

Induction of neutrophil degranulation by S100A9 via a MAPK-dependent mechanism

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ABSTRACT

S100A9 is a proinflammatory protein, expressed abundantly in the cytosol of neutrophils and monocytes. High extracellular S100A9 concentrations have been correlated with chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease, as well as with phagocyte extravasation. This study tested the hypothesis that S100A9 induces degranulation in human neutrophils. S100A9 was found to up-regulate the surface expression of CD35 and CD66b, proteins contained in secretory vesicles and specific/gelatinase granules, respectively. In addition, gelatinase and albumin, stored, respectively, in specific/gelatinase granules and secretory vesicles, were detected in the supernatants of neutrophils stimulated with S100A9. In contrast, stimulation with S100A9 had no effect on CD63 expression or MPO secretion, two proteins contained in azurophilic granules. S100A9 induced the phosphorylation of the MAPKs, ERK1/2, p38, and JNK. Inhibition of p38 and JNK but not ERK1/2, with specific inhibitors (SB203580, JNKII, and PD98059, respectively), blocked neutrophil degranulation induced by S100A9. Taken together, these results support the hypothesis and clearly indicate that S100A9 induces the degranulation of secretory and specific/gelatinase granules but not of azurophilic granules in a process involving p38 and JNK and further support its classification as a DAMP. *J. Leukoc. Biol.* 87: 905–914; 2010.

Introduction

The innate immune system is the first line of defense against infections. Cells constituting the innate immune system recognize pathogen-associated molecular patterns through receptors such as TLRs and mannose receptors. In addition,

damaged cells send signals to the immune system by releasing intracellular proteins, called alarmins [1] or DAMPs. These preformed molecules are released upon cell activation or damage and activate the innate and adaptive immune systems. IL-1, thioredoxin, galectins, and HMGB1 are examples of DAMPs.

Recently, S100A8, S100A9, and S100A12 have come to light as potent neutrophil activators. S100A8, S100A9, and S100A12 form a subfamily of the calcium-binding S100 protein family, called MRPs [2], mainly as a result of their restricted expression to cells of myeloid origin [3, 4]. They are expressed in the cytosol of phagocytes, where they are presumed to participate in intracellular calcium regulation as well as NADPH oxidase activation. Neutrophils and monocytes are rich in MRPs, although activated epithelial and endothelial cells also express these proteins [5, 6]. They form homodimers and a heterodimer, called calprotectin (S100A8/A9) [7]. S100A9, S100A8, and S100A12 are found at high concentrations in the serum and at inflammatory sites of patients suffering from multiple inflammatory diseases, such as Crohn's disease [8], rheumatoid arthritis [9], and cystic fibrosis [10], to name a few, indicating that they are secreted or released by damaged cells.

Once released to the extracellular milieu, MRPs are chemotactic for neutrophils, induce their adhesion to endothelium, and increase their transendothelial migration by activating the β 2-integrins Mac-1 (CD18) [11–14]. Recent studies also showed a potent destructive role for S100A8 and S100A9 in a mouse model of arthritis [15, 16]. In these studies, mice deficient in S100A9 showed reduced cartilage destruction compared with wild-type mice. Furthermore, addition of anti-S100A9 and anti-S100A8/A9, but not anti-S100A8 antibodies, reduced human monocyte migration across endothelial cells in vitro [17]. In summary, S100A9 seems to be a predominant component in inflammation. S100A8, S100A9, and S100A12 are therefore intracellular proteins released during inflamma-

Abbreviations: CR=complement receptor, DAMP=damage-associated molecular pattern, HMGB1=high-mobility group box 1, Mac-1=membrane-activated complex 1, MFI=mean fluorescence intensity, MMP-9=matrix metalloproteinase 9, MPO=myeloperoxidase, MRP=myeloid-related protein, RAGE=receptor for advanced glycation end-products, SAPK=stress-activated protein kinase, TREM-1=triggering receptor expressed on myeloid cell 1

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tory episodes with cytokine-like activities and as such, have been proposed as DAMPs.

Neutrophils are the first immune cells to reach an infection site and constitute the first line of defense against microbial invaders. To reach the inflammatory site, neutrophils must migrate across endothelial cells and the surrounding tissue. This migration necessitates the secretion of tissue-degrading enzymes such as gelatinase and elastase. Once at the inflammatory sites, neutrophils phagocytose the microorganisms and release copious amounts of oxygen radicals, enzymes, and antimicrobial peptides. Most of these proteins are preformed and stored in granules, which are exported rapidly to the cell surface in a process referred to as degranulation.

Neutrophils possess four types of granules containing overlapping sets of proteins. These granules are characterized by the presence of specific proteins in the granule membranes and lumen. Azurophilic granules contain high amounts of lysosomal enzymes [18] and are characterized by the presence of granulophysin (CD63) in their membranes [19]. Gelatinase granules, more easily exocytosed, are the main reservoir of tissue-degrading enzymes, such as metalloproteinases, and specific granules are rich in antimicrobial peptides and thus, participate in the antimicrobial activity of neutrophils [20–22]. The membrane of these two granules contains specific markers such as CD66, CD15, or CD67. Finally, secretory vesicles, presumed to be formed by endocytosis [23], constitute the main reservoir of plasma proteins and membrane-associated receptors essential for neutrophil inflammatory response and are released easily in response to inflammatory molecules [24, 25]. Neutrophil degranulation occurs in part after activation of pathogen recognition receptors such as TLRs. In addition, proinflammatory cytokines such as IL-8, fMLP, and TNF- α induce neutrophil degranulation [20, 26–28].

