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# Human S100A9 potentiates IL-8 production in response to GM-CSF or fMLP via activation of a different set of transcription factors in neutrophils

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## Highlights

S100A9 does not increase cytokine production by itself in human neutrophils.

S100A9 primes neutrophils to GM-CSF- or fMLP-induced cytokine production.

A different set of transcription factors is involved in the priming effect of S100A9 in response to GM-CSF or fMLP.

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## Abstract

Inflammation is highly regulated by various agents. Unexpectedly, we report here that the damage-associated molecular pattern S100A9 protein, a potent neutrophil activator and inducer of cytokine production in monocytes, is not a direct activator of cytokine production in human neutrophils. However, S100A9 primed IL-8 production in fMLP- and GM-CSF-stimulated neutrophils via NF- $\kappa$ B and CREB-1, and NF- $\kappa$ B, STAT3 and STAT5, respectively. Pharmacological inhibition confirmed the importance of these transcription factors by significantly decreasing IL-8 production. This is the first time that a different set of transcription factors are shown to be involved in S100A9-primed neutrophils in response to proinflammatory agonist.

## Keywords

- S100A9;
- NF- $\kappa$ B;
- CREB-1;
- STAT-3;
- STAT-5;

- Neutrophil
- 

## 1. Introduction

The innate immune system, in which neutrophils play a major role, is the first biological barrier against invading pathogens. Toll-like and NOD-like receptors are the main receptors responsible for recognition of pathogens. Engagement of these receptors initiates an inflammatory response via the secretion of inflammatory cytokines/chemokines and the recruitment of leukocytes. Production of cytokines and chemokines is largely dependent on transcription factors such as NF- $\kappa$ B and CREB-1, especially in neutrophils [1] and [2]. Initiation of the immune response is also amplified by secretion and liberation of Damage-Associated Molecular Patterns (DAMPs) such as HMGB-1 and S100/calgranulins, which modulate many inflammatory functions [3] and [4].

S100A8/calgranulin A, S100A9/calgranulin B and S100A12/calgranulin C belong to the S100 family of proteins comprising 21 members [5]. S100A8, S100A9 as well as the heterodimer S100A8/A9 (also named calprotectin) were previously found to bind to TLR-4, RAGE and some other putative receptors, thereby modulating various functions of phagocytes and endothelial cells [6], [7], [8] and [9]. S100A8, S100A8/A9 and S100A9 are chemoattractant for neutrophils and increase their adhesion [10]. S100A9 also induce the expression and activation of  $\beta$ 2-integrins [11] and [12]. We recently demonstrated that S100A9 stimulates neutrophil degranulation and phagocytosis [13] and [14]. In addition, S100A8 and S100A9 stimulate the secretion of several inflammatory cytokines and regulate the NLRP-3 inflammasome, resulting in IL-1 $\beta$  processing and secretion by human monocytes [15].

Neutrophils are exposed to bioactive lipids, cytokines and DAMPs as they migrate to the inflammatory site and, each of these, stimulate or inhibit their activity. As S100A8 and S100A9 are found on the endothelium near sites of inflammation, we examined the effect of S100A9 on neutrophil cytokine production. Unexpectedly, we show here that S100A8 and S100A9 do not directly induce cytokine production in human neutrophils. However, we demonstrate that S100A9 potentiates IL-8 production by fMLP- or GM-CSF-

stimulated neutrophils following NF- $\kappa$ B, CREB-1 and STAT3/STAT5 activation.

## 2. Materials and methods

### 2.1. Reagents

Recombinant human S100A8 and S100A9 were produced as previously described [16]. Cycloheximide, fMLP and anti- $\beta$ -actin were purchased from Sigma–Aldrich (St. Louis, MO). BAY-117082, AG-490 and the CBP–CREB interaction inhibitor were purchased from Calbiochem (San Diego, CA). Phospho-IKK- $\alpha/\beta$  (Ser176/177), phospho-IKK- $\gamma$  (Ser376), phospho-CREB-1/ATF-1, phospho-STAT-3, phospho-STAT-5, phospho-STAT-6 and I $\kappa$ B- $\alpha$  antibodies were purchased from Cell Signaling Technology (Danvers, MA). GAPDH (FL), STAT-3 and CREB-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). RPMI 1640, HEPES, penicillin/streptomycin (Pen/Strep), heat-inactivated fetal bovine serum (FBS) was from Life Technologies (Camarillo, CA). Ficoll-Paque was obtained from GE Healthcare Bio-Science AB (Uppsala, Sweden). All secondary antibodies were purchased from Jackson Immuno-Research Laboratories (West Grove, PA). Proteome Profiler Array (Human Cytokine Array Panel A) was purchased from R&D Systems (Minneapolis, MN). Ultrapure-LPS (from *Escherichia coli*) were purchased from Invivogen (San Diego, CA). GM-CSF was from Peprotech (Rocky Hill, NJ).

