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IL-10-Dependent S100A8 Gene Induction in Monocytes/Macrophages by Double-Stranded RNA

Yasumi Endoh,* Yuen Ming Chung,† Ian A. Clark,‡ Carolyn L. Geczy,* and Kenneth Hsu‡,*

The S100 calcium-binding proteins S100A8 and S100A9 are elevated systemically in patients with viral infections. The S100A8-S100A9 complex facilitated viral replication in human CD4+ T lymphocytes latently infected with HIV-1 and S100A8-induced HIV-1 transcriptional activity. Mechanisms inducing the S100 genes and the potential source of these proteins following viral activation are unknown. In this study, we show that S100A8 was induced in murine macrophages, and S100A8 and S100A9 in human monocytes and macrophages, by polyinosinic:polycytidylic acid, a dsRNA mimetic. Induction was at the transcriptional level and was IL-10 dependent. Similar to LPS-induced S100A8, induction by dsRNA was dependent on p38 and ERK MAPK. Protein kinase R (PKR) mediates antiviral defense and participates in MyD88-dependent/independent signaling triggered by TLR4 or TLR3. Like IL-10, S100 induction by polyinosinic:polycytidylic acid and by LPS was inhibited by the specific PKR inhibitor 2-aminopurine, indicating a novel IL-10, PKR-dependent pathway. Other mediators such as IFN-β, which synergized with dsRNA, may also be involved. C/EBPβ bound the defined promoter region in response to dsRNA. S100A8 was expressed in lungs of mice infected with influenza virus and was maximal at day 8 with strong immunoreactivity in epithelial cells lining the airways and in mononuclear cells and declined early in the recovery phase, implying down-regulation by mediator(s) up-regulated during resolution of the infection. IL-10 is implicated in viral persistence. Since S100A8/S100A9 levels are likely to be maintained in conditions where IL-10 is raised, these proteins may contribute to viral persistence in patients infected by some RNA viruses. *The Journal of Immunology, 2009, 182: 2258–2268.

Calcium-binding proteins S100A8 and S100A9 are expressed constitutively in large amounts in neutrophils and are inducible in monocytes and macrophages. S100A8/S100A9-positive macrophages are found in numerous autoimmune diseases (1) and in atherosclerotic plaque (2). They are also inducible in other cell types such as epithelial cells (3) and fibroblasts (4) by appropriate stimulants and their expression is associated with several tumors. S100A8 and S100A9 have intracellular and extracellular functions and although the S100A8-S100A9 complex is considered the major functional form, separate functions for the individual proteins are reported (reviewed in Refs. 5 and 6). These proteins are proposed to function as novel damage-associated molecular pattern molecules (7). The complex is antimicrobial, apoptotic for some cells, and inhibits matrix metalloproteinase (MMP)3 activities (8) by virtue of its ability to chelate zinc. Recently, S100A8 was reported to activate cytokine production from murine bone marrow cells via TLR4 ligation; S100A9 negated this activity and S100A9-null mice are protected from endotoxic shock (9). S100A8 and S100A8/S100A9 were recently shown to induce MMP-2, 3, 9, and particularly 13, in murine bone marrow-derived macrophages (10), suggesting that the S100 proteins mediate macrophage migration and matrix degradation via MMP production. Intracellularly, the complex is implicated in arachidonic acid transport (11) assembly of the NADPH oxidase complex (12) and may regulate cytoskeletal rearrangement of phagocyte membranes during migration by integrating MAPK and calcium-dependent signals that influence tubulin polymerization (13, 14).

Elevated systemic levels of S100A8/S100A9 are associated with viral infections such as human papillomavirus (15), and microarray of PBMC from patients with severe acute respiratory syndrome, caused by a coronavirus, identified ~60-fold increase in the S100A9 gene (16). S100A8/A9 levels in patients infected with a lentivirus, HIV-1, correlate with disease progression and low CD4+ counts (17), and in HIV-1-seropositive patients with advanced immunodeficiency (18, 19) correlate with the onset of and with opportunistic infections (20–22). The 27E10 Ag, (detects the S100A8/A9 heterocomplex) was suggested to be a potential marker for different stages of HIV (19). S100A8 was isolated from cervico-vaginal secretions from women at high risk of HIV infection that contained HIV-inducing activity for an HIV-infected monocyte cell line, and recombinant S100A8 mimicked this activity (23). The source of this protein was suggested as being derived from granulocytes, macrophages, and epithelial cells present as a consequence of localized inflammation of the genital mucosa. In another study, S100A8, S100A9, and S100A8/S100A9 facilitated viral replication in human CD4+ T lymphocytes latently infected with HIV-1 and S100A8-induced HIV-1 transcriptional activity via an NF-κB-dependent pathway (24). It was proposed that these S100 proteins may particularly contribute to HIV-1 progression in seropositive patients with opportunistic infections and/or
inflammatory conditions because such conditions would likely have elevated systemic levels.

IL-10 inhibits adaptive T cell-mediated responses, primarily by anti-inflammatory functions through effects on Ag-presenting dendritic cells and in the generation of regulatory T cells. It down-regulates proinflammatory cytokine production in macrophages. On the other hand, IL-10 can stimulate B lymphocyte proliferation and activation of NK cells and CD8⁺ CTLs (reviewed in Ref. 25). Recent studies implicate IL-10 in the pathogenesis of viral persistence, possibly because it induces a generalized immunosuppression. Clinical observations indicate that a diminished capacity to produce IL-10 is associated with improved viral clearance and antiviral immunity can be promoted by targeting the IL-10 signaling pathway or by neutralizing IL-10 (reviewed in Ref. 26). Blockade of IL-10 receptor prevented functional exhaustion of memory T cells and promoted resolution of a chronic viral infection (reviewed in Ref. 27).

