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Damage-Associated Molecular Pattern S100A9 Increases Bactericidal Activity of Human Neutrophils by Enhancing Phagocytosis

Jean-Christophe Simard,* Marie-Michelle Simon,* Philippe A. Tessier,†‡ and Denis Girard*

The damage-associated molecular-pattern S100A9 is found at inflammatory sites in infections and various autoimmune diseases. It is released at very high concentrations in the extracellular milieu by activated neutrophils and monocytes in response to various agents. This proinflammatory protein is found in infected mucosae and tissue abscesses where it acts notably as a potent neutrophil activator. In this study, we examined the role of S100A9 in the control of infections. S100A9 was found to increase human neutrophil bactericidal activity toward Escherichia coli. Although S100A9 induced the accumulation of reactive oxygen species over time through the activation of NADPH oxidase, its antimicrobial activity was mediated mainly by enhancing the efficiency of neutrophil phagocytosis. Interestingly, S100A9 did not act by increasing cell surface expression of CD16, CD32, or CD64 in neutrophils, indicating that its biological effect in FcR-mediated phagocytosis is independent of upregulation of FcγR levels. However, S100A9-induced phagocytic activity required the phosphorylation of Erk1/2, Akt, and Syk. Taken together, our results demonstrate that S100A9 stimulates neutrophil microbicidal activity by promoting phagocytosis. The Journal of Immunology, 2011, 186: 000–000.
accumulation over time through NADPH oxidase activation, that damage-associated molecular pattern protein mainly mediated its effect by increasing the rate of neutrophil phagocytosis via the activation of Syk, PI3K/Akt, and Erk1/2. These results further support a crucial role for S100A9 in the control of microbial infection.

Materials and Methods

Reagents

Human recombinant S100A8, S100A9, and S100A12 were produced as previously described (20, 30) and found to contain <1 pg endotoxin/μg protein. PD98059, SB203580, piceatannol, PMA, and superoxide dismutase (bovine erythrocytes) were purchased from Sigma-Aldrich (St. Louis, MO). JNK inhibitor II and U0126 were purchased from Calbiochem (San Diego, CA). Wortmannin and Syk inhibitor (Syki) II were obtained from EMD Biosciences (San Diego, CA). Mouse IgG1 isotype-FITC, mouse anti-human CD64-FITC, mouse anti-human CD32-FITC, and mouse anti-human CD64-ITC were all obtained from BD Biosciences (San Diego, CA). SRBCs were purchased from Qoolab (Montreal, Quebec, Canada). Luria-Bertani broth (Lennox) and Luria-Bertani agar (Lennox) were obtained from BD Difco (Franklin Lakes, NJ). Fluoresbrite YG carboxylate microspheres (1.75 μm) were bought from Polyscience (Warrington, PA). Anti-total Akt clone (C-20) and rabbit anti-phospho-specific Ab directed against tyrosine residues were purchased from Cell Signaling Technology (Danvers, MA). Anti–phospho-specific Erk1/2 and anti-phosphoserine Abs were purchased from BioSource International (Camarillo, CA). Rabbit polyclonal Erk1/2 and anti–phospho-specific tryosine Abs were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal Syk Ab (clone 4D10), as well as goat polyclonal anti-p47phox (C-20) and rabbit polyclonal anti-p47phox (H-195) Abs, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), Mitotox Red mitochondrial superoxide indicator, Escherichia coli (K-12 strain), Alexa Fluor 488 conjugate, and phallolidin-Alexa Fluor 568 were obtained from Invitrogen/ Molecular Probes (Camarillo, CA). CaCl2 was purchased from Fisher Scientific (Fair Lawn, NJ). Cytochrome c (horse heart) was obtained from ICN Biomedicals (Aurora, OH). GM-CSF was obtained from PeproTech (Rocky Hill, NJ). E. coli MG1655 strain was a gift from Prof. Eric Déziel (Institut National de la Recherche Scientifique–Institut Armand-Frappier, Laval, Quebec, Canada).

Neutrophil isolation

Neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Hypaque cushion (Pharmacia Biotech, Quebec, Canada) as described previously (22). Blood donations were obtained from informed, consenting individuals according to institutionally approved procedures. Cell viability was monitored by trypan blue exclusion and found to be consistently >98%. Cell purity (>98%) was monitored by cytometry from cytocentrifuged preparations treated with Wright-Giemsa stain (Fisher Scientific, Ottawa, Ontario, Canada). Cell viability was systematically evaluated before and after each treatment and was always >98%. Neutrophils were re-suspended at 10 × 106 cells/ml in HBSS (1×) unless specified.