### 2.2. Neutrophil isolation

Blood samples were obtained from informed, consenting individuals according to institutionally approved procedures as previously described [13]. Cell viability was monitored by trypan blue exclusion and found to be consistently higher than 99%. Neutrophils were suspended at  $10 \times 10^6$  cells/ml in RPMI 1640 containing 10% autologous deplete serum.

### 2.3. Cell stimulation

Neutrophils were stimulated with S100A8 (10  $\mu$ g/ml), S100A9 (10  $\mu$ g/ml), LPS (100 ng/ml) or the equivalent volume of diluent (Hank's Balanced Salt Solution 1X) at 37 °C for various periods of time, as specified. In some

experiments, cells were primed for 30 min with S100A8 (10 µg/ml) or S100A9 (10 µg/ml) and then stimulated with fMLP ( $10^{-7}$ M), GM-CSF (65 ng/mL) or the equivalent volume of diluent for 30 min at 37 °C for Western-blots or 24 h for measurement of cytokines.

#### 2.4. Western blot analysis

After stimulation, the cells were lysed in Laemmli's sample buffer (0.25 M Tris-HCl [pH 6.8], 8% SDS, 40% glycerol, and 20% 2-ME), and aliquots of extracts corresponding to  $1 \times 10^6$  cells were loaded onto 10% SDS-PAGE and transferred to nitrocellulose membranes for the detection of specific proteins. Membranes were blocked for 1 h at room temperature in 3% dry fat milk. The primary antibodies were added at a final dilution of (1:1000) 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 Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:20 000 in TBS-Tween or a goat anti-mouse IgG HRP secondary Ab (Jackson ImmunoResearch Laboratories) diluted at 1:20 000 in TBS-Tween followed by several washes. Protein expression was revealed with Luminata Forte Western HRP Substrate (Millipore) using ChemiDoc™ MP Imaging System (Bio-Rad). Membranes were stripped with ReBlot Plus Strong (Millipore) and stained with Amido-black to confirm equal loading of proteins.

#### 2.5. Electromobility shift assay

Nuclear extracts from cells stimulated for 1 h were prepared using NucBuster protein extraction kit from Novagen. Electrophoretic mobility shift assay (EMSA) reaction was done using Gel Shift Assay System from Promega, as previously described [\[15\]](#).

#### 2.6. NF-κB p50 TransFactor ELISA assay

Assays were done according to the Colorimetric TransFactor ELISA kit Procedure (Clontech Laboratories) as previously described [\[15\]](#).

#### 2.7. Proteome profiler™ array

Human Cytokine Array Panel A were purchased from R&D Systems (Minneapolis, MN, USA) and all the steps were performed within 2 weeks following experiments. Cells from 7 different donors of PMNs were stimulated for 24 h with the corresponding agonists. Supernatants were then pooled together and used to probe the membranes as previously described [15]. The chemiluminescent signal from the bound cytokines present in the supernatants was detected by chemiluminescence using ChemiDoc™ MP Imaging System.

## 2.8. IL-8 production

The measurement IL-8 was done using a commercially available enzyme-linked-immunosorbent assay (ELISA) kit (Life Technologies) according to the manufacturer's instructions.

## 2.9. Statistical analysis

Experimental data are expressed as mean  $\pm$  S.E.M. One-way ANOVA (Dunnett multiple-comparison test) and two-way ANOVA (Bonferroni post-test) were performed using Graph-Pad Prism (version 5.01). Differences were considered statistically significant as follows:  $*P \leq 0.05$ ,  $**P \leq 0.01$ , and  $***P \leq 0.005$  versus buffer or the appropriate diluent.