Our earlier studies showed that S100A8 expression in murine macrophages induced by LPS is a late-phase response that is neutralized by anti-IL-10 Abs, although IL-10 does not directly induce the gene (28). Moreover, corticosteroids potentiate S100A8 induction by LPS in an IL-10-dependent manner (29), suggesting that S100A8 may have anti-inflammatory properties. IL-10 down-regulates release of reactive oxygen and nitrogen species in macrophages (30) and this may be facilitated by S100A8, which we showed to be a potent scavenger of peroxide and hypochlorite generated by the myeloperoxidase system (31). These effects are reminiscent of induction of heme oxygenase 1, the rate-limiting enzyme in heme catabolism by IL-10, that generates carbon monoxide and other metabolites that contribute to the anti-inflammatory responses attributed to IL-10 (32).

Table 1. Primers and conditions used for real-time RT-PCR amplification and ChIP

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<th>Gene</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
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</tr>
<tr>
<td>β-actin</td>
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<td>CTCTTAAATGTACACGACAGT</td>
</tr>
</tbody>
</table>

FIGURE 1. Induction of S100A8 mRNA by poly(I:C), poly(C), and poly(I). A, RAW 264.7 macrophages were incubated with 2.0, 10, 50, or 100 μg/ml poly(I:C), poly(C), or poly(I). Controls include untreated or 20 ng/ml LPS. Cells were harvested 24 h poststimulation and S100A8 mRNA was quantitated by real-time RT-PCR; HPRT was endogenous control. Data represent means (relative to HPRT mRNA levels) ± SD of duplicate measurements from at least three independent experiments. * p < 0.05 compared with relative S100A8:HPRT mRNA ratio of untreated cells. B, S100A8 in RAW cell supernatants quantitated by ELISA. RAW cells were stimulated with poly(I:C), poly(C), poly(I)(10 or 50 μg/ml), or LPS (20 or 100 ng/ml) for 36 h. ** p < 0.01 compared with untreated cells. C, RAW cells were stimulated with 10 μg/ml poly(I:C) ± 20 ng/ml LPS for 24 h. mRNA levels relative to HPRT were quantitated. Data represent means ± SD of duplicate measurements from at least three independent experiments. Ctr, Control.
In this study, we demonstrate induction of S100A8 in murine macrophages and of S100A8 and S100A9 in human monocytes by poly(I:C), a dsRNA mimic. Induction was via a novel IL-10 and PKR-dependent pathway and required p38 and ERK MAPK. S100A8 was expressed in lungs of mice infected with influenza virus and declined early in the recovery phase, implying downregulation by mediator(s) up-regulated during resolution. Since S100A8/S100A9 levels are likely to be maintained in conditions where IL-10 is raised, these proteins may contribute to viral persistence in patients infected by some RNA viruses.

Materials and Methods

Reagents

LPS (Escherichia coli O111:B4), poly(I:C), poly(I), and poly(C) (all potassium salts) were from Sigma-Aldrich. Pathway inhibitors U0126 and SP600125 were from Calbiochem; all others were from Sigma-Aldrich. TRIzol reagent, glycogen, and a SuperScript III First-Strand Synthesis system were from Invitrogen; other RNA extraction reagents were from Sigma-Aldrich and all reagents for real-time RT-PCR were from Invitrogen, except for TURBO-DNase which was from Ambion). Affinity-purified rabbit anti-murine IL-10, anti-human IL-10 mAb, and mouse IgG2b isotype control were from R&D Systems.

Monocyte and macrophage preparation

Tissue culture medium for all experiments was filtered through 0.22-µm Zetapore filters (Cuno) to remove traces of endotoxin. Medium and mediators were only used if endotoxin levels were <20 pg/ml (chromogenic Limulus amebocyte assay; Associates of Cape Cod) (4, 29). Bovine calf serum (HyClone) had been pretreated before purchase.

Murine RAW 264.7 macrophages were cultured as previously described (28) and activated 24 h after seeding. Animal experiments were done with the approval of the Animal Care and Ethics Committees (University New South Wales, Australia). C57BL/6 mice or C57BL/6-IL-10−/− mice (33), 6–8 wk old, were maintained under specific-pathogen free conditions. Resident peritoneal cells were lavaged with cold RPMI 1640, washed, and 0.5 × 10⁶ cells in 500 µl of RPMI 1640 containing 2% bovine calf serum, 2 mM l-glutamine, and penicillin/streptomycin dispensed into 24-well plates (Nunc). Plates were incubated for 2 h at 37°C in 5% CO₂ in air, washed three times with warm PBS to remove nonadherent cells, and equilibrated in the same medium overnight. Medium was replenished before activation. Populations contained ~98% macrophages (~98% viable by trypan blue exclusion) and ~0.3% neutrophils by differential staining.

Human monocytes isolated from peripheral blood of healthy subjects as previously described (29) were analyzed using a Beckman Coulter counter. Preparations generally contained ~10% monocytes, ~90% lymphocytes, and <1.5% granulocytes. PMBC (2.5 × 10⁶/well) were dispensed into 24-well plates (Costar), then incubated in RPMI 1640 containing 10% heated (56°C, 30 min) autologous serum, 2 mM l-glutamine, and penicillin/streptomycin at 37°C in 5% CO₂ in air for 4 h. After rinsing with warm medium to remove nonadherent lymphocytes, monocytes were cultured overnight in the same medium. To generate macrophages, monocyte culture in 24-well Costar plates was continued for 7 days; cells were replenished with fresh medium with 10% autologous serum on day 4. Monocytes/macrophages were activated with particular stimulants after replenishing with fresh culture medium. For experiments using pathway inhibitors, cells were generally pretreated with inhibitors for 30 min, then untreated or stimulated for 24 h, unless specifically designated.