Phagocytosis of SRBCs

SRBCs were opsonized with a 1:200 dilution of a rabbit IgG anti-SRBC Ab (Sigma-Aldrich) for 45 min at 37°C as published (31). Neutrophils were treated 30 min with the indicated agonists and then incubated in a 1:5 ratio of opsonized SRBCs for 30 min at 37°C. In some experiments, cells were treated with specific inhibitors as indicated. Cells were washed twice after incubation with inhibitors. After incubation with SRBCs, the samples were centrifuged at 200 × g for 10 min at 4°C. Supernatants were discarded and an osmotic shock was performed on the pellets by resuspending the cells with 400 μl H2O for 15 s, followed by the addition of 4.5 ml PBS. The samples were then washed twice and the final pellets were suspended in 100 μl PBS. Phagocytosis is expressed as the percentage of neutrophils having ingested at least one opsonized SRBC or neutrophils having ingested two or more opsonized SRBCs (phagocytosis index). GM-CSF (65 ng/ml) was used as a positive control (32).

Phagocytosis of latex microspheres or Alexa Fluor 488-labeled E. coli

Phagocytosis was assessed by flow cytometry using FITC-labeled latex microspheres or Alexa Fluor 488 conjugate E. coli. Cells were stimulated with the indicated agonists at 37°C for 30 min before incubation with latex beads or serum-opsonized E. coli. Cells were then centrifuged and supernatant was discarded. Beads or bacteria (10:1 neutrophil) were diluted in HBSS and were then added to neutrophils. After 30 min, latex beads or bacteria that were not uptaken by the cells were washed twice with PBS and removed by centrifugation onto a 4.5-ml gradient of RPMI 1640 medium containing 5% BSA. Latex beads or bacteria remaining at the surface were then removed and cells located in the pellet were quenched with trypan blue. After three washes, the cell viability was monitored. Cell phagocytosis was monitored by flow cytometry at 525 nm. A negative control was performed in parallel by incubating cells with latex beads or bacteria at 4°C instead of 37°C. Phagocytosis of latex microspheres and Alexa Fluor 488 conjugate E. coli were expressed as the difference in the mean fluorescence intensity (ΔMFI) measured between 37°C (uptake at 37°C) and 4°C (uptake at 4°C) (33).

Cell surface expression of CD16, CD32, and CD64

Cell surface expression of CD16, CD32, and CD64 was monitored by flow cytometry. Neutrophils were resuspended in a final volume of 100 μl following addition of agonists. After stimulation, the cells were centrifuged and washed twice with cold PBS. Non-specific binding of Abs was prevented by incubating cells with PBS plus 20% decompemented human autologous serum for 30 min on ice. After several washes with PBS, the cells were incubated with FITC-coupled Abs (CD16, CD32, CD64, or IgG isotype) for 30 min on ice. After three washes, the cells were resuspended in PBS for FACS analysis on a BD FACScan apparatus (BD Biosciences).

Immunoprecipitation

Polymorphonuclear leukocytes (7 × 106 cells/condition) were treated with the indicated agonists for the indicated period of time at 37°C, centrifuged, and lysed in non-denaturing cold lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1% Triton X-100, 0.01% SDS, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml trypsin inhibitor, aprotinin, leupeptin, and pepstatin) for 1 h on ice and sonicated three times for 20 s. The lysates were preincubated with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). After 1 h, samples were centrifuged to remove Sepharose beads and then incubated with 2 μg/ml goat anti-human p47phox at 4°C with gentle agitation overnight. Protein G-Sepharose was then added for 4 h with gentle agitation at 4°C. The solid matrix was collected and washed three times with lysis buffer before adding an equivalent volume of sample buffer. The samples were then boiled at 100°C for 10 min. Immunoprecipitates were electrophoresed on an SDS-polyacrylamide gel, followed by Western blot analysis.

Western blot analysis

Neutrophils (40 × 106 cells/ml in RPMI 1640 supplemented with 25 mM HEPES, 100 μM penicillin, and 100 μg/ml streptomycin) were stimulated with PMA (10−7 M) for 5 min, GM-CSF (65 ng/ml) for 10 min, and with S100A9 (40 μg/ml) or the diluent HBSS for 1–60 min at 37°C. In some experiments, cells were preincubated for 30 min at 37°C with the following inhibitors: wortmannin (10 μM), piceatannol (30 μM), Syki II (1 μM), PD98059 (5 μM), or U0126 (5 μM). At the end of the incubation period, the cells were lysed in 4× Laemmli’s sample buffer (0.25 M Tris-HCl [pH 6.8], 8% SDS, 40% glycerol, and 2% 2-ME), and aliquots of extracts corresponding to 1 × 105 cells were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes for the detection of p-Syk, p-Akt, Akt, p-Erk1/2, Erk1/2, and p-Tyr. For detecting the phosphorylated form of p47phox immunoprecipitates were electroblotted onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in TBS-Tween containing 5% nonfat dry milk or 3% BSA. After washing, the anti–phospho-specific Syk, Erk1/2, and Akt Abs were added at a final dilution of 1:1000 in TBS-Tween 0.15% containing 3% BSA. The anti–phospho-specific Ab directed against tyrosine residues and the Ab against total Syk were added at a final dilution of 1:750. A dilution of 1:1000 in 3% BSA was used for total Akt Ab. The membranes were kept overnight at 4°C, then washed with TBS-Tween and incubated for 1 h at room temperature with a goat anti-rabbit IgG HRP secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:20,000 in TBS-Tween plus 5% nonfat dry milk, or a goat anti-mouse IgG HRP secondary Ab (Jackson ImmunoResearch Laboratories) diluted 1:20,000 in TBS-Tween plus 5% nonfat dry milk. Protein expression was revealed using an ECL Western blot analysis detection system (Amersham Biosciences). Membranes were stripped and reprobed to confirm equal loading of proteins.
Preparation of bacteria