## 3. Results

### 3.1. S100A8 and S100A9 do not induce cytokine production in human neutrophils

We recently reported that S100A8 and S100A9 induce inflammatory cytokine expression and secretion by human monocytes [15]. As both of these DAMPs are potent neutrophil activators [13], [14] and [15], we investigated their effects on cytokine production. Surprisingly, neither S100A8 nor S100A9 were found to induce cytokine secretion as assessed by an antibody array assay (Fig. 1A). To confirm this, we next quantified the IL-8 production using a specific ELISA kit. As illustrated in Fig. 1B, unlike LPS, S100A8 or S100A9 did not significantly increase the IL-8 production.

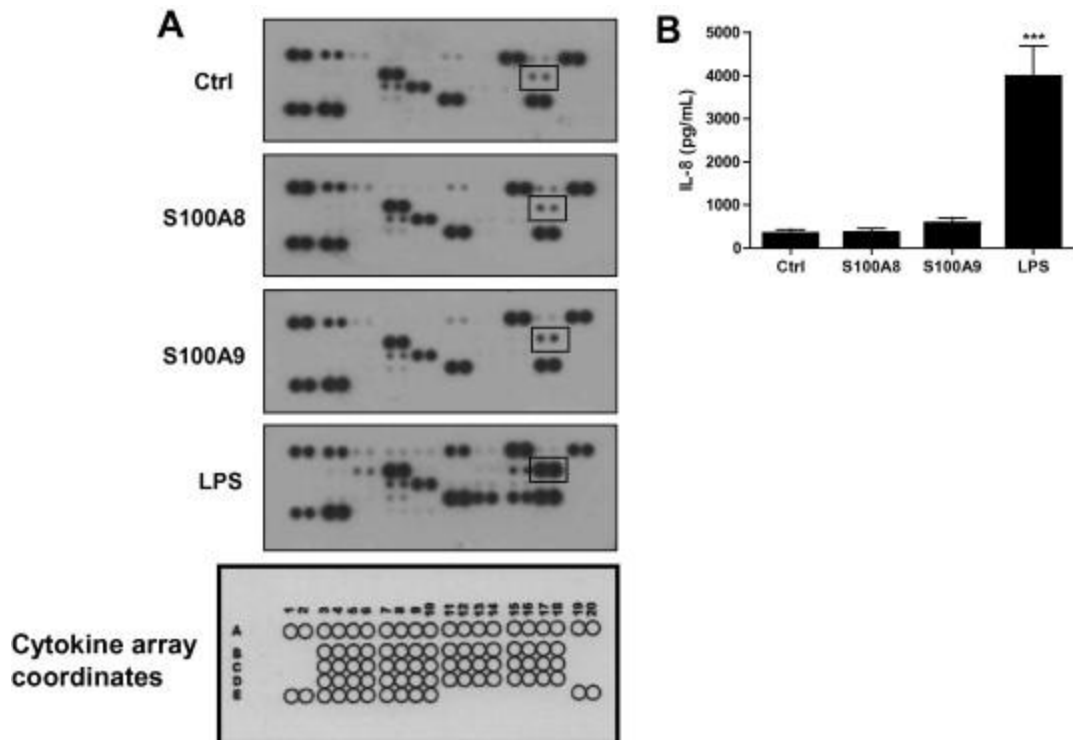


Fig. 1.

S100A8 and S100A9 do not induce cytokine and chemokine expression in human neutrophils. Cytokine production was evaluated using proteome profiler arrays (A) or ELISA (B). Neutrophils ( $10^6$  cells/mL) were stimulated with S100A8 (10  $\mu$ g/mL), S100A9 (10  $\mu$ g/mL), LPS (1  $\mu$ g/mL) or the equivalent volume of diluent (Ctrl) for 24 h. Supernatants were harvested and used for experiments. Data represent supernatants pooled from 10 different blood donors (A). Data are mean  $\pm$  S.E.M. of four experiments performed on cells from different donors (B). Boxes, localization of IL-8 (duplicate) on the membrane. The cytokine array coordinates are: Positive Ctrl (A1,A2); C5a (A3,A4); CD40 ligand (A5–6); G-CSF (A7,A8); GM-CSF (A9,A10); CXCL1/GRO $\alpha$  (A11,A12); CCL1/I-309 (A13,A14); sICAM-1 (A15,A16); IFN- $\gamma$  (A17,A18); Positive Ctrl (A19,A20); IL-1 $\alpha$  (B3,B4); IL-1 $\beta$  (B5,B6); IL-1ra (B7,B8); IL-2 (B9,B10); IL-4 (B11,B12); IL-5 (B13,B14); IL-6 (B15,B16); IL-8 (B17,B18); IL-10 (C3,C4); IL-12p70 (C5,C6); IL-13 (C7,C8); IL-16 (C9,C10); IL-17 (C11,C12); IL-17E (C13,C14); IL-23 (C15,C16); IL-27 (C17,C18); IL-32 $\alpha$  (D3,D4); CXCL10/IP-10 (C5,D6); CXCL11/I-TAC (D7,D8); CCL2/MCP-1 (D9,D10); MIF (D11,D12); CCL3/MIP-1 $\alpha$  (D13,D14); CCL4/MIP-1 $\beta$  (D15,D16); Serpin E1/PAI-1 (D17,D18); Positive Ctrl (E1,E2); CCL5/RANTES (E3,E4); CXCL12/SDF-1 (E5,E6); TNF- $\alpha$  (E7,E8); sTREM-1 (E9,E10); Negative Ctrl (E19,E20).

