

S100A12 provokes mast cell activation: A potential amplification pathway in asthma and innate immunity

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Background: The calcium-binding protein S100A12 might provoke inflammation and monocyte recruitment through the receptor for advanced glycation end products.

Objective: Because inflammation elicited by S100A12 *in vivo* had characteristics of mast cell (MC) activation, we aimed to define the mechanism.

Methods: Various MC populations were used to test S100A12 activation assessed on the basis of morphology, histamine release, leukotriene production, and cytokine induction. MC dependence of S100A12-provoked inflammation was tested in mice and on the rat microcirculation by means of intravital microscopy. Immunohistochemistry localized S100A12 in the asthmatic lung, and levels in sputum from asthmatic patients were quantitated by means of ELISA. Expression of the receptor for advanced glycation end products was evaluated by means of RT-PCR and Western blotting.

Results: S100A12 provoked degranulation of mucosal and tissue MCs *in vitro* and *in vivo* and amplified IgE-mediated responses. It induced a cytokine profile indicating a role in innate/T_H1-mediated responses. S100A12-induced edema and leukocyte rolling, adhesion, and transmigration in the microcirculation were MC dependent. Eosinophils in airway

tissue from asthmatic patients were S100A12 positive, and levels were increased in sputum. S100A12 responses were partially blocked by an antagonist to the receptor for advanced glycation end products, but MCs did not express mRNA or protein, suggesting an alternate receptor.

Conclusion: This novel pathway highlights the potential importance of S100A12 in allergic responses and in infectious and chronic inflammatory diseases.

Clinical implications: MC activation by S100A12 might exacerbate allergic inflammation and asthma. S100A12 might provide a novel marker for eosinophilic asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: S100A12, inflammation, mast cell activation, leukocyte migration, asthma, eosinophils, sputum, receptor for advanced glycation end products

Mast cells (MCs) are critical to the pathogenesis of asthma, allergy, and parasitic infection; are initiators and effectors of innate immunity; and might contribute to the transition to acquired immunity.¹⁻⁴ MC activation could be particularly important in autoimmune diseases and modulated by inflammation,^{1,2} but the activators in these settings are poorly understood. MCs are classically activated through antigen cross-linking of IgE bound to FcεRI. Ig-independent pathways include ligation of Toll-like receptors 2, 3, 4, 5, 7, and 9; complement components; some cytokines^{1,2}; and some positively charged peptides.⁵ Some stimulants upregulate various cytokine and chemokine genes without provoking degranulation.¹ The stored mediators released on degranulation increase blood vessel permeability and leukocyte extravasation, and newly synthesized mediators can also promulgate vascular changes that contribute to wound healing and chronic inflammation.¹⁻³

There is evidence for communication between MCs and neutrophils in inflammatory responses, and neutrophil recruitment to sites of infection is MC dependent.^{4,6} Histamine-releasing factors from diverse cell types, including activated mononuclear cells and neutrophils, are implicated.⁵ S100A12 (also known as calgranulin C; extracellular newly identified RAGE-binding protein [EN-RAGE]) is a member of the S100 family of acidic

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Abbreviations used

BMMC:	Murine bone marrow–derived mast cell
CBMC:	Human cord blood–derived mast cell
EN-RAGE:	Extracellular newly identified RAGE-binding protein
EP:	Eosinophil peroxidase
LTC ₄ :	Leukotriene C ₄
MC:	Mast cell
MCP:	Monocyte chemoattractant protein
MIP:	Macrophage inflammatory protein
PMC:	Murine peritoneal mast cell
RAGE:	Receptor for advanced glycosylation end products
WT:	Wild-type

calcium-binding proteins expressed in the human, but not in the mouse or rat genomes.⁷ It comprises approximately 5% of neutrophil cytoplasmic protein and is induced in monocytes by LPS, TNF,⁸ and IL-6.⁹ S100A12 was proposed to affect MC-mediated responses by binding MC-stabilizing agents.¹⁰

S100A12 is a potent monocyte chemoattractant,^{8,11} and S100A8 might be the functional ortholog of human S100A12 in the mouse.⁷ We showed that murine S100A8 (previously called CP-10, MRP8) generated a leukocyte infiltrate with composition and kinetics similar to delayed-type hypersensitivity responses to antigen.^{12,13} S100A12 enhances neutrophil adhesion, and intravenous injection into mice stimulates neutrophil mobilization from bone marrow to inflammatory sites.¹⁴ Bovine S100A12 injection caused edema, leukocyte recruitment, and upregulation of vascular cell adhesion molecule 1 on endothelium,¹¹ effects that were partially blocked by antagonists to the receptor for advanced glycosylation end products (RAGE), which is proposed as a pan-S100 protein receptor. RAGE interacts with diverse ligands implicated in inflammation, particularly in diabetes.¹⁵ S100A12/RAGE ligation induces TNF and IL-1 β in monocytes and adhesion receptors on endothelial cells through nuclear factor κ B activation.^{11,15} S100A12 is implicated in the pathogenesis of inflammatory bowel disease,¹⁶ rheumatoid arthritis,^{8,14,17} and vascular complications of diabetes¹⁸ and plays a role in parasite defense.¹⁹

Here we show that S100A12 provoked MC degranulation and activation *in vitro* and potentiated MC responses to Fc ϵ RI cross-linking. S100A12 provoked leukocyte adhesion and extravasation in the microcirculation *in vivo*, and responses elicited *in vivo* were MC dependent. Importantly, eosinophils in lung lesions from patients with asthma expressed S100A12, and the protein level was significantly increased in sputum from patients with eosinophilic asthma compared with that seen in sputum from healthy subjects and subjects with other asthma subtypes. The S100A12-induced cytokine profile overlapped but was distinct from that induced by IgE cross-linking. The types and levels of chemokines produced indicate that

S100A12 is a novel physiologic activator of MCs that might potentiate innate and allergic responses.