Bacteria were prepared essentially as previously described (34), with few modifications. Briefly, E. coli MG1655 was grown overnight in a shaking incubator at 37°C. Bacteria were centrifuged at 1000 × g for 5 min and washed twice with PBS. The final pellet was resuspended in HBSS and the concentration of bacteria was calculated by measuring the OD at 550 nm using an established standard curve. Bacteria were opsonized by suspending 1 × 10^7 CFU in 1 ml HBSS containing 10% autologous serum and by rotating the solution end over end for 20 min at 37°C.

One-step and two-step killing assay

To measure neutrophil bactericidal activity, a one-step assay was used in which neutrophils were not separated from uningested bacteria (34). Neutrophils (3 × 10^6 cells) were stimulated with 40 μg/ml S100A8, S100A9, S100A12, or the equivalent volume of the diluent (HBSS) for 30 min at 37°C. Following phagocytosis of Alexa Fluor 488-labeled E. coli (1 × 10^7 cells/ml) and incubated for various periods of time at 37°C. Fifty-microliter aliquots were removed at 10, 20, and 30 min and diluted into 2.45 ml H2O with one drop of 1 mM NaOH (pH 11). Neutrophils were lysed for 5–10 min at room temperature, then vortexed vigorously for 10 s to disperse bacteria. Each sample was diluted further into water (pH 11) and spread on Luria-Bertani agar plates at 5 cm/plate in duplicate. Plates were incubated overnight at 37°C and the numbers of colonies formed were counted. Results were expressed as percentage of survival. This killing assay provided a composite measure of both phagocytosis and killing. For the two-step assay, 50-μl samples were taken from 10, 20, and 30 min and diluted with 950 μl ice-cold PBS to stop neutrophil activity. Samples were centrifuged at 100 × g for 5 min at 4°C. Supernatants were collected and pellets were resuspended in ice-cold PBS while pooling the supernatants (unopsonized bacteria). The pellets were then resuspended in 2.5 ml H2O (pH 11). Neutrophils were lysed for 10 min and then vortexed to disperse bacteria (ingested bacteria). Each sample was diluted further into water. Pellets and supernatants were spread separately on Luria-Bertani agar plates at ∼100–150 CFU per plate (i.e., two plates were used for each sample). Plates were incubated overnight at 37°C and the number of colonies formed was counted. Colony counts were converted to original bacterial densities by multiplying with the appropriate dilution factor. Growth adjustment, phagocytosis rate constant (Kp), and killing rate constant (Kb) values were calculated according to the formula described by Green et al. (34).

Detection of intracellular and mitochondrial ROS

Cells were resuspended in HBSS containing 10 μM CM-H2DCFDA or 10 μM mitochondrial superoxide indicator MitoSOX at 1 × 10^6 cells/ml for 15 min at 37°C. Cells were then washed twice with PBS before being incubated in the presence or absence of S100A8 (40 μg/ml) for the indicated period of time. PMA (10−7 M) was used as a positive control. For some experiments, S100A9-stimulated cells were incubated with opsonized SRBCs as phagocytic stimuli, and ROS production was measured for the indicated periods of time. Fluorescence was recorded using a FACScan. ROS production was expressed as MFI.

Detection of extracellular O2•−

Superoxide production was monitored by the reduction of ferricytochrome c, as previously reported (35, 36) with few modifications. Briefly, neutrophils (1 × 10^6 cells/ml) were suspended in HBSS (supplemented with 1.6 mM CaCl2) with or without 10 μM superoxide dismutase with 150 μM ferricytochrome c for 5–180 min at 37°C in the presence of 40 μg/ml S100A8, S100A9, S100A12, buffer, diluent, or PMA (10−7 M). The reduction of ferricytochrome c was monitored at 550 nm, and the concentration of superoxide anions (O2•−) produced was calculated from the difference between corresponding wells either with or without superoxide dismutase using a molar coefficient extinction of 21.11 mol−1 cm−1.

