IFN-γ and TNF Regulate Macrophage Expression of the Chemotactic S100 Protein S100A8

Ken Xu and Carolyn L. Geczy

J Immunol 2000; 164:4916-4923; doi: 10.4049/jimmunol.164.9.4916

http://www.jimmunol.org/content/164/9/4916

References

This article cites 44 articles, 18 of which you can access for free at: http://www.jimmunol.org/content/164/9/4916.full#ref-list-1

Why The JI? Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
IFN-γ and TNF Regulate Macrophage Expression of the Chemotactic S100 Protein S100A8¹

Ken Xu and Carolyn L. Geczy²

The murine calcium-binding protein S100A8 is a potent chemoattractant for neutrophils and monocytes in vivo and in vitro but may also play a protective role. We show that the kinetics of induction of S100A8 mRNA in elicited murine macrophages (Mac) by LPS, IFN-γ, and TNF were distinct from the C-C chemokines monocyte chemotactant protein-1 (MCP-1), macrophage-inflammatory protein-1α (MIP-1α), and RANTES. Monomeric S100A8 was predominantly secreted. IFN substantially increased S100A8 mRNA levels after 1 h with optimal induction after 12 h; induction by TNF was slower and more sustained. TNF did not up-regulate MCP-1 and MIP-1α mRNA in these cells. Luciferase reporter assays confirmed that LPS and IFN induce S100A8 gene transcription and mRNA in LPS-treated Mac showed little decay over 16 h, whereas transcripts induced by IFN and TNF were markedly less stable. Newly synthesized proteins may be required for mRNA transcription and stabilization in response to LPS. S100A9 associates with A8 in neutrophils, but was not coinduced with S100A8. S100A8 gene induction in Mac stimulated with LPS and IFN may be modulated by mobilization of intracellular Ca²⁺ concentration from distinct intracellular stores and/or the extracellular compartment and by distinct pathways involving protein kinase C and leading to activation of mitogen-activated protein kinase. The Journal of Immunology, 2000, 164: 4916–4923.

M̄urine S100A8 (mS100A8),³ formerly known as CP-10, is a 10-kDa acidic protein containing two Ca²⁺-binding EF hands belonging to the highly conserved S100 protein family (1). Originally isolated as a soluble product of activated spleen cells (2), mS100A8 is a constitutive cytoplasmic protein in neutrophils (3) and is expressed by LPS-activated macrophage (Mac) cell lines (4) and microvascular endothelial cells (5). S100 proteins have diverse functions and are postulated to regulate cell migration, cytoskeletal-membrane interactions, neutrophil activation, and kinase activities (1, 6, 7).

mS100A8 stimulates myeloid cell chemotaxis in vitro and sustains leukocyte recruitment, with monocytes following an early influx of PMN, and kinetics similar to that of a delayed-type hypersensitivity (DTH) response in vivo (2, 8–10). Moreover, mS100A8 and A9 are associated with granuloma formation and injection of agarse-bound complexes into mice causes severe infiltration of neutrophils and Mac over 7–14 days (11). S100A8 is a more potent chemoattractant than most chemokines (8) and does not activate degranulation, enzyme release, or provoke an oxidative burst (12). S100A8 and A9 may influence leukocyte margination and transmigration into tissues (13) by increasing leukocyte deformability (12) and integrin-mediated adhesion (14). Expression of the proteins by microvascular endothelial cells activated by IL-1 and TNF (5) may facilitate these processes.

Mac recruited by mS100A8 in vivo have a particular phenotype which would favor bacterial clearance by virtue of increased levels of scavenger receptor, Fc receptor, and phagocytosis (10). Our recent evidence that S100A8 can efficiently scavenge hypochlorite anions produced by activated neutrophils (15) suggests that it can promote and modulate inflammatory responses. TGF-β1 and mS100A8 share apparently paradoxical functions in immune and inflammatory processes. TGF-β1 is also chemoattractant at picomolar levels but fails to activate leukocytes and both are implicated in embryogenesis. Deletion of the S100A8 gene is lethal to embryos at mid-gestation when it is expressed by migrating trophoblasts (16).

