ANTIOXIDANTS & REDOX SIGNALING
Volume 14, Number 9, 2011

DOI: 10.1089/ars.2010.3583

ORIGINAL RESEARCH COMMUNICATION

S100A8 Modulates Mast Cell Function and Suppresses Eosinophil Migration in Acute Asthma

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Abstract

S100A8 is implicated in the pathogenesis of inflammatory diseases. S100A8 is upregulated in macrophages by Toll-like receptors (TLR)-3, 4, and 9 agonists in an IL-10-dependent manner, and by corticosteroids *in vitro* and *in vivo*, and scavenges oxidants generated by activated phagocytes. Because if its elevated expression in various lung disorders, we asked whether S100A8 was protective in allergic inflammation. S100A8, but not Cys^{41}-Ala S100A8, in which the single reactive Cys residue was replaced by Ala, reduced mast cell (MC) degranulation and production of particular cytokines (IL-6, IL-4, and granulocyte macrophage colony-stimulating factor) in response to IgE-crosslinking *in vitro*, likely by inhibiting intracellular reactive oxygen species production, thereby reducing downstream linker for activation of T cells and extracellular signal regulated kinase/mitogen-activated protein kinase phosphorylation. In lungs of mice with acute asthma, S100A8, but not Cys^{41}-Ala S100A8, reduced MC degranulation, production of eosinophil chemoattractants (IL-5, eotaxin, and monocyte chemoattractant protein-1), and eosinophil infiltration. Suppression of IL-6 and IL-13 could have contributed to reduced mucus production seen in lungs of S100A8-treated mice. IgE production was unaffected. In asthma, there is an imbalance of anti-oxidant systems that are generally protective. Our results strongly support a protective role for S100A8 in allergic inflammation by modulating MC activation and eosinophil recruitment, and by scavenging oxidants generated by activated leukocytes, in processes reliant on its thiol-scavenging capacity. *Antioxid. Redox Signal.* 14, 1589–1600.

Introduction

Myeloid-associated S100 proteins S100A8 and S100A9 (MRP8 and MRP14) and S100A12 (collectively known as the calgranulins) play key roles in inflammation. S100A8 scavenges reactive oxygen species (ROS) (19, 39) and this property may protect the host against excessive oxidative damage. We showed that S100A8 is readily S-nitrosylated and S100A8-SNO inhibited mast cell (MC) activation by nonspecific agonists and suppressed leukocyte transmigration triggered by MC activation in the inflamed microcirculation; S100A8 had little effect (30). S100A8 is oxidized in bronchoalveolar lavage fluid (BALF) from lungs of mice given lipopolysaccharide (LPS) and disulfide-linked homodimerization inhibited its chemotactic function (19). Murine S100A8 is also an avid scavenger of reactive halides generated by activated granulocytes *via* the myeloperoxidase or eosinophil peroxidase systems, forming covalent intra- and inter-protein disulfide (19), sulfenamide, and sulfinamide cross-linked forms (39). In keeping with a protective function, the S100A8/S100A9 complex (known as calprotectin) has anti-microbial and anti-fungal properties (21).

The calgranulins may also act as damage-associated molecular patterns possibly by interacting with TLRs and/or the receptor for advanced glycation end products (3, 12). Indeed, S100A12, expressed by infiltrating macrophages in human asthmatic lung, provokes MC sequestration and degranulation and upregulates expression of certain cytokines and chemokines (49, 50). However, our studies strongly suggested an alternate role for S100A8.

S100A8 and S100A9 are abundant in neutrophil cytosol, and macrophages in inflamed tissues generally express high amounts [reviewed in (12)]. Induction of S100A8 in monocytes/macrophages is IL-10-dependent and enhanced by corticosteroids (CS) (13, 22) and CS potentiate S100A8 generation in mice given LPS in the lung (6), suggesting an anti-inflammatory role. Epithelial cells and tracheal goblet cells from patients with cystic fibrosis express S100A8/A9 (25, 40), and the genes are upregulated by LPS in bronchial epithelial cells (20), and by oxidative stress in some cells (18). The

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calgranulins are increased in BALF or sputum from patients with a variety of lung disorders and their differential expression may regulate distinct mechanisms in respiratory inflammation (32).

MCs are key effector cells in acute allergic responses and immunoglobulin E (IgE) receptor (FcεRI) cross-linking causes degranulation, release of preformed mediators, and induction of numerous cytokines, chemokines, lipid mediators, and growth factors (1, 15, 33). The role of ROS in MC activation, and mechanisms regulating its generation are still a matter of debate. ROS, as reflected by 8-isoprostane concentrations in lung perfusates, are increased in sensitized guinea-pig lung after allergen-challenge (34). Pretreatment of lungs with indomethacin, a nonselective cyclo-oxygenase (COX) inhibitor, but not with a selective COX-2 inhibitor, abolished the 8-isoprostane increase observed during lung anaphylaxis, indicating that in this experimental system, 8-isoprostane seems to be derived from COX-1 activity (34). Consistent with these data, ROS are generated in murine MCs after IgE aggregation, in an NADPH oxidase (NOX)-independent, 5-lipoxygenase (5-LO), and (COX-1)-dependent process (43) although some studies implicate flavoenzyme-dependant H_2O_2 generation. Activated MCs do not generate extracellular H_2O_2 and exogenous H_2O_2 does not directly promote activation (44). MCs also produce small amounts of NO when activated with selected stimulants although exogenous NO downmodulates MC activation (16, 23).

Allergic inflammation is exacerbated by release of contents of eosinophil granules, comprising chemokines, cytokines, growth factors, toxic cationic and basic proteins, and eosinophil peroxidase. Excessive ROS generated in asthma can exacerbate inflammation, vascular permeability, epithelial damage, mucus hyper-secretion, smooth muscle contraction, and airway hyper-responsiveness (35, 41). The thiol scavenger N-acetyl cysteine (NAC) suppresses MC activation and attenuates asthma (5). Anti-oxidant systems are generally protective during normal lung homeostasis and include, in addition to mucin and surfactants, low-molecular-weight antioxidants in lung lining fluid, enzymes such as superoxide dismutase, catalase, thioredoxin, *etc*, and the coordinated expression of antioxidative and cytoprotective genes [reviewed in (8, 35, 38)]. However, an imbalance of oxidants and anti-oxidants may contribute to pathology. For example, decreased superoxide dismutase activity is related to airway hyperresponsiveness and remodeling (9), whereas its overexpression in mice rendered them resistant to allergen-induced changes in airway control (29).

Because S100A8 can scavenge hypohalous acid oxidants (39) and NO (30), we proposed that it may be protective in asthma. Here we show that S100A8, but not Cys^{41}-Ala mutant S100A8, reduced FcεRI-mediated MC degranulation and activation *in vitro*, likely by inhibiting intracellular ROS production and the subsequent phosphorylation of linker for activation of T cells (LAT) and extracellular signal regulated kinase (ERK)/mitogen-activated protein (MAP)-kinase. In mice with acute asthma, S100A8 suppressed expression of eosinophil chemoattractants and eosinophil infiltration into the lung, and reduced IL-6 and IL-13 levels may have contributed to decreased mucus production. Results describe novel anti-inflammatory effects of S100A8 that were likely reliant on its thiol-scavenging capacity.

Materials and Methods

Generation and culture of MCs

Murine bone marrow-derived MCs (BMMCs) were differentiated from BALB/c bone marrow cells as described (50). Maturity and purity was assessed weekly by flow cytometry using fluorochrome-conjugated rat anti-mouse c-kit (CD117) mAb (Dako) and by metachromatic staining of cytospin preparations with toluidine blue. Cells passaged weekly and differentiated until >98% MC (~3 weeks) and >95% maturity ($CD117^{high}$, toluidine blue positive) were used for functional studies. The murine MC line, Galli (derived from C57BL/6 mice), constitutively expresses c-kit and FcεRIα and growth is cytokine-independent (52). Galli cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum, 1 m*M* sodium pyruvate, 0.1 m*M* MEM containing nonessential amino acids and vitamins, 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 m*M* 2-mercaptoethanol, supplemented with 6 mg/l folic acid, 36 mg/l L-asparagine, and 106 mg/l L-arginine hydrochloride.