Human lung epithelial A549 cell culture

Human A549 pulmonary type II epithelial cells, used to study gene induction by viral infection (34), were obtained from Prof. P. Thomas (Department of Respiratory Medicine, Prince of Wales Hospital, Sydney, Australia). Cells were cultured in F12 medium (Invitrogen) containing 10% FCS, 100 IU penicillin/ml, and 100 µg/ml streptomycin at 37°C in 5% CO₂ in air. Cells (2 × 10⁵) were seeded into 12-well plates (Nunc) in fresh medium containing 2% FCS and stimulated with the indicated dose of poly(I:C) or LPS for 4 or 24 h.

RNA preparation and analysis

Adherent macrophages grown as monolayers in 24-well Costar plates were lysed directly after stimulation with TRIzol reagent and RNA prepared as described elsewhere (29). Total RNA (1 µg) was treated with TURBO DNase and reverse transcribed using random hexamers and the Superscript III First-Strand Synthesis System for RT-PCR according to the manufacturer’s instructions. Negative controls (no first-strand synthesis) were prepared by performing reverse transcription reactions in the absence of reverse transcriptase. PCR amplification was performed with Platinum SYBR Green qPCR SuperMix UDG. Reactions were performed in duplicate, and contained 2X SYBR Green qPCR SuperMix, 1 µl of template cDNA or control, and 100 nM primers (Table I) in a final volume of 25 µl and analyzed in 96-well optical reaction plates (Applied Biosystems). Reactions were amplified and quantified using an Applied Biosystems 7700 sequence detector with standard cycle conditions and the Applied Biosystems software. Relative quantities of mRNA in duplicate samples were obtained using the ΔΔCT method and normalized against murine hypoxanthine-guanine phosphoribosyltransferase (HPRT) or human β-actin as endogenous controls.

Dual luciferase reporter assay

Construction of the murine S100A8 promoter-luciferase-fused reporter plasmids was described previously (28). RAW 264.7 cells were transiently transfected as described elsewhere (35) using 3.0 × 10⁵ cells/500 µl seeded into 24-well plates (Nunc) 72 h before transfection, and then 0.5 µg of luciferase reporter plasmid or 0.05 µg of reference plasmid (pRL-TK) was transfected in the presence of DEAE-dextran (300 µg/ml; Sigma-Aldrich). After 24 h, cells were stimulated for 20 h with poly(I:C) and firefly and Renilla luciferase activities were assayed with 15 µl of extract using the Luciferase Assay System (Promega) according to the manufacturer’s instructions and measured using a TD-20/20 Luminometer. Results are expressed as mean ± SD of luciferase activity normalized to the reference Renilla luciferase from three separate experiments. Database searching (TFSEARCH; http://www.cbrc.jp/research/db/TFSEARCH.html) was used to search putative consensus
binding sequences of transcription factors in the murine S100A8 promoter.

**Chromatin immunoprecipitation (ChIP) assays**

RAW 264.7 cells ($5 \times 10^6$) were untreated or stimulated with LPS (500 ng/ml) or poly(I:C) (50 µg/ml); means ± SD of duplicate measurements of cells from five donors. *, $p < 0.05$, compared with relative S100β-actin mRNA ratios of control. B, Monocytes were untreated or stimulated with poly(I:C) (50 µg/ml) with or without IL-10 (2 or 10 ng/ml). Data represent S100 mRNA relative to poly(I:C)-stimulated samples without IL-10; means ± SD of three separate experiments. *, $p < 0.05$, compared with relative S100β-actin mRNA ratios of poly(I:C) alone. Human macrophages (C and D) or monocytes (E and F) were untreated or stimulated with LPS (500 ng/ml) or poly(I:C) (50 µg/ml) with or without anti-IL-10 mAb (5 µg/ml) or IgG isotype control for 24 h, or pretreated with 2-AP (4 mM) for 4 h. S100A8 (C and E) and S100A9 (D and F) mRNA levels relative to β-actin mRNA were quantitated. Data represent means ± SD of duplicate measurements and are representative of two to three independent experiments. IL-10 mRNA levels relative to β-actin mRNA were also quantitated. Ctr, Control.

**FIGURE 3.** Poly(I:C) induces S100 mRNA in human monocytes and macrophages and IL-10 is involved. A, Monocytes were untreated or stimulated with LPS (500 ng/ml) or poly(I:C) (50 µg/ml); means ± SD of duplicate measurements of cells from five donors. *, $p < 0.05$, compared with relative S100β-actin mRNA ratios of control. B, Monocytes were untreated or stimulated with poly(I:C) (50 µg/ml) with or without IL-10 (2 or 10 ng/ml). Data represent S100 mRNA relative to poly(I:C)-stimulated samples without IL-10; means ± SD of three separate experiments. *, $p < 0.05$, compared with relative S100β-actin mRNA ratios of poly(I:C) alone. Human macrophages (C and D) or monocytes (E and F) were untreated or stimulated with LPS (500 ng/ml) or poly(I:C) (50 µg/ml) with or without anti-IL-10 mAb (5 µg/ml) or IgG isotype control for 24 h, or pretreated with 2-AP (4 mM) for 4 h. S100A8 (C and E) and S100A9 (D and F) mRNA levels relative to β-actin mRNA were quantitated. Data represent means ± SD of duplicate measurements and are representative of two to three independent experiments. IL-10 mRNA levels relative to β-actin mRNA were also quantitated. Ctr, Control.