Although there is increasing interest in S100A8/A9 in inflammatory disease, little is known concerning their regulation by appropriate mediators. S100A8/A9 are not expressed by tissue Mac (17, 18) whereas Mac in inflammatory lesions may do so (19–22) and, although not always coordinately expressed (21, 22), S100A8/9 +ve Mac produce high amounts of TNF and IL-1, suggesting a particular proinflammatory phenotype (23).

Leukocyte recruitment is mediated by a variety of chemoattractants, and specific temporal and differential induction may regulate the composition of inflammatory exudates. Activated Mac are a primary source of chemokines that bear no amino acid sequence similarities and that are located within different chromosomal clusters (murine chromosome 5 for CXC and 11 for CC chemokines (reviewed in Ref. 24)) to the S100 chemotactant proteins (murine chromosome 3 (Ref. 25)). In addition, S100A8 has a single Cys at position 41 (2) and, unlike the chemokines, binds calcium with high affinity. Here, we present evidence that mS100A8, but not mS100A9, is up-regulated by some key mediators of Mac function, including IFN, IL-1, and TNF and compare induction with some C-C chemokine genes considered important in monocyte/Mac recruitment in cell-mediated immune responses.
Materials and Methods

Reagents

RPMI 1640 from Life Technologies (Grand Island, NY) was supplemented with 126.6 μg/ml penicillin, 126.6 μg/ml streptomycin (Sigma, St. Louis, MO), and 2% heated (56°C, 30 min) bovine calf serum (HyClone, Logan, UT), hereafter referred to as culture medium (CM). Plastic flasks and plates were obtained from Falcon (Lincoln Park, NJ). Thioglycolate broth (TG) and LPS (E. coli 055:B5) were purchased from Difco (Detroit, MI). IFN-γ was obtained from Genzyme (Cambridge, MA; endotoxin content <0.01 ng/μg; sp. act., 1.14 × 107 U/mg) or Genentech (San Francisco, CA; 0.032 endotoxin units/mg; sp. act., 0.5 × 107 U/mg). TNF was purchased from Genzyme (0.51 endotoxin units/mg; sp. act., 1.2 × 107 U/mg) or Sigma (≤0.1 EU/μg; L929 cell inhibition EC50: 0.04 ng/ml). IL-1β (sp. act., 3.5 × 107 U/mg), IL-6, IL-12, and recombinant human IFN-α were obtained from Genzyme. Neutralizing hamster anti-murine TNF-Ab (10–100 ng/ml) and 1 U mouse TNF in vitro was a generous gift from Dr. Ian Clarke (Australia National University, Canberra, Australia). Human IL-1 receptor antagonist (sp. act., 1000 U/ml) was purchased from Boehringer Mannheim (Manheim, Germany). Native murine S100A8C and S100A9 from bone marrow were purified in our laboratory by Dr. Mark Raferty. Sodium periodate was purchased from BDH Laboratory Supplies (Merck, Elyshiy, Victoria, Australia). Calcium ionophore A23187, PMA, and EDTA were obtained from Sigma, 1,2-bis[(N,N,N,N-tetratoctylammonium)bromide] [CTAB] (Bio-Rad, Richmond, CA). The relative magnitude of expression by TG-elicited Mac. A. Control unstimulated (−); cells stimulated with two different samples of IFN-γ (300 U/ml) for 12 h, LPS (100 ng/ml) for 24 h, and TNF (25 ng/ml) for 24 h; and IL-1β (300 U/ml), IL-6 (500 U/ml), IFN-α (25 ng/ml) and IL-12 (500 U/ml) for 24 h. Mac were primed with LPS (0.1 ng/ml) or IFN-γ (10 U/ml) for 8 h, washed, and incubated with LPS (0.1 ng/ml), IFN-γ (10 U/ml), IL-1 (300 U/ml), or TNF (25 ng/ml) for 24 h. S100A8 mRNA was analyzed using the riboprobe. Filters were rehybridized with the S100A9 riboprobe or the 18S rRNA oligonucleotide after stripping. Data are representative of three experiments.