MCs were passively sensitized with 5–100 ng/ml mouse anti-2,4-dinitrophenol (DNP) monoclonal IgE (Sigma) overnight at 37°C, washed with phosphate-buffered saline (PBS), and resuspended in PGE buffer (25 m*M* PIPES, 120 m*M* NaCl, 5 m*M* KCl, 40 m*M* NaOH, 5.6 m*M* glucose, 0.1% BSA, and 1 m*M* $CaCl_2$). MCs were then challenged with 40 ng/ml DNP-human serum albumin (DNP-HSA; Sigma) for the times indicated with or without preincubation with 1 μ*M* S100A8 or $S100A8Ala^{41}$ at 37°C for 30 min. MCs were also nonspecifically activated with Compound 48/80 (60 μg/ml) or A23187 (1 μ*M*) for 30 min at 37°C in some experiments. Doses were optimized to induce degranulation equivalent to that induced by DNP-IgE cross-linking.

MC degranulation was assessed as β-hexosaminidase (β-hex) release. At 0.5–2 h postactivation, plates were centrifuged at 1000 rpm for 5 min and supernatants harvested. For total β-hex content, cells were lysed with 100 μl 1% Triton X-100. Supernatants or lysates (25 μl) were incubated with 25 μl 5 m*M* p-nitrophenyl N-acetyl-β-D-glucosaminide in 50 m*M* sodium citrate buffer (pH 4.5) at 37°C for 1 h. Reactions were terminated with 250 μl 0.2 M glycine-NaOH, pH 10.6, and A_{405nm} determined and calculated as follows: % β-hex release = β-hex in supernatants × 100/(β-hex in supernatants + cell lysates).

FcεRIα and c-kit expression

IgE-sensitized BMMCs (2×10^5/200 μl) in PGE buffer were incubated with DNP-HSA (100 ng/ml) ± S100A8 (1 μ*M*) for 0.5 h, and then washed with cold PBS containing 0.05% NaN_3 and 1% BSA. Cell suspensions (10^5) were incubated at 4°C for 30 min with 1 μg/ml FITC-conjugated mouse FcεRIα mAb (eBioscience), saturating amounts of PE-conjugated mouse IgG1 mAb to c-kit (CD117) (BD Pharmingen), or with isotype PE- mouse IgG1 (BD Pharmingen) and FITC-mouse IgG1 (eBioscience). After washing in cold PAB buffer, cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry (Becton Dickinson).

Viability, apoptosis, and necrosis were evaluated using double staining with FITC-conjugated annexin V and PE-conjugated propidium iodide (BD Pharmingen) following the manufacturer's instructions. IgE-sensitized BMMCs (10^6/ml)

were incubated for 6 h ± S100A8 1 (μM) after DNP-HSA challenge. Cell suspensions (100 μl) were transferred to FACS tubes (Becton Dickinson) and incubated with 5 μl FITC-anti-annexin V and 5 μl PE-anti-PI for 15 min at room temperature in the dark then immediately analyzed by flow cytometry.

Measurement of intracellular ROS

A fluorometric assay was used as described (42). Briefly, anti-DNP IgE-sensitized Galli cells (2×10^5) were incubated with 5 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes) for 30 min at 37°C; cells washed and then incubated with 1 μM S100A8, S100A8Ala[41], or NAC (2.5 mM) for 30 min at 37°C. Trapped DCFH-DA is rapidly oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular peroxides; DCF was analyzed at 10 s intervals in a microplate using a SpectraMax M2 fluorometer (Molecular Devices) at $\lambda_{ex} = 485$ nm, $\lambda_{em} = 527$ nm immediately after DNP-HSA (40 ng/ml) was added into DCFH-DA-loaded cells.

Modulation of antigen-induced phosphorylation

Murine MC line (Galli) cells (10^6) were preincubated for 30 min with S100A8 or S100A8 Ala[41] (1 μM), and then challenged with DNP-HSA for 3, 5, 15, and 30 min and immediately solubilised in cold lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM Na_3VO_4, 20 mM NaF, protease inhibitor cocktail; Roche Diagnostics) for 30 min on ice. Equal amounts of protein (40 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and then incubated with antibodies against β-actin (1:5000 v/v; Abcam), LAT (1:400 v/v; Biosource), phospho-LAT (1:1000 v/v; Upstate), P44/42 MAPK (ERK1/2), phospho-ERK1/2, phospho-P38 MAPK, phosphor-phospholipase Cγ1 (PLCγ1) (all from Cell Signaling Technology), and phosphor-protein kinase C (PKC)α (Abcam), followed by HRP-conjugated goat-anti-rabbit IgG (1:3000v/v; Bio-Rad). Positive reactivity was observed with the FLA5000 imaging system (FUJI) after development in ECL Western Blotting Substrate (Pierce). Images were quantified using Gel-Pro Analyzer software (Media Cybernetics).

Murine asthma model

Specific pathogen-free female BALB/c mice (6–10 weeks of age) were from the Biological Resource Centre, University of New South Wales. Experiments were performed according to ethics guidelines of the National Health and Medical Research Council of Australia with specific institutional approval. Mice were sensitized by intraperitoneal injections of 20 μg ovalbumin (OVA; Grade V; Sigma) with 4 mg $Al(OH)_3$ in 0.1 ml saline on days 0 and 14. On days 18, 19, and 20, animals were challenged with OVA (20 μg/40 μl saline) intranasally. Control mice were sensitized and challenged with saline. S100A8 or Ala41S100A8 (10 μg/30 μl saline) were given 2 h before each challenge; mice were sacrificed 24 h after the last OVA challenge. Tracheotomy was performed, a cannula (18G) inserted into the trachea, and ice-cold PBS (0.5 mL×2) instilled into the lungs and BALF collected. Total cell numbers were counted and differential staining performed with Diff-Quik (Lab Aids). At least 500 cells were counted microscopically. Total serum IgE and OVA-specific IgE were measured using ELISA kits (BD Pharmingen).

Formalin-fixed, paraffin-embedded lung tissue was sectioned (4 μm) and stained with hematoxylin and eosin to evaluate general morphology and cell infiltration. Evaluation of hyperplasia of goblet cells and degree of peribronchial and perivascular inflammation was performed using coded samples and was based on a 5-point scoring system (11). Briefly, the scoring system for periodic acid-Schiff-positive goblet cells was as follows: 0, none positive; 1, <25%; 2, 25%–50%; 3, 50%–75%; 4, >75%.

Cytokine measurement

mRNA levels of murine IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, monocyte chemoattractant protein 1 (MCP-1, CCL2), tumor necrosis factor (TNF)α, transforming growth factor-β (TGF-β), eotaxin, and granulocyte macrophage colony-stimulating factor (GM-CSF) were measured by real-time reverse transcriptase–polymerase chain reaction (PCR) as described (22). Anti-DNP IgE-sensitized Galli cells (5×10^5) in 24-well plates (NUNC) were challenged with DNP-HSA after 30 min preincubation with S100A8 or Ala[41]S100A8, and harvested 2 h later. Total RNA was extracted with Trizol (Invitrogen) and reverse transcribed (1 μg) using random hexamers and then the Superscript III First-Strand Synthesis System (Invitrogen). Total RNA from murine lung tissue (2×10^6/well) was also extracted. PCR amplification was carried out with Platinum SYBR Green quantitative PCR SuperMix UDG (Invitrogen) and using ABI 7700 sequence detector (Applied Biosystems). Primers used are listed in Table 1. mRNA levels were normalized to the housekeeping gene, β-actin mRNA, and relative levels expressed as ratios of levels in control samples.

IL-6, TNF, GM-CSF, and MCP-1 (CCL2) protein levels in supernatants or BALF were measured by ELISA following the manufacturer's instructions (R&D Systems). Sensitivities were as follows: IL-6 > 15 pg/ml, TNF >31 pg/ml, and CCL2 >3.9 pg/ml.

mRNA expression of enzymes relevant to ROS generation

Galli cells were sensitized and challenged with DNP-HSA as described for cytokine measurement. The murine macrophage cell line RAW 264.7 (ATCC) was seeded into 24-well tissue culture plates (Nunc) (1.5×10^5) in RPMI-1640 (Invitrogen) supplemented with 10% heated (56°C, 30 min) bovine calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Some were stimulated with 100 ng/ml LPS (Sigma) for 20 h before harvest. mRNA (measured as above) of primary enzymes relevant to intracellular ROS generation was quantitated: NOX family (NOX-1, NOX-2 (gp91phox), NOX-3, and NOX-4), dual oxidase (DUOX) 1 and DUOX2, and 5–LO, COX-1, and inducible nitric oxide synthase (iNOS). Primers used are listed in Table 1.