In this study, we report a new role for S100A9 in leukocyte migration and function. We prove for the first time that S100A9, but not S100A8 or S100A12, induces neutrophil degranulation. More specifically, S100A9 induced the release of the secretory vesicles, the specific/gelatinase granules, but not azurophilic granules. This degranulation was found to be dependent on p38 and JNK but not ERK1/2 kinases. These findings suggest a crucial role of S100A9 in neutrophil physiology and support its classification as a DAMP.

MATERIALS AND METHODS

Reagents

Human rS100A8, rS100A9, and rS100A12 were produced as described previously [29] and found to contain <1 pg endotoxin/ μ g protein, as we reported previously [30]. PD98059, SB203580, BAPTA, EGTA, TNF- α , and mAb anti-albumin (clone HSA-11) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GM-CSF and IL-4 were obtained from PeproTech (Rocky Hill, NJ, USA). Antiphospho-specific JNK1/2/SAPK G9 (pTpY^{183/185}), Erk-1/2 (pTpY^{185/187}), and p38 (pTpY^{180/182}) were obtained from BioSource (Camarillo, CA, USA). mAb against total p38 (sc-535) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit poly-

clonal antibody against Erk-1/2 was obtained from Upstate Biotechnology (Lake Placid, NY, USA), and rabbit polyclonal anti-SAPK/JNK was obtained from Cell Signaling Technology (Danvers, MA, USA). mAb against CD35 (clone E11) and CD63 (clone H5C6) were purchased from BD PharMingen (San Diego, CA, USA). The anti-CD66b mAb (clone 80H3) was obtained from AbD Serotec (Raleigh, NC, USA). Rabbit polyclonal antibody against MMP-9 and mouse mAb against MPO (clone 2C7) were obtained from Abcam (Cambridge, MA, USA). JNK inhibitor II, p38 MAPK inhibitor III, and U0126 inhibitor were purchased from Calbiochem/Merck KGaA (Darmstadt, Germany). All other reagents were obtained from Sigma-Aldrich.

Neutrophil isolation

Neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation, followed by centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Inc., Quebec, Canada) as described previously [31]. Blood donations were obtained from informed and 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 verified by cytology from cytocentrifuged preparations colored by Wright-Giemsa staining (Fisher Scientific, Ottawa, Canada). Cell viability was evaluated systematically before and after each treatment. Neutrophils were then resuspended [10×10^6 cells/mL in RPMI-HEPES (25 mM), penicillin (100 U/mL)/streptomycin (100 μ g/mL)] for all experiments.

Flow cytometry

Cell surface expression of CD35, CD63, and CD66b was monitored for assessing degranulation of secretory, azurophilic, and specific granules, respectively, by flow cytometry, as published previously [32], with a few modifications. Briefly, nonspecific binding of the antibodies was prevented by incubating the cells with PBS + 20% de complemented autologous serum for 30 min on ice. After several washes with PBS, primary antibodies or an IgG₁ isotype control antibody were added at a concentration of 1 μ g/mL in PBS for 30 min on ice. Cells were washed twice in PBS and incubated with 1 μ g/mL FITC-conjugated goat anti-mouse IgG antibody for 30 min on ice. After three washes, cells were resuspended in PBS, and analysis was performed with a FACScan (BD Biosciences, San Jose, CA, USA). In some experiments, cells were preincubated for 30 min at 37°C with the inhibitors SB203580 (2 μ M), p38 MAPK inhibitor III (500 nM), PD98059 (10 μ M), JNKII (10 μ M), U0126 (5 μ M), BAPTA (30 μ M), or EGTA (1 mM).

Western blot analysis

Neutrophils [10×10^6 cells/mL in RPMI-HEPES (25 mM), penicillin (100 U/mL)/streptomycin (100 μ g/mL)] were stimulated with fMLP (10^{-6} M), S100A9 (40 μ g/mL), GM-CSF (65 ng/mL), IL-4 (250 ng/mL), or their diluents HBSS for 1–60 min at 37°C. In some experiments, cells were preincubated for 30 min at 37°C with the inhibitors SB203580 (2 μ M), p38 MAPK inhibitor III (500 nM), PD98059 (10 μ M), or JNKII (10 μ M). At the end of the incubation period, the cells were lysed in 4 $\times$ Laemmli's sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, and 20% 2 β -ME), and aliquots corresponding to 1×10^6 cells were loaded onto 10% SDS-PAGE and transferred to nitrocellulose (p38 and Erk-1/2) or polyvinylidene difluoride membranes (JNK). For detection of MMP-9, MPO, and albumin, cells were preincubated with or without inhibitors and were stimulated for 1 h at the concentration mentioned above. Cells were then centrifuged at 13,000 RPM for 10 min at 4°C. Pellets were discarded, and supernatants equivalent to 1×10^6 cells for MMP-9 and albumin or 20×10^6 cells for MPO were separated on a 7.5% SDS-PAGE gel before being transferred to nitrocellulose membranes, which were blocked for 1 h at room temperature in TBS-Tween containing 5% non-fat dry milk (Carnation, Don Mills, Ontario, Canada). After washing, the antiphospho-specific p38, Erk-1/2, or JNK antibody was added at a final dilution of 1:1000 in TBS-Tween 0.15%. The antibody directed

against total p38, Erk1/2, or JNK was added at a final concentration of 1:400 in TBS-Tween 0.15%. A dilution of 1:2000 was used for antibodies MMP-9/gelatinase and albumin. Antibodies against MPO were used at 1:500 in TBS-Tween 0.15%. 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 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), diluted 1:20,000 in TBS-Tween + 5% nonfat dry milk, or a goat anti-mouse IgG HRP secondary antibody (Jackson ImmunoResearch Laboratories), diluted 1:20,000 in TBS-Tween + 5% nonfat dry milk, followed by several washes. Protein expression was revealed using an enhanced chemiluminescence Western blot analysis detection system (Amersham Biosciences, Piscataway, NJ, USA).