Northern blot analysis

Total cellular RNA (from 5 × 106 Mac) (4) was size fractionated on a 1% agarose-2.2 M formaldehyde gel and transferred onto Hybond N+ membranes (Amersham, Buckinghamshire, U.K.) with alkali fixing in 0.05 M NaOH (4). Hybridizations were performed for 16 h at 58°C for ribonucleic acid (RNA) and 36°C for the oligoprobe in formamide-containing buffer. S100A8 and A9 riboprobes and an 18S rRNA oligoprobe were used as described previously (5). pBluescript containing murine MCP-1, RANTES, and MIP-1α (kindly provided by Dr. T. Yoshimura, National Cancer Institute, Frederick, MD) were used to produce riboprobes. Membranes were washed twice at 48°C for 10 min in 2× standard saline citrate (SSC) with two different samples of IFN-γ (300 μg/ml). After 24 h, cells were stimulated with LPS (500 ng/ml), IFN (300 μg/ml), or TNF (25 ng/ml) for 20 h and firefly and Renilla luciferase activities were assayed with 20 μl extract using Promega reagents according to the manufacturer’s instructions.

Characterization of S100A8 protein

TG-elicited Mac (5 × 107) were cultured in 4-well plates with or without stimuli and S100A8 in supernatants or cell lysates was quantitated with a double-sandwich ELISA and rabbit polyclonal anti-S100A8 IgG, as described elsewhere (4, 26), using recombinant S100A8 (0.1–50 ng/ml) as standard.
that stimulated by LPS. TNF and IL-1 were not involved in induction by IFN because the anti-TNF or IL-1 receptor antagonist did not alter S100A8 mRNA induction (data not shown). IFN-α, IL-6, IL-12 (Fig. 1A), and mS100A8 and/or S100A9 (10⁻⁸ M) did not induce S100A8 mRNA (data not shown). There were no obvious differences in responses of periodate- or TG-elicited Mac. IFN-primed Mac did not exhibit enhanced responses to LPS whereas cells primed with suboptimal amounts of LPS showed elevated responses to IFN; priming with either mediator did not influence TNF-induced mRNA levels (Fig. 1B).

Elicited Mac were sensitive to as little as 0.3 ng/ml LPS; mRNA levels increased >8-fold with 100 ng/ml, with little additional effect at higher concentrations (data not shown). IFN increased S100A8 mRNA (Fig. 2) with 10 U/ml; maximal expression occurred at 50 U/ml and maximal at 10 ng/ml (Fig. 2). Different sources of the stimulants produced similar results (Fig. 1A).

Kinetics of induction of S100A8 mRNA were compared with those of MCP-1, RANTES and MIP-1α mRNA stimulated with predetermined optimal amounts of LPS, IFN, and TNF. S100A8 mRNA was evident 6 h after addition of LPS, peaked at 24–28 h, and declined slowly over 96 h (Fig. 3A), whereas the chemokines exhibited high mRNA levels 3 h after onset of stimulation (data not shown). MCP-1 mRNA declined more rapidly than S100A8 mRNA but RANTES was sustained over 96 h. Constitutive expression of MIP-1α mRNA was always obvious and the high levels induced between 3 and 12 h slowly declined thereafter. IFN substantially increased S100A8 mRNA after only 1 h, with optimal induction after 12 h, and gradually decreased over 48 h. Fig. 3A emphasizes the rapid induction by IFN compared with the later and more sustained responses to LPS and TNF. MCP-1 and MIP-1α mRNA induced by IFN was maximal within 3 h and not detected after 48 h, whereas RANTES increased slowly to maximal levels at 24 h (data not shown). Major differences in induction patterns were evident with TNF. S100A8 mRNA induction was strong and followed a time course similar to that provoked by LPS (Fig. 3A). In marked contrast, constitutive levels of MCP-1 and MIP-1α mRNAs were barely altered by TNF and RANTES mRNA was optimal only after 48 h (Fig. 3B).