Statistical analysis

Comparisons between groups and of results of ELISA or densitometry were analyzed using one-way analysis of variance with Dunnett's *post-hoc t*-test and the Mann–Whitney *U*-test, respectively. Real-time PCR results were analyzed using one-way analysis of variance in conjunction with Dunnett's

Table 1. Primers for Real-Time Reverse Transcriptase–Polymerase Chain Reaction

	Forward	*Reverse*
β-actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
IL-4	ACTTGAGAGAGATCATCGGCA	AGCTCCATGAGAACACTAGAGTT
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-13	GGATATTGCATGGCCTCTGTAAC	AACAGTTGCTTTGTGTAGCTGA
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Eotaxin-1	GAATCACCAACAACAGATGCAC	ATCCTGGACCCACTTCTTCTT
IL-17	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
IL-5	ACTGTCCGTGGGGGTACTG	AGGAACTCTTGCAGGTAATCCA
TGF-β1	ATCCTGTCCAAACTAAGGCTCG	ACCTCTTTAGCATAGTAGTCCGC
GM-CSF	TCGTCTCTAACGAGTTCTCCTT	GCAGTATGTCTGGTAGTAGCTGG
NOX-1	GGTTGGGGCTGAACATTTTTC	TCGACACACAGGAATCAGGAT
NOX-2	TGAATGCCAGAGTCGGGATTT	CCCCCTTCAGGGTTCTTGATTT
NOX-3	ACCTCCGCTCCCCAGGAGGA	TGCAATGCACACGCTCACGGG
NOX-4	AGGATTGTGTTTAAGCAGAGCAT	CCGGCACATAGGTAAAAGGATG
DUOX1	CTCCTGGTTGGGACACTGGCTTCTT	TAGCTGGCTGGAACAAGGCGCT
DUOX2	CCTGGGAAGTGCAGCGCTACGA	CGGCGAGGGTTGGGCAGTAGC
5-LO	ACTACATCTACCTCAGCCTCATT	GGTGACATCGTAGGAGTCCAC
COX-1	ATGAGTCGAAGGAGTCTCTCG	GCACGGATAGTAACAACAGGGA
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC

Primers used for real-time reverse transcriptase–polymerase chain reaction to detect mRNA levels of murine cytokines and enzymes.

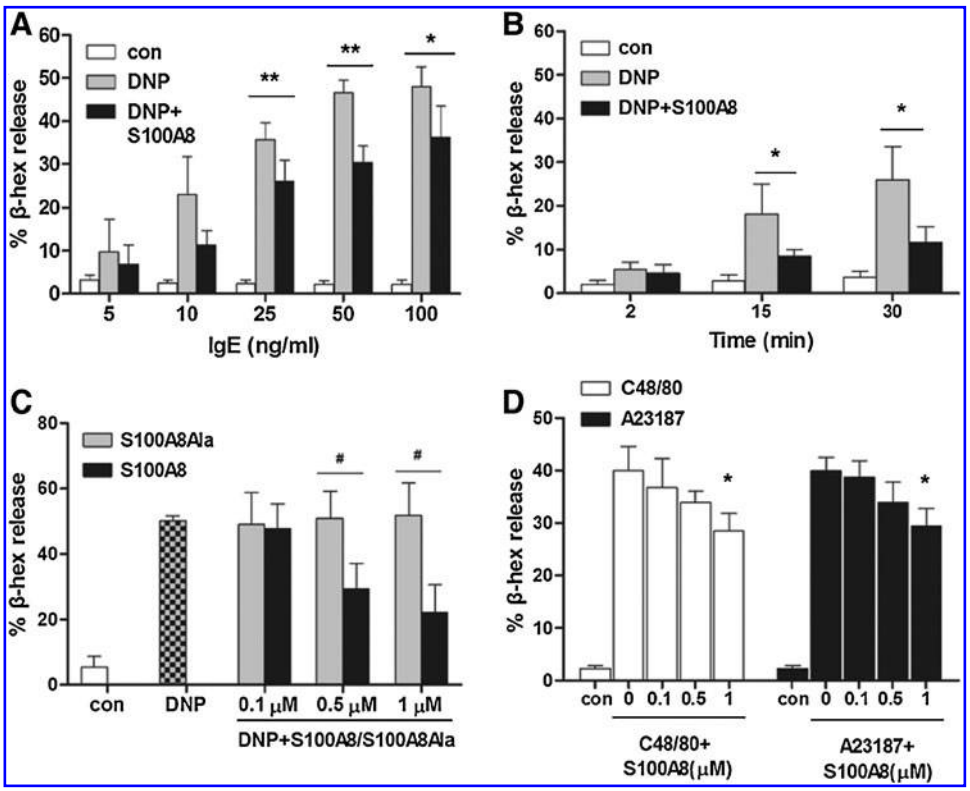

FIG. 1. S100A8 inhibited MC degranulation. (A) Galli cells primed with the given concentrations of anti-DNP IgE overnight were challenged with DNP-HSA (40 ng/ml) with (*black bars*), or without (*gray bars*) S100A8 (0.5 μ*M*) pretreatment for 30 min and β-hex levels in supernatants measured as an indicator of degranulation. **(B)** IgE-primed BMMCs were challenged with vehicle (control, *open bars*) or with 100 ng/ml DNP-HSA with (*black bars*), or without (*gray bars*) S100A8 (0.5 μ*M*) pretreatment and supernatants collected over 2–30 min for β-hex measurement. Responses of DNP compared with DNP + S100A8: $^{*}p < 0.05$ $^{**}p < 0.01$. **(C)** IgE-primed BMMCs were challenged with DNP-HSA (100 ng/ml) alone (*spotted*) or after pretreatment for 30 min with the given doses of S100A8 (*black bars*) or S100A8Ala[41] (gray) and β-hex levels measured in supernatants collected 30 min after DNP-HSA challenge. Responses of DNP+S100A8 compared to DNP+ S100A8Ala[41] $^{\#}p < 0.05$. **(D)** S100A8 weakly suppressed nonspecific MC degranulation. BMMCs were challenged with Compound 48/80 (60 μg/ml; *open bars*) or calcium ionophore A23187 (1 μ*M*; *black bars*) after preincubation with/without 0.1, 0.5, or 1 μ*M* S100A8 for 30 min. Responses to Compound 48/80 or A23187 ± S100A8, compared: $^{*}p < 0.05$. All data expressed as mean% β-hex release ± SD, $n = 4$. MC, mast cell; BMMC, bone marrow-derived mast cell; β-hex, β-hexosaminidase; DNP-HSA, dinitrophenylated human serum albumin.

post-hoc test, which allowed two-way comparisons. For two group comparisons, the *t*-test was used. Values are reported as means ± SD or SEM where indicated, and statistical significance was set at $p < 0.05$.

Results

S100A8 inhibited FcεRI-mediated MC degranulation and activation

S100A8 is a thiol scavenger and effects on IgE-sensitized murine BMMC degranulation in response to DNP challenge were tested. Degranulation, assessed as β-hex, increased with IgE dose (Fig. 1A) and over 30 min post-challenge (Fig. 1B). Pretreatment for 30 min with S100A8 (0.01–1 μM) had little direct effect (up to 5.0% ± 7.3%) compared to vehicle controls (3.3% ± 2.9%, $n = 4$, not shown) but significantly inhibited β-hex release provoked by DNP (Fig. 1A, B). Optimal dose-dependent suppression was seen 15–30 min postactivation when responses were reduced by ~50% ($n = 4$, $p < 0.05$) with 0.5 and 1 μM S100A8 (Fig. 1C). Although murine S100A8-SNO markedly inhibits MC activation provoked by Compound 48/80 (30), only 1 μM S100A8 significantly inhibited nonspecific MC activation by ~25% (Fig. 1D).

