JLB

Article

Serum amyloid A1α induces paracrine IL-8/CXCL8 via TLR2 and directly synergizes with this chemokine via CXCR2 and formyl peptide receptor 2 to recruit neutrophils

Mieke De Buck, Nele Berghmans,* Noëmie Pörtner,* Lotte Vanbrabant,* Maaike Cockx,* Sofie Struyf,* Ghislain Opdenakker,† Paul Proost,* Jo Van Damme,*,[1,2] and Mieke Gouwy*,[1]*

*Laboratory of Molecular Immunology and †Laboratory of Immunobiology, Rega Institute for Medical Research, Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium

RECEIVED MARCH 4, 2015; REVISED JULY 10, 2015; ACCEPTED JULY 23, 2015. DOI: 10.1189/jlb.3A0315-085R

ABSTRACT

Cell migration depends on the ability of leukocytes to sense an external gradient of chemotactic proteins produced during inflammation. These proteins include chemokines, complement factors, and some acute phase proteins, such as serum amyloid A. Serum amyloid A chemoattracts neutrophils, monocytes, and T lymphocytes via its G protein-coupled receptor formyl peptide receptor 2. We demonstrate that serum amyloid A1α more potently chemoattracts neutrophils in vivo than in vitro. In contrast to CD14$^+$ monocytes, no rapid (within 2 h) induction of interleukin-8/CXC chemokine ligand 8 or macrophage-inflammatory protein-1α/CC chemokine ligand 3 was observed in purified human neutrophils after stimulation of the cells with serum amyloid A1α or lipopolysaccharide. Moreover, interleukin-8/CXC chemokine ligand 8 induction in monocytes by serum amyloid A1α was mediated by toll-like receptor 2 and was inhibited by association of serum amyloid A1α with high density lipoprotein. This indicates that the potent chemotactic response of neutrophils toward intraperitoneally injected serum amyloid A1α is indirectly enhanced by rapid induction of chemokines in peritoneal cells, synergizing in a paracrine manner with serum amyloid A1α. We observed direct synergy between IL-8/CXC chemokine ligand 8 and serum amyloid A1α, but not lipopolysaccharide, in chemotaxis and shape change assays with neutrophils. Furthermore, the selective CXC chemokine receptor 2 and formyl peptide receptor 2 antagonists, SB225002 and WRW4, respectively, blocked the synergy between IL-8/CXC chemokine ligand 8 and serum amyloid A1α in neutrophil chemotaxis in vitro, indicating that for synergy their corresponding G protein-coupled receptors are required. Additionally, SB225002 significantly inhibited serum amyloid A1α-mediated peritoneal neutrophil influx. Taken together, endogenous (e.g., IL-1β) and exogenous (e.g., lipopolysaccharide) inflammatory mediators induce primary chemoattractants such as serum amyloid A that synergize in an autocrine (monocyte) or a paracrine (neutrophil) fashion with secondary chemokines induced in stromal cells.
***J. Leukoc. Biol.* 98: 1049–1060; 2015.**

Introduction

The acute phase protein SAA is an apolipoprotein associated with HDL in the blood circulation [1]. The family consists of the inducible SAA1 and SAA2, the locally expressed (in mammary glands) SAA3, and the constitutively produced SAA4 [2–4]. SAA1 (-α, -β, and -γ) and SAA2 (-α and -β) are mainly produced in the liver on stimulation with IL-1β, IL-6, and TNF-α when inflammation, infection, or injury occurs [5]. Because its primary structure is highly conserved during evolution [6], one might expect that SAA plays an important role in life. However, the exact role of SAA has, until now, not been fully understood, although several functions have been ascribed to SAA. First, SAA is involved in transport of cholesterol to the liver, where it is excreted into the bile [2, 7–9]. Second, SAA has anti-inflammatory properties by inhibiting platelet aggregation [10] and by reducing the oxidative burst in neutrophils [11, 12]. However, SAA also has proinflammatory effects, such as induction of extracellular matrix degrading enzymes, allowing the repair of tissue damage [13–15]. Furthermore, SAA mediates an increase in local inflammation by chemoattracting neutrophils, monocytes, immature dendritic cells, mast cells, and T cells to sites of inflammation [16–19]. SAA also stimulates the induction of cytokines, including chemokines (e.g., IL-8/CXCL8, MCP-1/CCL2), in leukocytes [20–25]. Chemokines are small

Abbreviations: CI = chemotactic index, DPBS = Dulbecco's phosphate-buffered saline, FPR = formyl peptide receptor, GCP = granulocyte chemotactic protein, GPCR = G protein-coupled receptor, RP-HPLC = reversed phase HPLC, SAA = serum amyloid A, WRW4 = WRWWWW (selective FPR2 antagonist)

1. These authors contributed equally to this work.
2. Correspondence: Laboratory of Molecular Immunology, Rega Institute for Medical Research, Department of Microbiology and Immunology, University of Leuven, Minderbroedersstraat 10, Leuven 3000, Belgium. E-mail: jo.vandamme@rega.kuleuven.be

(7–12 kDa) cytokines, attracting specific leukocyte subsets via interaction with their GPCR. According to the position of the first 2 highly conserved cysteine residues in their primary structure, they are classified mainly into 2 groups: CC chemokines, in which the cysteine residues are adjacent, and CXC chemokines, in which the residues are separated by 1 amino acid. This nomenclature has also been applied for their corresponding receptors (CCR and CXCR) [26–29].

The chemotactic activity of SAA is mediated by the GPCR FPR2 [30–32], a low affinity receptor for the bacterial tripeptide fMLP [33]. For SAA-induced production of cytokines, the implication of different receptors has been suggested. Although most investigators have evidenced the use of FPR2, alternative receptors have also been proposed, including TLR2, CD36, CLA-1 (CD36 and LIMP II analogous-1, which is the human ortholog of the murine scavenger receptor class B type I) and the receptor for advanced glycation end products [22, 23, 30, 34–41]. In particular, TLR2 has recently gained interest as a receptor for cytokine induction by SAA. TLRs recognize a wide range of pathogen-associated molecular patterns and, therefore, play an important role in the clearance of pathogens from living organisms [42]. LPS is also a potent cytokine inducer through binding to TLR4 (just as SAA is via TLR2) [43–46].

Gouwy et al. [47] showed that LPS-induced IL-8/CXCL8 can synergize with the CC chemokine MCP-1/CCL2 to attract monocytes. Likewise, we have recently shown that SAA1α-induced chemokines in monocytes and immature dendritic cells synergize with each other and possibly also with SAA1α to attract these cells in an autocrine way [17]. In neutrophil chemotaxis assays, synergy has also been demonstrated between inflammatory IL-8/CXCL8 and other inducible (MCP1/CCL2, MCP-2/CCL8, and MCP-3/CCL7) or constitutive chemokines (regakine-1 and stromal cell-derived factor-1α/CXCL12) [48]. IL-8/CXCL8 is a major chemoattractant for neutrophils [49] and signals through CXCR1 and CXCR2 [50]. Synergy between chemoattractants occurs when combining these at suboptimal concentrations, which might be lower than the concentrations required for being chemotactic on their own. This cooperation between chemotactic GPCR ligands increases the recruitment of leukocytes to sites of inflammation and hence strengthens the inflammatory response [51].

We provide evidence that SAA1α is a more potent neutrophil chemoattractant in vivo than in vitro, because it synergizes in a paracrine way with chemokines induced in peritoneal cells by SAA1α. For chemotaxis in vitro, a direct and rapid synergistic interaction occurs between SAA1α and IL-8/CXCL8, which is mediated via their corresponding receptors FPR2 and CXCR2. Furthermore, owing to the synergy between SAA1α-induced chemokines in peritoneal cells, the inflammatory response can be prolonged.

MATERIALS AND METHODS

Cell cultures

Neutrophils were isolated from fresh whole blood samples from healthy donors. After density gradient centrifugation on Ficoll-sodium diatrizoate (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway), the pellet, consisting of RBCs and granulocytes, was incubated (37°C, 30 min) with equal volumes of PBS and 6% dextran (Sigma-Aldrich, St. Louis, MO, USA) in PBS. The supernatant was collected and centrifuged (250*g*, 15°C, 10 min). After washing the cell pellet with PBS, a hypotonic shock was performed. After centrifugation and another 2 washing steps, the granulocytes were suspended in DPBS (Lonza, Verviers, Belgium) and differentially counted (>95% neutrophils).

Human $CD14^+$ monocytes were isolated from 1-d-old buffy coats, derived from healthy donors (Blood Transfusion Center, Leuven, Belgium) via density gradient centrifugation on Ficoll-sodium diatrizoate and MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described by De Buck et al. [52].

Bone marrow cells from NMRI mice were isolated from both femurs and tibias after excision and debridement of soft tissue attachments. The bone marrow was flushed with PBS supplemented with 1 mg/ml HSA (Belgian Red Cross, Brussels, Belgium). Bone marrow neutrophils were isolated by density gradient centrifugation on Nycodenz, a tri-iodinated derivative of benzoic acid (Nycoprep; Axis-Shield PoC AS, Dundee, United Kingdom). After centrifugation (250*g*, 4°C, 10 min), the erythrocytes were hypotonically lysed and then washed in PBS and counted.

The HEK293 cell line transfected with FPR2 was kindly provided by Dr. J. M. Wang (National Cancer Institute, National Institutes of Health, Frederick, MD, USA) and cultured in DMEM (Lonza) enriched with 10%, Sigma-Aldrich), 1 mM glutamine, and 800 μg/ml G418 (Life Technologies, Paisley, United Kingdom).

Reagents

Recombinant human apo-SAA1 (SAA1α; catalog no. 300-53; endotoxin level <30 pg/μg SAA1α), IL-8/CXCL8 (6-77; catalog no. 200-08M), and IL-1β (catalog no. 200-01B) were purchased from PeproTech (Rocky Hill, NJ, USA). LPS from *Escherichia coli* (0111:B4) and the bacterial tripeptide fMLP were obtained from Sigma-Aldrich. The selective CXCR2 antagonist SB225002 and the selective FPR2 antagonist WRW4 [33, 53] were from Calbiochem (San Diego, CA, USA). Purified human HDL was obtained from EMD Millipore Corporation (Temecula, CA, USA). Anti-human/mouse TLR2 was purchased from Affimetrix eBioscience (San Diego, CA, USA). Recombinant murine GCP-2/CXCL6 (9-78) was expressed in *E. coli* and purified by subsequent heparin-Sepharose (Sigma-Aldrich) affinity chromatography, cation-exchange chromatography, and RP-HPLC [54].