S100A9 mRNA was not constitutively expressed by elicited Mac and was not induced by any of the stimulants at any time point tested. The same membranes used above, or newly prepared samples, tested with neutrophil-derived mRNA as positive control (data not shown), were consistently negative (Figs. 1 and 3).

S100A8 protein production by stimulated Mac

Unstimulated elicited Mac lysates contained ~0.7 ng S100A8/10⁶ cells, possibly derived from neutrophils originally contaminating Mac exudates. Cell-associated protein in lysates of IFN- and TNF-stimulated cells decreased by 5–20% after a 76-h culture. The low levels of S100A8 released into supernatants of unstimulated cells (Fig. 4) may represent amounts released by cells dying over this period. Secreted S100A8 increased steadily over 76 h and remained elevated 96 h after stimulation with the mediators shown. In contrast to the high mRNA levels (Fig. 3A) in LPS-activated cells, secreted S100A8 was approximately half that produced by

![Figure 2](http://www.jimmunol.org/) Induction of S100A8 mRNA by different doses of IFN-γ and TNF. Periodate-elicited Mac were cultured with the indicated doses of IFN-γ or TNF for 12 or 36 h, respectively, and S100A8 mRNA was analyzed by Northern blotting. Data are representative of three experiments.

![Figure 3](http://www.jimmunol.org/) Kinetics of LPS, IFN-γ, and TNF-mediated up-regulation of S100A8 mRNA. A, TG-elicited Mac were stimulated with 100 ng/ml LPS, 300 U/ml IFN-γ, or 25 ng/ml TNF for the times indicated and Northern blot analysis was performed. Comparison of the time course of S100A8 mRNA induction by LPS (○), IFN-γ (□), and TNF (□), determined by densitometry and normalized to 18S rRNA content, of autoradiograms of Northern blots. The unstimulated control (●) was compared with maximum induction of S100A8 mRNA by LPS to give 100% maximal response. B, mRNA induction of S100A8, MCP-1, RANTES, and MIP-1α by TNF. The same membranes were sequentially reprobed with S100A9, MCP-1, RANTES, and MIP-1α riboprobes and the 18S RNA oligoprobe. Results are representative of three experiments.
IFN- or TNF-activated Mac. S100A8 from stimulated supernatants enriched by immunoaffinity (5) eluted from C4 RP-HPLC at 20.1 min, the same time as native S100A8 standard and Western blotting confirmed monomeric S100A8 (10 KDa) as the dominant structural form. S100A9, S100A8/A9 heterodimers, or covalent S100A8 homodimers were not detected in any sample tested (data not shown).

**Regulation of S100A8 gene expression**

To assess whether RNA stabilization contributed to mRNA accumulation, Mac incubated with LPS for 24 h, IFN for 12 h, or TNF for 36 h were treated with ActD for various times to block further transcription. The half-lives of S100A8 mRNA induced by IFN or TNF were similar (t1/2, 9 h for IFN; 7 h for TNF; Fig. 5) and transcripts were undetectable after 16 h. S100A8 mRNA in LPS-treated cells decayed with markedly different kinetics (Fig. 5) with levels maintained over 16 h, with little decay.

To determine the requirement for protein synthesis in S100A8 mRNA induction, Mac were activated for 20 h with CHX (2 μg/ml). CHX superinduced S100A8 mRNA and completely abrogated up-regulation of S100A8 mRNA by LPS (Fig. 6). In contrast, IFN- and TNF-induced responses were reduced by 30 and 55%, respectively, indicating different requirements for gene transcription. Similar responses were evident with higher doses of CHX, although superinduction made results difficult to interpret.