To determine effects of S100A8 on cytokine gene induction, mRNA levels of IL-4, IL-6, IL-10, TNF-α, MCP-1, and GM-CSF were measured using IgE-sensitized MCs pretreated with S100A8 and harvested 2 h post-DNP challenge. S100A8 had little direct effect but significantly reduced IL-6 (Fig. 2A), IL-4 (Fig. 2B), and GM-CSF mRNAs (Fig. 2C). IL-6 protein in supernatants was also reduced (Fig. 2D). Effects of S100A8 were apparently selective. TNF-α mRNA (Fig. 2E) was somewhat reduced although TNF protein in supernatants of S100A8-treated MCs was significantly less than levels produced by DNP challenge (Fig. 2F). Because TNF-α is stored in granules (15),

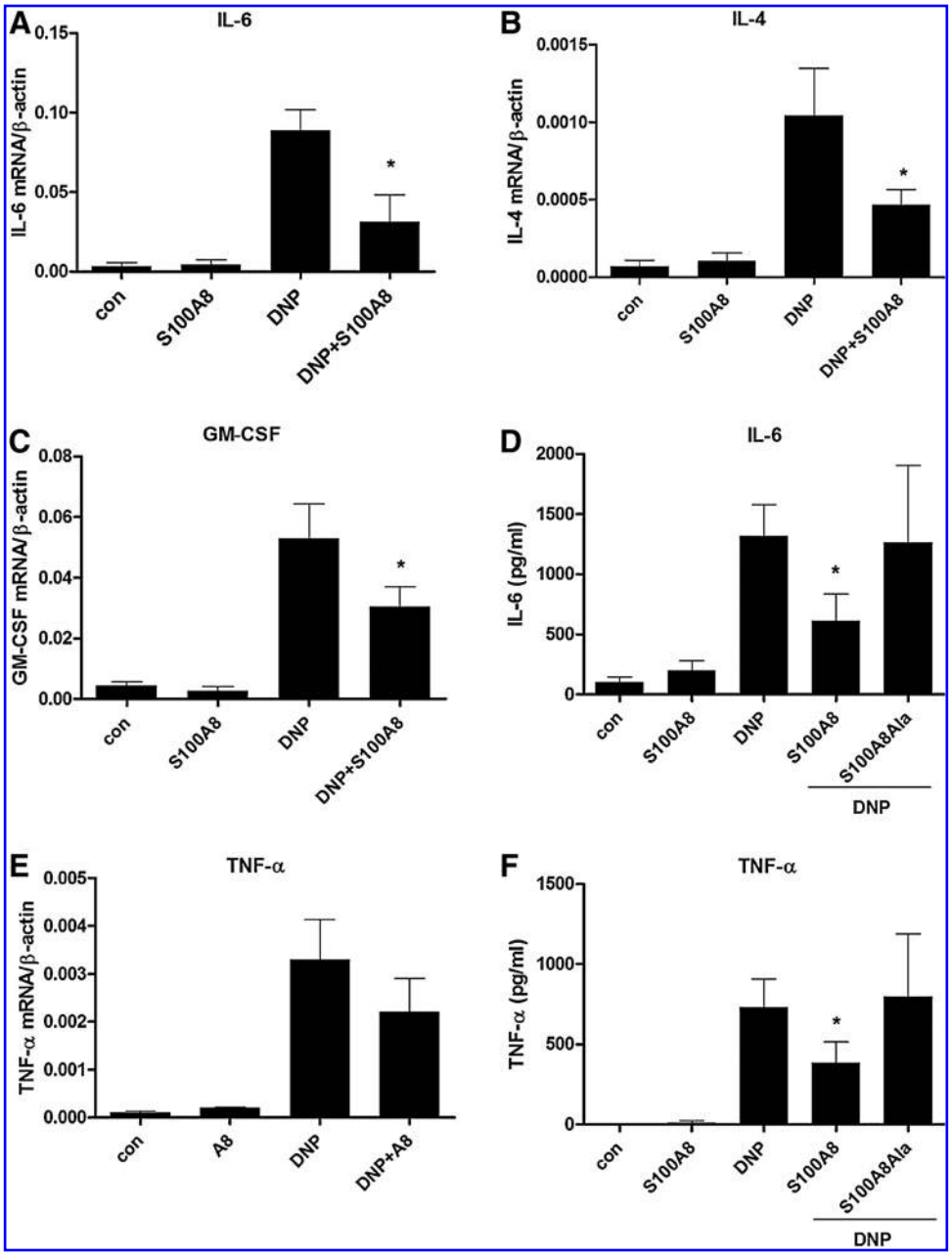

FIG. 2. Inhibition of MC cytokine production by S100A8. Anti-DNP IgE-primed Galli cells ± pretreatment with S100A8 or S100A8Ala[41] (both 1 μM) were activated by DNP-HSA (40 ng/ml)) for 2 h, and **(A)** IL-6 mRNA, **(B)** IL-4 mRNA, **(C)** GM-CSF mRNA, and **(E)** TNF-α mRNA quantitated. **(D)** IL-6 and **(F)** TNF-α levels in supernatants collected after 6 h. Results are means ± SD of 4 independent experiments; responses of DNP compared with DNP + S100A8: *$p < 0.05$. GM-CSF, granulocyte macrophage colony-stimulating factor; TNF, tumor necrosis factor.

reduced levels may reflect suppression of degranulation rather than effects on its *de novo* synthesis. MCP-1 mRNA or protein, TGF-β, or IL-10 mRNA levels were not altered (not shown).

S100A8 suppressed MC activation by scavenging ROS

To identify mechanisms of S100A8 suppression, we first tested the possibility that S100A8 might affect MC function by altering FcεRI expression; flow cytometry studies indicated no surface changes in FcεRI levels on BMMC after S100A8 incubation (not shown). Moreover, S100A8 did not alter MC viability or cause apoptosis (not shown). FcεRI cross-linking generates intracellular ROS that is essential for downstream signaling (45). As expected (42), diphenyleneiodonium chloride (DPI), a cell-permeable inhibitor of several oxidoreductases, only weakly suppressed IgE-mediated degranulation (Fig. 3A). Substantial suppression was seen with 2.5 m*M* NAC (Fig. 3A), to levels comparable with 0.5–1 μM S100A8 (Fig. 1C). S100A8Ala[41] is a mutant form in which the single reactive Cys residue in S100A8 is mutated to Ala. This did not affect degranulation (Fig. 1C), or induction of IL-6 or TNF-α at any concentration tested (Fig. 2D, F), confirming the importance of the reactive thiol in S100A8-mediated suppression of FcεRI-activated MCs. We next asked whether S100A8 inhibited MC activation by suppressing ROS generation. Intracellular ROS was rapidly produced by challenge of IgE-sensitized MCs with DNP, with maximal levels evident within 2–4 min. S100A8 had little direct effect, as ROS levels were similar to those of vehicle-challenged MCs, whereas it completely inhibited intracellular ROS generated by FcεRI cross-linking (Fig. 3B). NAC had a similar effect. In marked contrast, S100A8Ala[41] did not significantly alter ROS levels of DNP-challenged MCs, confirming that suppression by S100A8 was likely due to its intracellular ROS scavenging capacity.