METHODS

Animals

Specific pathogen-free male Sprague-Dawley rats (6–8 weeks) and BALB/c female mice (6–10 weeks) were from the Biological Resource Centre, University of New South Wales. MC-deficient *W^f/W^f* mice and MC-replete mice C57Bl6/DBA(+/+; wild-type [WT]) were from Dr P. Hart. Experiments were performed according to ethical guidelines of the National Health and Medical Research Council of Australia.

S100A12 preparation

Recombinant and native S100A12 from human neutrophils were produced and purified as previously described.⁸ Endotoxin content was less than 10 pg/10 μ g of S100A12.

MC activation *in vitro*

Tissue culture media and reagents were filtered through Zetapor 0.2- μ m membranes (Cuno, Meriden, Conn) to minimize endotoxin levels. Endotoxin levels were monitored with the chromogenic limulus amoebocyte lysate assay (Cape Cod Assoc, Wood Hole, Mass) and only used if levels were less than 10 pg/mL.

Human cord blood–derived MCs (CBMCs) were derived from human umbilical cord blood mononuclear cells, as previously described.²⁰ Nonadherent cells were transferred weekly for 9 to 10 weeks into media containing fresh cytokines. Maturity from 3 weeks was assessed weekly by means of tryptase, chymase, c-kit, and toluidine blue staining, and cells were used when more than 95% were metachromatic, c-kit high, and tryptase and chymase positive.

CBMCs (2×10^5 /100 μ L of RPMI/0.2% BSA) dispensed in duplicate into 96-well plates were incubated at 37°C for 1 hour with S100A12. For Fc ϵ RI cross-linking, CBMCs were primed with human myeloma IgE (2 μ g/mL; Chemicon International, Temecula, Calif) for 5 days in the presence of IL-4 (10 ng/mL; Endogen, Rockford, Ill) and SCF (100 ng/mL; R&D Systems, Minneapolis, Minn), washed, and stimulated with or without rabbit anti-human IgE (1 μ g/mL; ICN Pharmaceuticals, Costa Mesa, Calif) for 30 minutes, and histamine and leukotriene C₄ (LTC₄) levels were determined. For S100A12 potentiation, IgE-primed CBMCs were incubated with 0.01 to 10 μ M of S100A12 for 1 hour before activation with a predetermined suboptimal dose of anti-IgE (0.07 μ g/mL). A23187 (5 μ mol/L) was used as the positive control. Histamine was measured by means of ELISA (Immuno-Biological Laboratories, Hamburg, Germany), and levels were calculated as above. LTC₄ levels determined by means of EIA (Cayman Chemicals, Ann Arbor, Mich) were expressed as nanograms per 10^6 cells. Means $\pm$ SD of duplicate assays from at least 3 donors are given.

IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, monocyte chemoattractant protein (MCP) 1, macrophage inflammatory protein (MIP) 1 α , IFN- γ , TNF, granulocyte colony-stimulating factor, and GM-CSF in supernatants of CBMCs (2×10^5 , $n = 4$) were measured with the Bio-plex system (Bio-Rad Laboratories, Hercules, Calif), and results were analyzed with Bio-plex Manager software. Sensitivity was 2.0 pg/mL for each cytokine (upper limit, 32 ng/mL).

Activation of human lung MCs

Samples of apparently normal lung tissue from patients ($n = 2$) undergoing surgery for lung cancer in the Central Area Health Service, Sydney, Australia, were obtained with consent under ethical

guidelines. Duplicate samples of chopped parenchyma (50 mg each) in 0.5 mL of Krebs-Henseleit solution, pH 7.4, at 37°C were treated with S100A12 or 1 μ M of A23187 for 45 or 90 minutes, placed on ice, and 0.3 mL mixed with 0.3 mL HClO₄. Histamine in supernatants and total histamine in 0.5-mL supernatant mixed with lung tissue/0.8 mol/L HClO₄ boiled for 15 minutes in 1.0 mL Krebs-Henseleit solution was measured by means of ELISA.

S100A12 levels in sputum

Subjects with airway disease ($n = 21$) attending the Department of Respiratory and Sleep Medicine, John Hunter Hospital (Newcastle), comprised 13 (62%) female subjects, and 16 (76%) were atopic. These included patients with neutrophilic asthma ($n = 4$), paucigranulocytic asthma ($n = 4$), bronchiectasis ($n = 6$), and eosinophilic asthma ($n = 8$). Their mean age was 57 years (range, 21–72 years), and 9 (43%) were exsmokers who had a median of a 28-pack-year (interquartile range, 8–43 pack-years) smoking history. All patients except for 2 with eosinophilic asthma were taking inhaled corticosteroids with a median dose of 1600 μ g (interquartile range, 800–2000 μ g) beclomethasone equivalents. Subjects with airway disease had evidence of airflow obstruction, with a mean FEV₁ predicted value of $74.7\% \pm 23.4\%$ and an FEV₁/forced vital capacity of $65.3\% \pm 10.9\%$ after bronchodilator. Induced sputum from these subjects and from 10 healthy nonasthmatic subjects was collected as previously described,²¹ and S100A12 levels were quantitated by using a double-sandwich ELISA as previously detailed⁸; recombinant S100A12 standards contained the same concentration of dithiothreitol (Sputolysin, Calbiochem) as samples. Values for duplicate samples were calibrated against the standard curve, and data were expressed as means $\pm$ SD in micrograms per milliliter.