Luciferase reporter assays confirmed that LPS and IFN induced transcription of the S100A8 gene (Table I). The construct pCP-178/-465 (−178 to +465 of the S100A8 gene) was up-regulated 7-fold by LPS and 3-fold by IFN compared with control. Levels of induction were less than those observed by Northern blot analysis, indicating that this construct may not contain all of the elements necessary for maximal expression. Induction by TNF was not significantly above control levels and preliminary Northern blot analysis indicated that these cells were unresponsive to TNF (data not shown). Low basal expression in the absence of stimulants indicates that pCP-178/-465 either lacks some elements required to suppress the gene in unstimulated cells or that a low level of expression occurs naturally in RAW cells.

Some S100 genes are activated via PKC, PKA, and/or Ca2+-dependent pathways (28, 29). Preliminary experiments to determine intracellular signaling mechanisms involved in S100A8 expression in Mac were performed using agents affecting these pathways. PMA weakly induced S100A8 mRNA which reached optimal levels after 12 h (Fig. 7B) but strongly amplified responses to LPS or IFN. The PKC inhibitor H-7 and the more specific antagonist calphostin C maximally reduced induction by LPS and IFN at the time of optimal gene expression. (Fig. 7, A and B). In contrast, PMA strongly induced MCP-1 mRNA after 12 h, and an additive response was evident with PMA cultured with LPS or IFN; H-7 reduced induction by the stimulants (data not shown). H-89 and Rp-cAMP decrease cAMP and PKA activity but had little effect on S100A8 mRNA in the presence or absence of activators. Mac activation can result in rapid tyrosine phosphorylation via activation of the mitogen-activated protein (MAP) kinase

![FIGURE 4](image-url)  
**FIGURE 4.** S100A8 protein in supernatants of elicited Mac stimulated with LPS ( ■ ), IFN-γ ( ■ ), or TNF ( ○ ) or unstimulated ( ○ ). Protein levels of supernatants collected at the times indicated after the start of stimulation were determined by ELISA. Results (expressed as ng S100A8 generated by 10⁶ cells) represent the mean of two separate experiments. The SE was <20% of the mean for all values.

![FIGURE 5](image-url)  
**FIGURE 5.** Effect of LPS, IFN-γ, and TNF on mRNA stability. TG-elicited Mac were stimulated with LPS (100 ng/ml) for 24 h, IFN-γ (300 U/ml) 12 h, or TNF (25 ng/ml) for 36 h. mRNA expression was tested immediately or after incubation with ActD (1 μg/ml) for the times indicated (A). Densitometric analysis of signal intensities from LPS ( ■ ), IFN-γ ( ■ ), or TNF ( ▲ )-treated cells (B) are expressed as amounts relative to 18S rRNA signal intensities. Results are representative of three experiments.
pathways. Fig. 7C shows that PD98059, a selective inhibitor of MAP/extracellular signal-related kinase 1 kinase, and the highly specific p38 kinase inhibitor SB202190 both markedly reduced LPS- and IFN-stimulated S100A8 mRNA, suggesting involvement of members of the MAP kinase (MAPK) subfamily in S100A8 induction in elicited Mac.

Depletion of extracellular Ca\(^{2+}\) by EGTA and of cytosolic Ca\(^{2+}\) with the chelator TMB-8, which blocks Ca\(^{2+}\) influx from the extracellular space, inhibited S100A8 mRNA expression by LPS and IFN at the time points optimal for induction (Fig. 8). The ionophore A23187 completely abolished S100A8 mRNA induced by both stimulants whereas reduction of resting cytoplasmic Ca\(^{2+}\) levels by BAPTA-AM had no effect (Fig. 8). In contrast, MCP-1 mRNA induction was somewhat enhanced 1.5- to 1.9-fold by EGTA and reduced by changes in cytosolic Ca\(^{2+}\) provoked by BAPTA-AM and A23187 (Fig. 8). Thapsigargin, an inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase, dramatically inhibited S100A8 mRNA induction by LPS but not by IFN, indicating divergent pathways of activation. The phospholipase C (PLC) inhibitor U73122 reduced S100A8 and MCP-1 mRNAs induced by IFN but amplified the genes in cells activated with LPS, indicating a regulatory role for PLC-mediated pathways in the transcription of these genes.