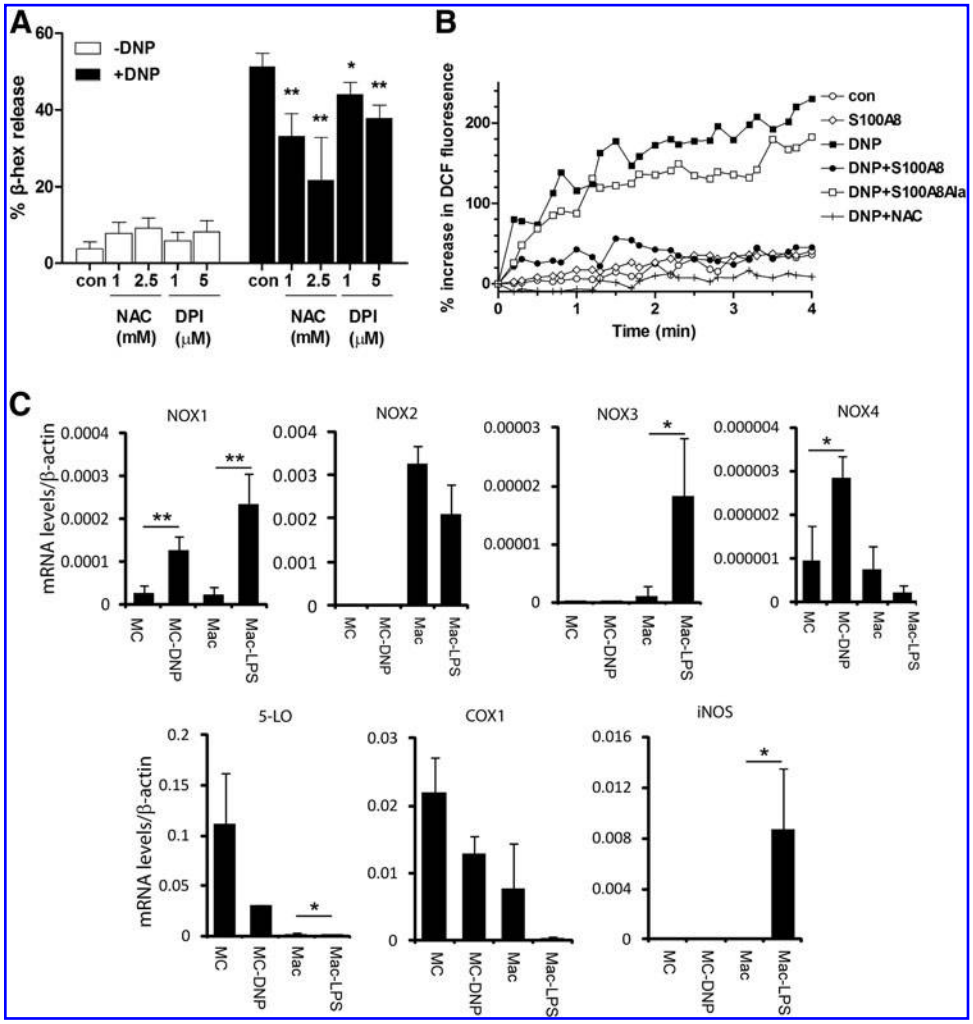

FIG. 3. S100A8 suppressed MC activation by scavenging ROS. (A) β-hex release from anti-DNP IgE-sensitized Galli cells preincubated with N-acetyl cysteine (1 or 2.5 m*M*), DPI (1 or 5 μM) with (solid) or without (open) DNP-HSA (40 ng/ml) challenge for 30 min. Data represent means ± SD of 3–4 independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with DNP-HSA challenge of IgE-sensitized MCs. **(B)** To measure intracellular ROS, Galli cells loaded with 5 μM DCFH-DA for 30 min after sensitization with anti-DNP IgE were incubated with medium, S100A8, S100A8Ala[41] (both 1 μM) alone, or with DNP-HSA (40 ng/ml) stimulation. S100A8 preparations did not directly affect intracellular ROS levels. S100A8 suppressed ROS generation after antigen challenge to levels comparable to those observed with N-acetyl cysteine (2.5 m*M*). ROS-mediated DCF oxidation was measured immediately, and at 10 s intervals for 4 min at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 527$ nm. Data are representative of three independent experiments. **(C)** Anti-DNP IgE-primed Galli MCs were activated with DNP-HSA (40 ng/ml)) for 2 h; RAW 264.7 macrophages were stimulated with LPS (100 ng/ml) for 20 h. Treated and untreated cells were analyzed for mRNA expression by quantitative reverse transcriptase–polymerase chain reaction. mRNA level of primary enzymes related to ROS generation were normalized to β-actin mRNA levels. NOX-1, NOX-2, NOX-3, NOX-4, 5-LO, COX-1, and iNOS mRNA levels are given; dual oxidase 1 and dual oxidase 2 mRNAs were below detection limits and are not shown. Results are means ± SD of duplicates of 2–4 independent experiments; comparisons of responses of untreated *versus* treated samples of the same cell types: $^*p < 0.05$; $^{**}p < 0.01$. DCFH-DA, dichlorodihydrofluorescein diacetate; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; LPS, lipopolysaccharide; NOX, NADPH oxidase; COX, cyclo-oxygenase; 5-LO, 5-lipoxygenase; DPI, diphenyleneiodonium chloride.

We made a preliminary attempt to define likely pathways of ROS generation by examining expression of relevant genes and compared this in RAW-267 macrophages using quantitative reverse transcriptase–PCR. Figure 3C shows that NOX-2 and NOX-3 mRNA levels were absent in MCs, although present in macrophages. Constitutive amounts of NOX-1 and NOX-4 were low; NOX-1 mRNA increased significantly after activation of both cell types. On the other hand, NOX-4 increased some threefold after DNP challenge of IgE-sensitized MCs, whereas levels decreased in activated macrophages. Neither cell type expressed the Duox1 or Duox2 genes (not shown). In contrast to the NOX genes, constitutive levels of COX-1 and 5-LO mRNA levels were high in MCs, and tended to decline after FcεRI cross-linking. Only activated macrophages expressed iNOS. Gene expression in MCs contrasted with those in resting or LPS-activated macrophages, in which ROS generation *via* NADPH-oxidase is important. Although endogenous levels of NOX-1 and NOX-4 were low, these could have contributed to the slight suppression of MC degranulation seen with DPI (Fig. 3A). However, induction of these genes 2 h after MC activation may contribute to more sustained ROS generation that could mediate cytokine production. On the other hand, the high endogenous levels of COX-1 and 5-LO may be important in the immediate response required for degranulation and are in keeping with the earlier proposal that ROS generated by FcεRI cross-linking occurs principally *via* COX-1 and 5-LO (44).

FcεRI cross-linking induces phosphorylation of a cluster of proteins, including the adaptor protein LAT and downstream MAP kinases ERK1/2 (p44/42) and p38 kinase, and PKCα and PLCγ1 (24). Phosphorylation was maximal after 3 min for LAT; 5 min for ERK, P38, and PLCγ1; and 15 min for PKCα (data not shown). NAC substantially suppressed LAT phosphorylation. S100A8 reduced this by ~40% ($p < 0.05$ compared to MCs, not treated with S100A8), ERK1/2 by 28% ($p < 0.05$) (Fig. 4A, B) and p38 kinase 14% ($p > 0.05$). Changes in phosphorylation of PKCα and PLCγ1 were not statistically significant (Fig. 4C). In keeping with a ROS-dependent mechanism, S100A8Ala[41] had no effect on the phosphorylation status of any of these proteins (Fig. 4A–C).

S100A8 suppressed some features of acute murine asthma

We next compared some parameters of asthma pathogenesis in an acute asthma model using mice treated with S100A8 or S100A8Ala[41] before OVA challenge. Total cell numbers in BALF 24 h post OVA-challenge were significantly higher