Zymography assay

Neutrophils were stimulated as described previously [26] and then centrifuged at 13,000 rpm for 10 min at 4°C, and the pellets were discarded. The supernatants (5 μ l corresponding to 50,000 cells) were mixed with a nonreducing buffer (40% glycerol, Tris-HCl 1 M, pH 6.8, SDS 8%) and separated on 10% acrylamide gels containing 0.2% gelatin. Gels were washed twice for 30 min with 2.5% Triton X-100 and incubated overnight in digestion buffer (Tris-HCl 50 mM, pH 7.4, NaCl 150 mM, CaCl₂ 5 mM). The gels were then stained with Coomassie blue 0.1% and destained.

Statistical analysis

Experimental data are expressed as means $\pm$ SEM. Repeated measures ANOVA (Dunnnett multicomparison test) were performed using GraphPad Prism, Version 5.01. Differences were considered statistically significant as followed: *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.005$, versus buffer or appropriated diluent. All densitometric analyses were performed with a Fluor S MultiImager, Quantity One, Version 4.6.6 (Bio-Rad, Hercules, CA, USA).

RESULTS

S100A9 up-regulates cell surface expression of granule markers

S100A9, S100A8, and S100A12 are highly regulated in inflammatory pathologies [8–10, 33–36]. We and others [12, 13, 30] have demonstrated major roles for these proteins in neutrophil adhesion, migration, and chemotaxis. In addition, recent data from our laboratory indicate that S100A9 increases neutrophil migration across endothelial cells in response to IL-8. As neutrophil migration is associated with degranulation, these results suggested a potential role for S100A9 in inducing the release of soluble factors by neutrophils. Secretion of proteins by neutrophils often requires degranulation [18, 37]. We therefore determined whether S100A9 increased cell surface expression of CD35, CD66b, and CD63 as markers of secretory, specific/gelatinase, and azurophilic granules, respectively. Cell surface expression was monitored by flow cytometry. As shown in **Figure 1**, S100A9 induced cell surface expression of CD35 and CD66b but not CD63. In contrast, fMLP induced CD35, CD66b, and CD63 expression significantly (the latter, after 5 min of stimulation). S100A9 induced CD35 up-regulation at concentrations as low as 1 μ g/ml (data not shown). However,

maximal up-regulation was observed at concentrations of 10–40 μ g/ml, similar to those observed in rheumatoid or gout synovial fluids [38]. rS100A8 and rS100A12 (purified using the same method) have no effect, thereby demonstrating that the activity of S100A9 was not a result of a contamination of the protein preparation (**Fig. 1C**).

We next investigated the possibility that S100A9 induced transient expression of CD63 by performing time-course experiments. Maximal response to S100A9 stimulation was observed at 30 min for CD35 (**Fig. 2A**) and 60 min for CD66b (**Fig. 2B**). Again, no effect on cell surface expression of CD63 was observed following stimulation with S100A9 (**Fig. 2C**), even after 2 h of stimulation (data not shown). In contrast, fMLP induced rapid and transient expression of CD63. These experiments suggested that S100A9 induces the release of secretory and specific or gelatinase granules.

S100A9 induces the release of gelatinase B

Gelatinase B (MMP-9) is a main component of specific and gelatinase granules known to be actively involved in leukocyte migration and tissue remodeling [39–41]. As we demonstrated that S100A9 increased cell surface expression of CD66b (contained in gelatinase granules), we next investigated whether S100A9 could induce the release and activation of MMP-9 by zymography. As illustrated in **Figure 3**, S100A9 induced the release of enzymatically active MMP-9. Although this secretion was delayed compared with TNF- α (detected after 30 min instead of 15 min), it closely correlated with the kinetics of CD66b surface expression. Interestingly, the intensity of the response was similar for S100A9 and TNF- α . As expected, S100A8 and S100A12 did not induce the release and activation of MMP-9 as assessed by zymography (**Fig. 3C**).

S100A9 induces neutrophil degranulation by a MAPK-dependent mechanism

MAPKs are known to be actively involved in the inflammatory process and to participate in many neutrophil functions, including cell adhesion, chemotaxis, apoptosis, production of cytokines and chemokines, and degranulation [42–47]. We thus investigated the activation of p38, Erk-1/2, and JNK MAPKs in S100A9-induced neutrophils by examining phosphorylation of these signaling molecules. As illustrated in **Figure 4**, S100A9 induced the phosphorylation of the three MAPKs. This phosphorylation was reversed by pretreatment with the MAPK inhibitors SB203580 (p38 inhibitor, **Fig. 4A**), PD98059 (MEK-Erk1/2 inhibitor, **Fig. 4B**), and JNKII (JNK inhibitor, **Fig. 4C**).