Immunohistochemistry

Formalin-fixed and paraffin-embedded human lung tissue from asthmatic patients ($n = 5$) was serially sectioned (2 μ m), and S100A12 was localized, as previously detailed.⁸ MCs were identified with mouse anti-tryptase IgG1 mAb (1.0 μ g/mL, DakoCytomation, Glostrup, Germany) captured with biotin-conjugated rabbit anti-mouse IgG (DakoCytomation). Controls were normal rabbit IgG or mouse IgG1.

Processing and immunostaining were performed as previously reported²² with mouse anti-human neutrophil elastase (1:20 vol/vol; DakoCytomation), mouse anti-human eosinophil peroxidase (EP; 1:50 vol/vol; Chemicon), mouse anti-human CD68 (1:50 vol/vol; Dako), and rabbit anti-S100A12 IgG (10 μ g/mL) to confirm S100A12 expression in eosinophils. Controls were nonimmune mouse or rabbit IgG. Elastase and EP reactions were visualized with avidin biotin complex–alkaline phosphatase and fast red (Vector Laboratories, Burlingame, Calif), S100A12, and CD68, with avidin biotin complex–horseradish peroxidase and diaminobenzidine substrate on sections pretreated with 3% H₂O₂ to block endogenous peroxidase. Counterstaining was with Mayer's hematoxylin. Magnification for visualization was $\times 200$ or $\times 400$.

Inflammation induced by S100A12 *in vivo*

The effects of S100A12 on vascular permeability were determined as previously described²³ by using Evans blue injected into the tail vein 30 minutes before challenge of one footpad with 2 μ g of S100A12 in 30 μ L of HBSS and the other with HBSS. Footpad thickness (in millimeters) was measured with an electronic caliper (Mitutoyo) at 30-minute intervals for 2 hours. For MC stabilization, mice were injected intraperitoneally with sodium cromoglycate (5 mg/kg/1 mL HBSS) 2 hours before challenge. Four mice per group were tested.

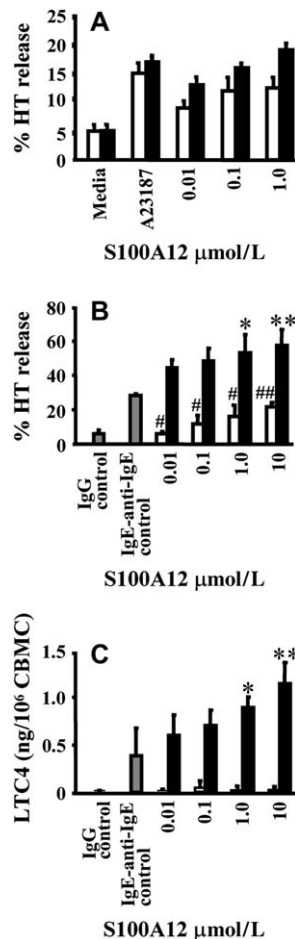


FIG 1. A, S100A12 provoked histamine (HT) release from human lung MCs. Lung tissue was incubated with S100A12 for 45 minutes (open bars) or 90 minutes (solid bars). Duplicates from tissues from 2 donors are given. B and C, IgE-primed CBMCs were incubated with S100A12 1 hour before challenge with control rabbit IgG (open bars) or anti-IgE (0.07 μ g/mL, solid bars), IgE-anti-IgE without S100A12, or control IgG (gray bars). Fig 1, B, shows histamine (HT) levels from 4 donors, and Fig 1, C, shows LTC₄ levels from 3 donors. * $P < .05$ and ** $P < .01$ compared with IgE-anti-IgE; # $P < .05$ and ## $P < .01$ compared with media control.

MC-deficient *Wf/Wf* mice ($n = 5$ per group) were reconstituted with MCs by injecting 10^7 murine bone marrow–derived mast cell (BMMC)s from congenic C57BL/6/DBA(+/+) mice through the tail vein²⁴ and challenged in the footpad with 2 μ g of S100A12 10 weeks later. MC reconstitution in skin confirmed successful engraftment.

Intravital microscopy

The effects of S100A12 on the rat mesenteric microcirculation were evaluated by means of intravital microscopy, as previously described.²⁵ A segment of midjejunum from anaesthetized and fasted rats (5 per group) was exteriorized, and the mesentery draped over a viewing pedestal was superfused (2.0 mL/min) with bicarbonate-buffered saline (37°C, pH 7.4; Po₂, 40 mm Hg). Single unbranched venules (25–45 μ m in diameter and 250 μ m in length) were selected for study. Vessels were videotaped for 7 minutes to establish baseline