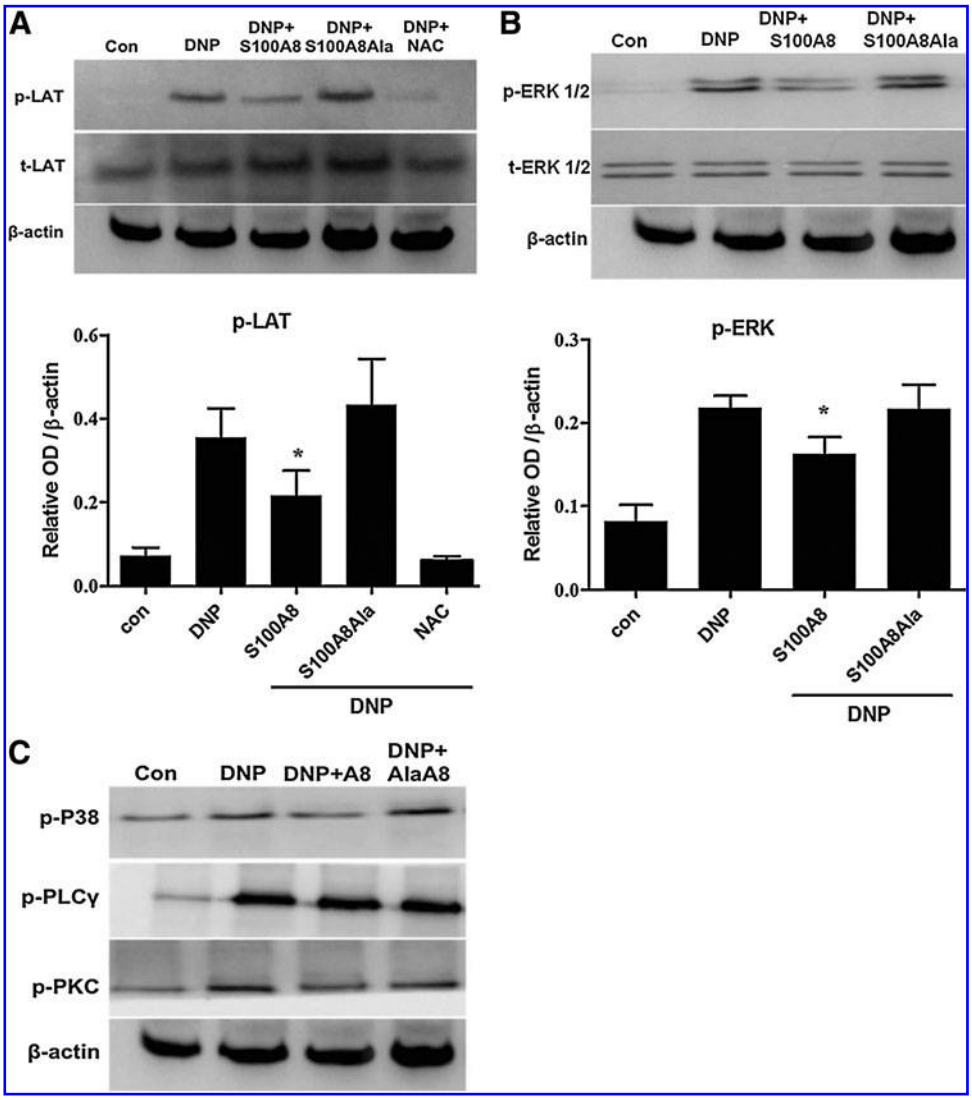

FIG. 4. S100A8 suppressed FcεRI-induced phosphorylation of LAT and ERK. IgE-sensitized Galli cells were incubated with vehicle control, S100A8, or S100A8Ala[41] (1 μM) for 30 min at 37°C and then stimulated with DNP-HSA (40 ng/ml) for 3 min for analysis of LAT, 5 min for ERK1/2, P38, and PLCγ1, and 15 min for PKCα phosphorylation. Cell lysates (40 μg) were Western blotted with anti-phospho-LAT, -ERK1/2, -phospho-P38, -PLCγ1, -PKCα, or β-actin antibodies. Images were quantified by densitometry and analyzed using Gel-Pro Analyzer software. S100A8 significantly suppressed **(A)** LAT and **(B)** ERK1/2 phosphorylation, whereas S100A8Ala[41] had no effect. **(C)** S100A8 did not suppress phosphorylation of P38, PKCα, or PLCγ1. Data from 4 independent experiments expressed as means of ratios of ODs to ODs of β-actin ± SD. *$p < 0.05$, compared with DNP-activated MCs and with DNP-activated MCs pretreated with S100A8Ala[41]. ERK, extracellular signal regulated kinase; PKC, protein kinase C; PLC, phospholipase C; LAT, linker for activation of T cells.

($1.34 \pm 0.26 \times 10^6$) than in BALF from vehicle-challenged mice ($0.2 \pm 0.02 \times 10^6$; Fig. 5A) and comprised predominantly eosinophils (74.3% ± 2.6% in OVA *vs.* 0.1% ± 0% in control). S100A8 alone did not increase cell numbers in BALF from sensitized mice challenged with vehicle, although, as expected (27), low levels of leukocyte recruitment were apparent within the lung parenchyma (Fig. 5B). In BALF from mice pretreated with S100A8, total leukocyte numbers, in particular eosinophils, were reduced by 65.65%. In marked contrast, S100A8Ala[41] did not reduce OVA-provoked leukocyte infiltration, and eosinophil numbers were similar to those in BALF from untreated, OVA-challenged mice (Fig. 5A). Mucus-positive goblet cells in the airways of mice treated with S100A8 were some 30% less than seen in lungs from OVA-challenged mice, or those from mice pretreated with S100A8Ala[41] than those challenged with OVA (Fig. 5C). MC degranulation was assessed as β-hex levels in BALF; S100A8 pretreatment did not alter basal levels in unchallenged mice. OVA challenge increased β-hex levels some fivefold; S100A8 pretreatment significantly reduced β-hex generation, whereas S100A8Ala[41] had no effect (Fig. 5D).

S100A8 also significantly reduced IL-6 protein levels (Fig. 6A), but had no effect on GM-CSF or TNF-α (Fig. 6B–E). Importantly, MCP-1, IL-5, IL-13, and eotaxin mRNA levels in lung tissue were significantly reduced by S100A8-pretreatment compared to lungs from sensitized mice challenged OVA (Fig. 6F–I). Interestingly, S100A8Ala[41] pretreatment did not suppress these, whereas eotaxin mRNA in lungs from these mice was significantly reduced, to levels similar to those after S100A8 pretreatment, indicating an alternate, somewhat selective