Immunofluorescence microscopy

Following phagocytosis of Alexa Fluor 488-labeled E. coli, as described before, cells were washed twice in ice-cold PBS and then cytocentrifuged on glass coverslips (Fisher Scientific). Cells were fixed and permeabilized in 3% paraformaldehyde plus 0.1% digitonin at room temperature for 20 min. After three washes, cells were incubated with 2 U phallolidin-Alexa Fluor 568 at 37°C for 30 min to detect F-actin filaments. After three washes, coverslips were mounted with Vectashield plus DAPI (Vector Laboratories, Burlington, Ontario, Canada). Fluorescent-labeled cells were captured from high-power field (×400) and observed with a Leica microscope equipped with an eob 100 dc epifluorescent condenser. Images were taken with a Cooke Sensicam high-performance camera (Applied Scientific Instrumentation) coupled to the Image-Pro Plus program (version 4.0; Media Cybernetics).

Statistical Analysis

Experimental data are expressed as means ± SEM. Repeated-measures ANOVA (Dunnett multiple-comparison test) were performed using GraphPad Prism (version 5.01). Differences were considered statistically significant as follows: *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.005 versus buffer or the appropriate diluent. Densitometric analyses were performed using Quantity One, version 4.6.6 (Bio-Rad, Hercules, CA).

Results

S100A9 induces ROS production through NADPH oxidase activation

Neutrophils are key components of innate immunity against bacterial infections. In addition to sensing invading pathogens, neutrophils have developed efficient mechanisms for extracellular killing and for processing of phagocytosed bacteria. One of the key mechanisms involved in bacterial killing is the production of ROS, which can occur through NADPH oxidase activation or triggered by an alteration of the mitochondria transmembrane potential in response to diverse stimuli (1). Because S100A9 is secreted during infections and is a potent neutrophil agonist, we investigated its effect on neutrophil bactericidal activity through the production of ROS. As shown in Fig. 1, S100A9 induced a significant intracellular accumulation of ROS over time (Fig. 1A), but little or no accumulation of O2•− in the extracellular medium (Fig. 1B). This contrasts with the rapid burst of ROS detected when neutrophils were stimulated with PMA, which occurred both in the intracellular and extracellular compartments (Fig. 1A, 1B). Unlike S100A9, S100A8 or S100A12 had no effect on ROS production even when neutrophils were stimulated for longer periods of time (Fig. 1B, inset). Because S100A8/A9 was previously found to induce mitochondrial ROS production in cancer cells (37), we then investigated the origin of ROS in S100A9-induced neutrophils. Based on our results, S100A9, as well as S100A8 and S100A12, do not induce damage in mitochondria since no O2•− has been detected in S100A9-stimulated neutrophils compared with PMA-treated cells (Fig. 1C). We next investigated whether the observed ROS production in S100A9-primed neutrophils was from the activation of NADPH oxidase. For this purpose, we measured the phosphorylation of p47phox which is the initial step in the activation of the enzyme. We demonstrated in this case that signaling cascade triggers by S100A9 led to phosphorylation of p47phox, suggesting an activation of NADPH oxidase (Fig. 1D). Uptake of phagocytic stimuli often triggers the activation of NADPH oxidase and ROS production to kill pathogens. According to our previous data, we were interested to know whether S100A9-primed neutrophils can further produce ROS in response to phagocytic stimuli. Interestingly, S100A9-primed neutrophils have an increased capacity to generate ROS in response to opsonized SRBCs (Fig. 1E), suggesting that the bactericidal activity of those cells is more powerful.

S100A9 increases neutrophil bactericidal activity

ROS production is associated with neutrophil bactericidal activity (38, 39), and patients with deficiency in NADPH oxidase components suffer from repeated bacterial infections (40). Because we demonstrated that S100A9 induces an accumulation of intracel-
lular ROS and increases the oxidative burst in response to a phagocytic stimulus, we next investigated the possibility that it could potentiate neutrophil bactericidal activity. As illustrated in Fig. 2, S100A9-primed neutrophils showed an enhanced capacity to kill \textit{E. coli} as early as 10 min after incubation with bacteria, and this effect was sustained over time. Again, this effect was restricted to S100A9, as other S100 proteins failed to potentiate neutrophil bacterial killing (Fig. 2). Because this assay does not discriminate between phagocytosis and killing, we next proceeded to a two-step assay, allowing the distinction between intracellular killing (phagocytosis) and extracellular killing. As shown in Fig. 3, cells treated with S100A9 showed more intracellular bacteria after 10 min as compared with control. Intracellular and extracellular bacteria analyses allowed the determination of $K_p$ and $K_k$. Interestingly, S100A9 increased the $K_p$ of neutrophils, but had no effect on their $K_k$ (Fig. 3B). The concentration of S100A8, S100A9, and S100A12 used in our study had no effect on \textit{E. coli} growth (data not shown). This is in agreement with a previous study showing that higher concentrations of S100A8/A9 were required to inhibit \textit{E. coli} growth (41). Because we previously demonstrated that S100A9 induced the degranulation of human neutrophils mainly through activation of the MAPK p38 (22) and that released granules can have antimicrobial activities, we tested the involvement of this pathway in S100A9-induced extracellular killing. Interestingly, inhibition experiments revealed that this pathway is not involved in overall S100A9-treated neutrophil bactericidal activity (Supplemental Fig. 1).