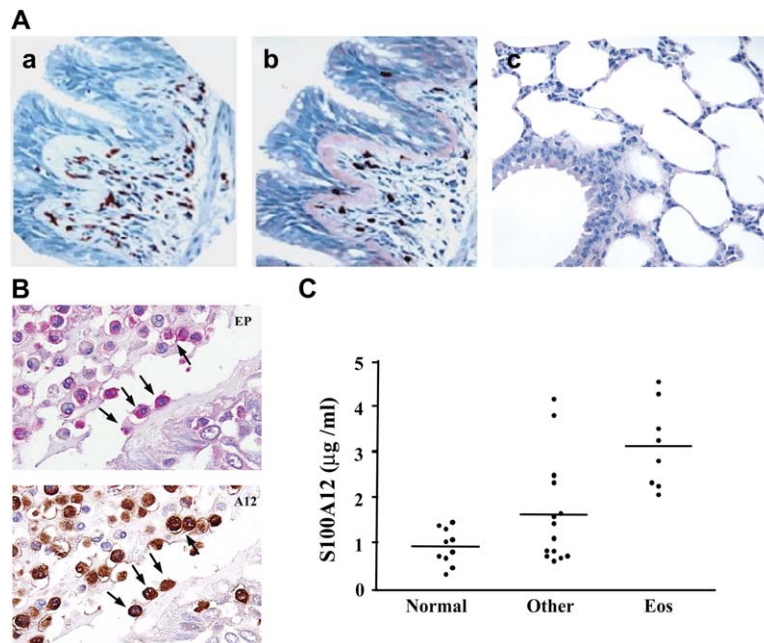


FIG 2. MCs colocalize in human asthmatic airways/bronchial tissue. **A**, S100A12-positive leukocytes in the sub-mucosal region (*a*) were in close proximity to tryptase-positive MCs (*b*) in adjacent serial sections. (*c*) No S100A12-positive cells were evident in apparently normal lung tissue. **B**, S100A12-positive eosinophils were prominent within a mucus plug in asthmatic airways. Adjacent sections show EP-positive (*red*) and S100A12-positive (*brown*) eosinophils (*arrows*). Scale bar = 20 μm. **C**, S100A12 levels in sputum from subjects with eosinophilic asthma were higher than those in healthy subjects ($P < .0001$) and subjects with other asthma subtypes ($P = .0086$).

conditions, and then S100A12 (10 μg/0.5 mL 0.9% NaCl) was injected into the cannulated femoral vein, 250 mL of S100A12 (3.6×10^{-8} mol/L) was superfused for up to 120 minutes, and the vessel was again videotaped; compound 48/80 (1.3 mmol/L) was used as the positive control. For MC stabilization, cromoglycate (5 mg/kg/2 mL HBSS) was injected intraperitoneally 2 hours before and into the femoral vein 20 minutes before S100A12. Adherent leukocytes remaining stationary for more than 30 seconds counted from videotaped images were expressed as numbers per 100-μm venule over 2 minutes of playback. Flux represents the numbers of leukocytes rolling past a defined reference point per minute. The same reference point was used throughout. Extravasated leukocytes were leukocytes outside the venule within an area of 100×200 -μm field of view.

RAGE involvement in S100A12 activation

RAGE₄₂₋₅₉ and retro-RAGE₄₂₋₅₉ (reverse sequence) were synthesized by means of manual solid-phase synthesis²⁶ and purified by means of C₁₈ preparative RP-HPLC. Fractions containing pure RAGE₄₂₋₅₉ and retro-RAGE₄₂₋₅₉ were identified by means of analytic RP-HPLC, and masses were confirmed by means of electrospray ionization mass spectrometry (2176.50 ± 1.0 d; calculated, 2177.21 d). S100A12 (5 μmol/L) was preincubated with various concentrations of RAGE₄₂₋₅₉ peptide, and histamine release from CBMCs from 3 donors performed in duplicate was determined.

CBMCs from 4 separate donors were used to detect RAGE mRNA or protein. Western blotting of cell extracts (10^6 cells/50 μL of lysis buffer) was performed as previously described⁸ by using goat anti-RAGE serum (1:2000 vol/vol, Chemicon), goat anti-RAGE IgG (0.4 μg/mL; Santa Cruz Biotechnology, Santa Cruz, Calif), or mouse anti-RAGE IgG MAb (2 μg/mL; Chemicon). Reactivity detected with horseradish peroxidase-conjugated rabbit anti-goat or goat

anti-mouse IgG (Bio-Rad), respectively, was visualized by means of enhanced chemiluminescence. For RT-PCR, total RNA (2 μg) from THP-1 monocytoid cells (express RAGE²⁷), HL60 promyelomonocytic cells differentiated to monocytes with dimethyl sulfoxide, and CBMCs from 4 donors was reverse transcribed to the first-strand cDNA, as previously described.⁸ The cDNA mix (3/20 of cDNA reaction volume) was amplified by using 10 pmol of 2 different RAGE primer pairs,^{28,29} and cDNA synthesis efficiency was monitored by amplifying glyceraldehyde-3-phosphate dehydrogenase. PCR was performed in a GeneAmp PCR System 2400 (Perkin-Elmer, Wellesley, Calif) with 2.5 U of Taq DNA polymerase. Products separated on 1.0% agarose were stained with ethidium bromide.

Statistical analysis

Data were analyzed with the standard Student *t* test and 1-way ANOVA with the Bonferroni correction for multiple comparisons where appropriate. For ELISA analysis, the Mann-Whitney test was used. Values are reported as means $\pm$ SD or SEM when indicated, and statistical significance was set at a *P* value of less than .05.

RESULTS

S100A12 provokes MC degranulation *in vitro*

Responses of MCs from various sources were evaluated to address MC heterogeneity. β-Hexosaminidase levels released in response to native or recombinant S100A12 approached those generated by an optimal dose of compound 48/80 from murine BMMC (see Fig E1 in the Online Repository at www.jacionline.org). Murine