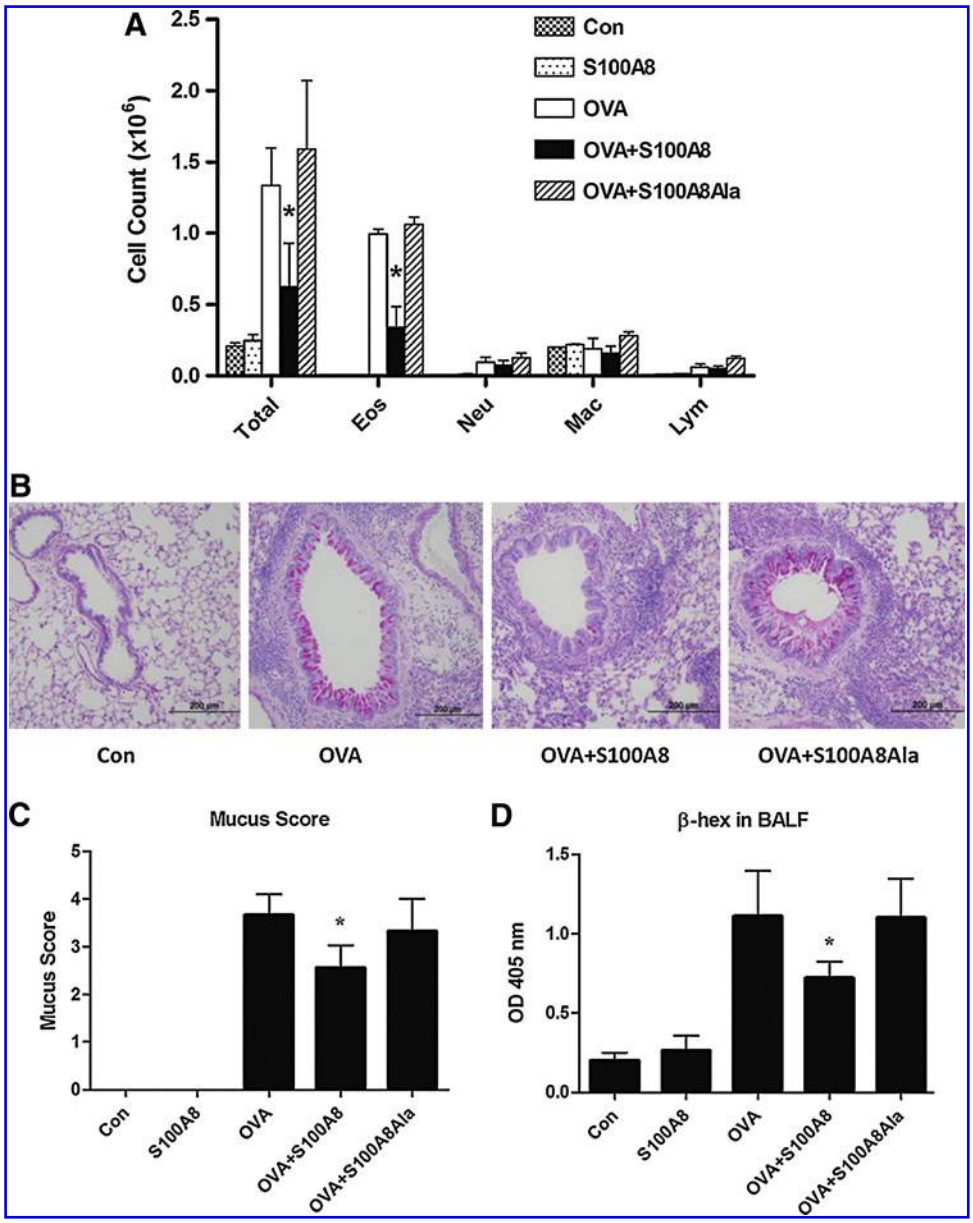

FIG. 5. S100A8 reduced inflammation in an acute murine asthma model. (A) Total and differential cell counts in BALF collected 24 h after the last OVA challenge of sensitized mice. Eos, eosinophils; Neu, neutrophils; Mac, macrophages; Lym, lymphocytes. **(B)** Lungs from OVA-challenged mice had significantly more PAS-positive goblet cells than seen in vehicle-challenged mice (Con), whereas this was significantly reduced in lungs from mice pretreated with S100A8 before OVA challenge; PAS-positive goblet cells in lungs from mice pretreated with S100A8Ala[41] were similar to those in lungs from mice challenged with OVA. Magnification: 400×. Results are representative of at least six mice/group. **(C)** The bar graph shows significantly reduced PAS-positive goblet cells in S100A8 pretreated compared to OVA-challenged mice, or mice pretreated with S100A8Ala[41] and challenged with OVA (*$p < 0.05$). **(D)** β-hex in BALF was significantly less (*$p < 0.05$) in samples from mice pretreated with S100A8 compared to samples from OVA-challenged mice or from mice pretreated with S100A8Ala[41], and then OVA challenged. Values are means ± SD (con, $n = 6$; S100A8, $n = 6$; OVA, $n = 7$; OVA+S100A8, $n = 9$; OVA+S100A8Ala[41], $n = 6$). BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; PAS, periodic acid-Schiff reagent. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

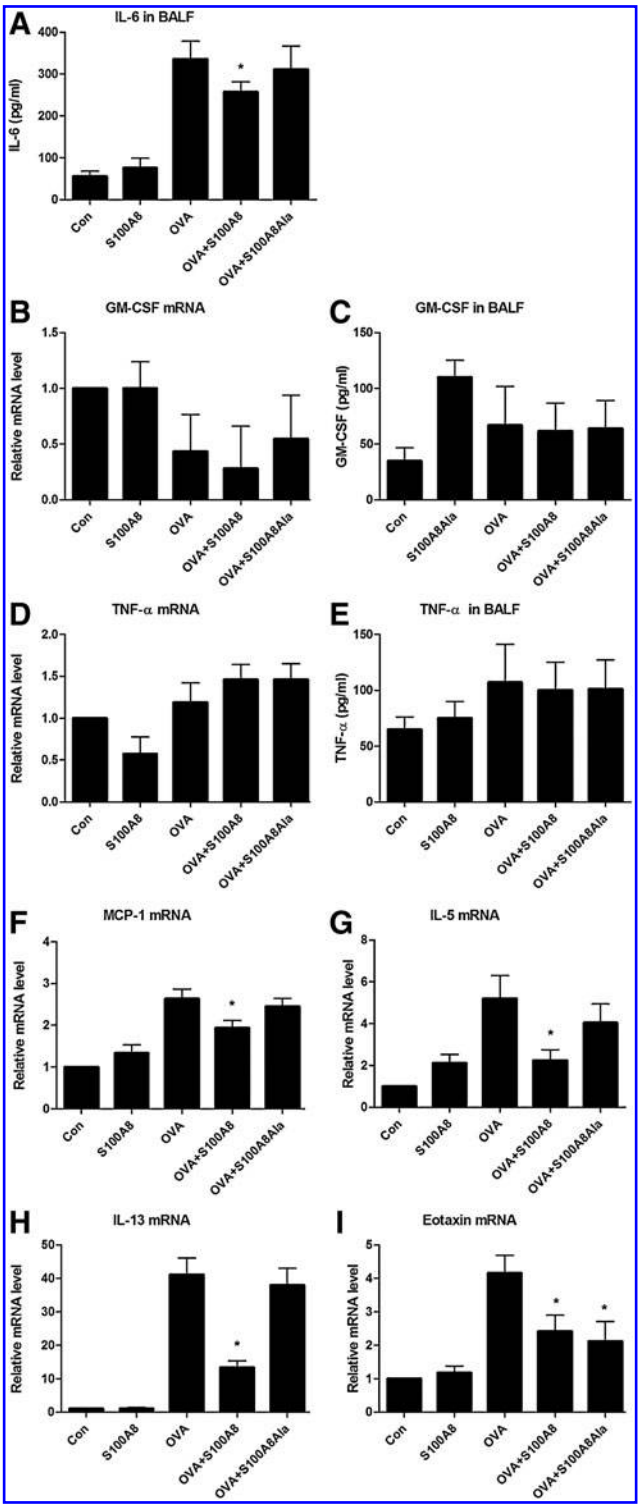

FIG. 6. S100A8 suppressed production of some cytokines in acute murine asthma. (A) IL-6 in BALF was modestly, but significantly reduced by S100A8 pretreatment. **(B, C)** GM-CSF and **(D, E)** TNF-α mRNA/protein levels were not altered. S100A8 pretreatment significantly reduced **(F)** MCP-1, **(G)** IL-5, **(H)** IL-13, and **(I)** eotaxin mRNA levels. Values are means $\pm$ SD (con, $n=6$; S100A8, $n=6$; OVA, $n=7$; OVA+S100A8, $n=9$; OVA+S100A8Ala[41], $n=6$); OVA-challenged mice, or mice pretreated with S100A8Ala[41] and challenged with OVA, compared to mice pretreated with S100A8, $^*p<0.05$. MCP, monocyte chemoattractant protein.

mechanism. Some functions of S100A8 are mediated by the "hinge" domain that resides between the two calcium-binding regions (27) and this could be responsible here. Tissue mRNA levels of IL-4, IL-10, TGF-β, and IL-17 were not significantly different in lungs from mice challenged with OVA, or from those pretreated with S100A8 or S100A8Ala[41] (not shown).

To determine whether S100A8 altered IgE levels, we assessed total IgE and OVA-specific IgE. These were substantially elevated in OVA-challenged mice compared to vehicle challenge but were not significantly affected by S100A8 or S100A8Ala[41] preincubation (Fig. 7A, B), indicating that S100A8 principally mediated changes to the inflammatory arm of this response.

Discussion

Several reports implicate S100A8/S100A9 in respiratory diseases (17, 20, 25, 32, 40) although their functions in these conditions are largely unexplored. Our earlier studies strongly implicate S100A8 in antioxidant defense (31) and some modifications have anti-inflammatory effects (19, 30, 39). Reactivity is predominantly dependent on a single reactive thiol residue in this protein. S100A8 can be readily S-nitrosylated; S100A8-SNO suppressed nonspecific MC activation, and leukocyte adhesion and extravasation in the microcirculation of rats treated with the MC secretagogue, Compound 48/80, whereas native S100A8 had little effect. Because S100A8 is induced in activated microvessels (51), and can shuttle NO to hemoglobin, we proposed that this protein may regulate vessel tone in inflamed lesions. Here we show that native S100A8 suppressed classical MC activation.

Intracellular ROS mediates downstream signaling in MC activation by FcεRI cross-linking (45). S100A8, but not S100A8Ala,[41] significantly inhibited IgE-mediated MC degranulation and production of some proinflammatory cytokines, in particular TNF (due to reduced degranulation), IL-4, IL-6, and GM-CSF. MC degranulation in lungs of sensitized mice challenged with OVA was also suppressed by S100A8 pretreatment, which significantly reduced β-hex levels in BALF, whereas S100A8Ala[41] had no effect. Products from MCs such as TGF-β and IL-10 can suppress the initiation, magnitude, and/or duration of responses (14), but S100A8 did not alter these, either from MCs *in vitro* or in acute asthma in mice, excluding the possibility of autocrine inhibition of inflammation *in vivo*.