\textbf{S100A9 increases FcR-mediated phagocytosis}

Because exposure to S100A9 increased $K_p$ in neutrophils, we then investigated how S100A9 modulates this process. Phagocytosis in neutrophils and macrophages generally occurs via two major distinct mechanisms, namely complement receptor-dependent and Fc receptor-dependent pathways (42). Accordingly, we first examined the effect of S100A9 pretreatment on FcR-mediated phagocytosis through the uptake of IgG-opsonized SRBCs (Fig. 4). Neutrophils pretreated with S100A9 showed an increased capacity to uptake IgG-tethered SRBCs as early as 10 min after incubation with antibody-opsonized bacteria, and this effect was sustained over time. Again, this effect was restricted to S100A9, as other S100 proteins failed to potentiate phagocytic activity (Fig. 4).

\textbf{FIGURE 1.} S100A9 induces intracellular ROS accumulation over time via NADPH oxidase activation in human neutrophils. Production of reactive oxygen species was assessed by (A) oxidation of carboxy-H2DCFDA, (B, E) reduction of ferrocytochrome c, and (C) oxidation of mitochondrial superoxide indicator, as described in Materials and Methods. Cells were incubated in the presence of buffer (Ctrl), diluent (0.1% DMSO), or 40 \mu g/ml S100A8, S100A9, or S100A12, or 10^{-7} M PMA for 5–180 min (A, inset in B and C) or 30 min (B). Results are from one experiment representative of four others (A, inset in B and C). NADPH oxidase activation was determined based on p47^{phox} phosphorylation by immunoprecipitation (IP) of p47^{phox} followed by immunoblotting with an anti-phosphoserine Ab, as described in Materials and Methods. Cells were treated in the presence of buffer (Ctrl), 40 \mu g/ml S100A9 for 60 min, or 10^{-7} M PMA for 5 min. E. Cells were treated with buffer (Ctrl) or S100A9 before being stimulated with opsonized SRBCs. Data represent the mean ± SEM of at least three experiments performed on cells from different donors (B, D, E).

\textbf{FIGURE 2.} S100A9 increases human neutrophil bactericidal activity. Killing of \textit{E. coli} was evaluated by a one-step assay, as described in Materials and Methods. Cells were incubated in the presence of buffer (Ctrl) or 40 \mu g/ml S100A9, S100A8, or S100A12 for 10–30 min before incubation with \textit{E. coli}. Data represent the mean ± SEM of four experiments performed on cells from different donors.

\textbf{FIGURE 3.} S100A9 increases FcR-mediated phagocytosis. Because exposure to S100A9 increased $K_p$ in neutrophils, we then investigated how S100A9 modulates this process. Phagocytosis in neutrophils and macrophages generally occurs via two major distinct mechanisms, namely complement receptor-dependent and Fc receptor-dependent pathways (42). Accordingly, we first examined the effect of S100A9 pretreatment on FcR-mediated phagocytosis through the uptake of IgG-opsonized SRBCs (Fig. 4). Neutrophils pretreated with S100A9 showed an increased capacity to uptake IgG-tethered SRBCs as early as 10 min after incubation with antibody-opsonized bacteria, and this effect was sustained over time. Again, this effect was restricted to S100A9, as other S100 proteins failed to potentiate phagocytic activity (Fig. 4).
Materials and Methods

Materials and Methods. Kp and Ks were evaluated using bacterial concentrations from the two-step assay (B). Cells were treated with buffer (Ctrl) or 40 μg/ml S100A9 for 30 min before incubation with E. coli. Data represent the mean ± SEM of three experiments performed with cells from different donors.

4) Treatment with S100A9 increased not only the phagocytosis rate (one or more SRBCs) (Fig. 4A), but also the phagocytosis index (three or more SRBCs/cell) (Fig. 4B). Interestingly, the effect of S100A9 was stronger than the positive control, GM-CSF (32, 43). Although concentrations of 40–100 μg/ml S100A9 were found to be optimal, concentrations as low as 1 μg/ml showed a significant effect on phagocytosis (Fig. 4C).

Because S100A9 induces neutrophil degranulation and FcyRIII (CD16) is a known component of neutrophil granules (44), we next investigated the cell surface expression of FcyRs. Fig. 5 shows that CD16 was not significantly increased in cells treated with S100A9 as compared with unstimulated cells. Additionally, S100A9 had no effect on either CD32 or CD64 cell surface expression.