Enriched DNA was detected by real-time quantitative PCR (LightCycle 480; Roche) and immunoprecipitated overnight at 4°C using the Ab-coated beads. Magnetic-separated beads were washed five times with high-salt radioimmunoprecipitation assay wash buffer (50 mM HEPES (pH 7.9), 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, and 0.7% sodium deoxycholate) and once with Tris-EDTA buffer. Extraction of DNA and reversal of cross-linking were performed in 150 µl of elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS) supplemented with proteinase K (50 µg/ml) in a hybridization oven with rotation at 65°C for 2 h. The DNA was purified using a PCR purification kit (Qiagen).
FIGURE 4. PKR is implicated in S100A8 and IL-10 up-regulation. A, RAW cells were untreated or pretreated with 2-AP (4 mM) and then untreated or stimulated with poly(I:C) (50 µg/ml) for 8 h (IL-10 mRNA; ■) or 24 h (S100A8 mRNA; □). B, S100A8 in supernatants of RAW cell pretreated with 2-AP, then stimulated with poly(I:C) for 36 h. Data represent the S100A8 protein level relative to poly(I:C)-stimulated samples; means ± SD of duplicate measurements from at least three independent experiments. *, p < 0.05 compared with poly(I:C) alone. C, RAW cell pretreated with 2-AP were untreated or stimulated with poly(I:C) in the presence (■) or absence (□) of IL-10 (10 ng/ml) for 24 h. mRNA levels relative to HPRT were quantitated. For A and C, data represent mRNA levels relative to poly(I:C)-stimulated samples without 2-AP; means ± SD of three separate experiments are given. *, p < 0.05 compared with poly(I:C) alone. D, Human monocytes were pretreated with 2-AP (4 or 10 mM), then untreated or stimulated with poly(I:C) (50 µg/ml). Data represent mRNA levels relative to poly(I:C)-stimulated samples without 2-AP; means ± SD of three separate experiments. *, p < 0.05 and ***, p < 0.001 compared with relative S100A8:β-actin mRNA ratios of poly(I:C) alone. E, Human macrophages were untreated or stimulated with LPS (500 ng/ml) or poly(I:C) (50 µg/ml) with or without 2-AP (4 mM) for 4 h. IL-10 mRNA levels relative to β-actin mRNA were quantitated.

ELISA

A double sandwich ELISA was used to detect murine S100A8 as described elsewhere (35, 37) using recombinant S100A8 as standard (38).

Influenza virus infection of BALB/c mice and localization of S100A8 in lung

Viral stock and infection procedures were as described previously (39). Briefly, stocks of influenza virus A/Japan/305/57 (A/Jap, H2N2), a negative ssRNA virus, were grown in embryonated eggs. Virus-containing allantoic fluid was harvested and stored in aliquots at −70°C. Virus content was determined by hemagglutination assay using erythrocytes from Gallus domesticus. Influenza virus infection was established by inoculating 2,2,2-tribromoethanol (Avertin)-anesthetized BALB/c female mice, 6–8 wk old, intranasally with 50 hemagglutination units of virus. Mice were weighed before infection and then daily. Survival was monitored for 30 days.

Procedures for immunohistochemistry were as described elsewhere (40). Briefly, formalin-fixed tissue samples from murine lung embedded in paraffin were sectioned onto poly-t-lysine-coated slides and stained with H&E for routine morphology. Rabbit anti-murine S100A8 IgG and control IgG were used; in another control, the primary Ab was omitted. The Abs to murine S100A8 do not cross-react with S100A9 (38). Ag retrieval was performed by immersion in 0.01 M citrate buffer (pH 6.0) in a water bath at 95°C for 20 min and cooled to room temperature while still immersed in buffer (41). After quenching with 3% H2O2 and treating with primary Ab (dilution of stock solution, 1/500 to 1/4000, v/v) at room temperature for 1 h, biotin-conjugated secondary Ab and streptavidin-conjugated HRP were added at room temperature to amplify the Ag signal for subsequent 3,3’-diaminobenzidine staining. Known positive controls (murine bone marrow cells) were stained in each run and runs were duplicated on different days to confirm repeatability. Sections were counterstained with hematoxylin.

Statistical analysis

Values in figures are expressed as means ± the SD of the indicated number of observations. Statistical analyses were performed using the Student t test.

Results

Synthetic dsRNA induces S100A8, but not S100A9, in murine RAW cells

This study was initiated because our initial attempts to silence induction of the S100A8 gene in murine RAW 264.7 macrophages with small interfering RNAs (siRNAs) were unsuccessful. In fact, transfection of enzymatically synthesized S100A8 siRNAs (four constructs) potentiated S100A8 mRNA levels induced by LPS 21- to 180-fold (data not shown). In the absence of stimulation, transfection of enzymatically synthesized GAPDH siRNA suppressed GAPDH mRNA by 62%, compared with untransfected RAW cells. However, enzymatically synthesized GAPDH siRNA caused 28-fold induction of S100A8 mRNA (data not shown), indicating broad induction of the gene by enzymatically synthesized siRNA.