Figure options

### 3.2. Lack of NF- $\kappa$ B activation by S100A8 and S100A9, although S100A9 increased IKK- $\gamma$ phosphorylation

Since we have previously shown that S100A8 and S100A9 induced NF- $\kappa$ B activation in human monocytes, we next investigated the effect of S100A8 and S100A9 on NF- $\kappa$ B translocation/activation in human neutrophils. EMSA analyses revealed that neither S100A8 nor S100A9 induced NF- $\kappa$ B translocation/activation (Fig. 2A). These results were confirmed using the NF- $\kappa$ B p50 TransFactor Assay (Fig. 2B). Curiously, S100A9, but not S100A8, significantly increased the phosphorylation of IKK- $\gamma$ . However, phosphorylation of IKK- $\alpha/\beta$  and degradation of I $\kappa$ B- $\alpha$  was not observed in S100A8 and S100A9-treated cells (Fig. 2C). In addition, kinetic experiments in S100A8 and S100A9-treated cells revealed that the expression level of I $\kappa$ B- $\alpha$  remained unchanged at 30, 60, 120 and 180 min (*data not shown*). Only the positive control LPS led to phosphorylation of all three IKK subunits, resulting in degradation of I $\kappa$ B- $\alpha$  and translocation of NF- $\kappa$ B.

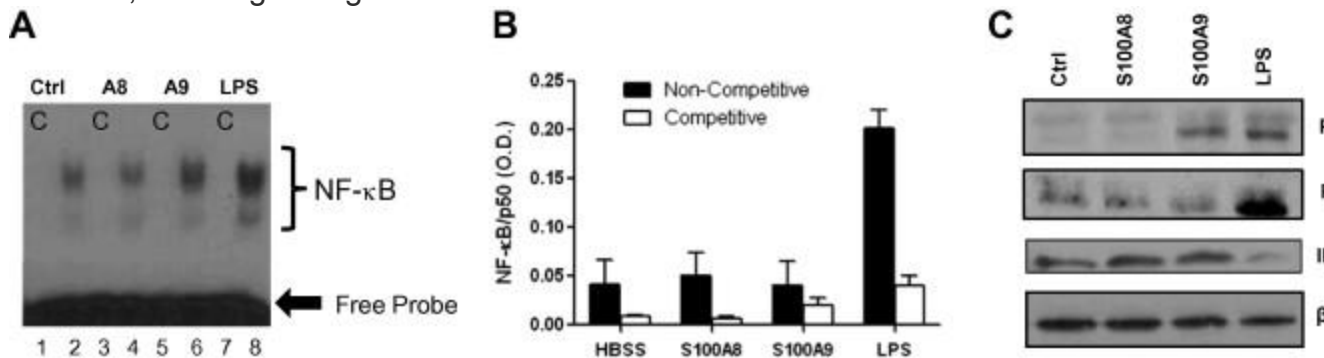


Fig. 2.

S100A8 and S100A9 do not increase translocation of NF- $\kappa$ B in human neutrophils. Translocation and binding of NF- $\kappa$ B was assessed by EMSA (A) and with a NF- $\kappa$ B p50 TransFactor ELISA kit (B), as described in Section 2. Nuclear proteins were isolated from cells stimulated for 1 h. Lanes 1, 3, 5 and 7 (c, competitive assay). Lanes 2, 4, 6 and 8 (non-competitive assay). Phosphorylation of the IKK complex and degradation of I $\kappa$ B- $\alpha$  was assessed by Western blot experiments (C). Results are from one representative experiment of at least four others (A and C). Data are means  $\pm$  S.E.M. of three experiments performed on cells from different blood donors (B).