Chemotaxis assays

Neutrophil migration was assessed in 48-well Boyden microchambers (Neuro Probe, Gaithersburg, MD, USA), as previously described by De Buck et al. [52]. In brief, SAA1α, IL-8/CXCL8, LPS, or a combination of SAA1α or LPS with IL-8/CXCL8 were added at different concentrations in triplicate to the lower wells of the microchamber (30 μl/well). Neutrophils (1×10^6 cells/ml; 50 μl/well) were added to the upper compartment, which was separated from the lower compartment by a polyvinylpyrrolidone-free polycarbonate membrane (5-μm pore size; GE Water & Process Technologies, Manchester, United Kingdom). After an incubation period of 45 min, the polycarbonate membrane was stained using Hemacolor solutions (Merck, Darmstadt, Germany), and the migrated cells, adhering to the lower surface of the membrane, were counted microscopically (magnification ×500) in 10 high power fields/well. The CI was calculated by dividing the average number of cells that had migrated to the chemotactic factor by the average number of cells that had migrated to the chemotaxis control buffer, HBSS (Life Technologies), containing 1 mg/ml HSA. Synergy was obtained when the net CI (CI − 1) of the 2 chemotactic substances together was superior to the sum of the net CI of the chemotactic substances added separately to the microchamber. For the antagonizing experiments, the upper wells of the Boyden microchamber were loaded with neutrophils in the presence of the selective CXCR2 antagonist SB225002 (3.5 μg/ml) or the selective FPR2 antagonist WRW4 (10 μg/ml).

Shape change assays

Shape change assays were performed to verify the morphologic shape changes that neutrophils undergo rapidly after stimulation with chemotactic agents.

Therefore, 50 μl of SAA1α or IL-8/CXCL8 alone, a combination of SAA1α and IL-8/CXCL8, or control buffer was added to a 96-well plate with a flat bottom. All dilutions and cell suspensions were made in shape change buffer (HBSS without calcium and magnesium, supplemented with 10 mM HEPES; Life Technologies) at 37°C, that was also used as a negative control. After adding 3×10^4 cells/50 μl, the neutrophils were fixed with 100 μl 4% formaldehyde in buffer at time points 0 and 2.5 min. For each condition, 100 cells, divided in round, blebbed, and elongated cells, were counted microscopically (magnification ×200) independently by 2 individuals in a blinded manner. For the assessment of synergy, the net percentages of the neutrophils undergoing shape change were used (i.e., the percentage of round, blebbed, and elongated neutrophils after stimulation with SAA1α or IL-8/CXCL8 minus the percentage of round, blebbed, and elongated cells after stimulation with buffer).

Induction experiments

Freshly isolated human neutrophils (3×10^6 cells/ml in RPMI 1640 medium [Lonza] supplemented with 1 mg/ml HSAs), human $CD14^+$ monocytes (2×10^6 cells/ml in chemotaxis control buffer), or FPR2-transfected HEK293 cells were seeded in 24-well (1 ml/well) or 48-well (500 μl/well) plates, respectively. The cells were stimulated with different doses of SAA1α, LPS, or IL-1β or were left untreated, and the plates were incubated at 37°C in 5% carbon dioxide. To test the effect of HDL on SAA1α-induced chemokine production in $CD14^+$ monocytes, SAA1α at 100 and 1000 ng/ml was preincubated (1 h at 37°C) with HDL at a concentration of 0.2 and 2 mg/ml, respectively. To test whether TLR2 and/or FPR2 are involved in SAA1α-induced production of chemokines, $CD14^+$ monocytes were preincubated (1 h at 37°C) with the anti-human/mouse TLR2 antibody (5 μg/ml) or the FPR2 antagonist WRW4 (10 μg/ml). Supernatant samples were taken 1 h, 2 h (neutrophils), 3 h (monocytes), and 24 h after induction. After centrifugation (250 *g*, 5 min), cell supernatants were stored at −20°C until determination of IL-8/CXCL8 (sensitivity of the ELISA: 0.03 ng/ml) and MIP-1α/CCL3 (sensitivity of the ELISA: 1 ng/ml) concentrations via specific ELISAs developed in our laboratory [55].

In vivo mobilization of neutrophils

The local animal ethics committee (University of Leuven) approved the in vivo experiments in mice, which were conducted in conformity with animal use for scientific purposes. Female NMRI mice (7–8 wk old; 3 mice per group per experiment), kept in a specific pathogen-free environment (Elevage Janvier, Le Genest Saint Isle, France), were injected intraperitoneally with PBS, SAA1α, GCP-2/CXCL6, SB225002, or a combination of SAA1α or GCP-2/CXCL6 with SB225002. After 2 h, the mice were killed by a subcutaneous injection of 30 mg of sodium pentobarbital (Nembutal; CEVA, Libourne, France), and the peritoneal cavity was washed for 1 min with 5 ml DPBS, supplemented with 2% FCS and 20 U/ml heparin (LEO Pharma A/S, Ballerup, Denmark). The amount of mouse GCP-2/CXCL6 in the peritoneal washes was measured by ELISA [56]. The cells were diluted 2-fold in Türk's solution (Merck), and the cell concentrations were determined using a hemocytometer. Cytospins were prepared for differential leukocyte counting by diluting 5×10^4 cells in 500 μl of peritoneal lavage buffer. After staining with Hemacolor solutions (Merck), 5 times 100 cells were counted microscopically (magnification ×500) by 2 persons independently in a blinded manner. The infiltration of neutrophils into the peritoneal cavity was represented as the average percentage of neutrophils and the average number of neutrophils/ml present in the peritoneal lavages. In the antagonizing experiments, the percentage of inhibition was calculated from the average percentage of neutrophils or the average neutrophil count minus the neutrophil influx caused by SB225002 alone (equals net count). For the ex vivo chemokine induction experiments, freshly isolated peritoneal cells from healthy untreated mice were stimulated with SAA1α or LPS for 24 h.

Statistical analysis

Statistical data analysis was performed using the Mann-Whitney *U* test (Statistica, version 12, software; StatSoft, Dell, Aliso Viejo, CA, USA). Statistically significant values are indicated by an asterisk for $P \leq 0.05$, 2 asterisks for $P \leq 0.01$, and three asterisks for $P \leq 0.001$; by a dollar sign for synergy, with 1 dollar sign indicating $P \leq 0.05$ and 2 indicating $P \leq 0.01$; and by dagger for inhibition of the effects of agonists or synergy by receptor antagonists, with one dagger indicating $P \leq 0.05$.

RESULTS

SAA1α is a more potent chemoattractant for neutrophils in vivo than in vitro

We investigated the chemotactic activity of SAA1α on neutrophils, as previously observed for SAA (a hybrid of SAA1α and SAA2β) by Badolato et al. [16]. In a direct comparison, SAA and SAA1α behaved similarly in chemotaxis assays with human neutrophils (i.e., CI = 2.9 ± 0.8 for SAA1α and CI = 2.1 ± 0.6 for SAA at 1000 ng/ml). In a different set of experiments, SAA1α significantly stimulated the migration of neutrophils from a concentration of 1000 ng/ml onward. In contrast, IL-8/CXCL8 was already chemotactic at 0.2 ng/ml (**Fig. 1A**). For SAA1α, a statistically significant higher number of migrated neutrophils was reached at 1000 ng/ml (47.3 ± 7.9 cells/10 fields; $n = 28$, $P = 0.04$) compared with the number of spontaneously migrating cells to the buffer control (24.1 ± 3.8 cells/10 fields). In contrast, for IL-8/CXCL8, a maximal number of migrated cells had already been obtained at 3 ng/ml (501.3 ± 36.7 cells/10 fields; $n = 22$). Moreover, fMLP at 10^{-9} M significantly chemoattracted human neutrophils (153.8 ± 55.3 cells/10 fields; $n = 6$). In the shape change assay, neutrophils (stimulated for 2.5 min) were significantly activated by SAA1α at a concentration of 3000 ng/ml (45 ± 7% blebbed cells, $P = 0.0041$; 4 ± 1% elongated cells, $P = 0.0002$; $n = 10$). In contrast, compared with the chemotaxis results, a substantially higher concentration (25 ng/ml) of IL-8/CXCL8 ($n = 6$) was needed to obtain a similar effect as that with SAA1α on shape change (Fig. 1B). For in vivo migration, a relatively high (100 ng) i.p. dose of a most potent N-terminally truncated form of mouse GCP-2/CXCL6, which is the murine counterpart of IL-8/CXCL8 recognizing CXCR1 and CXCR2 [57], was needed to acquire an optimal effect after 2 h (Fig. 1C). The peritoneal lavages (5 ml) of GCP-2/CXCL6-treated NMRI mice contained 14.6 ± 1.8% neutrophils, equivalent to $24.0 \pm 3.1 \times 10^4$ neutrophils/ml ($n = 9$, $P = 0.0006$). Injection of 1000 ng of SAA1α into mice caused a significant increase in the number of neutrophils in the peritoneal lavages (from 3.0 ± 0.4% or $3.9 \pm 0.8 \times 10^4$ neutrophils/ml in the control mice to 23.2 ± 2.9% or $42.5 \pm 5.9 \times 10^4$ neutrophils/ml in the SAA1α-treated mice; $n = 9$, $P = 0.0006$; Fig. 1C). The effects on the neutrophil count by injection of GCP-2/CXCL6 were significantly lower than those obtained by i.p. injection of mice with SAA1α ($P = 0.0341$ and $P = 0.0171$, respectively).

SAA1α is neither a rapid nor a potent inducer of IL-8/CXCL8 and MIP-1α/CCL3 in human neutrophils but is a potent chemokine inducer in monocytes via TLR2 and is inhibited by HDL

The experiments detailed in the previous section showed that a similar amount of SAA1α provoked chemotaxis or activation of neutrophils in vitro that was more pronounced in vivo. In contrast, the chemokine (IL-8/CXCL8 or mouse GCP-2/CXCL6) concentration had to be increased in vivo to reach a significant effect. This observation could have resulted from the rapid production of chemokines by peritoneal cells in the in vivo