**Discussion**

mS100A8 is a potent myeloid cell chemoattractant that is up-regulated by LPS in Mac (4) and microvascular endothelial cells (5). Expression of S100A8 in a subpopulation of microvascular endothelial cells (5) and Mac in DTH lesions (11) prompted us to examine the cytokines potentially involved in modulating the Mac response because there is little information regarding mechanisms of their regulation in this setting.

TG- or periodate-elicited murine Mac do not express S100A8 (4, 17) but respond directly to LPS, and rechallenge markedly down-regulates mRNA expression (4). We chose elicited Mac as a reference system to study the regulation of S100A8 expression in Mac. We report here that S100A8 expression by Mac is modulated by LPS, IFN, or TNF, and that this modulation involves multiple pathways, including MAPK and PLC signaling pathways.

### Table I. Relative activity of the S100A8-luciferase construct pCP-178/ +465 in the presence of LPS, IFN, or TNF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Luciferase Activity (fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>pGL2-Basic</td>
<td>1.14 ± 0.15</td>
</tr>
<tr>
<td>pCP-178/ +465</td>
<td>7.05 ± 2.37</td>
</tr>
</tbody>
</table>

* The luciferase reporter construct was assayed in transiently transfected RAW cells as described in Materials and Methods. Relative luciferase activity for each plasmid construct is expressed as fold induction of luciferase activity in stimulated compared to unstimulated medium controls. Data represent means ± SD of triplicate determinations from three experiments.
responses (Fig. 1B). S100A8 may represent another mechanism of regulating monocyte recruitment in situations in which TNF plays a major role. S100A8 mRNA was strongly up-regulated within 6 h by TNF in a dose-dependent manner (Fig. 2); expression was maximal at 36 h and slowly decreased over 96 h (Fig. 3, A and B). Here, we demonstrate differential induction of S100A8 mRNA and MCP-1 mRNA by TNF which did not alter MCP-1 gene expression (Fig. 3B). Moreover, TNF had relatively little effect on endogenous MIP-1α mRNA levels, and induction of RANTES mRNA was substantially slower than that of S100A8. In contrast, MCP-1 mRNA was more rapidly stimulated by LPS and IFN (data not shown), although expression was less sustained than that of S100A8. Differences in the state of activation and/or differentiation may be important determinants in induction of MCP-1 by TNF in Mac (33, 34) and may determine the nature of the response.

S100 proteins have no structural sequences required for secretion by the classical endoplasmic reticulum-Golgi pathway, but extracellular functions are well accepted (1). The human S100A8/9 complex may be released via a novel tubulin-dependent mechanism following leukocyte activation (35), suggesting active secretion. We demonstrated mS100A8 in supernatants from LPS-activated Mac cell lines (4) and from IFN and TNF-activated elicited Mac (Fig. 4). S100A9 mRNA or protein was not induced by any mediator tested (Figs. 1 and 3), supporting the notion that coexpression with S100A9 is not essential for S100A8 function or secretion (35). S100A8 protein levels secreted by elicited Mac after stimulation with predetermined optimal amounts of IFN and TNF were higher than those produced by LPS (Fig. 4), suggesting that these mediators may also activate pathways involved in S100A8 release.

The high levels (≈10^−10 M) of S100A8 produced 8 h after stimulation with TNF or IFN were above the chemotactically optimal dose of 10^−12 M (8). The monomer was the predominant structural form in supernatants (data not shown). S100A8 is exquisitely sensitive to oxidation by hypochlorite generated by activated neutrophils (15), and, in situations where concentrations within the range generated at the later time points are potentially liberated, S100A8 may be protective by virtue of its ability to scavenge reactive oxygen species. Moreover, oxidation of mS100A8 to the covalent homodimer negates its chemotactic capacity and, along with the ability of hypochlorite to reduce the chemotactic activity of C5a and fMLP (36), strengthens the notion that this mechanism may be physiologically relevant in limiting leukocyte recruitment by these stimuli.