S100A9 increases complement receptor-mediated phagocytosis in human neutrophils

Next, we determined whether the effect of S100A9 was restricted to FcR-mediated phagocytosis. We thus investigated the possibility that this protein might also modulate complement receptor (CR)-mediated phagocytosis. First, the internalization of FITC-labeled microspheres was examined by flow cytometry. Exposure of neutrophils to S100A9 markedly increased the uptake of FITC-labeled microspheres as compared with untreated cells. More than 61% of neutrophils had internalized at least five latex beads compared with 35% in buffer-treated cells (see markers in Fig. 6A). To further support these findings, we evaluated the effect of S100A9 on the uptake of E. coli incubated in the presence of serum. Again, S100A9 markedly increased the internalization of bacteria based on flow cytometry (Fig. 6B) and immunofluorescence microscopy experiments (Fig. 6C). Therefore, stimulation of neutrophils with S100A9 enhances phagocytic processes independently of the receptors used to engulf opsonized Ags.

S100A9 induces phosphorylation events in human neutrophils

Phagocytosis and bacterial killing are complex processes involving, among other things, cytoskeleton reorganization (4) and phosphorylation of different proteins, including the Syk tyrosine kinase (45), PI3K/Akt, and Erk1/2 (46). Consequently, phosphorylation events were evaluated in S100A9-stimulated neutrophils. S100A9 induced a strong phosphorylation of total tyrosine residues after 30 min (Fig. 7A). Phosphorylation was sustained and further increased after 60 min. Among phosphorylated targets, S100A9 induced the phosphorylation of Syk. This phosphorylation was reversed by two Syk inhibitors, namely piceatannol and Syki II (Fig. 7B). Additionally, neutrophils treated with S100A9 displayed increased levels of phosphorylated Erk1/2 (Fig. 7C) and Akt (Fig. 7D). S100A9-induced Erk1/2 phosphorylation was partially reversed by preincubation with the MEK inhibitors PD98059 and U0126, whereas Akt phosphorylation was completely inhibited by wortmannin.

S100A9 enhances human neutrophil phagocytosis by a Syk-, Erk1/2-, and PI3K/Akt-dependent mechanism

To further decipher the mechanisms of action of S100A9, we next examined the involvement of the Syk, MEK/Erk1/2, and PI3K/Akt pathways in the phagocytic process. We chose to investigate the roles of the Syk, MEK/Erk1/2, and PI3K/Akt pathways in the phagocytic process because these pathways are known to be involved in phagocytosis and bacterial killing.

FIGURE 4. S100A9 increases human neutrophil phagocytosis dependent of FcyR in a concentration-dependent manner. Percentage of phagocytosis was assessed by counting the uptake of opsonized SRBCs, as described in Materials and Methods. Cells were stimulated with 40 μg/ml S100A8, S100A9, or S100A12 or 65 ng/ml GM-CSF (A, B) or 0.1–100 μg/ml S100A9 (C) for 30 min. SRBCs were then added at a 5:1 ratio for 30 min and the phagocytosis rate (A, C) and index (B) were determined. Data represent the mean ± SEM of four experiments performed on cells from different donors. Arrows point to ingested SRBCs. Images were obtained by cytology from cytocentrifuged preparations colored by Wright–Giemsa staining (original magnification ×400).
pathways in S100A9-primed phagocytosis. Cells were preincubated in the presence or absence of specific inhibitors of Syk (piceatannol and Syki II), MEK1/2 (PD98059 and U0126), and PI3K/Akt (wortmannin) for 30 min and then stimulated with S100A9. Because we had previously demonstrated that S100A9 also induces the phosphorylation of p38 and JNK in human neutrophils (22), SB203580 and JNK II were also tested for their ability to block S100A9-induced phagocytosis as specific inhibitors of p38 and JNK MAPKs, respectively (22). However, both SB203580 (Supplemental Fig. 1) and JNK II had no effect on the ability of S100A9 to enhance phagocytosis. In contrast, the MEK1/2 (PD98059 and U0126) inhibitors partly blocked the effect of S100A9 on phagocytosis without affecting the control (Fig. 8). Interestingly, the Syk inhibitors (piceatannol and Syki II)

FIGURE 5. Cell surface expression of FcyRs in human neutrophils stimulated with S100A9. Cell surface expression of CD16 (A), CD32 (B), and CD64 (C) were monitored by flow cytometry, as described in Materials and Methods. Cells were incubated in the presence of 40 μg/ml S100A9 or the equivalent volume of buffer (Ctrl) for 30 min at 37°C. Data represent the mean ± SEM of three experiments performed on cells from different donors. For the inset, results are from one experiment representative of three others.

FIGURE 6. S100A9 increases CR-mediated phagocytosis in human neutrophils. Phagocytosis was assessed by flow cytometry (A, B) by monitoring fluorescence intensity or by immunofluorescence microscopy (C), as described in Materials and Methods. Cells were stimulated with 40 μg/ml S100A9 or the equivalent volume of buffer (Ctrl) for 30 min. FITC-labeled microspheres (A) or Alexa Fluor 488-labeled E. coli (B), in a 10:1 ratio, were then added to neutrophils for 30 min at 37°C or 4°C (negative control) to measure phagocytosis. Results are representative of one out of at least three experiments. Uptakes, expressed as ∆MFI (∆MFI = MFI_{37°C} - MFI_{4°C}), are means ± SEM of four experiments performed on cells from different donors.
blocked the S100A9-enhanced phagocytosis by 90%, whereas phagocytosis in control cells was not significantly affected by the inhibitors. Finally, the PI3K inhibitor (wortmannin) reduced phagocytosis rate by ~25% in both control and S100A9-stimulated neutrophils. Collectively, these results demonstrate that the S100A9-enhanced phagocytic activity in neutrophils is strongly mediated by the activation of Syk and its downstream substrates, and partly by the activation of MEK1/2.