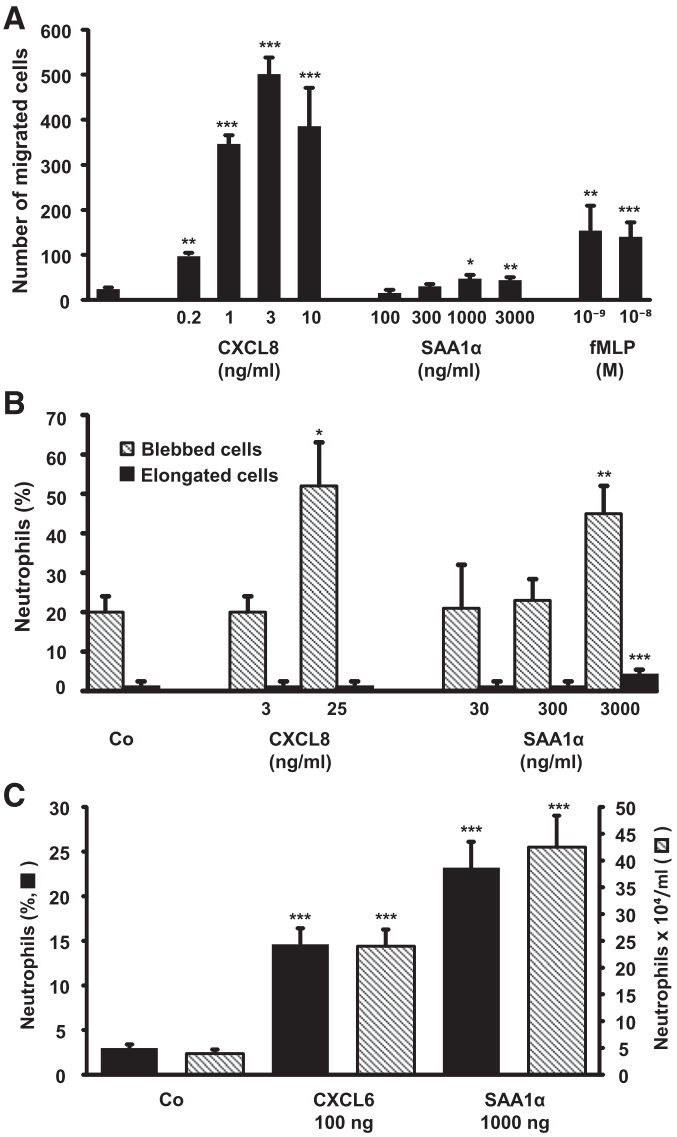

Figure 1. SAA1α chemoattracts and activates neutrophils in vitro and in vivo. A) The chemotactic activity of IL-8/CXCL8 (0.2–10 ng/ml), SAA1α (100–3000 ng/ml), and fMLP (10^{-8} and 10^{-9} M) was evaluated on human neutrophils in the Boyden microchamber assay. Data represent the mean number of migrated cells ± SEM/10 high power fields from 5–20 independent experiments. B) Activation of neutrophils by IL-8/CXCL8 (3 and 25 ng/ml) and SAA1α (30–3000 ng/ml) was assessed after 2.5 min in the shape change assay. The mean percentages of blebbed (hatched bars) and elongated (black bars) cells ± SEM from 4–10 independent experiments are shown. C) In vivo migration of neutrophils into the peritoneal cavity was tested by intraperitoneal injection of GCP-2/CXCL6 (100 ng), SAA1α (1000 ng), or PBS in female NMRI mice (9 mice/group). Peritoneal lavages (5 ml) were obtained 2 h after injection. Cells were counted using a hemocytometer, and differential cell counts were performed microscopically. The mean percentage of neutrophils ± SEM (black bars) and the mean number of neutrophils ± SEM × 10^4/ml (hatched bars) in the peritoneal lavages are shown. A–C) Statistically significant differences compared with controls (Co), determined by the Mann-Whitney *U* test, are indicated by asterisks (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

experimental setting on stimulation with SAA1α, as was previously shown in vitro in monocytes and immature monocyte-derived dendritic cells [17]. To verify a similar autocrine mechanism, induction experiments on human neutrophils were performed, and the amounts of IL-8/CXCL8 and MIP-1α/CCL3 in the cell supernatants were measured by specific ELISAs (**Figs. 2 and 3**). However, after 1 or 2 h, no induction of IL-8/CXCL8 (≤0.1 ng/ml) could be observed in SAA1α-stimulated neutrophils (Fig. 2A and B). In contrast, the maximal duration needed for in vitro and in vivo cell migration was 45 min and 2 h, respectively. LPS and IL-1β also failed to rapidly induce IL-8/CXCL8 in neutrophils. After stimulation for 24 h, only weak induction of IL-8/CXCL8 was observed, with a maximal concentration of 1.6 ± 1.2 ng/ml ($n = 4$, $P = 0.4705$) and 1.1 ± 0.5 ng/ml IL-8/CXCL8 produced on stimulation of neutrophils with 100 ng/ml SAA1α or LPS, respectively (Fig. 2B). In monocytes, however, 100 ng/ml SAA1α was able to induce significant production of IL-8/CXCL8, yielding 1.2 ± 0.5 ng/ml ($n = 4$, $P = 0.0606$) and 16.9 ± 10.4 ng/ml ($n = 4$, $P = 0.0304$) after 1 and 3 h, respectively (Fig. 2C and D). After 24 h (Fig. 2D), monocytes produced >400 ng/ml IL-8/CXCL8 on stimulation with SAA1α (100 ng/ml) or LPS (50 ng/ml), which is ≥100-fold more than neutrophils. In line with these results, no induction of MIP-1α/CCL3 (≤1.0 ng/ml) was detectable in neutrophils stimulated with SAA1α (1000 ng/ml), LPS (1000 ng/ml), or IL-1β (100 ng/ml) from 1 h onward to 24 h after induction (Fig. 3A and B). In contrast, from 1 h onward, monocytes had produced already a detectable amount of MIP-1α/CCL3 on stimulation with LPS or SAA1α (Fig. 3C and D). These results suggest that the improved chemotactic response of neutrophils to SAA1α in vivo (Fig. 1A and C) is not mediated by the induction of IL-8/CXCL8 or MIP-1α/CCL3 in neutrophils but might indirectly be affected by rapid induction of chemokines in peritoneal macrophages.

Because HDL can inhibit SAA-induced chemotaxis [16, 19, 58], we studied the effect of HDL on SAA1α-induced production of IL-8/CXCL8 in $CD14^+$ monocytes. SAA1α at 100 and 1000 ng/ml was preincubated (1 h at 37°C) with HDL at a concentration of 0.2 and 2 mg/ml, respectively. Cell supernatant samples were taken at 24 h after induction, and the IL-8/CXCL8 concentrations were determined by ELISA. The results shown in **Fig. 4A** confirm that after stimulation of $CD14^+$ monocytes with 100 and 1000 ng/ml SAA1α for 24 h, a significant induction of IL-8/CXCL8 in monocytes was observed (52.3 ± 10.0 and 461.1 ± 28.8 ng/ml, respectively; $n = 4$). In addition, HDL (2 mg/ml) was able to inhibit (96.1 ± 3.2%) the production of IL-8/CXCL8 in $CD14^+$ monocytes stimulated with 1000 ng/ml SAA1α (19.2 ± 9.4 ng/ml; $n = 3$, $P = 0.049$). In parallel, the effect of anti-human TLR2 antibody (5 μg/ml) and the FPR2 antagonist WRW4 (10 μg/ml) on the SAA1α-induced production of IL-8/CXCL8 in $CD14^+$ monocytes was determined (Fig. 4A). Although WRW4 could inhibit SAA1α-induced chemotaxis of neutrophils (see "Synergy between SAA1α and IL-8/CXCL8 in neutrophil chemotaxis in vitro can be blocked by antagonizing their corresponding receptors FPR2 and CXCR2"), this FPR2 antagonist failed to inhibit SAA1α-induced IL-8/CXCL8 production. In contrast, the TLR2 antibody significantly reduced the SAA1α-induced IL-8/CXCL8 production (61.7 ± 6.1% inhibition; $P = 0.03$; Fig. 4A). As a control, HDL (2 mg/ml), anti-human TLR2 antibody (5 μg/ml), and WRW4 (10 μg/ml) did not inhibit the constitutive production of IL-8/CXCL8 after treatment of $CD14^+$ monocytes with medium. Furthermore, FPR2-transfected human embryonic kidney 293 cells

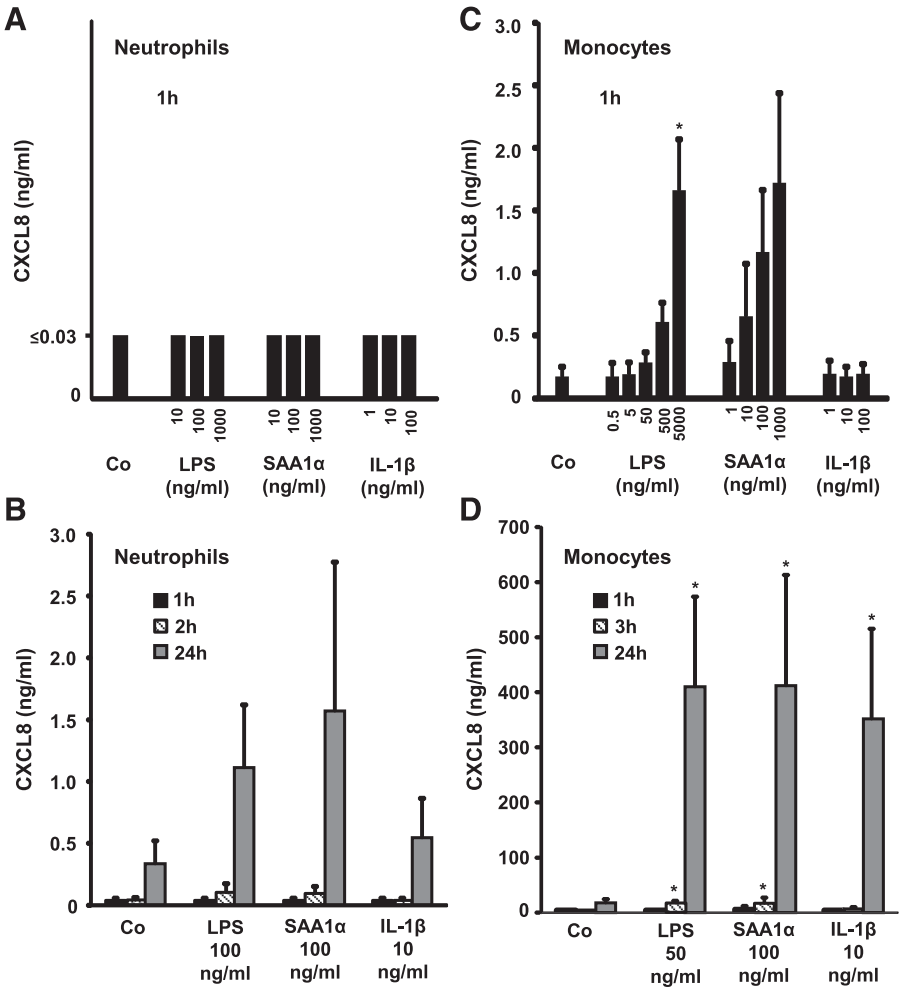

Figure 2. SAA1α rapidly induces the production of IL-8/CXCL8 in monocytes but not in neutrophils. Purified neutrophils (A and B) and $CD14^+$ monocytes (C and D) were stimulated with different concentrations of LPS (0.5–5000 ng/ml), SAA1α (1–1000 ng/ml), IL-1β (1–100 ng/ml), or control buffer (Co). Cell supernatants were taken 1 h (black bars), 2 h (neutrophils; hatched bars), 3 h (monocytes; hatched bars), and 24 h (gray bars) after stimulation, and the amount of IL-8/CXCL8 in the supernatants was measured by ELISA. Data represent the mean concentration ± SEM from 3 or 4 independent experiments. Averages below the lowest detection limit are shown without SEM. Statistically significant differences compared with controls, determined by the Mann-Whitney *U* test, are indicated by asterisks (*$P \leq 0.05$).