Knowing that S100A9 induced degranulation and MAPK activation, we next examined the involvement of MAPKs in S100A9-induced degranulation. Cells were preincubated in the presence or absence of MAPK inhibitors for 30 min and then stimulated with or without S100A9. Only cell surface expression of CD35 and CD66b was studied, as we demonstrated previously, CD63 is not modulated by S100A9 (**Fig. 2C**). The MEK/Erk-1/2 PD98059 and U0126 inhibitors have no effect on the ability of S100A9 to induce cell surface ex-

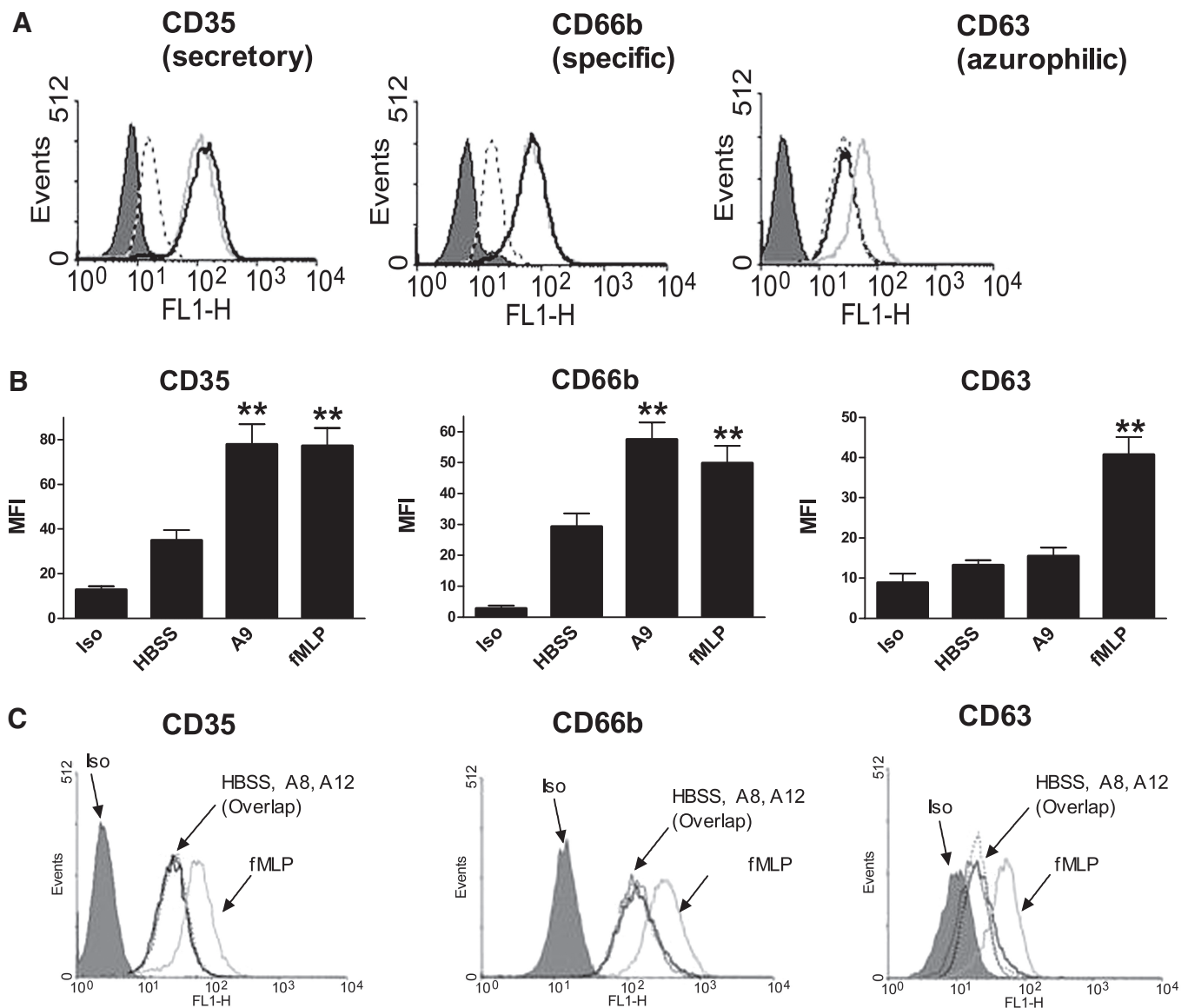


Figure 1. S100A9 induces degranulation of specific/gelatinase and secretory but not azurophilic granules in human neutrophils. (A) Release of subset types of granules was tested by flow cytometry following cell surface expression of CD35, CD66b, and CD63, as described in Materials and Methods. Cells were incubated in the presence of HBSS (control), 40 $\mu\text{g}/\text{mL}$ S100A9 (A9) for 30 min, or fMLP (10^{-6} M) for 5 min. Legend is as follows: Isotypic antibody (solid), control (dotted), S100A9 (open), and fMLP (gray). Results are from one representative experiment of three others. FL1-H, Fluorescence 1-height. (B) Data represent the mean $\pm$ SEM of at least three experiments done on cells from different donors. (C) S100A8 and S100A12 did not induce cell surface expression of CD35, CD66b, and CD63, as assessed by flow cytometry. Results are from one representative experiment of three others. Iso, Isotype.

pression of both markers (Fig. 5). In contrast, the p38 (SB203580) and JNK (JNKII) inhibitors significantly inhibited the S100A9-induced expression of CD35 and CD66b by $\sim 80\%$, indicating an important role for these kinases in S100A9-induced neutrophil degranulation. p38 MAPK inhibitor III was also tested in parallel with SB203580. Both inhibitors blocked equally the effect of S100A9 on CD35 and CD66b expression (data not shown). In addition, using BAPTA and EGTA, we have determined the role of intracellular and extracellular calcium in S100A9-induced CD35

and CD66b cell surface expression and found that only intracellular calcium is involved (Fig. 5, C and D).

