FEBS Letters 588 (2014) 2141–2146

journal homepage: www.FEBSLetters.org

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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ARTICLE INFO

Article history:
Received 4 March 2014
Revised 14 April 2014
Accepted 17 April 2014
Available online 2 May 2014

Edited by Bing Sun

Keywords:
S100A9
NF-κB
CREB-1
STAT-3
STAT-5
Neutrophil

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 neutrophiles via NF-κB and CREB-1, and NF-κB, 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.

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-κB and CREB-1, especially in neutrophils [1,2]. 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 [3,4].

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 [6–9]. S100A8, S100A8/A9 and S100A9 are chemoattractant for neutrophils and increase their adhesion [10]. S100A9 also induce the expression and activation of β2-integrins [11,12]. We recently demonstrated that S100A9 stimulates neutrophil degranulation and phagocytosis [13,14]. In addition, S100A8 and S100A9 stimulate the secretion of several inflammatory cytokines and regulate the NLRP-3 inflammasome, resulting in IL-1β 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-κB, 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-β-actin

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http://dx.doi.org/10.1016/j.febslet.2014.04.027
0014-5793/

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-α/β (Ser176/177), phospho-IKK-γ (Ser376), phospho-CREB-1/ATF-1, phospho-STAT-3, phospho-STAT-5, phospho-STAT-6 and IκB-α 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 10×10^6 cells/ml in RPMI 1640 containing 10% autologous decomplemented serum.

2.3. Cell stimulation

Neutrophil were stimulated with S100A8 (10 μg/ml), S100A9 (10 μg/ml), LPS (100 ng/ml) or the equivalent volume of diluent (Hank's Balanced Salt Solution 1X) at 37 °C for various period 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 1×10^6 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 ± 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: $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, and $^{***}P \leqslant 0.005$ 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 [13–15], 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.

3.2. Lack of NF-κB activation by S100A8 and S100A9, although S100A9 increased IKK-γ phosphorylation

Since we have previously shown that S100A8 and S100A9 induced NF-κB activation in human monocytes, we next investigated the effect of S100A8 and S100A9 on NF-κB translocation/activation in human neutrophils. EMSA analyses revealed that neither S100A8 nor S100A9 induced NF-κB translocation/activation (Fig. 2A). These results were confirmed using the NF-κB p50 TransFactor Assay (Fig. 2B). Curiously, S100A9, but not S100A8, significantly increased the phosphorylation of IKK-γ. However, phosphorylation of IKK-α/β and degradation of IκB-α 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 IκB-α remained unchanged at 30, 60, 120 and 180 min (*data not shown*). Only the positive control LPS