We next tested whether S100 proteins were up-regulated by stimulation of murine macrophages with synthetic dsRNA. Poly(I:C) at 2 µg/ml did not induce gene expression; 10 µg/ml increased S100A8 mRNA levels ~31-fold and ~466-fold with 50 µg/ml (Fig. 1A). Although 100 µg/ml poly(I:C) increased mRNA further, this dose can cause some toxicity, and 50 µg/ml was used in subsequent experiments. S100A8 in supernatants (0.48 ±
FIGURE 5. Pathways involved in poly(I:C)-activated S100A8 induction. A, RAW cells were untreated or stimulated with poly(I:C) (50 μg/ml) with or without anti-IFN-β mAb (4000 neutralization units/ml). One neutralization unit is the amount of antiserum required to neutralize 1 U of mouse IFN-β. Eight hours poststimulation, iNOS mRNA (■) or S100A8 mRNA (▲) levels relative to HPRT were quantitated. B, To determine the effects of IFN-β, RAW cells were incubated with the indicated dose of IFN-β (U/ml) with or without poly(I:C). Data represent S100A8 mRNA relative to poly(I:C)-stimulated samples; means ± SD of three separate experiments given. * p < 0.05 compared with poly(I:C) alone. C, RAW cells were pretreated with DMSO (vehicle control), NS389 (10 μM), SB202190 (2.5 μM), and SP600125 (10 μM), then untreated or stimulated with poly(I:C) (50 μg/ml); mRNA levels relative to HPRT were quantitated. Data represent S100A8 mRNA relative to poly(I:C)-stimulated samples without inhibitors; means ± SD of three separate experiments given. * p < 0.05 compared with relative S100A8:HPRT mRNA ratios of poly(I:C) stimulation alone. D, RAW cells were pretreated with SP600125 (10 μM), then untreated or stimulated with poly(I:C) (50 μg/ml) for 4 h; mRNA levels relative to HPRT were quantitated. Data represent COX-2 (▲) or IL-10 (■) mRNA relative to poly(I:C)-stimulated samples without inhibitors; means ± SD of three separate experiments given.

0.68 μg/10⁶ cells with 10 μg/ml poly(I:C) and 1.98 ± 0.72 μg/mg/ml poly (I:C) reflected this increase; the latter were 3-fold higher than protein levels in supernatants and reflected mRNA levels from cells stimulated with 20 ng/ml LPS (Fig. 1B), a control, positive activator (29). Poly(I:C) markedly amplified S100A8 mRNA when cultured together with LPS (Fig. 1C). S100A8 mRNA levels in RAW cells stimulated with 10 μg/ml poly(I:C) alone or 20 ng/ml LPS alone were 4- and 49-fold above control levels, respectively; when combined, induction increased ~447-fold.

Fig. 1A shows that synthetic mRNA at 50 μg/ml induced only low amounts of S100A8; poly(C) and poly(I) increased mRNA levels up to ~9- and ~29-fold above control, respectively, whereas poly(I:C) promoted a ~470-fold increase. S100A9 mRNA was not induced in RAW cells by poly(I:C) at any time point or in any experiment (data not shown).

S100A8 induction by poly(I:C) is IL-10 dependent

IL-10 does not directly induce S100A8 mRNA but is essential for, and increases, the LPS-activated response (28). S100A8 mRNA induction by poly(I:C) in RAW cells was evident after 8 h, maximal at 24 h, and gradually declined over 48 h, whereas IL-10 mRNA induction was evident within 2 h and maximal at 8 h, with mRNA increases ~30-fold above control levels, then declined over 16 h (Fig. 2A). IL-10 significantly enhanced the poly(I:C)-activated response ~5-fold at the mRNA (Fig. 2B) and ~3-fold at the protein level (Fig. 2C). An optimized dose (28) of a neutralizing anti-IL-10 mAb suppressed relative S100A8 mRNA levels by ~70% (Fig. 2B). To confirm the role of IL-10, peritoneal macrophages from IL-10−/− mice were tested. Fig. 2D shows significant S100A8 mRNA induction by LPS and poly(I:C) in macrophages from wild-type mice. In marked contrast, induction by poly(I:C) or LPS in IL-10−/− macrophages was significantly less than that in wild type (p < 0.01 compared with wild-type macrophages). S100A8 mRNA levels induced by these agents in IL-10−/− macrophages were elevated ~2-fold, but not significantly different to those in unstimulated samples. This confirmed that S100A8 induction by LPS and poly(I:C) was IL-10 dependent.

Mechanisms of gene induction of these S100s in human monocytes/macrophages have not been as carefully characterized as in murine macrophages. S100A8 mRNA was induced with poly(I:C) and by LPS in human monocytes (Fig. 3A), but unlike RAW cells (Fig. 1C), the combination of poly(I:C) and LPS was not synergistic (data not shown). Poly(I:C) also increased S100A9 and S100A12 mRNA; poly(I:C)- or LPS-induced S100A12 mRNA levels were 20- to 40-fold less than those of S100A8 or S100A9 (Fig. 3A). Ratios of
S100A9 mRNA levels relative to S100B in monocytes stimulated with LPS or poly(I:C) varied among individuals (from 0.2 to 5; n = 5). IL-10 enhanced poly(I:C)-activated S100A8 and S100A9 mRNA induction 2- to 3-fold (Fig. 3B). In this system, IL-10 directly induced low levels of S100A9 mRNA, although these were not significantly more than levels in unstimulated monocytes.