S100A9 induces the release of albumin and MMP-9 (gelatinase B)

To support these findings further, the release of albumin and MMP-9 in S100A9-induced supernatants was investigated next, as representative proteins contained in secretory (CD35) and specific and gelatinase (CD66b) granules, respectively [18, 37]. As shown in Figure 6, supernatant of neutrophils stimulated

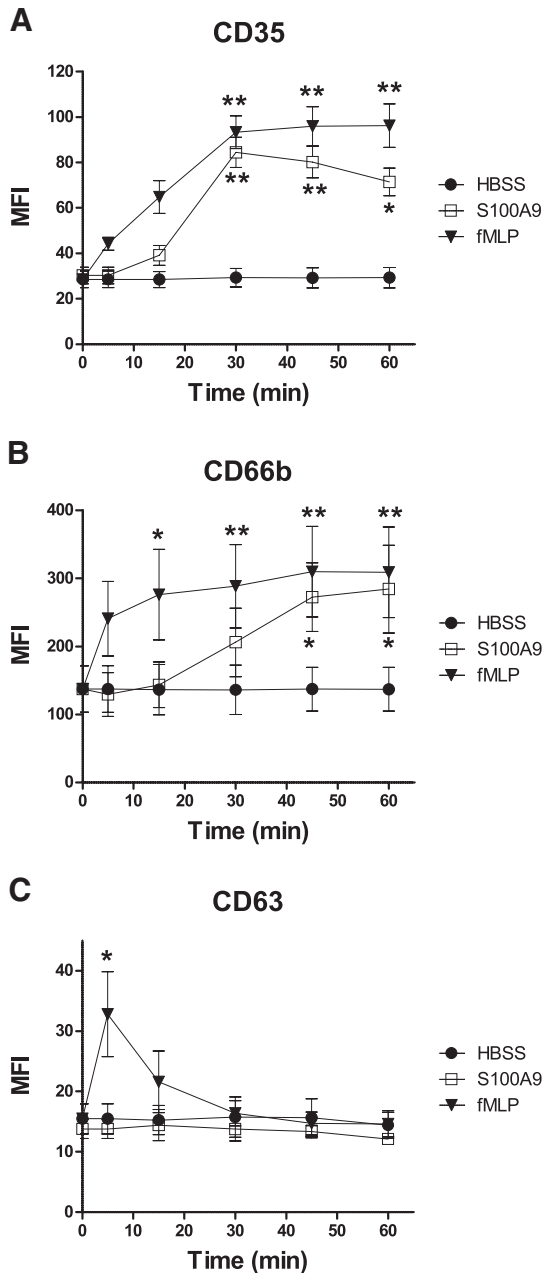


Figure 2. Occurrence of different granule markers of human neutrophils. Neutrophils were stimulated with 40 $\mu\text{g}/\text{mL}$ S100A9 and were then tested at various times for cell surface expression of (A) CD35, (B) CD66b, and (C) CD63. MFI of cells was quantified by flow cytometry. Data represent the mean $\pm$ SEM of at least three different experiments.

with S100A9 contained elevated levels of albumin and MMP-9, as assessed by immunoblotting. As expected, inhibition of Erk1/2 with PD98059 had no effect on the secretion of these proteins by S100A9-stimulated neutrophils. In contrast, pretreatment with p38 and JNK inhibitors markedly decreased the level of protein expression of albumin and MMP-9 (Fig. 6). Treatment with the JNKII inhibitor alone was found to increase the basal level of MMP-9, an observation reported previ-

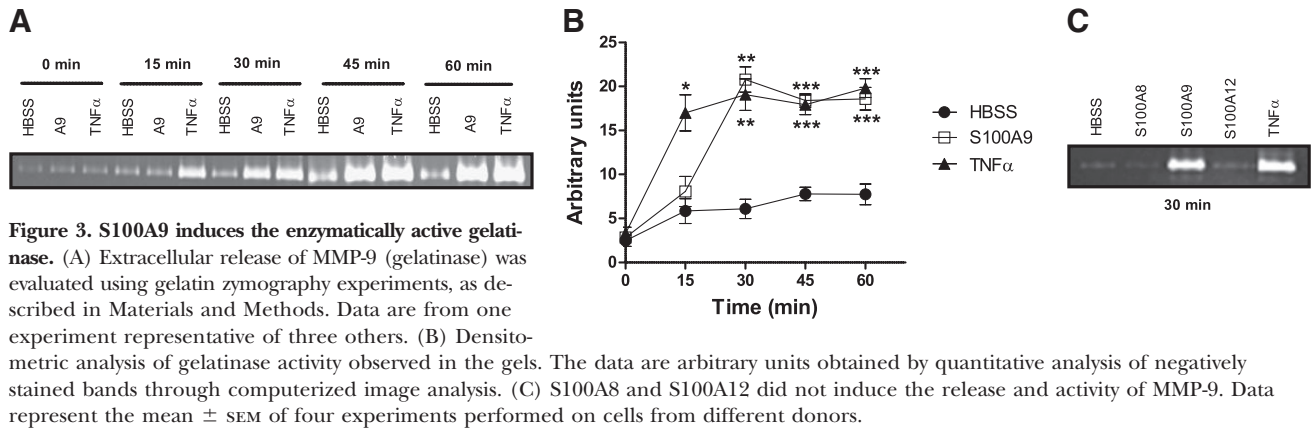
ously [26]. Taken together, these results demonstrate that S100A9 induces the release of two granule proteins in the external milieu via p38 and JNK activation, corroborating the observed, increased cell surface expression of CD35 and CD66b (Fig. 5).