[Figure options](#)

### 3.3. S100A9 potentiates the fMLP- or GM-CSF-induced cytokine production in neutrophils



Because IKK- $\gamma$  is the non-catalytic regulatory subunit of the IKK complex [17] and since S100A9 increased IKK- $\gamma$  phosphorylation, we reasoned that this protein could be involved in the regulation of cytokine production. S100A9 is found on endothelium near sites of extravasation and could therefore prime neutrophils to respond to cytokines found at the inflammatory site. To mimic the presence of S100A9 in inflammatory settings, neutrophils were primed with S100A9 prior to treatment with GM-CSF or fMLP. Interestingly, although S100A9 had no effect on cytokine secretion, it primed the effect of GM-CSF and fMLP on IL-8 secretion (Fig. 3). In contrast, S100A8 did not affect the secretion of IL-8 induced by either fMLP or GM-CSF (*data not shown*).

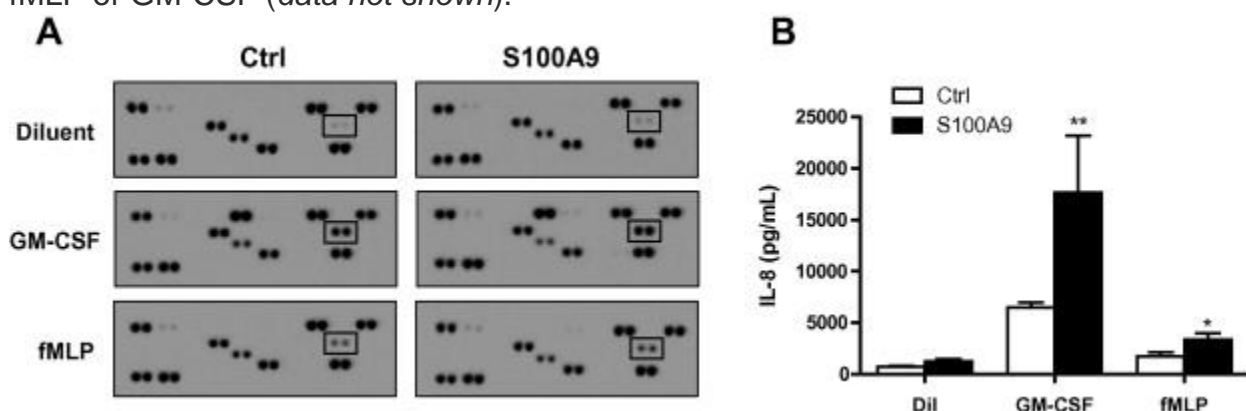


Fig. 3.

S100A9 potentiates the IL-8 secretion in human neutrophils treated with fMLP and GM-CSF. Cytokine production was evaluated using proteome profiler arrays (A) or ELISA (B). Neutrophils ( $10 \times 10^6$  cells/mL) were pre-treated S100A9 (10  $\mu$ g/mL) or the equivalent volume of diluent (Ctrl) for 30 min. Cells were then stimulated with fMLP ( $10^{-7}$  M) or GM-CSF (65 ng/mL) or the equivalent volume of diluent for a total of 24 h. Supernatants were then harvested and used for experiments. Data represent supernatants pooled from 7 different blood donors (A). Data are mean  $\pm$  S.E.M. of four experiments performed on cells from different blood donors (B). Boxes, localization of IL-8 (duplicate) on the membrane.

[Figure options](#)

### 3.4. Role of the transcription factors in response to GM-CSF or fMLP-induced IL-8 production in S100A9-primed neutrophils

We next determined the effect of S100A9 on various transcription factors known to be involved in the regulation of IL-8. Priming of neutrophils with

S100A9 promoted the degradation of I $\kappa$ B- $\alpha$  and translocation of the NF- $\kappa$ B p50 subunit to the nucleus in response to GM-CSF and fMLP (Fig. 4A and B). In addition, S100A9 led to the phosphorylation of CREB-1/ATF-1 (Fig. 4C). However, S100A9, by itself, did not induce STAT-3 or STAT-5 phosphorylation directly but induced a significant increase in the phosphorylation levels of these proteins following GM-CSF activation. Phosphorylation levels of STAT-6 remained unchanged in all tested conditions.