Fig. 3, C and D, shows that induction of S100A8 and S100A9 mRNAs in human macrophages by LPS and by poly(I:C) was suppressed by anti-IL-10. Similarly, poly(I:C) induced both genes in human monocytes and anti-IL-10 reduced mRNA to control levels (Fig. 3, E and F), strongly supporting the findings with murine macrophages that induction was via an IL-10-dependent mechanism.

**PKR mediates poly(I:C)-induced IL-10 and S100A8 mRNA**

Poly(I:C) can also signal through a TLR3-independent pathway via PKR (reviewed in Ref. 42). To test its involvement, 2-aminopurine (2-AP), which competes for ATP at the ATP binding site of PKR and thereby inhibits its autophosphorylation (43), was used. S100A8 mRNA levels induced by poly(I:C) in RAW cells were significantly inhibited by 2-AP by 90% (Fig. 4A) and protein levels by 90% (Fig. 4B). IL-10 mRNA was almost abolished by 2-AP by 8 h poststimulation (Fig. 4A). Suppression of S100A8 mRNA induction in RAW cells by 2-AP was restored to levels induced by poly(I:C) and were ~34% of the levels induced by poly(I:C) plus exogenous IL-10 (Fig. 4C). Fig. 4D shows the significant inhibition of S100A8 and S100A9 induction by poly(I:C) in human monocytes by 2-AP by 82 and 69%, respectively; 2-AP alone did not directly alter mRNA levels. Similarly, IL-10 levels induced by poly(I:C) or LPS were totally suppressed by 2-AP treatment of these cells (Fig. 4E). Interpretation of suppression of S100A12 mRNA was difficult because of its low expression levels. These experiments strongly indicate that PKR may contribute to S100A8/S100A9 gene up-regulation via its effects on IL-10 rather than through direct activation by dsRNA.

**Other signaling pathways**

Because dsRNA activates production of type 1 IFNs, we tested their potential involvement in S100A8 expression. IFN-β enhanced S100A8 mRNA induction by LPS (data not shown). RAW cells were incubated with poly(I:C) in the presence of anti-IFN-β mAb; the dose was initially optimized by measuring effects on inducible NO synthase (iNOS) mRNA induction by poly(I:C). Fig. 5A shows that anti-IFN-β suppressed iNOS mRNA by 81.1%, whereas S100A8 mRNA levels were reduced by only 37%, although this was statistically less (p < 0.05) than with poly(I:C) alone. S100A8 mRNA levels were potentiated by 20 or 100 U/ml IFN-β, increasing 1.9- or 2.6-fold (Fig. 5B). Thus, IFN-β may contribute to the poly(I:C)-mediated induction of S100A8 and can synergize with dsRNA and LPS.

LPS induction of S100A8 in murine macrophages is also PGE2/cAMP dependent (28). We next tested whether the poly(I:C) response was dependent on cyclooxygenase-2 (COX-2) using the specific inhibitor NS398, which reduces the LPS-stimulated response by 60% (28); however, changes in mRNA levels were not significant (Fig. 5C). Among the MAPK inhibitors, the JNK inhibitor SP600125, which partially blocked COX-2 and IL-10 mRNA induction (Fig. 5D), did not suppress poly(I:C)-induced S100A8 mRNA but caused some enhancement (Fig. 5C). As reported for induction by LPS (29), Fig. 5C shows that the p38 (SB202190) and ERK inhibitors (U0126) almost abolished S100A8 induction by poly(I:C). These results indicate that S100A8 mRNA induction by poly(I:C) was likely to be PGE2 independent but dependent on a p38- and ERK-mediated pathway; the JNK pathway was unlikely to be involved.

**Identification of poly(I:C)-responsive regions in the S100A8 promoter**

To examine mechanisms of transcriptional regulation of the S100A8 gene by poly(I:C), 5′-flanking sequences upstream of the transcription initiation site, untranslated exon 1, intron 1, and sequences upstream of the translation start site on exon 2 were used to evaluate activities of deletion constructs after transient transfection into RAW cells. Fig. 6 shows that levels of luciferase activity after poly(I:C) stimulation were similar for all positive constructs, with 5- to 11-fold increases compared with unstimulated cells. The region −94 to −34 bp contained the essential promoter because its deletion completely abrogated luciferase activity. The region −94 to −34 bp contained the essential promoter because deletion strongly reduced activity...
and lost enhancement. Consensus motifs for a number of transcription factors, including C/EBP and E26 transformation specific, are located within this region. Constructs not containing the first exon and intron (H11002/H11001 178 bp) generated positive, although somewhat weak luciferase activities and may contain elements essential for gene induction by poly(I:C).

Because C/EBPα and -β bind a similar region in the human S100A9 promoter (44, 45) and to further confirm functional activity of this region, ChIP assays were performed. Fig. 6B indicates specific time-dependent binding of C/EBPβ to the −178/+1 region in response to poly(I:C), whereas there was little enrichment of DNA in exon 2, located approximately +493 bp downstream of the promoter region and used as a specificity control.

**Influenza A virus induced S100A8 in epithelial cells in murine lung in vivo**

To assess whether S100A8 was up-regulated by a viral infection in vivo, we infected mice with influenza A virus (an RNA virus) and examined lungs histologically over a time course. Anti-S100A8 did not react with normal lung epithelial cells or interstitial cells (Fig. 7A) but, as expected, neutrophils within blood vessels were strongly reactive (Fig. 7B). Increased numbers of neutrophils and S100A8+ mononuclear cells were seen in interstitial tissue (Fig. 7B); the cytoplasm of epithelial cells reacted positively and was independent of the localization of neutrophils. During the acute phase (days 6 and 7), patchy staining of S100A8+ interstitial cells was seen, in particular within interstitial lesions (Fig. 7C). At day 8, peribronchial lesions were strongly S100A8+, particularly around the rims of the bronchioles (Fig. 7D). Destruction of the epithelial lining was obvious at this time (data not shown). At day 12, when mice had recovered, no cells strongly expressing S100A8 were seen (Fig. 7E). This time course suggests that S100A8 expression may reflect the severity of influenza infection.