DISCUSSION

In this study, S100A9, but not S100A8 or S100A12, was found to induce degranulation in human neutrophils. This was demonstrated by the fact that S100A9 increased the cell surface expression of CD35 and CD66b, two markers of secretory and specific/gelatinase granules, respectively. Also, the expression of albumin and MMP-9, known to be localized in secretory and specific/gelatinase granules, was increased in the supernatant of S100A9-induced neutrophils. Moreover, zymography experiments clearly indicated that MMP-9 preserved its enzymatic activity. In addition to the identification of this novel property for S100A9 in general neutrophil cell physiology, we provide the first evidence that this occurs by a MAPK-dependent mechanism.

Over the years, several studies showed potent proinflammatory roles for MRPs. For example, S100A8, S100A9, and S100A12 were shown to be chemotactic for neutrophils and to induce their release from the bone marrow [12, 13, 30]. Newton and Hogg [14] also demonstrated the activation of the $\beta 2$ integrin Mac-1 (CD18) after exposure to S100A9, suggesting a role for this protein in neutrophil adhesion and migration. In that study, S100A8 negatively regulated S100A9 activity on neutrophil adhesion to fibrinogen, showing an anti-inflammatory role of S100A8 [14]. Similarly, murine S100A8, also known as CP-10, although chemotactic for neutrophils, exhibits anti-inflammatory properties when oxidized [48]. In addition, recent results demonstrated that S100A9, but not S100A8 or S100A12, stimulates neutrophil adhesion to fibronectin and vitronectin [49]. S100A12, on the other hand, induces IL-2 and TNF- α secretion from macrophages and lymphocytes [50]. Taken together, these data suggest that S100A8, S100A9, and S100A12 are proinflammatory cytokines with multiple activities on cells of innate and adaptive immunity. However, their respective functions still need further analysis. Of interest, in *in vivo* experiments, S100A8 homodimers, S100A9 homodimers, and S100A8/A9 heterodimers are routinely found to be released separately [11, 13]. Interestingly, we find consistently that S100A9 is a more potent activator of neutrophils than S100A8 or S100A8/A9. This fact is corroborated by a study from Nancy Hogg and R. A. Newton [14] reporting that S100A8 negatively regulates S100A9 activity by forming the heterodimer S100A8/A9.

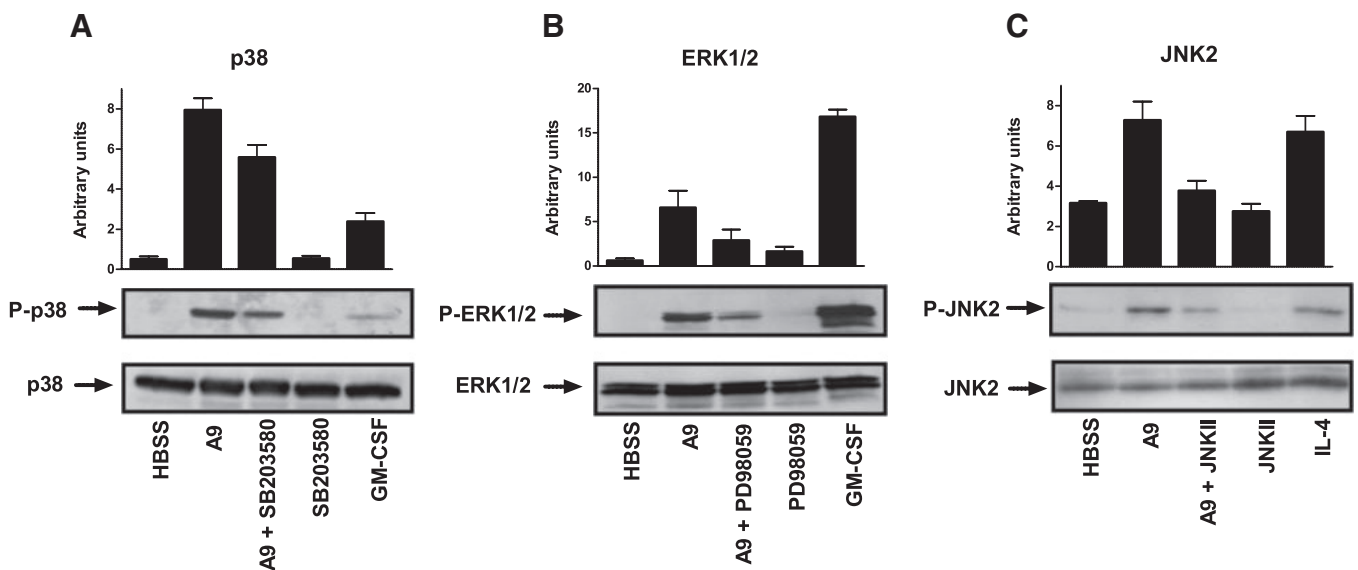
In this study, we demonstrate that S100A9, but not S100A8 or S100A12, induces neutrophil degranulation. During inflammation, neutrophils leave the blood to migrate to the tissue, where they phagocytose microbial invaders and release reactive oxygen species, antimicrobial peptides, and enzymes. During their migration, neutrophils encounter various cytokines that will cause the release of their granules [37]. Neutrophil degranulation is indeed important for the release of tissue-degrading enzymes such as elastase and gelatinase, which will