(HEK293/FPR2) did not produce IL-8/CXCL8 after stimulation with SAA1α (3–3000 ng/ml) for 24 h. However, as a positive control, IL-1β (0.1–100 ng/ml) induced IL-8/CXCL8 production in HEK293/FPR2 cells (Fig. 4B). In addition, SAA1α and SAA induced a similar increase in the intracellular calcium concentration in HEK293/FPR2 cells, indicating that both SAA and SAA1α activate the FPR2-signaling pathway (data not shown).

SAA1α but not LPS directly synergizes with IL-8/CXCL8 in in vitro neutrophil migration

We investigated whether the SAA1α-induced IL-8/CXCL8 from monocytes could take part in the migration of neutrophils toward SAA1α. Therefore, SAA1α and IL-8/CXCL8 were combined at different concentrations in the lower compartment of the Boyden microchamber (for 45 min), using highly purified human neutrophils (>95%). **Figure 5A** shows that suboptimal concentrations of SAA1α (300 ng/ml) and IL-8/CXCL8 (0.2 ng/ml) synergized with each other in neutrophil chemotaxis. A significantly higher CI (8.2 ± 0.3; $n = 7$, $P = 0.0298$) was reached when combining these 2 molecules compared with the sum of the chemotactic responses to SAA1α (300 ng/ml; CI = 1.1 ± 0.1) and IL-8/CXCL8 (0.2 ng/ml; CI = 6.3 ± 0.6) tested separately. In addition, we were able to confirm the synergistic effect between SAA1α at 300 ng/ml and IL-8/CXCL8 at 3 ng/ml in a different set of experiments (CI = 44.8 ± 12.0; $n = 5$, $P = 0.0122$ vs. CI = 1.2 ± 0.1 and 22.2 ± 1.1 for SAA1α and IL-8/CXCL8, respectively, added separately to the lower compartment of the Boyden microchamber). Furthermore, the chemotactic activity of SAA1α on murine bone marrow neutrophils was tested by adding 300 ng/ml SAA1α to the lower compartment of the chemotaxis assay. SAA1α weakly chemoattracted murine bone marrow neutrophils (CI = 1.4 ± 0.5 at a concentration of 300 ng/ml; $n = 7$). For comparison, murine GCP-2/CXCL6 (9–78) chemoattracted bone marrow neutrophils to reach a CI of 2.1 ± 0.2 at 1 ng/ml ($n = 6$). Furthermore, SAA1α (300 ng/ml) enhanced the chemotactic activity of murine bone marrow neutrophils toward murine GCP-2/CXCL6 (9–78) at 1 ng/ml (CI = 4.2 ± 0.5; $n = 6$, $P = 0.04$). We can conclude that the responsiveness of murine bone marrow neutrophils toward SAA1α is similar to that of human peripheral blood neutrophils (i.e., SAA1α is a weak chemoattractant for murine and human neutrophils in vitro and synergizes with murine GCP-2/CXCL6 or IL-8/CXCL8 to chemoattract murine or human neutrophils, respectively).

Such synergy was confirmed in human neutrophil shape change assays (stimulation for 2.5 min; Fig. 5B). In these experiments, a concentration of 3000 ng/ml SAA1α was required

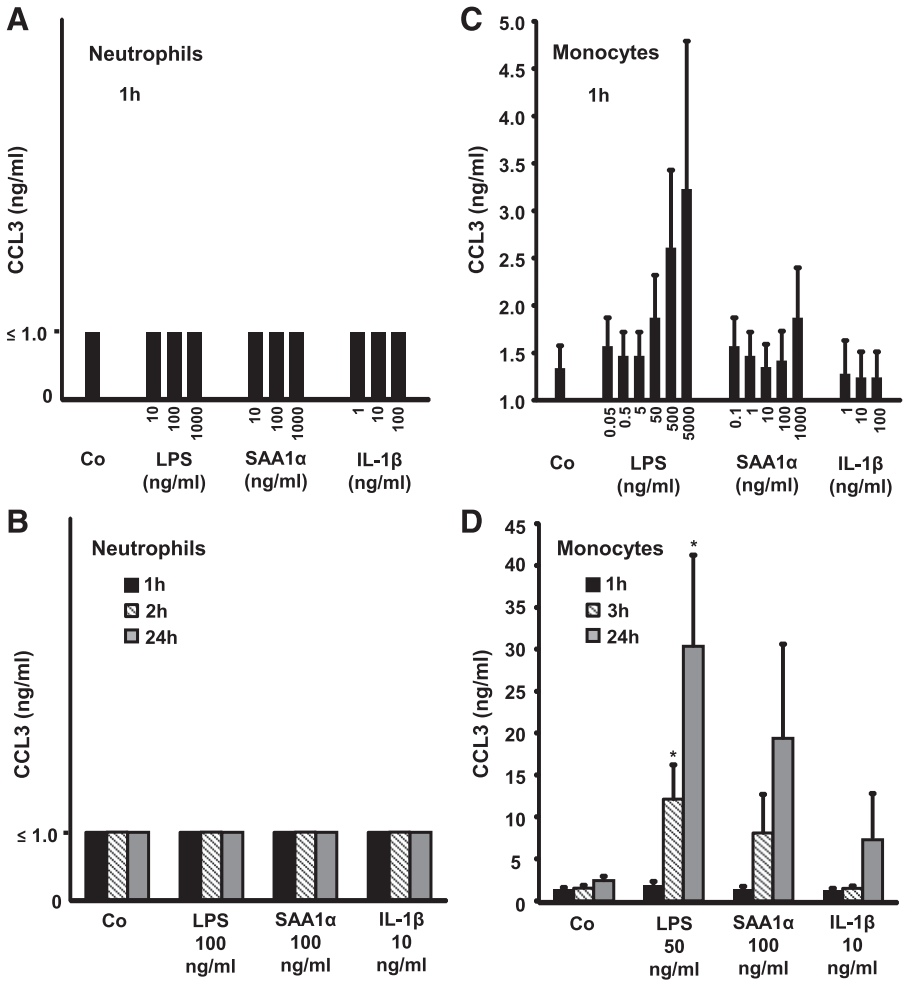

Figure 3. SAA1α induces MIP-1α/CCL3 in monocytes but not in neutrophils. Purified neutrophils (A and B) and CD14$^+$ monocytes (C and D) were incubated with different concentrations of LPS (0.05–5000 ng/ml), SAA1α (0.1–1000 ng/ml), IL-1β (1–100 ng/ml) or control buffer (Co). Cell supernatants were taken 1 h (black bars), 2 h (neutrophils; hatched bars), 3 h (monocytes; hatched bars), and 24 h (gray bars) after stimulation, and the amount of MIP-1α/CCL3 in the supernatants was measured by ELISA. Data represent the mean concentration ± SEM from 2–6 independent experiments. Averages below the lowest detection limit are shown without SEM. Statistically significant differences compared with controls, determined by the Mann-Whitney *U* test, are indicated by asterisks (*$P \leq 0.05$).

to synergize with 3 ng/ml IL-8/CXCL8 (58 ± 4 net blebbed neutrophils; $n = 10$, $P = 0.0028$ vs. 29 ± 5 and 5 ± 2 net blebbed neutrophils for SAA1α and IL-8/CXCL8, respectively, when added separately). Because the TLR4 agonist LPS has previously been shown to be chemotactic for monocytes through autocrine induction of chemokines [47], we also tested its capacity to synergize with IL-8/CXCL8 on neutrophils in the Boyden microchamber. However, as expected, no synergy between LPS and IL-8/CXCL8 was observed in neutrophil chemotaxis (Fig. 5C). This indicates that the GPCR (FPR2) ligand SAA1α, but not the TLR4 ligand LPS, can directly synergize with paracrine IL-8/CXCL8 in neutrophil chemotaxis. However, in monocyte chemotaxis, synergy also occurs via rapid autocrine chemokine induction by SAA1α [17].

Synergy between SAA1α and IL-8/CXCL8 in neutrophil chemotaxis in vitro can be blocked by antagonizing their corresponding receptors FPR2 and CXCR2

To evaluate whether the synergy between the GPCR ligands SAA1α and IL-8/CXCL8 in neutrophil chemotaxis is mediated through their main receptors (i.e., the formyl peptide receptor FPR2 and CXCR2, respectively), receptor antagonizing experiments were performed. Therefore, neutrophils were added to the upper compartment of the Boyden microchamber in the presence of the selective FPR2 and CXCR2 antagonists WRW4 (10 μg/ml) and SB225002 (3.5 μg/ml), respectively (Fig. 6). SAA1α (300 ng/ml) and IL-8/CXCL8 (5 ng/ml) again synergized significantly with each other (CI = 51.0 ± 6.2 vs. 1.2 ± 0.1 for SAA1α and 29.3 ± 2.3 for IL-8/CXCL8 when added separately; $n = 4$, $P = 0.03$), confirming the data shown in Figure 5A. This synergistic effect was inhibited for 93.9 ± 1.2% ($n = 4$, $P = 0.03$) on treatment of neutrophils with the CXCR2 antagonist SB225002 (**Fig. 6A**). The synergistic effect between SAA1α and IL-8/CXCL8 was totally lost in that the chemotactic response (CI = 51.0 ± 6.2) was reduced to a CI of 3.9 ± 0.4 in the presence of the CXCR2 antagonist. As a positive control, the chemotactic activity of the CXCR1/2 agonist IL-8/CXCL8 (5 ng/ml) was inhibited for 76.0 ± 9.3% ($n = 4$, $P = 0.03$) by SB225002. In contrast, the neutrophil response to SAA1α and the FPR1 ligand fMLP (10^{-8} M) remained unaltered (Fig. 6A). The CXCR1/2 agonist IL-8/CXCL8 had some remaining activity after the treatment of neutrophils with SB225002, for which its CXCR1 signaling capacity might be responsible. Because SAA1α was only very weakly chemoattracting neutrophils at 300 ng/ml (CI = 1.4 ± 0.2), the FPR2 antagonist WRW4 could not significantly inhibit its activity. As a control, WRW4