Experiments reported here indicate differences in transcriptional and posttranscriptional regulation of the S100A8 gene by LPS and IFN. Transcriptional regulation was measured using transient transfection of luciferase reporter constructs into the RAW monocyte cell line (Table I). Half-lives of mRNA induced by IFN and TNF were similar (9 and 7 h, respectively; Fig. 4) but markedly shorter than that induced by LPS (>16 h). The 3′ untranslated region does not contain known AU-rich sequences which destabilize the mRNA of numerous cytokine genes (37), and mechanisms regulating S100A8 mRNA stability may depend on inducible factors. CHX superinduced low and variable levels of S100A8 mRNA, but induction by IFN and TNF was relatively resistant whereas CHX completely inhibited the LPS response (Fig. 6 and Ref. 4), suggesting regulation by an inducible component.

Results presented in Fig. 7 implicate PKC and MAP kinase pathways in the regulation of transcriptional events leading to S100A8 gene expression. PKC regulates human S100A8/A9 in myelocytic differentiation (38), and H9, and the specific antagonist calphostin C (39), inhibited induction by LPS and IFN whereas PKA inhibitors had little effect. Although direct activation was weak, PMA synergized with LPS and IFN to markedly elevate

**FIGURE 8.** Northern blot analysis of S100A8 and MCP-1 mRNA expression by TG-elicited Mac after incubation for 12 h (A) or 24 h (B) with media (med), EGTA (5 mM), BAPTA-AM (10 μM), A23187 (5 μM), thapsigargin (0.5 μM), TMB-8 (50 μM), and U73122 (5 μM) in the presence or absence of IFN (300 U/ml) or LPS (100 ng/ml). Results represent three experiments.

model because transmigration of these cells from the blood into the peritoneal cavity may more closely resemble an inflammatory setting. The Th1 cytokine IFN plays a central role in Mac activation associated with bactericidal activity, Ag presentation, and orchestration of leukocyte-endothelial cell interactions and is a key mediator of DTH reactions (reviewed in Ref. 30). In contrast, to the inability of IFN to up-regulate mS100A8 mRNA in the murine microvascular cell lines (5), it was strongly up-regulated in elicited Mac by as little as 10 U/ml IFN (Fig. 2). Kinetics was rapid, with mRNA apparent within 1–3 h and optimal expression between 6 and 12 h (Fig. 3A). IFN primes Mac for a number of functional responses (31) but IFN-primed Mac were not hyper-responsive to TNF or IFN were above the chemotactically optimal dose of 10^−12 M (8). The monomer was the predominant structural form in supernatants (data not shown). S100A8 is exquisitely sensitive to oxidation by hypochlorite generated by activated neutrophils (15), and, in situations where concentrations within the range generated at the later time points are potentially liberated, S100A8 may be protective by virtue of its ability to scavenge reactive oxygen species. Moreover, oxidation of mS100A8 to the covalent homodimer negates its chemotactic capacity and, along with the ability of hypochlorite to reduce the chemotactic activity of C5a and fMLP (36), strengthens the notion that this mechanism may be physiologically relevant in limiting leukocyte recruitment by these stimuli.

Experiments reported here indicate differences in transcriptional and posttranscriptional regulation of the S100A8 gene by LPS and IFN. Transcriptional regulation was measured using transient transfection of luciferase reporter constructs into the RAW monocyte cell line (Table I). Half-lives of mRNA induced by IFN and TNF were similar (9 and 7 h, respectively; Fig. 4) but markedly shorter than that induced by LPS (>16 h). The 3′ untranslated region does not contain known AU-rich sequences which destabilize the mRNA of numerous cytokine genes (37), and mechanisms regulating S100A8 mRNA stability may depend on inducible factors. CHX superinduced low and variable levels of S100A8 mRNA, but induction by IFN and TNF was relatively resistant whereas CHX completely inhibited the LPS response (Fig. 6 and Ref. 4), suggesting regulation by an inducible component.