MC activation pathways vary with the source of MCs and the agonist, and may occur *via* multiple pathways (4). Sources of intracellular ROS that mediate downstream signaling after FcεRI cross-linking are not clearly understood, but the COX-1/5-LO pathway is likely to contribute (Fig. 3C) (45). Here we show that S100A8 reduced intracellular ROS production to a similar extent as NAC when primed MCs were challenged with DNP although suppression of degranulation was never >60%, implicating additional ROS-independent activation pathways. We present for the first time, a screen of expression of genes potentially involved in ROS generation by MCs. We found very low constitutive amounts of NOX-1 and NOX-4 mRNAs compared to the high endogenous 5-LO and COX-1 that decreased somewhat after antigen challenge. Interestingly, NOX-1 and NOX-4 gene levels increased 2 h after MC activation with DNP. A recent report implicates NOX-1 in antigen-mediated MC

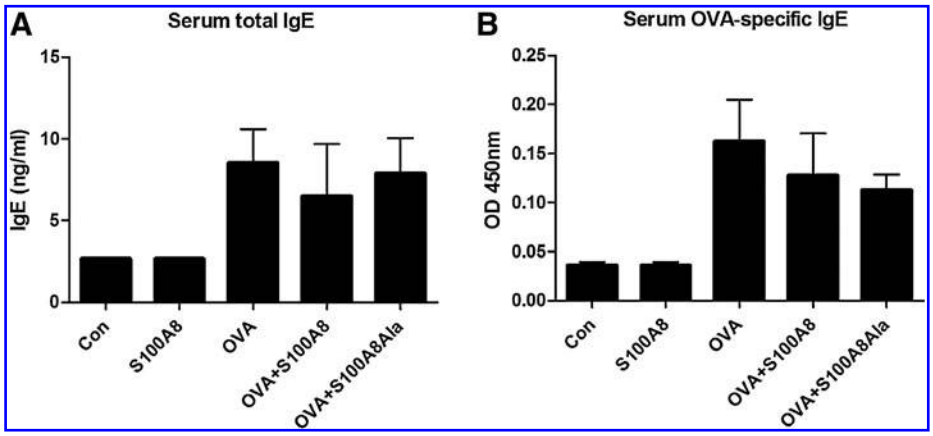

FIG. 7. S100A8 did not alter IgE levels in acute murine asthma. Serum levels of **(A)** total IgE and **(B)** OVA-specific IgE were quantitated by ELISA. As expected, OVA challenge increased total IgE, and OVA-specific IgE levels in serum from sensitized mice. Although there was a tendency toward reduced OVA-specific IgE in serum from S100A8 or S100A8Ala[41]-pretreated mice, differences were not statistically different from levels in serum from OVA-challenged mice. Data expressed as means ± SD in control (con, $n = 6$; S100A8, $n = 6$; OVA, $n = 7$; OVA+S100A8, $n = 9$; OVA+S100A8Ala[41], $n = 6$).

signaling (7). As shown in Figure 3C, Cho *et al.* also detected increased NOX-1 mRNA 1 h after antigen challenge and its deletion reduced Th-2 cytokine production. Likewise, S100A8 suppressed generation of some cytokines (Fig. 2). We propose that the high endogenous levels of COX-1 and 5-LO may be important in the immediate response required for degranulation. This response was only weakly reduced by DPI. However, induction of the NOX-1 and NOX-4 genes 1–2 h after MC activation may contribute to more sustained ROS generation that could mediate cytokine production. Because S100A8 suppressed degranulation and cytokine generation its effects were sustained, and more detailed analyses of potential pathways are warranted. Although S-nitrosylation of S100A8 generates a strongly suppressive adduct (30), MCs do not produce NO after FcεRI aggregation (46), making this possibility unlikely, and we found no iNOS induction by antigen challenge.

Syk-mediated phosphorylation and/or activation of PLCγ, LAT, SLP-76, and PI3K are important for calcium mobilization and degranulation in MCs (4), and phosphorylation of LAT, promoted by IgE cross-linking, is ROS dependent (42). S100A8 inhibited LAT phosphorylation by ~50%, a level reflected by reductions in degranulation although phosphorylation of PLCγ was unaffected. Increases in intracellular calcium levels lead to activation of Erk and p38, which are involved in IgE-mediated synthesis of some cytokines. S100A8 inhibited Erk1/2 phosphorylation and had minor effects on p38 that may have contributed to the reduced levels of IL-4, IL-6, and GM-CSF mRNAs. Several facets of S100A8 suppression appeared similar to those produced by the antioxidant NAC, and effects of S100A8 on intracellular glutathione levels are currently being investigated.

S100A8, but not S100A8Ala,[41] significantly inhibited IgE-mediated MC degranulation and production of some proinflammatory cytokines, in particular TNF (due to reduced degranulation), IL-4, IL-6, and GM-CSF. MC degranulation in lungs of sensitized mice challenged with OVA was also suppressed by S100A8 pretreatment, which significantly reduced β-hex levels in BALF, whereas S100A8Ala[41] had no effect. Products from MCs such as TGF-β and IL-10 can suppress the initiation, magnitude, and/or duration of responses (14), but S100A8 did not alter these, either from MCs *in vitro* or in acute asthma in mice, excluding the possibility of autocrine inhibition of inflammation *in vivo*.

S100A8, but not S100A8Ala[41], suppressed symptoms of acute murine asthma, particularly recruitment of eosinophils found in BALF from antigen-challenged mice. Reduced levels of eosinophil chemoattractants IL-5, eotaxin, and MCP-1 found in lung tissue of S100A8-treated mice challenged with OVA may have contributed. Numbers of mucus-producing goblet cells were also less, possibly due to suppression of IL-6 (36) and IL-13 (28), key mediators of mucus production in allergic airway inflammation. Activation of redox-sensitive transcription factors such as NF-κB in epithelial cells, and subsequent production of eotaxin promotes eosinophil infiltration into the lung (10), and NAC inhibits eotaxin and MCP-1 induction in airway smooth muscle cells *via* a p38 MAPK and ROS-driven pathway (47). Although exact mechanisms of S100A8-mediated suppression of symptoms of acute asthma are unclear, reduced MC degranulation (Fig. 5D) and activation, and suppression of chemokine generation by other cell types may have contributed.

Induction of S100A8 in macrophages is IL-10 dependent (13, 48). Interestingly, like S100A8, IL-10 suppresses allergic inflammation and eosinophilia by downregulating IL-5 and GM-CSF (37), functions described here for S100A8. We found no effects of S100A8 on IL-10 gene levels at the time-point studied (24 h post-OVA challenge), but it is conceivable that some suppressive affects attributed to IL-10 may be mediated through S100A8. CS therapy elevates endogenous IL-10 levels and inhaled CS are the main treatment for asthmatic subjects. We described increases in S100A8-positive macrophages in the synovium of patients with rheumatoid arthritis treated with high-dose steroids and found that S100A8 levels increased in several cell types treated with CS (22); induction by CS was IL-10-dependent in monocytes/macrophages. We also found higher S100A8 levels in lungs from mice treated with LPS plus CS compared to LPS alone (6, 22). Together with the results reported here, we propose that increased S100A8 production may contribute to the effectiveness of CS therapy in asthmatics (26). Drugs, such as inhaled p38 MAPK inhibitors and anti-oxidants that target specific pathways, and agents directed against the interleukin-13 pathway are also suggested as useful adjunct therapies for asthma treatment (2). Here we propose a novel mechanism whereby S100A8 may contribute to the resolution by reducing ROS-mediated pathways that regulate MC activation, and eosinophil recruitment and production of key target cytokines.

Acknowledgment

This study was funded by grant #455307 from the National Health and Medical Research Council of Australia.

Author Disclosure Statement

There are no commercial associations that might create a conflict of interest in connection with this article. Each author acknowledges that there are no competing financial interests.

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Date of first submission to ARS Central, August 22, 2010; date of final revised submission, December 6, 2010; date of acceptance, December 8, 2010.

Abbreviations Used

5-LO = 5-lipoxygenase
β-hex = β-hexosaminidase
BALF = bronchoalveolar lavage fluid
BMMC = bone marrow-derived mast cell
COX = cyclo-oxygenase
CS = corticosteroids
DCFH-DA = dichlorodihydrofluorescein diacetate
DNP = 2,4-dinitrophenol
DNP-HAS = dinitrophenylated human serum albumin
DPI = diphenyleneiodonium chloride
DUOX = dual oxidase
ERK = extracellular signal regulated kinase
FcεRI = immunoglobulin E (IgE) receptor 1
GM-CSF = granulocyte macrophage colony-stimulating factor
iNOs = inducible nitric oxide synthase
LAT = linker for activation of T cells
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
MC = mast cell
MCP = monocyte chemoattractant protein
NAC = N-acetyl cysteine
NOX = NADPH oxidase
OVA = ovalbumin
PAS = periodic acid-Schiff reagent
PBS = phosphate-buffered saline
PCR = polymerase chain reaction
PKC = protein kinase C
PLC = phospholipase C
ROS = reactive oxygen species
TGF-β = transforming growth factor-β
TLR = Toll-like receptors
TNF = tumor necrosis factor

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