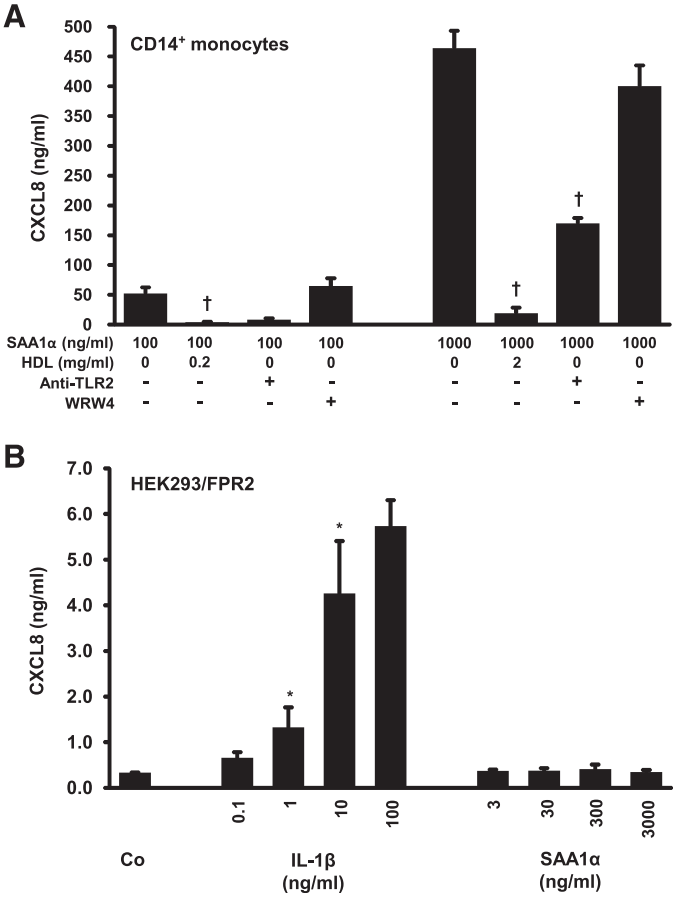

Figure 4. HDL and TLR2 antibody inhibit SAA1α-induced IL-8/CXCL8 production in monocytes. A) SAA1α (100 and 1000 ng/ml) was preincubated (1 h at 37°C) with 0.2 and 2 mg/ml HDL, respectively. $CD14^+$ monocytes were preincubated (1 h at 37°C) with TLR2 antibody (5 μg/ml) or WRW4 (10 μg/ml) before stimulation with SAA1α (100 and 1000 ng/ml). Cell supernatants were taken 24 h after stimulation, and the amount of IL-8/CXCL8 in the supernatants was measured by ELISA. Data represent the mean concentration ± SEM from 3 or 4 independent experiments. Statistically significant inhibition of IL-8/CXCL8 production, determined by the Mann-Whitney *U* test, is indicated by daggers ($^{\dagger}P \leq 0.05$). B) FPR2-transfected HEK293 cells were stimulated with different concentrations of SAA1α (3–3000 ng/ml), IL-1β (0.1–100 ng/ml), or control buffer (Co). Cell supernatants were taken 24 h after stimulation, and the amount of IL-8/CXCL8 in the supernatants was measured by ELISA. Data represent the mean concentration ± SEM from 2–4 independent experiments. Statistically significant differences compared with controls, determined by the Mann-Whitney *U* test, are indicated by asterisks ($^{*}P \leq 0.05$).

could not influence the spontaneous migration of neutrophils to control buffer (data not shown). However, the treatment of neutrophils with WRW4 significantly inhibited the synergistic effect between SAA1α (300 ng/ml) and IL-8/CXCL8 (5 ng/ml) for 50.3 ± 12.7% ($n = 5$, $P = 0.012$; Fig. 6B). The weak FPR2 agonist fMLP (10^{-9} M) was inhibited for 40.5 ± 13.3% ($n = 5$, $P = 0.2$). Furthermore, the CI of the already weak chemoattractant SAA1α (300 ng/ml) had diminished from 1.4 ± 0.2 to 1.1 ± 0.1. As expected, WRW4 did not influence the chemotactic activity of IL-8/CXCL8 (Fig. 6B). These data indicate that both the FPR2 ligand SAA1α and the CXCR2 ligand IL-8/CXCL8 are synergizing via use of their proper GPCR.

In vivo neutrophil recruitment by SAA1α is counteracted by blocking the receptor of induced paracrine chemokines

First, we investigated whether SAA1α and exogenous GCP-2/CXCL6 could synergize in vivo by intraperitoneal coinjection of these GPCR ligands in mice. The number of neutrophils recruited

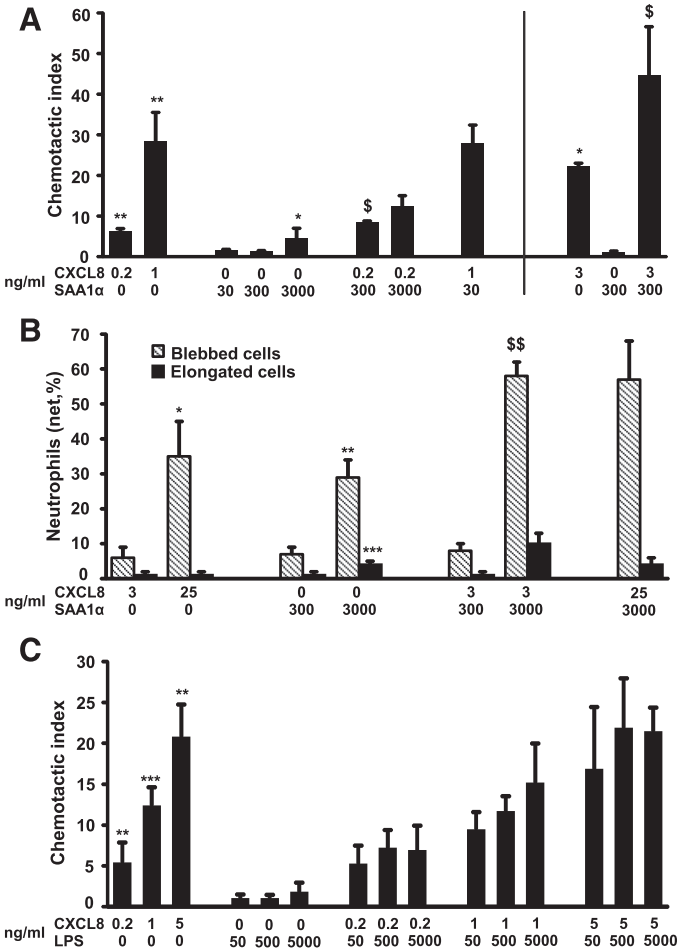

Figure 5. SAA1α but not LPS synergizes with IL-8/CXCL8 in neutrophil migration in vitro. A) The chemotactic potency of IL-8/CXCL8 (0.2 and 1 ng/ml), SAA1α (30–3000 ng/ml), and a combination of IL-8/CXCL8 and SAA1α was evaluated on neutrophils in the Boyden microchamber assay. In a similar, but separate, set of experiments (right from the vertical line), IL-8/CXCL8 (3 ng/ml) and SAA1α (300 ng/ml) were added individually or together to the lower compartment of the Boyden chamber. Data represent the mean CI ± SEM from 7 (left) or 3–5 (right) independent experiments. B) Shape change of neutrophils was determined after 2.5 min of stimulation of the cells with IL-8/CXCL8 (3 and 25 ng/ml), SAA1α (300 and 3000 ng/ml), or a combination of IL-8/CXCL8 and SAA1α. Data (5–10 independent experiments) are expressed as the net percentage of blebbed (hatched bars) and elongated (black bars) neutrophils ± SEM. C) Migration of neutrophils to IL-8/CXCL8 (0.2–5 ng/ml), LPS (50–5000 ng/ml), or a combination of IL-8/CXCL8 and LPS was measured using Boyden microchamber assays. Data are shown as mean CI ± SEM from 8 independent experiments. A–C) Statistically significant differences compared with controls, determined by the Mann-Whitney *U* test, are indicated by asterisks ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$). Dollar signs indicate statistically significant synergistic interactions (compared with the sum of the effect of both individual agonists; $\$P \leq 0.05$; $\$\$P \leq 0.01$).

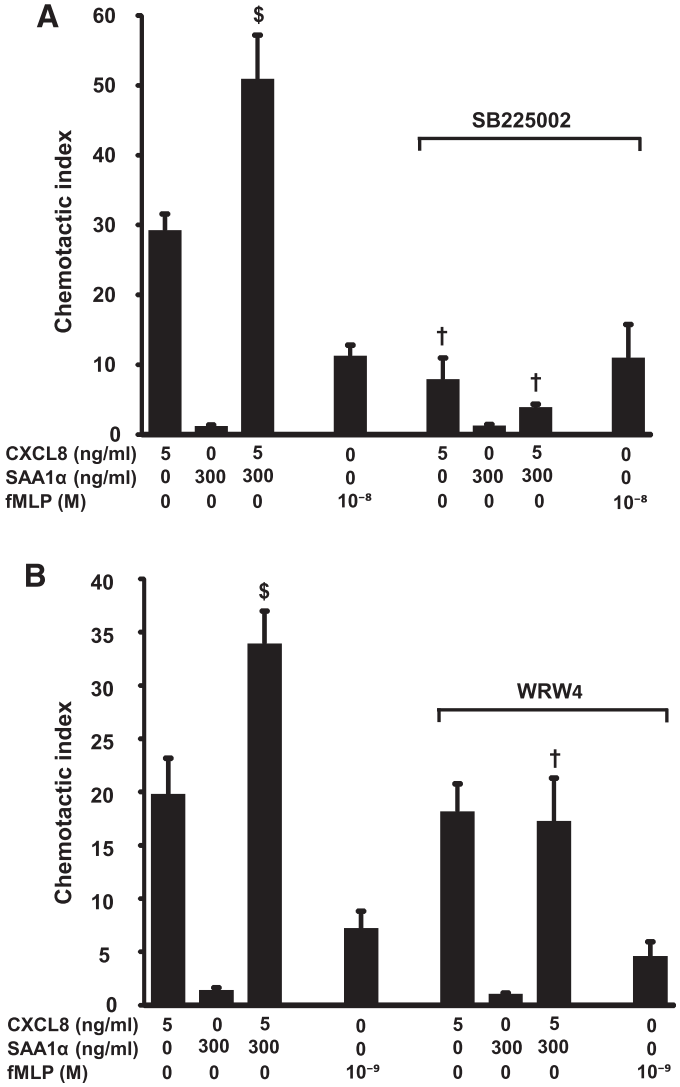

Figure 6. Synergy between SAA1α and IL-8/CXCL8 in neutrophil chemotaxis is mediated by their corresponding receptors, FPR2 and CXCR2. Neutrophils were treated with the CXCR2 antagonist SB225002 (3.5 μg/ml; A) or the FPR2 antagonist WRW4 (10 μg/ml; B) or were left untreated before loading the cells into the upper compartment of the Boyden microchamber. The migration of the cells to IL-8/CXCL8 (5 ng/ml), SAA1α (300 ng/ml), or a combination of both chemoattractants is shown as the mean CI ± SEM, obtained from 4 (A) or 5 (B) independent experiments. For comparison, the chemotactic potency ± SEM of neutrophils toward fMLP [10^{-8} M (A) or 10^{-9} M (B)] is shown. A and B) Statistically significant synergistic interactions (compared with the sum of the net migration indexes obtained by adding SAA1α and IL-8/CXCL8 separately to the lower compartment of the Boyden microchamber) and statistically significant inhibition of migration by antagonists (compared with the migration indexes of the individual or combined agonists), determined by the Mann-Whitney *U* test, are indicated by dollar signs ($\$P \leq 0.05$) and daggers ($\dagger P \leq 0.05$), respectively.