Airway epithelial cells express TLR3 (46) and because epithelial cells lining the airways of infected mice expressed S100A8, we tested its induction in lung epithelial A459 cells. Induction was low after 6 h (data not shown) and maximal 24 h poststimulation with 10μg/ml poly(I:C) but decreased with higher amounts (Fig. 7F). However, maximal induction in epithelial cells was ~10^4 times lower than responses induced in monocyte or macrophages when normalized to β-actin (cf with Fig. 3, C and E).

**Discussion**

Serum levels of S100A8/S100A9 are elevated in patients with viral infections (17–20), but mechanisms regulating this are unknown. Induction of S100A8 through activation of TLR4 by LPS is well studied in murine macrophages (28, 35). This study provides the first evidence that S100 proteins can be induced in monocytes/macrophages by RNAs. In this study, we show that murine and human monocytes and macrophages stimulated with dsRNA...
strongly expressed S100A8 mRNA. Synthetic ssRNAs also induced S100A8 mRNA, although this was comparatively weak. Activation was reflected by the elevated secreted levels of S100A8 from activated RAW cells. As reported for several activators of the gene in murine macrophages (28, 35), S100A9 was not co-expressed in murine macrophages but there was strong induction in the human cells, again confirming a divergence of S100A9 gene regulation in rodents and humans. Similarly, glucocorticoids only amplify LPS-induced S100A8 in murine macrophages, whereas S100A8 and S100A9 are both directly up-regulated in human monocytes and macrophages (29). S100A8 mRNA induced by dsRNA increased in synergy with LPS in murine macrophages, whereas no synergy was seen with human monocytes, possibly due to different expression levels of TLR3 and TLR4 (and/or CD14) between these cell types.

Kinetic studies showed that dsRNA-induced S100A8 mRNA in RAW cells was maximal 24 h poststimulation, similar to the time of optimal expression following LPS stimulation (29), suggesting a secondary event. Induction of S100A8 in murine macrophages by LPS is IL-10 and COX-2 dependent (28). IL-10 mRNA was also induced by dsRNA, peaking 8 h poststimulation (Fig. 2A). IL-10 alone only slightly induced S100A8 mRNA (2.5-fold), but synergistically enhanced dsRNA-induced S100A8 mRNA and protein (Fig. 2, B and C). Moreover, inhibition of endogenous IL-10 by anti-IL-10 mAb substantially reduced S100A8 mRNA levels in murine and human macrophages (Figs. 2B and 3C). Importantly, S100A8 mRNA levels induced by dsRNA or by LPS were significantly less in macrophages from IL-10−/− mice than those wild-type mice (Fig. 2D). Together, these results confirm that S100A8 is an IL-10-dependent secondary response gene product and indicate that IL-10 is a major enhancer, rather than an inducer of the gene.

Recognition of viral dsRNA and of poly(I:C) activates production of antiviral factors via multiple pathways including TLR3, PKR, and the helicase retinoic acid-induced protein I (46–48). Although dsRNAs and LPS are recognized by different TLR receptors, there is divergence and convergence in TLR4 and TLR3 signaling. TLR4 signaling can occur via MyD88-dependent and -independent pathways, whereas TLR3 signaling is generally MyD88 independent (49, 50) and the adaptor proteins more restricted in their TLR interactions. PKR can participate in MyD88-independent signaling triggered by TLR3 (51) and in MyD88-dependent and -independent pathways by TLR4 (52). Thus, PKR activation can be common to dsRNA and LPS signaling. In fact, LPS activation of murine alveolar macrophages induces rapid phosphorylation of PKR (53). The PKR inhibitor 2-AP almost totally abolished IL-10 mRNA induction in murine macrophages (Fig. 4), consistent with the reported role for PKR in IL-10 induction in human PBMC by HIV-Tat stimulation (54). Consequently, S100A8 mRNA and protein levels were also reduced (Fig. 4); S100A9 expression was also reduced in human monocytes, presumably because IL-10 production was suppressed. 2-AP-suppressed S100A8 induction was restored to just above levels induced by poly(I:C) alone and the potentiation seen when poly(I:C) and IL-10 were added together was not significant (Fig. 4C), suggesting that additional mediators such as IFN-β may contribute to full gene expression. Notwithstanding, the results reported here indicate a crucial and convergent role for PKR in S100A8 induction by LPS and by dsRNA.

dsRNA can activate two opposing antiviral strategies (55) and PKR plays a role in both. It contributes to self-elimination of infected cells via apoptosis by decreasing the rate of host cell protein synthesis to prevent viral replication and also regulates NF-κB and MAPK signaling, leading to expression of genes to combat viral invasion (56). Similar to LPS-activated RAW cells (35), IFN-β synergized with dsRNA to enhance S100A8 mRNA induction (Fig. 5B), PKR is an IFN-inducible gene but its expression is cell type dependent (57). RAW cells constitutively express PKR, whereas poly(I:C) or IFN-β induce it in numerous other cells (57, 58).