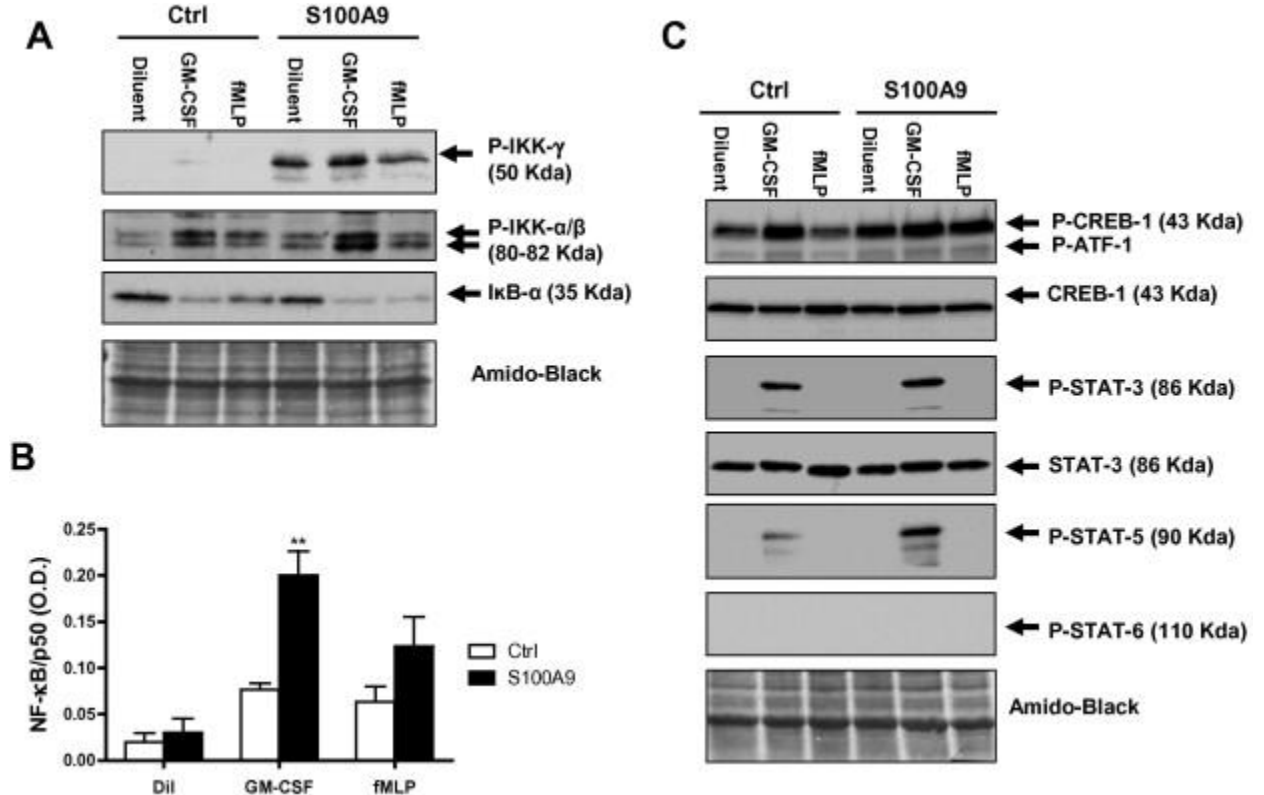


Fig. 4.

S100A9 potentiates the effect of fMLP or GM-CSF on transcription factors NF- $\kappa$ B, CREB-1/ATF-1, STAT-3 and STAT-5. Phosphorylation of the IKK complex, degradation of I $\kappa$ B- $\alpha$ , phosphorylation of CREB-1/ATF-1, STAT-3, STAT-5 and STAT-6 were assessed by Western blot experiments (A and C). Translocation of NF- $\kappa$ B with a NF- $\kappa$ B p50 TransFactor ELISA kit (B), as described in Section 2. Neutrophils ( $10 \times 10^6$  cells/mL) were pre-treated with S100A9 or the equivalent volume of diluent (Ctrl) for 30 min. fMLP ( $10^{-7}$  M), GM-CSF (65 ng/mL) or the diluent was then added for 30 min. Total proteins were then extracted for immunoblots while nuclear proteins were isolated for p50 transfactor assay, as described in Section 2. Results are from one representative

experiment of at least three others (A and C). Data are mean  $\pm$  S.E.M. of four experiments performed on cells from different blood donors (B).