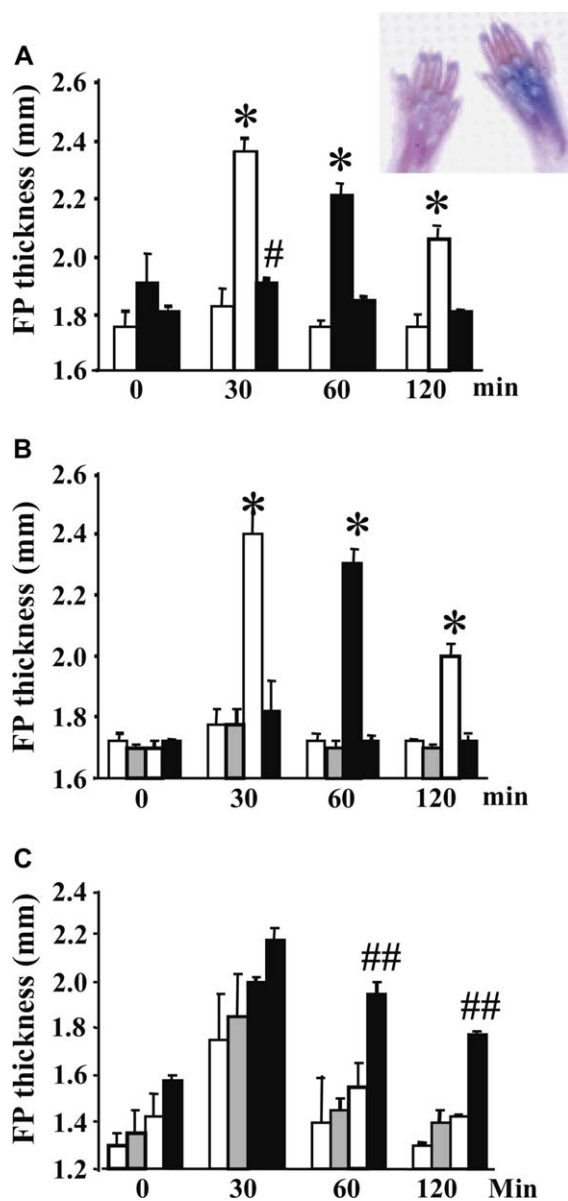


FIG 3. S100A12 activates MCs *in vivo*. **A**, Footpads (FP) injected with S100A12 (hatched bars) or vehicle (open bars) are shown; cro-moglycate pretreatment (solid bars) reduced edema. * $P < .0005$ compared with vehicle; # $P < .005$ compared with mice treated with S100A12 alone. Inset shows S100A12-induced plasma leakage in the “blue” right foot compared with the left foot (control). **B**, S100A12 did not induce edema in *Wf/Wf* mice. Footpad injection with vehicle (*Wf/Wf* mice, gray bars; WT mice, open bars) or S100A12 (*Wf/Wf* mice, solid bars; WT mice, hatched bars). # $P < .005$ compared with vehicle and with *Wf/Wf* mice injected with S100A12. **C**, *Wf/Wf* mice reconstituted with BMMCs from WT mice (solid bars) responded to S100A12 compared with vehicle (hatched bars), whereas no edema was seen in *Wf/Wf* mice in response to S100A12 (gray bars) or vehicle (open bars). ## $P < .005$ compared with unreconstituted mice.

peritoneal mast cells (PMCs) released $37.3\% \pm 1.38\%$ total histamine ($n = 3$) in response to $10 \mu\text{mol/L}$ of S100A12, about half that generated by compound 48/80 ($63.75\% \pm 20.9\%$). The morphology of S100A12-activated

BMMCs was typical of activated MCs in the process of exocytosis and discharge of granule contents (see Fig E1, C and D, in the Online Repository at www.jacionline.org).

Histamine released from MCs resident in human lung biopsy specimens (Fig 1, A) after 45 minutes was low but after 90 minutes ($10.83\% \pm 0.73\%$ with $0.1 \mu\text{mol/L}$; $14.52\% \pm 0.41\%$ with $1.0 \mu\text{mol/L}$ of S100A12) reached levels comparable with those induced by A23187 ($11.24\% \pm 0.11\%$). S100A12 provoked significant histamine release from human CBMCs; $32.6\% \pm 8.2\%$ total histamine was generated with an optimal dose of $1 \mu\text{mol/L}$ compared with a spontaneous release of $11.6\% \pm 3.2\%$ (see Fig E3 in the Online Repository at www.jacionline.org), although this was lower than levels induced by optimal FcεRI cross-linking ($90\% \pm 2.1\%$, $n = 5$) or A23187 (Fig E3).

S100A12 potentiates IgE receptor cross-linking

Sensitization of CBMCs with IgE did not influence S100A12 responses, whereas S100A12 significantly potentiated histamine release provoked by suboptimal FcεRI cross-linking. As little as $0.01 \mu\text{mol/L}$ of S100A12 increased histamine 1.6-fold (Fig 1, B; 44.6% compared with 28.1% with IgE/anti-IgE), which increased with higher S100A12 doses. FcεRI cross-linking generates LTC₄ that profoundly affects bronchial constriction in asthma and edema in allergy. S100A12 alone induced negligible LTC₄ levels (12.3 pg with $1 \mu\text{mol/L}$) compared with FcεRI cross-linking ($396 \text{ pg}/10^6 \text{ CBMCs}$), but when tested together, LTC₄ levels increased to 591 pg with $0.01 \mu\text{mol/L}$ and by 4.7-fold (1148 pg) with $10 \mu\text{mol/L}$ of S100A12 (Fig 1, C).

S100A12 in lung tissue and sputum from allergic asthmatic subjects

AS100A12 was not seen in alveolar macrophages or other cells in apparently normal lung tissue (Fig 2, A, c) apart from its expected expression in neutrophils within blood vessels. Lung tissue from asthmatic patients ($n = 5$) contained some S100A12-positive macrophages (CD68 positive) and neutrophils (elastase positive) within the alveolar and submucosal regions, often just below the epithelial basement membrane (Fig 2, A, a), where tryptase-positive MCs (Fig 2, A, b) colocalized. In specimens rich in eosinophils (EP positive), particularly within mucus plugs in the airways and adjacent lung parenchyma, S100A12 was heterogeneously expressed by eosinophils (Fig 2, B).