Discussion
S100A8, S100A9, and S100A12 form a subgroup of S100 proteins collectively referred to as myeloid-related proteins. They modulate neutrophil functions, including chemotaxis, adhesion, and transmigration via the activation of CD11b/CD18 (18–21). Recently, we demonstrated that S100A9, but neither S100A8 or S100A12, induces neutrophil degranulation (22). This suggests that these proteins have separate functions, a hypothesis corroborated by the fact that they are secreted separately by neutrophils (P. A. Tessier, unpublished observations). In this study, we further explored the activity of myeloid-related proteins and demonstrated that S100A9, but not S100A8 or S100A12, induces intracellular ROS accumulation over time through NADPH oxidase activation. We also found that S100A9 increases human neutrophil bactericidal activity against E. coli at least partly by increasing neutrophil FcR- and CR-mediated phagocytosis. The effect of S100A9 on phagocytosis was independent of the expression of FcγR since it did not upregulate the cell surface expression of CD16, CD32, and CD64.

Acute inflammation occurs a few hours following trauma or infection and is characterized by neutrophil adhesion to the endothelial barrier and migration toward affected tissues (47). Polymorphonuclear neutrophils are the first immune cells to arrive at the inflammatory site and they are crucial for the containment of pathogens within the infected site (1). In some cases, the acute response fails to eliminate pathogens and the response is exacerbated, leading to a chronic inflammatory state (47). This attests to the importance of an efficient and fast clearance of pathogens. During the inflammation process, different types of soluble molecules are secreted to regulate the immune response. Among these molecules, S100A8, S100A8/A9, and S100A9 are released in the extracellular milieu after phagocyte activation or cell necrosis. S100A8, S100A9, and S100A12 are potent antimicrobial factors inhibiting microorganismal growth (24, 48). The importance of these proteins is illustrated by the fact that S100A9-deficient mice had increased abscess lesions and mortality after s.c. challenge with C. albicans, probably due to the loss of S100A8/A9 (12). S100A8/A9 is thought to mediate its effect by chelating divalent ions, which are essential for microbial growth. Our results show that S100A9 also enhances neutrophil phagocytosis, corroborating its importance in the control of infection. Thus, in the context of
an infection, S100A9 secretion would favor clearance of pathogens and restore the normal homeostatic state of the tissue, thereby limiting or preventing excessive tissue injury and chronic inflammation. The results presented in this study demonstrate a new role for S100A9 in pathogen clearance and in the acute immune response.

Phagocytosis plays a crucial role in host defense leading to internalization and destruction of pathogens >0.5 μm. It is also involved in Ag presentation (42), although this latter function for neutrophils remains controversial. Albeit phagocytosis can occur in absence of opsonization, it is greatly enhanced by interactions between the Fc portion of Ig or complement components (iC3b) and FcRs or CRs at the phagocyte surface (3, 49). Interactions between receptors and their ligands trigger the formation of phagopodes and ultimately lead to the complete uptake of the particle. Several soluble mediators are known to enhance phagocytosis in human macrophages or neutrophils, including the cytokines GM-CSF, IL-4, and IL-15 (31, 43, 50), but the mechanisms underlying this biological response remain unclear. However, it has been demonstrated that Src proteins and Syk are essential for signal transduction leading to cytoskeleton remodeling and phagocytosis (46, 55). Interestingly, our results demonstrate that Src proteins and Syk are essential for signal transduction leading to cytoskeleton remodeling and phagocytosis, suggesting that this protein acts on both pathways or, at least, on common signaling molecules.

S100A9 induced tyrosine phosphorylation events, including the phosphorylation of Syk, Erk1/2, and Akt. Interestingly, this latter phenomenon is also pivotal for FcR-mediated phagocytosis (56), suggesting that S100A9 mediates its effect through phosphorylation-based mechanisms. It is noteworthy that tyrosine phosphorylation is associated with actin assembly, a key step in the process of cell adhesion and migration (57, 58), two functions that S100A9 also activates in human neutrophils (19, 21, 22). It is well known that phagocytosis mediated by FcyRIIa and/or FcyRIII depends on Syk, PI3K, and ERK1/2 (22). We have already

**FIGURE 8.** S100A9 increases human neutrophil phagocytosis by Syk-PI3K/Akt-Erk1/2-dependent mechanisms. The percentage of phagocytosis was assessed by counting the uptake of SRBCs as described in Materials and Methods. Cells (1 × 10⁷ cells/ml) were pretreated with specific inhibitors (30 μM piceatannol, 1 μM Syk II, 5 μM PD98059, 5 μM U0126, 5 μM SB203580, 10 μM JNKII, or 10 nM wortmannin) and then incubated with 40 μg/ml S100A9 or the equivalent volume of buffer (Ctrl) for 30 min. SRBCs were then added at a 5:1 ratio for 30 min and phagocytosis was assayed. Data represent the mean ± SEM of four experiments performed on cells from different donors.