Figure options

Pharmacological inhibitors were next used to determine the importance of these transcription factors in the priming effect of S100A9 on IL-8 production. Inhibition of NF- $\kappa$ B blocked almost completely the priming effect induced by S100A9 in both fMLP or GM-CSF treated neutrophils. Furthermore, while inhibition of the JAK/STAT pathway by the AG-490 inhibitor reversed specifically the effect of S100A9 on GM-CSF-induced IL-8 production, inhibition of CREB-1 by the CREB/EBP inhibitor resulted in a partial decreased level of IL-8 in response to fMLP (Fig. 5).

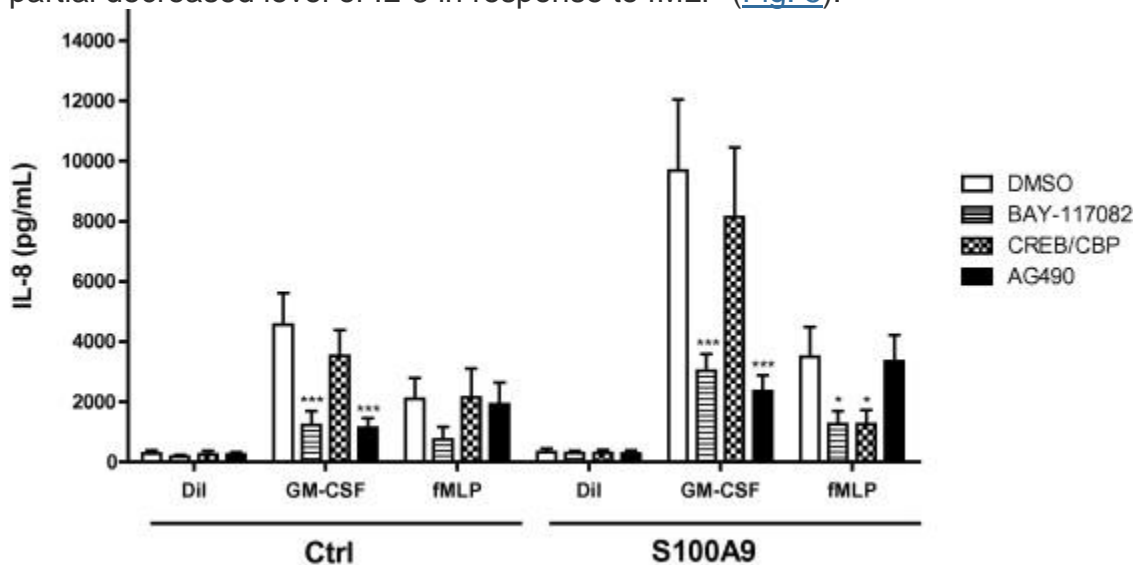


Fig. 5.

S100A9 potentiates the secretion of IL-8 in fMLP and GM-CSF-treated neutrophils by two distinct mechanisms. Cytokine production was evaluated by ELISA. Neutrophils ( $10 \times 10^6$  cells/mL) were incubated for 30 min with BAY-117082 (10  $\mu$ M), AG-490 (20  $\mu$ M), CREB/CBPi (5  $\mu$ M) or the equivalent volume of DMSO. Cells were then primed with S100A9 (10  $\mu$ g/mL) or the equivalent volume of diluent (Ctrl) for 30 min. Cells were then stimulated with fMLP ( $10^{-7}$  M), GM-CSF (65 ng/mL) or the equivalent volume of diluent for a total of 24 h. Supernatants were then harvested for quantification of IL-8 by ELISA. Data are mean  $\pm$  S.E.M. of four experiments performed on cells from different blood donors.