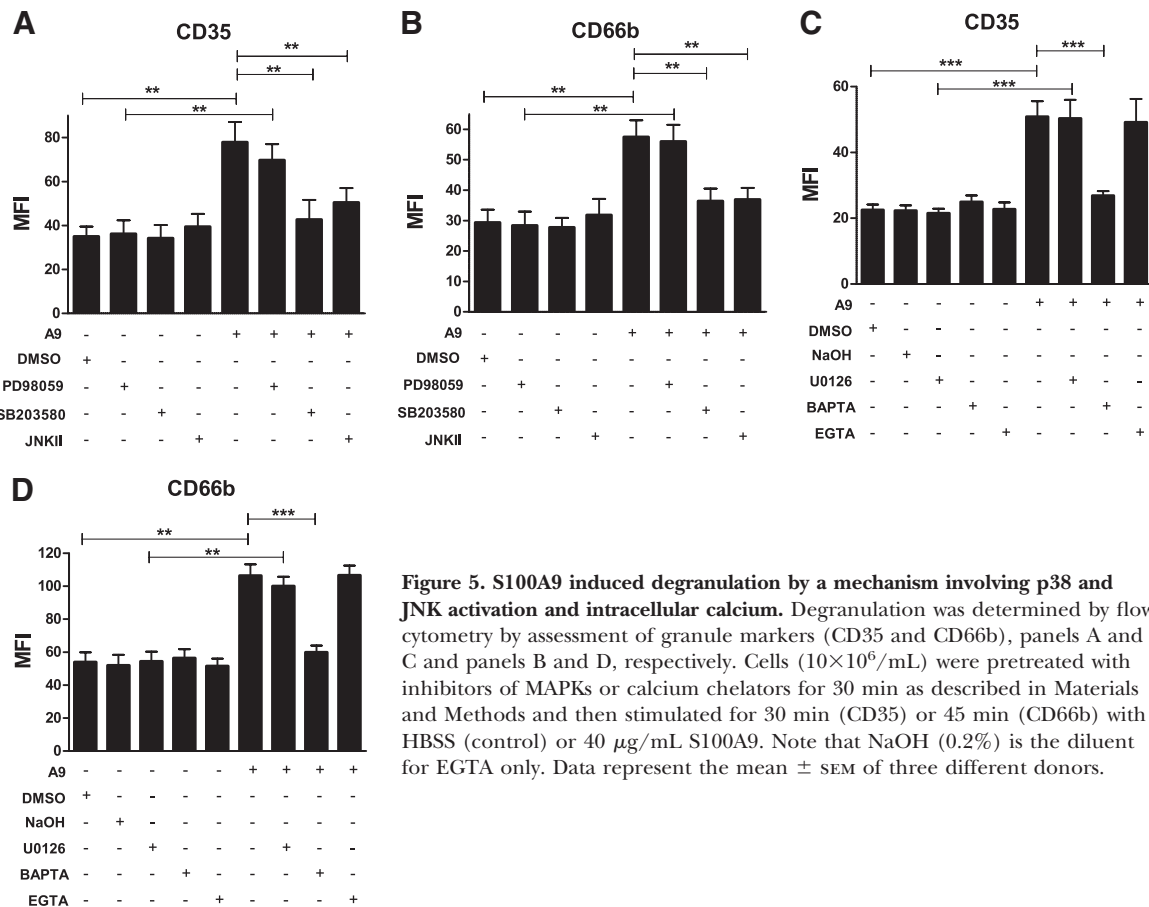


ease the migration of leukocytes across the endothelium and into the surrounding tissue. Neutrophils possess various kinds of granules released sequentially during inflammation [18]. We demonstrated that stimulation with S100A9 induces the release of secretory vesicles and specific/gelatinase granules. Secretory vesicles represent the main reservoir of multiple cell surface receptors including β 2-integrin Mac-1, CR1, fMLP receptor, and the LPS/lipoteichoic acid receptor (CD14), to name a few [24, 25, 51, 52]. These endocytic vesicles are mobilized in response to various stimuli at the earliest phase of the inflammatory response [23, 27]. By stimulating the release of secretory vesicles, S100A9 increases cell surface expression of the β 2-integrin Mac-1, thus leading to enhanced adhesion to the endothelium. These results corroborate previous data obtained by our laboratory and others, demonstrating increased adhesion of neutrophils following stimulation with

S100A9 [12–14]. Increasing levels of CRs on cell surface following stimulation with S100A9 also suggest that it could assist neutrophil phagocytosis, another important function of neutrophils for the control of infections [53]. However, the role of S100A9 in neutrophil phagocytosis remains to be properly elucidated.

By inducing the release of specific/gelatinase granules, S100A9 also promotes the secretion of enzymes playing an important role in tissue remodeling, destruction of cartilage, and leukocyte transmigration. For instance, gelatinase causes degradation of extracellular matrix by cleaving collagen, elastin, fibronectin, and laminin, to name a few [54]. Increased expressions of MMPs have been documented in fibroses and pulmonary diseases, conditions often associated with the presence of S100 proteins [10, 33, 35, 39, 55–57]. MMPs also need to be activated to fulfill their functions [41]. Our results show that





S100A9 induces not only the release of MMP-9 but also its activation (based on the zymography experiments). S100A8/A9 was shown recently to play an active role in the destruction of cartilage by increasing metalloproteinases [15, 16]. Furthermore, Viemann et al. [58] suggested that S100A8/A9 could induce the degradation of endothelial cell tight junctions, thereby increasing monocyte migration through the endothelium. Results presented here concur with these studies and give a better view about the mechanism of action of S100A9. Indeed, induction of degranulation through S100A9 stimulation could likely lead to erosion of cartilage and synovium. This hints that S100A9 could represent an interesting drug target for the control of inflammation in autoimmune diseases.