**FIGURE 9.** Proposed model of signalization involved in S100A9-induced degranulation and phagocytosis in human neutrophils. 1) S100A9 (A9) binds to its as yet unidentified receptor (possibly the receptor for advanced glycation end products and/or TLR-4) and induces early events (2) that are still unknown (represented here as a black box), ultimately leading to the activation of MAPKs (3). More specifically, S100A9 activates p38 (3) and JNK (6), which are both involved in its ability to induce degranulation. Unlike these two MAPKs, the activation of Erk-1/2 by S100A9 (8) leads to enhanced phagocytosis (8a). In addition to MAPKs, S100A9 activates Syk (9) and Akt (10), which are both involved in phagocytosis. The use of specific inhibitors of p38 (SB203580), JNK (JNKII), MEK1/2 (PD98059 and U0126), Syk (piceatannol [Pic] and SykiII)), and Akt (wortmannin [Wort]) leads to inhibition of S100A9-induced degranulation and phagocytosis (see boxed Xs). The dotted arrow indicates a probable association between Syk and Akt.
demonstrated that the MAPKs p38, JNK, and ERK1/2 are activated in neutrophils in response to S100A9 and that only p38 and JNK participate in the induction of degranulation (22). Therefore, we first tested the involvement of these kinases in S100A9-enhanced phagocytic activity. Unexpectedly, p38 and JNK inhibitors had no effects on the enhanced phagocytic activity of S100A9-primed neutrophils. However, abolishment of ERK1/2 activation, through the inhibition of MEK1/2, slightly diminished the priming effect of S100A9 on phagocytosis. Therefore, this pathway seems not to be largely implicated in S100A9-mediated signals rendering neutrophils more efficient to phagocyte pathogens.

Interestingly, using Syk-deficient neutrophils, it was recently demonstrated that specific deletion of Syk results in reduced host defense against bacterial infection, including E. coli (59). Corroborating the importance of Syk in neutrophil phagocytosis, we recently demonstrated that depletion of Syk by an antisense strategy dramatically diminished the ability of IL-4 to enhance phagocytosis of opsonized SRBCs (31). In this study, we demonstrate that S100A9 induces phosphorylation of Syk in neutrophils. Indeed, phosphorylation occurs between 15 and 30 min postexposure to S100A9. According to our data and considering the role of Syk in the phosphorylation of downstream cytoskeletal substrates and in F-actin remodeling (reviewed in Ref. 60), we suggest that Syk plays an important role in the phosphorylation of actin cytoskeleton before the addition of the phagocytic stimuli.

The evidence that reactive oxygen species act as signaling molecules suggests that S100A9 could transduce its effects, at least in part, via a ROS-dependent mechanism that needs to be further investigated. ROS production could occur via multiple routes in neutrophils. For example, the production of PGs and leukotrienes is a process known to generate ROS. Whether S100A9 induces the production of these proinflammatory mediators remains to be confirmed.

In conclusion, this study leads us to propose a novel role for S100A9 relevant to the control of infections. Early on during infections, S100A8, S100A9, and S100A12 are released upon phagocyte activation, for example, in tissue abscesses and infected mucosae (23–27). As they are highly expressed in the cytoplasm of neutrophils, the secretion of S100A9 even by a few neutrophils could easily lead to high concentrations of S100 proteins at the infection site. Upon their release, S100A8, S100A9, and S100A12 act as danger signals for the organism, inducing the recruitment of neutrophils from the blood to the inflammatory site. This activity is triggered by the stimulation of Mac-1 and the degranulation of neutrophils (20, 22, 30). Additionally, S100A8/A9 and S100A12 would inhibit the growth of bacteria at the infection site, allowing time for the migration of large numbers of neutrophils to occur.

Finally, as neutrophils reach the infection site, they become activated by S100A9, leading to the enhancement of their phagocytic activity and the rapid clearance of the pathogens.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary FIGURE S1 Legend: p38 is not involved in S100A9-induced bactericidal activity of human neutrophil. Killing of E. coli was evaluated by a one-step assay, as described in "Materials and Methods". Cells were pretreated with the p38 inhibitor (SB203580 5μM) for 30 min, then incubated in the presence of buffer (ctrl) or 40 μg/mL S100A9 for 30 min before being incubated with E. coli for increasing periods of time. Results are from one experiment representative of three others.