into the peritoneal cavity was lower than that of the additive effect when SAA1α (1000 ng) and GCP-2/CXCL6 (100 ng) were coinjected compared with the effect of SAA1α and GCP-2/CXCL6 alone (data not shown). To determine whether the strong influx of neutrophils into the peritoneal cavity in SAA1α-treated mice was obtained via synergistic interactions between SAA1α and endogenously SAA1α-induced CXCR2 ligands, CXCR2 was blocked in the experimental setting in vivo. In addition to intraperitoneal injection of GCP-2/CXCL6 (100 ng) or SAA1α (1000 ng) alone, the mice were injected with chemoattractant in the presence of the CXCR2 antagonist SB225002 (25 μg). **Figure 7** shows that, compared with PBS-treated mice, again (compare with the data shown in Fig. 1), a significant increase in neutrophil influx into the peritoneal cavity occurred after injection of mice with GCP-2/

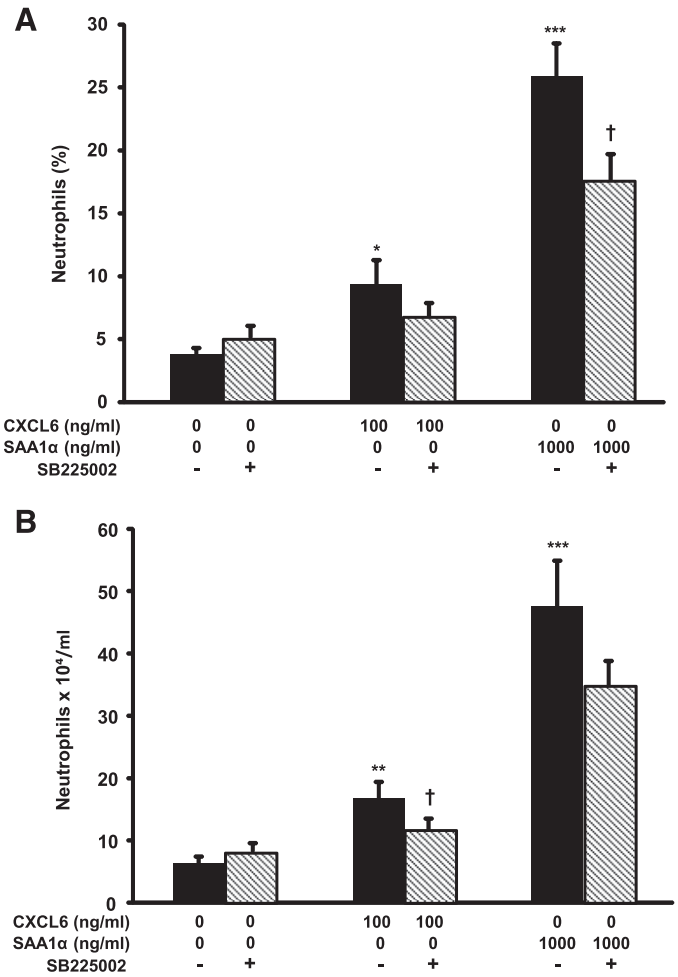

Figure 7. Inhibition of SAA1α-induced intraperitoneal neutrophil influx by antagonization of CXCR2. Female NMRI mice (9 mice per group) were injected intraperitoneally with PBS (200 μl), 1000 ng of SAA1α (100 μl + 100 μl PBS), 100 ng of murine GCP-2/CXCL6 (100 μl + 100 μl PBS), 25 μg of the CXCR2 antagonist SB225002 (100 μl + 100 μl PBS), or a combination of 1000 ng of SAA1α (100 μl) or 100 ng of GCP-2/CXCL6 (100 μl) and 25 μg of SB225002 (100 μl). Peritoneal lavages (5 ml) were obtained at 2 h after injection. Cells were counted using a hemocytometer, and differential cell counts were performed microscopically. The mean percentage of neutrophils ± SEM (A) and the mean number of neutrophils ± SEM × 10^4/ml (B) with (hatched histograms) and without (black bars) coinjection of SB225002 in the peritoneal lavages are shown. Statistically significant differences from control mice, not treated with SB225002, and statistically significant differences from mice treated with chemoattractant alone, determined by the Mann-Whitney *U* test, are indicated by asterisks ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$) and daggers ($\dagger P \leq 0.05$), respectively.

CXCL6 (9.4 ± 1.9% neutrophils; 16.8 ± 2.6 × 10^4 neutrophils/ml vs. 3.8 ± 0.5% neutrophils; 6.3 ± 1.1 × 10^4 neutrophils/ml in PBS-treated mice; $n = 11$, $P = 0.0269$ and $P = 0.0033$, respectively) or SAA1α (25.9 ± 2.6% neutrophils; 47.6 ± 7.3 × 10^4 neutrophils/ml vs. PBS-treated mice; $n = 11$, $P = 0.0006$) alone. SB225002 inhibited the neutrophil influx elicited by injection of SAA1α, because the net number of neutrophils in the peritoneal lavages from the mice treated with a combination of SAA1α and SB225002 diminished to 34.5 ± 4.3 × 10^4 neutrophils/ml ($n = 11$, $P = 0.0661$), equal to a 27.6% reduction in cell number. As a control, the GCP-2/CXCL6-mediated neutrophil influx was also affected by coinjection of the chemokine with SB225002 (from 16.8 ± 2.6 × 10^4 neutrophils/ml to 11.6 ± 1.9 × 10^4 neutrophils/ml; $n = 12$, $P = 0.0485$). The intraperitoneal injection of mice with SB225002 alone did not significantly affect neutrophil recruitment. Moreover, significantly greater amounts of mouse GCP-2/CXCL6 (mean OD ± SEM 0.141 ± 0.019; $P = 0.002$) were measured in the peritoneal washes after injection of SAA1α (1 μg) than in the PBS-treated mice (OD ± SEM 0.062 ± 0.002; 9 mice/group). In the GCP-2/CXCL6-treated mice (100 ng/mouse), no GCP-2/CXCL6 (mean OD ± SEM of 0.064 ± 0.003) was recovered in the peritoneal washes 2 h after injection, demonstrating that the intraperitoneal injected GCP-2/CXCL6 is probably firmly bound to the proteoglycans of the peritoneal cavity. These results indicate that the CXCR2 ligand GCP-2/CXCL6 is induced in peritoneal cells after intraperitoneal injection of mice with SAA1α. Moreover, significant mouse GCP-2/CXCL6 production (61.0 ± 22.0 pg/ml; $P = 0.013$; and 20.0 ± 3.0 pg/ml; $n = 6$–8, $P = 0.005$) was also measured ex vivo after stimulation of peritoneal lavage cells with SAA1α (1 μg) or LPS (500 ng/ml), respectively, compared with the nonstimulated cells.

DISCUSSION

The exact role of SAA in cell migration has until now not been fully elucidated. Other research groups have proved that SAA is directly chemotactic for neutrophils, monocytes, mast cells, and T cells [16, 18, 19]. However, for our investigations, we used a recombinant human SAA1 isoform (SAA1α), which is identical to natural SAA1α, except for an N-terminal methionine. In contrast, a non-natural hybrid between human SAA1α and SAA2β was mostly used in other studies. In a previous study, we showed that SAA1α is chemotactic in vitro for monocytes and also for immature dendritic cells, predominantly in an indirect manner via induction of chemokines [17]. We found that SAA1α-stimulated monocytes rapidly (within 3 h) produced MIP-1α/CCL3 and IL-8/CXCL8, which cooperated in an autocrine manner with each other and possibly with SAA1α to chemoattract monocytes. In neutrophil chemotaxis, SAA1α-induced CXCR2 ligands synergize with SAA1α in a paracrine way. Thus, the neutrophil chemotactic potency of the weak chemoattractant SAA1α, compared with IL-8/CXCL8, was increased significantly in vivo (Fig. 1). For our in vivo experiments, mouse GCP-2/CXCL6 was used as a murine counterpart for human IL-8/CXCL8, because GCP-2/CXCL6 is the only murine chemokine that signals through both CXCR1 and CXCR2, just as human IL-8/CXCL8 does [57, 59]. A 1000-fold higher concentration of SAA1α, compared with IL-8/CXCL8, was needed to acquire an equivalent chemotactic effect in the Boyden microchamber (1000 ng/ml for SAA1α vs. 0.2 ng/ml for IL-8/CXCL8). In contrast, only a 10-fold higher dose of SAA1α (1000 ng) than GCP-2/CXCL6 (100 ng) was required to reach an even more pronounced influx of neutrophils into the peritoneal cavity of SAA1α-treated mice. To explain this apparent discrepancy, we investigated whether neutrophils (like monocytes) could rapidly produce CXC or CC chemokines, such as IL-8/CXCL8 and MIP-1α/CCL3, which could in turn synergize with each other and with SAA1α in neutrophil chemotaxis. However, no induction (by SAA1α or LPS) of these chemokines was observed in neutrophils within the time period to reach maximal neutrophil chemotaxis in vitro and in vivo (2 h; Figs. 2A and B and 3A and B). In contrast, a significant amount of IL-8/CXCL8 (>15 ng/ml) was produced by SAA1α-stimulated monocytes within 3 h (Fig. 2C and D). As reported in published studies, we have confirmed that SAA-induced production of cytokines is TLR2 mediated (Fig. 4A) [30, 36, 38, 40, 41]. It is known that neutrophils have less potential than other leukocytes to produce cytokines [60–62], and IL-8/CXCL8 is the most abundantly produced chemokine by these cells [63]. Because several leukocyte types responsive to SAA1α [40] are present in the peritoneal cavity and because induction of IL-8/CXCL8 by monocytes occurred within the time limit (2 h) of the in vivo experimental setting, we assumed that mouse peritoneal macrophages, stimulated by the intraperitoneal injection of SAA1α, would quickly produce GCP-2/CXCL6, which synergizes in a paracrine manner with SAA1α to enhance neutrophil influx in the peritoneal cavity. We demonstrated that the CXCR2 ligand GCP-2/CXCL6 is rapidly (within 2 h) induced in the mouse peritoneal cavity after i.p. injection of mice with SAA1α.

This led us to further study the direct synergistic effect between SAA1α and IL-8/CXCL8 on purified neutrophils in vitro (Fig. 5). Synergy was obtained by combining suboptimal concentrations of SAA1α (300 ng/ml) and IL-8/CXCL8 (0.2 ng/ml) in the Boyden chemotaxis microchamber (Fig. 5A). In the neutrophil shape change assays, a higher SAA1α concentration (3000 ng/ml) was necessary to obtain synergy with IL-8/CXCL8 (3 ng/ml). This probably resulted from the relatively lower sensitivity of this assay, compared with that of the Boyden chemotaxis assays, because ≥10-fold higher concentrations of IL-8/CXCL8 were required for a significant effect on shape change.