Sputum from patients with eosinophilic asthma had significantly higher S100A12 levels ($3.11 \pm 0.93 \mu\text{g/mL}$, $n = 8$) than normal sputum ($0.91 \pm 0.39 \mu\text{g/mL}$, $n = 10$; Fig 2, C) and sputum from patients with neutrophilic or paucigranulocytic asthma; levels in samples from 3 of 6 patients with bronchiectasis were increased. S100A12 levels in sputum from patients with eosinophilic asthma were significantly greater than in all other asthmatic patients ($1.62 \pm 1.1 \mu\text{g/mL}$), which were not significantly

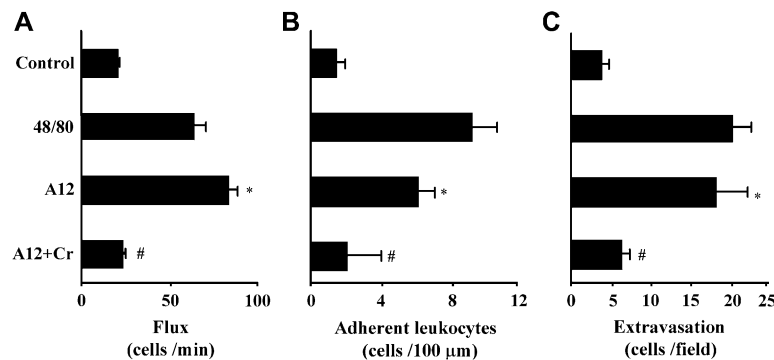


FIG 4. S100A12 provokes MC-dependent leukocyte transmigration in the mesenteric circulation. Superfusion of S100A12 (3.6×10^{-8} mol/L) or compound 48/80 (1.3 μ mol/L) significantly increased leukocyte flux (**A**), adhesion (**B**), and extravasation (**C**), as assessed by means of intravital microscopy. Cromoglycate negated responses. Results are means $\pm$ SD from groups of 5 rats. * $P < .001$ compared with buffer control; # $P < .01$ compared with S100A12 alone.

TABLE I. Cytokines produced by S100A12-stimulated CBMCs

	6 h				24 h			
	Media	A12 (1 μ mol/L)	A12 (10 μ mol/L)	Anti-IgE	Media	A12 (1 μ mol/L)	A12 (10 μ mol/L)	Anti-IgE
TNF- α	0.6 $\pm$ 0.8	97.6 $\pm$ 15.5*	206.6 $\pm$ 85.0†	36.2 $\pm$ 6.7	0.4 $\pm$ 0.5	69.8 $\pm$ 69.4*	125.0 $\pm$ 26.2†	1241.3 $\pm$ 0.2
IL-6	14.8 $\pm$ 17.3	932.4 $\pm$ 212.2†	1259.7 $\pm$ 526†	517.1 $\pm$ 37.8	11.1 $\pm$ 12.9	2770 $\pm$ 2391*‡	5217.4 $\pm$ 144†§	>32,000
IL-8	225.8 $\pm$ 206.1	1734.7 $\pm$ 1583.6*	2284.8 $\pm$ 64.5**	27534 $\pm$ 1139	145.5 $\pm$ 32.1	36700 $\pm$ 400	53,100 $\pm$ 1000	39,400 $\pm$ 400
MCP-1	70.1 $\pm$ 32.7	ND	77.3 $\pm$ 9.6	377.3 $\pm$ 108.4	54.0 $\pm$ 12.2	ND	551.5 $\pm$ 204†‡	13,044 $\pm$ 3164
MIP-1 β	143.5 $\pm$ 128.7	ND	1431.8 $\pm$ 121†	>32,000	153.9 $\pm$ 65.6	ND	5379 $\pm$ 279.8†§	>32,000
IFN- γ	0.3 $\pm$ 0.3	16.0 $\pm$ 2.1*	9.8 $\pm$ 2.1*	0	0.6 $\pm$ 0.8	27.7 $\pm$ 11.4*	22.7 $\pm$ 5.3*	71.2 $\pm$ 1.4

CBMCs were stimulated for 6 or 24 hours with S100A12 or IgE-anti-IgE, as detailed in the Methods section. Cytokine levels (in picograms per milliliter) are expressed as means $\pm$ SD of duplicates from 4 experiments (4 donors).

ND, Not done.

* $P < .05$ and † $P < .01$ compared with media control.

‡ $P < .05$ and § $P < .01$ compared with S100A12 at 6 hours.

higher than levels in sputum from healthy subjects ($P = .095$).

S100A12 activates MCs *in vivo*

Because histamine and serotonin released by activated MCs increase vascular permeability, the ability of S100A12 to induce edema in mice was tested. Responses were maximal after 30 to 60 minutes, and footpads remained significantly more swollen than those treated with vehicle control over 2 hours (Fig 3, A); 0.5 μ g of S100A12 was active (not shown), 2 μ g was optimal, and 4 μ g was marginally enhanced ($P = .052$ between 2 μ g and 4 μ g of S100A12). Degranulating MCs were evident in S100A12-injected footpads (see Fig E2, A, in the Online Repository at www.jacionline.org); plasma leakage was confirmed by “blueing” of S100A12-injected footpads (Fig 3, A). Mac-1–positive macrophages increased (25.9 ± 2.2 per field compared with 5.8 ± 1.2 per field in control animals; 3 fields, $n = 4$ mice) 8 hours after S100A12 injection.

S100A12-induced edema was not apparent in mice in which MCs were stabilized with cromoglycate (Fig 3, A) or in *Wf/Wf* mice, whereas responses of WT (+/+) mice were similar to those of BALB/c mice (Fig 3, B). Leukocyte

infiltration into footpads of WT mice (Fig E2, A) was markedly greater than in *Wf/Wf* mice (see Fig E2, F, in the Online Repository at www.jacionline.org). Footpad edema in MC-reconstituted *Wf/Wf* mice 60 minutes after S100A12 challenge was significantly greater than in *Wf/Wf* mice (Fig 3, C), supporting MC involvement.