Azurophilic granules contain high amounts of microbicidal proteins such as defensins, presenilin, stomatin, and MPO [59, 60]. Granulophysin (CD63) is a platelet lysosomal membrane protein, commonly used as a marker of azurophilic granules [19, 61], which are secreted only following intense stimulation of neutrophils [27, 28]. In fact, exocytosis of azurophilic granules after a stimulation with PMA or fMLP (two potent leukocyte activators) is limited in time and only occurs to a minimal extent [62]. These observations explain the rapid and transient expression of CD63 at the surface of neutrophils after 5 min of stimulation with fMLP but not S100A9. Calcium mobilization is a crucial secondary signal responsible for granule

exocytosis, including azurophilic granules [63]. Interestingly, Sengelov et al. [27] showed that neutrophil degranulation varied according to the nature of the agonist and its capacity to induce a calcium burst. In the present study, stimulation with S100A9 was not potent enough to induce the release of azurophilic granules. This is in agreement with the weak calcium mobilization induced by S100A9 in neutrophils [11]. Of note, S100A9-induced secretory and specific/gelatinase granule release was inhibited by the intracellular calcium chelator BAPTA but not by the extracellular EGTA chelator (Fig. 5).

Increasing evidence suggests that cells release intracellular proteins to alert the innate and adaptive immune systems. These intracellular proteins, termed alarmins or DAMPs, are released upon cell necrosis or following cellular activation. They are presumed to play an important role in infection and autoinflammatory diseases. IL-1, HMGB1, and heat shock proteins are good examples of DAMPs. Several observations support S100A8, S100A9, and S100A12 as DAMPs. For example, although MRPs are cytoplasmic proteins, their presence in high concentrations in inflammatory pathologies has been noted. S100A8/A9 is found on the endothelium near sites of extravasations, in the serum and synovial fluids of rheumatoid arthritis patients [9, 38], as well as in the feces and serum of patients with inflammatory bowel disease. However, the mechanism of secretion of S100A8/A9 remains unclear. S100A8/A9

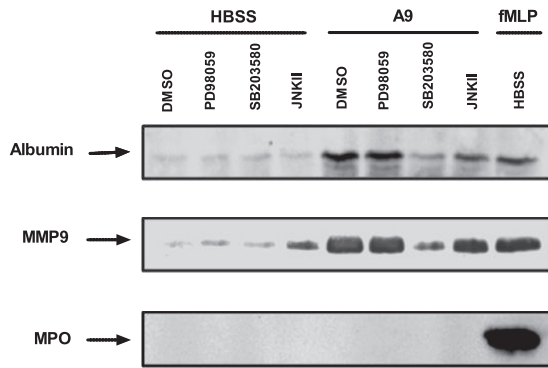


Figure 6. S100A9 induces the release of albumin, gelatinase (MMP-9), but not MPO by a p38/JNK-dependent mechanism. Degranulation was confirmed by assessment of secretory (albumin), azurophilic (MPO), and specific/gelatinase (MMP-9) markers in the medium. Cells ($10 \times 10^6/\text{mL}$) were pretreated with inhibitors of MAPKs for 30 min as described in Materials and Methods and then stimulated for 45 min with HBSS (control), $40 \mu\text{g}/\text{mL}$ S100A9, or fMLP ($1 \times 10^{-6} \text{ M}$), and supernatant was used for immunoblotting experiments. Shown here is one representative experiment out of three.

is known to be secreted by neutrophils interacting with endothelial cells and thus, presumed to play a role in leukocyte migration [64, 65]. In addition, it is secreted by neutrophils stimulated with monosodium urate crystals and *Porphyromonas gingivalis*, as well as PMA-stimulated monocytes [66–68]. Although its secretion pathway remains largely unexplored, it appears to necessitate tubulin polymerization [66]. Necrotic neutrophils have also been shown to release S100A8/A9 [69]. These modes of release are characteristics of DAMPs.

Several receptors appear to mediate the effects of DAMPs, including TLRs, RAGE, and TREM-1 [1, 70]. Interestingly, although the identity of the receptor for S100A9 is open for debate, RAGE and TLRs have been suggested as potential candidates. RAGE has been identified as a receptor for S100A12 [71] but not S100A9 [65]. S100A8/A9 has also been reported to bind to the scavenger receptor (CD36) on macrophages [72]. More recently, S100A8 was shown to be an endogenous activator of the TLR4 [73]. Interestingly, signal transduction induced by these receptors, as well as with the DAMP receptor TREM-1, involves MAPKs [47, 74, 75], which have been linked to many aspects of the inflammatory response, such as adhesion, chemotaxis, and degranulation [43, 44, 46]. MAPKs are also activated by numerous inflammatory cytokines and peptides such as fMLP, platelet factor 4, and $\text{TNF-}\alpha$ in human neutrophils [76–78]. Stimulation with S100A8/A9 has been linked to activation of NF- κB and MAPK in cancer cells [79, 80]. In the present study, we demonstrate activation of p38, ERK1/2, and JNK2 in human neutrophils in response to S100A9. In addition, we show the involvement of p38 and JNK, but not ERK1/2, in neutrophil degranulation induced by S100A9, further supporting the involvement of S100A9 as a DAMP. Despite the fact that we showed an important role of p38 and JNK in neutrophil S100A9-induced degranulation,

activation of ERK1/2 suggests an involvement of this kinase in other neutrophil functions.

In conclusion, we propose that S100A9, present at the inflammatory site, activates neutrophil degranulation, leading to the release of tissue-degrading enzymes and the up-regulation of several cell surface receptors. S100A9 would thus enhance neutrophil function during infections and could contribute to amplify the tissue destruction associated with neutrophil activation in several autoimmune diseases.

AUTHORSHIP

Conception and design: P. A. T., D. G., and J-C. S. Performed research: J-C. S. Analysis and interpretation of data: P. A. T., D. G., and J-C. S. P. A. T., D. G., and J-C. S. wrote the paper.

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