Poly(I:C) stimulates rapid activation of ERK, JNK, and p38 in RAW cells (59). Similar to LPS (35), dsRNA-induced S100A8 was dependent on p38 and ERK MAPK (Fig. 5C). Moreover, the PKR inhibitor blocks numerous antiviral responses (60) including reduction of MEK6 and p38 MAPK phosphorylation in RAW cells, since the p38 MAPK activator MEK6 has increased affinity for PKR, forming a catalytic complex following exposure of cells to dsRNA (61). Although there are other possible mechanisms, such as inhibition of other signaling molecules, this mechanism could be involved in induction of IL-10 and S100A8, because the p38 pathway was crucial for both (ref. 62 and Fig. 5C). On the other hand, pharmacological inhibition of JNK enhanced dsRNA-induced S100A8 mRNA (Fig. 5C), suggesting that JNK has a regulatory role. Interestingly, JNK inhibition increases TNF-α levels following dsRNA treatment in epithelial cells and enhances p38 MAPK phosphorylation, suggesting that activation of JNK by dsRNA negatively regulates TNF-α induction (63). Its negative effect on S100A8 regulation is indirectly supported by the strong induction of S100A8 and S100A9 seen in the epidermis of JunB/c-Jun double-knockout mice (64), because activated JNKs phosphorylate c-Jun to enhance its transcriptional activity (65). In contrast to its effects on S100A8 however, the JNK inhibitor suppressed IL-10 mRNA (Fig. 5D), suggesting additional mechanisms.

This study indicated divergence of pathways used by dsRNA and LPS to express S100A8. Unlike the LPS-provoked response (28), dsRNA-induced S100A8 was independent of COX-2 metabolites because suppression of the COX-2 gene had no effect on S1008 mRNA levels. Inhibition of JNK attenuates dsRNA-stimulated COX-2 mRNA accumulation and PGE2 production by RAW cells (59). Because JNK inhibition amplified S100A8 expression by dsRNA, this supports our finding that the COX-2 pathway is unlikely to be involved. Thus, at least two pathways may independently contribute to induction and/or enhancement of S100A8 in macrophages, depending on the stimulant.

Experiments with a S100A8 luciferase reporter construct confirmed that S100A8 induction by dsRNA is at the transcriptional level. Although deletion constructs had various basal responses, possibly due to activation by the transfection procedure, we narrowed the region containing response elements to poly(I:C) to −178 to −94 of the S100A8 promoter. This is also the reported LPS/IL-10 response region (28), which contains consensus sequences for c-E26 transformation specific, C/EBP, and atypical STAT motifs. The critical role of IL-10 in dsRNA induction indicates a potential role for STAT binding; cross-talk between Jak-STAT and TLR pathways in macrophage activation is reported (66). C/EBPα and -β bind the human S100A9 promoter in a myeloid differentiation-dependent manner (44, 45) and we identified several consensus C/EBP boxes that are conserved in the defined promoter regions of human and murine S100A8 and S100A9. As described here for S100A8, poly(I:C) induces iNOS in astroglia through activation of PKR, which induces p38-MAPK-coupled activation of C/EBPβ (67). Specific binding of C/EBPβ to the S100A8 promoter in RAW cells in response to dsRNA indicates its critical role in gene induction by poly(I:C). IFN-β potentiated the poly(I:C)-induced response (Fig. 5B), although no IFN-stimulated response element motif is obvious within the essential promoter, whereas a conserved motif is located within intron 1.
Most natural dsRNA activators of PKR are synthesized in virus-infected cells as by-products of viral replication or transcription. Particularly for RNA viruses, dsRNA replicative forms are obligatory intermediates for the synthesis of new genomic RNA copies and dsRNA may be critical for the outcome of influenza A infection. S100A8 was expressed in lungs of mice infected with influenza virus (Fig. 7), although it was not obvious on the day of infection. Our results strongly support in vivo induction of S100A8 in response to an RNA viral infection in mice, as well as related S100 proteins in human infections with a papillomavirus (16) and a lentivirus (18). Expression was observed in our mice early in infection and was maximal at day 8 when strong immunoreactivity in epithelial cells lining the airways and in mononuclear cells was obvious. Pulmonary epithelial cells express TLR3 (46, 68) and PKR (46, 68, 69). S100A8 is up-regulated in bronchial epithelial cells by LPS (3) and S100A8/S100A9 is expressed in tracheal epithelial cells from patients with cystic fibrosis (70). In this study, we show that poly(I:C) induced S100A8 mRNA in alveolar A549 epithelial cells (Fig. 7F), although levels were low and other mediators may be involved in vivo. Moreover, S100A8A9 induces IL-8 production by these cells, suggesting a mechanism for amplification of neutrophilic inflammation (71). S100A8 expression declined early in the recovery phase, implying that it may be down-regulated by mediator(s) up-regulated in the resolution phase of the infection. This is consistent with reports that serum levels correlate with the clinical course in patients with HIV infection (20–22).

Increased expression of S100A8 in HIV-1-infected patients could be induced by opportunistic pathogens or by direct HIV infection of monocytes. Alternatively, production could be subsequent to IL-10 secretion by HIV-infected cells. Recently, two groups independently identified an important role for the IL-10 in viral persistence. Blockade of IL-10 signaling increased virus-specific CD8\(^+\) and CD4\(^+\) T cells and enhanced their function, resulting in resolution (72, 73). Since S100A8 levels may be maintained in conditions where IL-10 is raised and human S100A8 accentuates HIV-1 transcription and virus production (23, 24), this amplification may reflect clinical manifestations in patients with HIV-1.

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Disclosures

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