We speculated that in the Boyden chemotaxis assay, the chemokine-inducing TLR4 agonist LPS [47] could also synergize with IL-8/CXCL8 in neutrophil chemotaxis, although LPS did not induce chemokines in neutrophils during the assay period (Figs. 2A and B and 3A and B). In contrast to SAA1α, LPS did not synergize with IL-8/CXCL8 in the Boyden microchamber (Fig. 5C), indicating that LPS contamination could not be responsible for the observed direct synergy between SAA1α and IL-8/CXCL8 on neutrophils. Furthermore, this allowed us to speculate that, in chemotaxis, direct synergy is not likely to occur between a GPCR agonist (IL-8/CXCL8) and a TLR agonist (LPS) that cannot bind GPCR. This suggests that SAA1α synergized with IL-8/CXCL8 in neutrophil chemotaxis through its GPCR (i.e., FPR2) but not via

TLR2. This was supported by the significant inhibition of synergy between SAA1α and IL-8/CXCL8 in neutrophil chemotaxis by the FPR2 antagonist WRW4 (Fig. 6B). Moreover, the CXCR2 antagonist SB225002 completely inhibited the synergy between SAA1α and IL-8/CXCL8 (Fig. 6A). Finally, i.p. injection of the CXCR2 antagonist together with SAA1α significantly reduced the i.p. neutrophil influx in mice (Fig. 7), indicating that the in vivo chemotactic effect of SAA1α is in part mediated through synergy with CXCR2 ligands induced in peritoneal cells (e.g., macrophages). The remaining neutrophil chemotactic effect of GCP-2/CXCL6 in mice coinjected with the chemokine and SB225002 could have been a result of interaction with CXCR1, which also binds GCP-2/CXCL6 [57]. In addition, SB225002 can inhibit other CXCR2 ligands, induced by SAA1α, which synergize with SAA1α and possibly also with induced CC chemokines such as MIP-1α/CCL3.

Taken together, no rapid induction of chemokines in SAA1α-stimulated neutrophils occurred; thus, fast synergy between SAA1α and autocrine-induced chemotactic ligands was not likely to increase neutrophil migration in vitro and in vivo. In contrast, human monocytes were able to rapidly produce IL-8/CXCL8 (and other chemokines) after stimulation with SAA1α within the time limit of a chemotaxis assay, as shown by Gouwy et al. [17]. Thus, these rapidly induced chemokines desensitized chemokines exogenously added to the assay, blocking further synergy between SAA1α and these exogenously added chemokines. These findings show that, in vitro, synergy was already present in monocyte chemotaxis between SAA1α and the induced IL-8/CXCL8 and MIP-1α/CCL3 in an autocrine way, such that no further synergy could be obtained between SAA1α and the exogenously added chemokine.

The receptor usage of SAA has mostly been investigated in relation to the induction of cytokines. In particular, FPR2 and TLR2 are often reported as signaling receptors, inducing the production of cytokines, including chemokines, such as IL-8/CXCL8 and MCP-1/CCL2 [22, 23, 30, 34, 38–41]. However, only FPR2 has been described as the SAA receptor mediating chemotaxis [30–32]. This corresponds with our finding that synergy between SAA1α and IL-8/CXCL8 in neutrophil chemotaxis is probably not mediated by TLR2, because it was blocked by an FPR2 antagonist. Nonetheless, because multiple receptors are involved in cytokine induction, additional studies should be conducted to elucidate the potential use of other receptors by SAA for chemotaxis.

In summary, with the development of inflammation, several chemokines are coproduced by leukocytes, fibroblasts, and endothelial cells, which cooperate or synergize with each other to enhance recruitment of circulating leukocytes. Such synergy on monocytes can be obtained in an autocrine [47] or, as we observed in the present study for neutrophils, in a paracrine way. Moreover, chemokines can synergize with other GPCR ligands that are generated on stimulation of the immune system by microbial agents [51]. For instance, we have shown direct synergy between IL-8/CXCL8 and the acute phase protein and chemokine inducer SAA1α in neutrophil chemotaxis. The synergy observed in the present study is clinically relevant. Shen et al. [64] demonstrated increased SAA serum levels in patients with rheumatoid arthritis. Moreover, SAA is also significantly increased in the serum of osteoarthritis patients compared with that in healthy volunteers [65]. In rheumatoid arthritis, macrophage-derived cytokines such as IL-1, TNF, IL-6, and IL-8/CXCL8 are all present in the synovial cavity and in the blood circulation and play a key role in amplifying inflammation. IL-8/CXCL8 recruits inflammatory cells into the joint. IL-1 and TNF are important mediators of the acute phase response in that they directly induce acute phase proteins and potently induce IL-6, itself a major mediator of hepatic synthesis of acute phase proteins. This acute phase response implies a rapid elevation of SAA in the blood [66]. Moreover, Urbanowska et al. [67] described IL-1β, IL-6, IL-8/CXCL8, MCP-1/CCL2, and SAA as potential markers of disease progression of rheumatoid arthritis. Hence, in inflammation, the induction of the acute phase protein SAA1α in the systemic circulation could be viewed as a general alarm bell. Once SAA1α enters peripheral inflammatory sites, it exerts the short-term local induction of chemokines, which synergize with each other and with SAA1α in neutrophil chemotaxis through GPCRs when the chemokine levels are still very low. SAA1α thus plays a critical role in innate immunity, both systemically and locally.

AUTHORSHIP

M.D.B. performed experiments, analyzed data, wrote part of the manuscript, and submitted the manuscript. N.B. performed the in vivo experiments and analyzed the data. N.P., L.V., and M.C. performed in vitro experiments. S.S. initiated the research line, analyzed the data, and corrected the manuscript. G.O. produced the murine GCP-2/CXCL6, was involved in the discussions, and corrected the manuscript. P.P. executed biochemical quality control of the reagents and corrected the manuscript. J.V.D. designed the study, planned the experiments, analyzed the data, and corrected the manuscript. M.G. gave technical advice, planned and performed the experiments, wrote part of the manuscript, and corrected the manuscript.

ACKNOWLEDGMENTS

The authors thank Dr. J. M. Wang (National Cancer Institute, National Institutes of Health, Frederick, MD, USA) for providing the FPR2-transfected HEK293 cells and S. Lepla for his technical assistance. This work was supported by the Concerted Research Actions of the Regional Government of Flanders, the Fund for Scientific Research of Flanders, and the Interuniversity Attraction Poles Program (P7/40)-Belgian Science Policy. The Hercules foundation of the Flemish government provided funding to purchase LC-MS/MS equipment (contract AKUL/11/31).

DISCLOSURES

The authors declare no competing financial interests.

REFERENCES

1. Eriksen, N., Benditt, E. P. (1980) Isolation and characterization of the amyloid-related apoprotein (SAA) from human high density lipoprotein. *Proc. Natl. Acad. Sci. USA* **77,** 6860–6864.
2. Larson, M. A., Wei, S. H., Weber, A., Weber, A. T., McDonald, T. L. (2003) Induction of human mammary-associated serum amyloid A3 expression by prolactin or lipopolysaccharide. *Biochem. Biophys. Res. Commun.* **301,** 1030–1037.