Results presented in Fig. 7 implicate PKC and MAP kinase pathways in the regulation of transcriptional events leading to S100A8 gene expression. PKC regulates human S100A8/A9 in myelocytic differentiation (38), and H9, and the specific antagonist calphostin C (39), inhibited induction by LPS and IFN whereas PKA inhibitors had little effect. Although direct activation was weak, PMA synergized with LPS and IFN to markedly elevate

**FIGURE 8.** Northern blot analysis of S100A8 and MCP-1 mRNA expression by TG-elicited Mac after incubation for 12 h (A) or 24 h (B) with media (med), EGTA (5 mM), BAPTA-AM (10 μM), A23187 (5 μM), thapsigargin (0.5 μM), TMB-8 (50 μM), and U73122 (5 μM) in the presence or absence of IFN (300 U/ml) or LPS (100 ng/ml). Results represent three experiments.
S100A8 mRNA (Fig. 7). MAP kinases regulate Mac activation in response to LPS, TNF, and IL-1; PD98059 inhibits p38 activity and both pathways can cooperatively regulate transcription of AP-1 components c-jun and c-fos (40). Results shown in Fig. 7C implicate both pathways in transcriptional events regulating S100A8 expression in LPS- or IFN-activated Mac. MAPK phosphorylation also modulates STAT and NF-κB activity (40). Consensus motifs for transcription factors located within the region of the promoter tested (Table I) include TATA, NF1, E box, GC box, NF-IL-6, NF-κB, SRE, IRE, Ets box, Myb, and AP-1, many of which are associated with LPS- and IFN-induced genes involved in myeloid-specific differentiation, activation, and inflammation.

Calcium regulates transcription of a number of S100 genes (1, 18) and changes in Ca^{2+} mobilization via release from intracellular stores and/or the extracellular space may regulate S100A8 gene expression in Mac (Fig. 8). In contrast to S100A8, elevated levels of cytosolic Ca^{2+} increase c-fos, TNF, and MCP-1 expression in Mac (Ref. 41 and Fig. 8). Chelation of extracellular Ca^{2+} with EGTA and inhibition of Ca^{2+} release from the endoplasmic reticulum by TMB-8 (42) suppressed LPS- and IFN-induced responses (Fig. 8). EGTA increased, but TMB-8 decreased MCP-1 mRNA and reduction of resting cytoplasmic Ca^{2+} levels with BAPTA-AM reduced MCP-1 but not S100A8 mRNA levels. A23187, which passively transfers extracellular calcium and triggers release from intracellular pools causing a calcium spike, was an effective inhibitor of MCP-1 and S100A8 genes (Fig. 8). Divergent pathways of S100A8 gene expression by LPS and IFN were indicated with thapsigargin. This causes influx of stored Ca^{2+} by inhibiting microsomal ATPases without producing inositol 1,4,5-trisphosphate and only inhibited the LPS-induced response. IFN-induced S100A8 and MCP-1 mRNAs, but not S100A8 mRNA induced by LPS, were suppressed by U73122 which inhibits PLC and the resultant conversion of phosphatidylinositol-4,5-bisposphate to inositol 1,4,5-trisphosphate and mobilization of intracellular Ca^{2+}.

Taken together, we suggest that S100A8 gene induction in Mac stimulated with LPS and IFN is modulated by mobilization of intracellular Ca^{2+} concentration from distinct intracellular stores and by converging pathways leading to phosphorylation of MAP kinase, an important event in stress-induced and inflammatory responses. LPS activation may occur via a calcium-independent kinase, an important event in stress-induced and inflammatory responses mediated by activated macrophages.


