The schematic above the graph shows the position of primers amplifying CXCL1 transcripts containing both intronic and exonic sequences. (B) BMDMs transfected with control or RIPK1 siRNAs were treated with DMSO, Tm, or Tg for 6 h followed by 6 h of LPS treatment. CXCL1 primary transcripts were determined as in (A). (C) BMDMs were treated with DMSO or Tm for 6 h and LPS for 5 h prior to addition of actinomycin D. RNA was prepared at the indicated time and used to determine the remaining CXCL1 mRNA by Northern hybridization. Autoradiographs were quantified as in Fig. 1A, and results are representative of two separate experiments. (D) BMDMs from TTP+/+ or TTP−/− mice were subjected to DMSO or Tm treatment for 6 h followed by LPS treatment for 6 h. CXCL1 mRNA was measured by real-time PCR using primers to detect mature mRNA and quantified as in (A). (E) BMDMs were treated with DMSO or Tm for 6 h followed by LPS for the indicated times. Nuclear extracts were prepared and used to analyze NF-κB p65 DNA binding activity as described in Materials and Methods. Results are presented as the means of duplicate determinations ± half of the range. (F) BMDMs were treated with DMSO or Tm for 6 h, then stimulated with LPS for indicated times. Nuclear extracts were prepared and used to determine levels of NF-κB p65 by Western blot. Results are representative of three separate experiments. (G) RAW264.7 cells transiently transfected with the indicated luciferase reporter plasmids were treated with DMSO or Tm for 6 h followed by LPS for 6 h. Total RNA was prepared and used to determine luciferase mRNA by Northern hybridization as in Fig. 1. Results are representative of three separate experiments.
was made in TRIF<sup>−/−</sup> macrophages (Figs. 2E, 4B). These observations suggest that TRIF/RIPK1 are not requisite for basal CXCL1 transcription in LPS-stimulated BMDMs. Moreover, stress engagement leads to an increase in cellular RIPK1 protein as well as ubiquitination status. The TRIF signaling pathway is thought to be responsible for prolonged NF-κB–mediated transcription in LPS-stimulated macrophages (72), and the effects of cell stress are most evident in this context (see Fig. 1B). It is noteworthy that RIPK1 depletion does not compromise the effects of stress on responses to TLR2 or TLR7 (data not shown).

Although mRNA half-life is well recognized as an important regulatory mechanism governing the expression of many cytokine and chemokine genes (67, 73), it does not appear to be the mechanistic route through which environmental stresses amplify chemokine expression levels. Rather, changes in target gene transcription, measured as an increase in the abundance of non-splitted primary transcripts, appear to be responsible. Furthermore, although prior reports have shown that both IRF3 and NF-κB exhibit sensitivity to stress pathways (42, 74–76), the stress-enhanced transcription of CXCL1 mRNA is observed in IRF3-deficient macrophages and does not appear to be a consequence of altered NF-κB activation. Multiple criteria support the latter conclusion. First, whereas cell stress has been linked to the activation of NF-κB through several mechanistic pathways (indeed, there is both modest activation of NF-κB and associated cytokine gene expression in macrophages treated with stress inducers), there is no significant amplification of κB binding activity or nuclear localization of p65 in stressed macrophages stimulated with LPS. Second, importantly, cell stress does not enhance LPS-stimulated NF-κB-dependent reporter gene expression in transiently transfected RAW264.7 cells. These findings support the conclusion that stress operates via alternate mechanisms.

Myeloid cells are among the sentinels that detect and provide first response to signals resulting from disruption of the local tissue microenvironment. The link between cell stress and inflammatory cytokine gene expression provides a mechanism for adjusting the magnitude and duration of proinflammatory gene expression and operates in vivo as evidenced by the dramatic change in chemokine expression when stress signaling is engaged at a site of inflammatory response (see Fig. 6). Sensitivity to such environmental cues does not, however, appear to be a property shared by all myeloid cell populations. Indeed, resident myeloid cells within the liver or peritoneal cavity do not appear to show altered LPS-induced CXCL1 expression following stress either in vitro or in vivo. This selectivity may reflect the phenotypic heterogeneity within myeloid cell populations (77–80). Importantly, recent
findings suggest the existence of at least two populations of circulatory monocytes, of which one is a precursor to inflammatory-infiltrating macrophages. Moreover, there is substantial evidence supporting the idea that inflammatory cytokine expression occurs selectively within infiltrating myeloid cell populations (81, 82). Our findings suggest that the sensitivity for stress-mediated amplification of cytokine and chemokine expression may be restricted to such inflammatory cell populations. Interestingly, a recent report identifies RIPK1 as a critical determinant of systemic inflammation that operates on infiltrating myeloid cells (83). Deficiency of RIPK1 in mice with this mutation protects against neutrophilia, tissue inflammation, and elevated systemic cytokine production. Hence, the pattern of chemokine and cytokine expression described in the present study may reflect a mechanism for controlling the intensity of inflammation that operates on infiltrating inflammatory macrophages in specific pathophysiologic contexts and depends on, at least in some circumstances, the stress-sensitive kinase RIPK1.

Disclosures
The authors have no financial conflicts of interest.

References