MC activation promotes inflammatory changes in the mesenteric microcirculation that can be assessed with intravital microscopy.³⁰ Superfusion of S100A12 for 1 hour significantly increased the flux of rolling leukocytes approximately 4-fold (Fig 4, A), and this was accompanied by an approximately 5-fold increase in numbers of adherent leukocytes (Fig 4, B) and extravasated cells (Fig 4, C); compound 48/80 provoked changes of similar magnitude. S100A12 did not alter vessel diameter (control, 33 ± 1 μ m; after superfusion with S100A12, 33 ± 1 μ m) or blood flow because shear rates after S100A12 superfusion (503 ± 21 /s) were similar to basal levels (498 ± 18 /s). S100A12-provoked responses in cromoglycate-treated rats approximated basal levels, confirming that MC activation mediated leukocyte transmigration. Likewise, the 3-fold increase in leukocyte numbers 8 hours after intraperitoneal injection of S100A12 (predominantly monocytes and neutrophils⁸)

was baseline in MC-stabilized mice (see Table E1 in the Online Repository at www.jacionline.org).

The reduced activity in animals in which MCs were stabilized with cromoglycate was not due to cromoglycate binding A12. Preincubation of S100A12 with tranilast or cromoglycate did not alter S100A12-provoked responses, whereas CBMCs pretreated with these stabilizing agents did not respond to S100A12 (see Fig E4 in the Online Repository at www.jacionline.org).

S100A12 induces proinflammatory cytokines

Upregulation of specific sets of MC genes might determine responses in allergy and host defense.^{1,2} There was a concentration and time-dependent induction of relatively high levels of IL-6, IL-8, MCP-1, and MIP-1 β (Table I) from CBMCs; IL-8 levels induced by 1 μ mol/L of S100A12 were similar to those induced by amounts of IgE-anti-IgE causing maximal degranulation. Cycloheximide (predetermined optimum, 0.25 μ g/mL) inhibited IL-6 and IL-8 induction by 60% to 90%, confirming a requirement for new protein synthesis. MIP-1 β levels increased 35-fold in 24 hours; MCP-1 levels were lower but approximately 10-fold greater than basal levels within 24 hours. TNF levels increased approximately 370-fold within 6 hours but decreased at 24 hours to levels some 10-fold less than those induced by Fc ϵ RI cross-linking, suggesting that S100A12 provoked TNF release of preformed stores. Both activators induced low amounts of IFN- γ . Only Fc ϵ RI cross-linking generated IL-1 β , IL-4, IL-5, granulocyte colony-stimulating factor, and GM-CSF (not shown).

Does RAGE mediate S100A12 activation of MCs?

RAGE is the putative S100A12 receptor,¹¹ and a synthetic peptide (RAGE₄₂₋₅₉) corresponding to the ligand-binding site of RAGE³¹ significantly suppressed histamine release, with RAGE₄₂₋₅₉/S100A12 molar ratios of 2:1 (Fig 5, A); the reverse control peptide (10 μ mol/L) weakly enhanced responses. RAGE₄₂₋₅₉ (10 μ mol/L) also reduced IL-8 or IL-6 levels produced by S100A12 (1 μ mol/L)-activated CBMCs by approximately 40% (not shown). However, S100A12-induced footpad edema was not affected by RAGE₄₂₋₅₉ administered *in vivo* (not shown).

RT-PCR with RAGE-specific primers²⁸ detected RAGE mRNA transcripts in THP-1 cells (Fig 5, B, lane 2) but not in CBMCs (one of 4 shown in lane 1). Another primer set²⁹ confirmed the absence of RAGE transcripts in CBMCs (not shown). Western blotting with 2 polyclonal antibodies (Chemicon and Santa Cruz, not shown) and anti-RAGE mAb (Chemicon; Fig 5, C) failed to detect RAGE in 4 different CBMC lysates (Fig 5, C, 2 shown in lanes 3 and 4). All antibodies reacted with components of the expected mass of RAGE (approximately 45 kd) in THP-1 lysates (lane 2). RAGE was not detected in HL60 promyelomonocytic cells, although, like THP-1 cells, higher mass components were obvious with all antibodies.

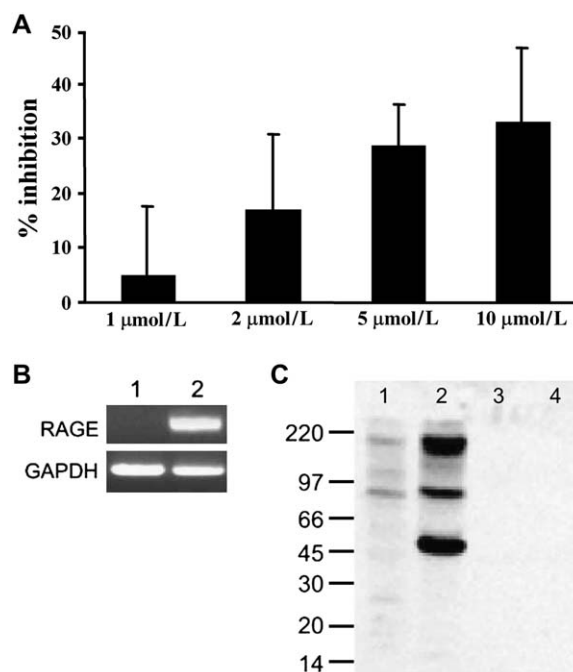


FIG 5. A, Histamine release provoked by S100A12 was partially suppressed by 1–10 μ mol/L RAGE₄₂₋₅₉. * P = .03 compared with S100A12. B, RAGE mRNA was not detected in CBMCs (lane 1); THP-1 cells (lane 2) expressed the gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is shown. C, Western blotting for RAGE in lysates of HL60 cells (lane 1), THP-1 cells (lane 2), and CBMCs from 2 donors (lanes 3 and 4). Molecular weight markers are indicated.