Figure options

#### 4. Discussion

Neutrophils play a crucial role in host defense and immunity, contributing to pathogen clearance and to the recruitment of other leukocytes through secretion of inflammatory mediators [18]. Among these, S100A8 (MRP8) and S100A9 (MRP14) are two inflammatory proteins highly expressed by neutrophils and found at inflammatory sites. Binding of S100A8 and S100A9 to TLR-4 leads to the activation of NF- $\kappa$ B and secretion of inflammatory cytokines in murine BMC and THP-1 cells [6] and [19]. In addition, studies have reported important roles for S100A8, S100A8/A9 and S100A9 in the regulation of cytokine secretion by monocytes [15], [19] and [20]. However, only one study reported an effect of S100A8 on the secretion of IL-8 and IL-16 by human neutrophils [21]. This discrepancy might be explained by the fact this study used GST-tagged-S100A8 with a molecular weight of ~36 kDa instead of the 8 kDa for human S100A8 protein. Also, S100A8 is very sensitive to oxidation, thereby modifying its inflammatory functions. In this study, we demonstrate that S100A8 and S100A9 do not have a direct effect on the secretion of cytokines. However, S100A9, but not S100A8, primed neutrophils to secrete IL-8 in response to GM-CSF or fMLP by partially activating NF- $\kappa$ B.

Intriguingly, S100A8 and S100A9 had no effect on the secretion of inflammatory cytokines or the activation of transcription factors in neutrophils, with the notable exception of S100A9 which led to the direct phosphorylation of CREB-1, without affecting IL-8 concentration. These results suggest that secretion of IL-8 is not solely dependent on phosphorylation of CREB-1 in neutrophils and that phosphorylation of CREB induced by S100A9 could be involved in other neutrophil functions.

Several transcription factors including CREB-1, NF- $\kappa$ B and STATs regulate cytokine production in neutrophils [2], [22] and [23]. A few studies reported a direct effect of MRPs on translocation of NF- $\kappa$ B in murine BMC, THP-1 and human prostate cancer cells [6],[19] and [24]. We have also shown that S100A8 and S100A9 induce NF- $\kappa$ B translocation and activation in human monocytes [15]. The NF- $\kappa$ B canonical pathway is regulated by members of the IKK family [17]. IKK- $\alpha$  and IKK- $\beta$  are two protein kinases that mediate the phosphorylation and the degradation of I $\kappa$ B- $\alpha$ , involved in NF- $\kappa$ B inhibition [25]. The IKK complex also comprises IKK- $\gamma$ , the essential modulator of this complex [26]. Our results show that S100A9 does not induce IKK- $\alpha$ / $\beta$  phosphorylation in neutrophils, explaining the lack of I $\kappa$ B- $\alpha$

degradation and the absence of the NF- $\kappa$ B p50 subunit in the nucleus. However, S100A9 induced a strong phosphorylation of IKK- $\gamma$ . Moreover, priming by S100A9 led to increased levels of NF- $\kappa$ B present in the nucleus in response to GM-CSF and fMLP. Our results suggest that S100A9 regulates NF- $\kappa$ B activity through regulation of the IKK- $\gamma$  subunits. Results in this study also indicate that even if a potent neutrophil activator such as S100A9 does not directly induce pro-inflammatory cytokine production by itself, it can potentiate the secretion induced by other neutrophil activators, including a cytokine like GM-CSF or bacterial products such as fMLP by recruiting a different set of transcription factors.

Previous work from our laboratory showed that S100A8 and especially S100A9 regulate various functions in neutrophils including adhesion, degranulation, migration and phagocytosis [10], [11], [13] and [14]. S100A8 and S100A9 were also shown to be potent chemoattractants for neutrophils [10] and [27] and are important for host defense and clearance of various pathogens [28], [29] and [30]. As IL-8 is one of the most potent chemoattractant for neutrophils and other leukocytes, data presented here suggest a new regulatory role for S100A9 by contributing to the secretion of this chemokine. IL-8 has important immunological roles other than the recruitment of neutrophils to the site of inflammation, it also contributes to the cross-talk between neutrophils and other immune cells such as Th17 [31] highly involved in the inflammatory process.

The above observations, together with the results of the present study, lead to a plausible scenario regarding the role of S100A9 in inflammation. It can be hypothesized that neutrophils encounter a series of stimuli as they migrate from the blood to the inflammatory site, one of the first being S100A9 deposited on the endothelium by migrating myeloid cells [32]. S100A9 would then activate neutrophils, although not sufficiently to induce the secretion of cytokines in the blood. However, primed neutrophils would secrete more IL-8 once activated by GM-CSF or fMLP in the tissue. This would in turn lead to an enhanced recruitment of neutrophils to the inflammatory site as observed in vivo in models of bacterial infection [27] and [28].