DISCUSSION

Recent studies support pivotal roles for MCs in innate immunity^{1,4,6,32} and autoimmunity^{2,33} in addition to their important contributions to allergy and asthma. Activation through the high-affinity IgE receptor causes degranulation and secretion of numerous mediators and induction of genes promoting allergic responses. In contrast, LPS, cytokines, and chemokines that activate MCs do not generally cause degranulation.^{1,34,35} S100A12 induced morphologic changes in murine BMMCs in keeping with activation, and various types of MCs responded *in vitro*. Taking into account the functional heterogeneity of mucosal and connective tissue MCs,³⁶ it is noteworthy that all MC types tested were responsive. Although levels of histamine released from CBMCs were relatively low compared with Fc ϵ RI cross-linking, they were significant, even at S100A12 concentrations as low as 10 nM. Immediate-early allergic reactions are also associated with LTC₄ production, but S100A12 induced little LTC₄, although it significantly increased responses provoked by Fc ϵ RI cross-linking. Increases in LTC₄ levels (4.7-fold) were comparable with those reported for potentiation by cytokines involved in allergic inflammation (IL-3, 4-fold; IL-5, 6-fold).³⁷ Colocalization of S100A12-positive leukocytes with MCs in the airways and in

eosinophils in biopsy specimens from patients with asthma strongly implicates S100A12 in MC activation mediated by IgE and antigen. S100A12 is constitutively expressed in neutrophils and might be particularly relevant in acute exacerbations of asthma common in viral infections associated with neutrophilic inflammation that can cause more severe clinical disease.³⁸ S100A12 expression in neutrophils and macrophages at sites of inflammation was expected, whereas S100A12 in blood eosinophils is not reported, and we found no constitutive expression in enriched populations (not shown). Here we show intracytoplasmic S100A12 in eosinophils in lung biopsy specimens from patients with allergic asthma, and levels in sputum from patients with eosinophilic asthma were significantly increased and greater than those in samples from patients with airway neutrophilia. Thus S100A12 could contribute to adverse outcomes in asthma by virtue of its potentiating effects on MC activation by allergens.

S100A12 is a chemoattractant for monocytes *in vitro*,^{8,11} and intraperitoneal injection into mice recruited monocytes, neutrophils, and some lymphocytes over 24 hours.⁸ Results reported here strongly implicate MC activation in this response. S100A12 caused pronounced edema and vascular permeability changes that were MC dependent. MC degranulation is sufficient to cause leukocyte rolling that persists for hours and is P-selectin dependent and largely histamine mediated, leading to adhesion and transmigration in the microcirculation.^{30,39} Platelet-activating factor³⁰ and chemokines, such as IL-8, stored in intracellular granules might mediate this response.⁴⁰ Here we show that the rapid MC-dependent leukocyte transmigration provoked by S100A12 in the rat mesenteric microcirculation was of similar magnitude to that reported for compound 48/80.³⁰ The more prolonged response⁸ could be mediated by chemokines produced by S100A12-activated MCs.

Although MC products are detrimental to the host in allergy and asthma, they are critical in initiating host defense to parasitic and bacterial infections^{4,6} and in cell-mediated immune responses⁴¹ mediating chronic diseases, such as RA, multiple sclerosis, and inflammatory bowel disease. After initial activation of MCs by microbial products, the rapid recruitment of neutrophils from the circulation^{4,6} that determines the outcome of an effective host response⁴² could generate a ready source of constitutive S100A12 that might promulgate responses. In addition to histamine, TNF from activated MCs can promote neutrophil influx,^{4,6} and because TNF upregulates S100A12 in monocytes/macrophages,⁸ this could represent an important feedback loop for MC activation in chronic inflammation. In addition to preformed cytokines, specific gene expression profiles, such as those mediated by Toll-like receptor 4 and FcεRI, suggest tailored pathogen-specific and antigen-specific MC responses.³⁵ S100A12 induced high amounts of IL-6 and chemokines important for acute-phase responses and leukocyte recruitment (Table I), whereas certain cytokines induced by LPS, FcεRI cross-linking, or both (eg, GM-CSF, IL-1β, IL-5, IL-4, IL-10,

and IL-13)³⁵ that favor T_H2-type responses were not induced.

Bovine S100A12 activation of various cell types is, at least partially, suppressed by RAGE antagonists.¹¹ Although S100A12-RAGE ligation might enhance adhesive interactions of transmigrating leukocytes,¹¹ the almost immediate response observed within the microcirculation after S100A12 superfusion was most likely mediated by histamine.³⁰ The partial suppression of histamine release from S100A12-stimulated CBMCs *in vitro* by the RAGE peptide antagonist implicated a RAGE-mediated mechanism, although no RAGE mRNA or protein was detected in CBMCs. RAGE is the putative S100 receptor, but others report RAGE-independent pathways.⁴³ Importantly, studies with RAGE-null mice show that RAGE plays little role in adaptive immune responses.⁴⁴ Although soluble RAGE reduced delayed-type hypersensitivity responses proposed to be mediated by S100A12,¹¹ it was similarly suppressive in RAGE-null mice, and undefined effects, other than simply blocking cell-surface RAGE function, were proposed.⁴⁴ The findings presented here indicate an alternate receptor for S100A12 on MCs.

Another key function of S100A12 is its antifilarial activity.¹⁹ MCs and eosinophils mediate parasite defense, and it is tempting to speculate that S100A12 might contribute to nonclassical MC activation in these conditions. We propose that the inflammatory responses provoked by S100A12 occur principally through MC activation. It might mediate responses in allergy, in host defense against infection, and in chronic cellular activation in inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, in which it is highly expressed.

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