3. Whitehead, A. S., de Beer, M. C., Steel, D. M., Rits, M., Lelias, J. M., Lane, W. S., de Beer, F. C. (1992) Identification of novel members of the serum amyloid A protein superfamily as constitutive apolipoproteins of high density lipoprotein. *J. Biol. Chem.* **267,** 3862–3867.
4. Woo, P., Sipe, J., Dinarello, C. A., Colten, H. R. (1987) Structure of a human serum amyloid A gene and modulation of its expression in transfected L cells. *J. Biol. Chem.* **262,** 15790–15795.
5. Uhlar, C. M., Whitehead, A. S. (1999) Serum amyloid A, the major vertebrate acute-phase reactant. *Eur. J. Biochem.* **265,** 501–523.
6. Uhlar, C. M., Burgess, C. J., Sharp, P. M., Whitehead, A. S. (1994) Evolution of the serum amyloid A (SAA) protein superfamily. *Genomics* **19,** 228–235.
7. Liang, J. S., Sipe, J. D. (1995) Recombinant human serum amyloid A (apoSAAp) binds cholesterol and modulates cholesterol flux. *J. Lipid Res.* **36,** 37–46.
8. Stonik, J. A., Remaley, A. T., Demosky, S. J., Neufeld, E. B., Bocharov, A., Brewer, H. B. (2004) Serum amyloid A promotes ABCA1-dependent and ABCA1-independent lipid efflux from cells. *Biochem. Biophys. Res. Commun.* **321,** 936–941.
9. Van der Westhuyzen, D. R., Cai, L., de Beer, M. C., de Beer, F. C. (2005) Serum amyloid A promotes cholesterol efflux mediated by scavenger receptor B-I. *J. Biol. Chem.* **280,** 35890–35895.
10. Zimlichman, S., Danon, A., Nathan, I., Mozes, G., Shainkin-Kestenbaum, R. (1990) Serum amyloid A, an acute phase protein, inhibits platelet activation. *J. Lab. Clin. Med.* **116,** 180–186.
11. Gatt, M. E., Urieli-Shoval, S., Preciado-Patt, L., Fridkin, M., Calco, S., Azar, Y., Matzner, Y. (1998) Effect of serum amyloid A on selected in vitro functions of isolated human neutrophils. *J. Lab. Clin. Med.* **132,** 414–420.
12. Linke, R. P., Bock, V., Valet, G., Rothe, G. (1991) Inhibition of the oxidative burst response of N-formyl peptide-stimulated neutrophils by serum amyloid-A protein. *Biochem. Biophys. Res. Commun.* **176,** 1100–1105.
13. Lee, H. Y., Kim, M. K., Park, K. S., Bae, Y. H., Yun, J., Park, J. I., Kwak, J. Y., Bae, Y. S. (2005) Serum amyloid A stimulates matrix-metalloproteinase-9 upregulation via formyl peptide receptor like-1-mediated signaling in human monocytic cells. *Biochem. Biophys. Res. Commun.* **330,** 989–998.
14. Migita, K., Kawabe, Y., Tominaga, M., Origuchi, T., Aoyagi, T., Eguchi, K. (1998) Serum amyloid A protein induces production of matrix metalloproteinases by human synovial fibroblasts. *Lab. Invest.* **78,** 535–539.
15. O'Hara, R., Murphy, E. P., Whitehead, A. S., FitzGerald, O., Bresnihan, B. (2004) Local expression of the serum amyloid A and formyl peptide receptor-like 1 genes in synovial tissue is associated with matrix metalloproteinase production in patients with inflammatory arthritis. *Arthritis Rheum.* **50,** 1788–1799.
16. Badolato, R., Wang, J. M., Murphy, W. J., Lloyd, A. R., Michiel, D. F., Bausserman, L. L., Kelvin, D. J., Oppenheim, J. J. (1994) Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J. Exp. Med.* **180,** 203–209.
17. Gouwy, M., De Buck, M., Pörtner, N., Opdenakker, G., Proost, P., Struyf, S., Van Damme, J. (2015) Serum amyloid A chemoattracts immature dendritic cells and indirectly provokes monocyte chemotaxis by induction of cooperating CC and CXC chemokines. *Eur. J. Immunol.* **45,** 101–112.
18. Olsson, N., Siegbahn, A., Nilsson, G. (1999) Serum amyloid A induces chemotaxis of human mast cells by activating a pertussis toxin-sensitive signal transduction pathway. *Biochem. Biophys. Res. Commun.* **254,** 143–146.
19. Xu, L., Badolato, R., Murphy, W. J., Longo, D. L., Anver, M., Hale, S., Oppenheim, J. J., Wang, J. M. (1995) A novel biologic function of serum amyloid A: induction of T lymphocyte migration and adhesion. *J. Immunol.* **155,** 1184–1190.
20. Christenson, K., Björkman, L., Ahlin, S., Olsson, M., Sjöholm, K., Karlsson, A., Bylund, J. (2013) Endogenous acute phase serum amyloid A lacks pro-inflammatory activity, contrasting the two recombinant variants that activate human neutrophils through different receptors. *Front. Immunol.* **4,** 92.
21. Gouwy, M., Struyf, S., Leutenez, L., Pörtner, N., Sozzani, S., Van Damme, J. (2014) Chemokines and other GPCR ligands synergize in receptor-mediated migration of monocyte-derived immature and mature dendritic cells. *Immunobiology* **219,** 218–229.
22. He, R., Sang, H., Ye, R. D. (2003) Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA4R. *Blood* **101,** 1572–1581.
23. Lee, H. Y., Kim, S. D., Shim, J. W., Lee, S. Y., Lee, H., Cho, K. H., Yun, J., Bae, Y. S. (2008) Serum amyloid A induces CCL2 production via formyl peptide receptor-like 1-mediated signaling in human monocytes. *J. Immunol.* **181,** 4332–4339.
24. Ribeiro, F. P., Furlaneto, C. J., Hatanaka, E., Ribeiro, W. B., Souza, G. M., Cassatella, M. A., Campa, A. (2003) mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes. *Mediators Inflamm.* **12,** 173–178.
25. Song, C., Hsu, K., Yamen, E., Yan, W., Fock, J., Witting, P. K., Geczy, C. L., Freedman, S. B. (2009) Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes. *Atherosclerosis* **207,** 374–383.
26. Locati, M., Murphy, P. M. (1999) Chemokines and chemokine receptors: biology and clinical relevance in inflammation and AIDS. *Annu. Rev. Med.* **50,** 425–440.
27. Rot, A., von Andrian, U. H. (2004) Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu. Rev. Immunol.* **22,** 891–928.
28. Struyf, S., Proost, P., Van Damme, J. (2003) Regulation of the immune response by the interaction of chemokines and proteases. *Adv. Immunol.* **81,** 1–44.
29. Thelen, M. (2001) Dancing to the tune of chemokines. *Nat. Immunol.* **2,** 129–134.
30. Chen, M., Zhou, H., Cheng, N., Qian, F., Ye, R. D. (2014) Serum amyloid A1 isoforms display different efficacy at Toll-like receptor 2 and formyl peptide receptor 2. *Immunobiology* **219,** 916–923.
31. Liang, T. S., Wang, J. M., Murphy, P. M., Gao, J. L. (2000) Serum amyloid A is a chemotactic agonist at FPR2, a low-affinity N-formylpeptide receptor on mouse neutrophils. *Biochem. Biophys. Res. Commun.* **270,** 331–335.
32. Su, S. B., Gong, W., Gao, J. L., Shen, W., Murphy, P. M., Oppenheim, J. J., Wang, J. M. (1999) A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J. Exp. Med.* **189,** 395–402.
33. Ye, R. D., Boulay, F., Wang, J. M., Dahlgren, C., Gerard, C., Parmentier, M., Serhan, C. N., Murphy, P. M. (2009) International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol. Rev.* **61,** 119–161.
34. Ather, J. L., Ckless, K., Martin, R., Foley, K. L., Suratt, B. T., Boyson, J. E., Fitzgerald, K. A., Flavell, R. A., Eisenbarth, S. C., Poynter, M. E. (2011) Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. *J. Immunol.* **187,** 64–73.
35. Baranova, I. N., Vishnyakova, T. G., Bocharov, A. V., Kurlander, R., Chen, Z., Kimelman, M. L., Remaley, A. T., Csako, G., Thomas, F., Eggerman, T. L., Patterson, A. P. (2005) Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. *J. Biol. Chem.* **280,** 8031–8040.
36. Baranova, I. N., Bocharov, A. V., Vishnyakova, T. G., Kurlander, R., Chen, Z., Fu, D., Arias, I. M., Csako, G., Patterson, A. P., Eggerman, T. L. (2010) CD36 is a novel serum amyloid A (SAA) receptor mediating SAA binding and SAA-induced signaling in human and rodent cells. *J. Biol. Chem.* **285,** 8492–8506.
37. Cai, H., Song, C., Endoh, I., Goyette, J., Jessup, W., Freedman, S. B., McNeil, H. P., Geczy, C. L. (2007) Serum amyloid A induces monocyte tissue factor. *J. Immunol.* **178,** 1852–1860.
38. Cheng, N., He, R., Tian, J., Ye, P. P., Ye, R. D. (2008) Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. *J. Immunol.* **181,** 22–26.
39. He, R. L., Zhou, J., Hanson, C. Z., Chen, J., Cheng, N., Ye, R. D. (2009) Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2. *Blood* **113,** 429–437.
40. O'Reilly, S., Cant, R., Ciechomska, M., Finnigan, J., Oakley, F., Hambleton, S., van Laar, J. M. (2014) Serum amyloid A induces interleukin-6 in dermal fibroblasts via Toll-like receptor 2, interleukin-1 receptor-associated kinase 4 and nuclear factor-κB. *Immunology* **143,** 331–340.
41. Sun, L., Zhu, Z., Cheng, N., Yan, Q., Ye, R. D. (2014) Serum amyloid A induces interleukin-33 expression through an IRF7-dependent pathway. *Eur. J. Immunol.* **44,** 2153–2164.
42. Janeway, C. A., Jr., Medzhitov, R. (2002) Innate immune recognition. *Annu. Rev. Immunol.* **20,** 197–216.
43. Dueñas, A. I., Aceves, M., Orduña, A., Díaz, R., Sánchez Crespo, M., García-Rodríguez, C. (2006) Francisella tularensis LPS induces the production of cytokines in human monocytes and signals via Toll-like receptor 4 with much lower potency than E. coli LPS. *Int. Immunol.* **18,** 785–795.
44. Kaufmann, A., Gemsa, D., Sprenger, H. (2000) Differential desensitization of lipopolysaccharide-inducible chemokine gene expression in human monocytes and macrophages. *Eur. J. Immunol.* **30,** 1562–1567.
45. Mariman, R., Tielen, F., Koning, F., Nagelkerken, L. (2014) The probiotic mixture VSL#3 dampens LPS-induced chemokine expression in human dendritic cells by inhibition of STAT-1 phosphorylation. *PLoS One* **9,** e115676.
46. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282,** 2085–2088.
47. Gouwy, M., Struyf, S., Verbeke, H., Put, W., Proost, P., Opdenakker, G., Van Damme, J. (2009) CC chemokine ligand-2 synergizes with the

nonchemokine G protein-coupled receptor ligand fMLP in monocyte chemotaxis, and it cooperates with the TLR ligand LPS via induction of CXCL8. *J. Leukoc. Biol.* **86,** 671–680.
48. Gouwy, M., Struyf, S., Catusse, J., Proost, P., Van Damme, J. (2004) Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration. *J. Leukoc. Biol.* **76,** 185–194.
49. Van Damme, J., Van Beeumen, J., Opdenakker, G., Billiau, A. (1988) A novel, NH2-terminal sequence-characterized human monokine possessing neutrophil chemotactic, skin-reactive, and granulocytosis-promoting activity. *J. Exp. Med.* **167,** 1364–1376.
50. Murphy, P. M., Tiffany, H. L. (1991) Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* **253,** 1280–1283.
51. Gouwy, M., Struyf, S., Proost, P., Van Damme, J. (2005) Synergy in cytokine and chemokine networks amplifies the inflammatory response. *Cytokine Growth Factor Rev.* **16,** 561–580.
52. De Buck, M., Gouwy, M., Proost, P., Struyf, S., Van Damme, J. (2013) Identification and characterization of MIP-1α/CCL3 isoform 2 from bovine serum as a potent monocyte/dendritic cell chemoattractant. *Biochem. Pharmacol.* **85,** 789–797.
53. Bae, Y. S., Lee, H. Y., Jo, E. J., Kim, J. I., Kang, H. K., Ye, R. D., Kwak, J. Y., Ryu, S. H. (2004) Identification of peptides that antagonize formyl peptide receptor-like 1-mediated signaling. *J. Immunol.* **173,** 607–614.
54. Van Coillie, E., Van Aelst, I., Wuyts, A., Vercauteren, R., Devos, R., De Wolf-Peeters, C., Van Damme, J., Opdenakker, G. (2001) Tumor angiogenesis induced by granulocyte chemotactic protein-2 as a countercurrent principle. *Am. J. Pathol.* **159,** 1405–1414.
55. Schutyser, E., Struyf, S., Proost, P., Opdenakker, G., Laureys, G., Verhasselt, B., Peperstraete, L., Van de Putte, I., Saccani, A., Allavena, P., Mantovani, A., Van Damme, J. (2002) Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma. *J. Biol. Chem.* **277,** 24584–24593.
56. Starckx, S., Wuyts, A., Opsomer, I., Van Coillie, E., Proost, P., Arnold, B., Van Damme, J., Opdenakker, G. (2002) Recombinant mouse granulocyte chemotactic protein-2: production in bacteria, characterization, and systemic effects on leukocytes. *J. Interferon Cytokine Res.* **22,** 965–974.
57. Fan, X., Patera, A. C., Pong-Kennedy, A., Deno, G., Gonsiorek, W., Manfra, D. J., Vassileva, G., Zeng, M., Jackson, C., Sullivan, L., Sharif-Rodriguez, W., Opdenakker, G., Van Damme, J., Hedrick, J. A., Lundell, D., Lira, S. A., Hipkin, R. W. (2007) Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8. *J. Biol. Chem.* **282,** 11658–11666.
58. Patel, H., Fellowes, R., Coade, S., Woo, P. (1998) Human serum amyloid A has cytokine-like properties. *Scand. J. Immunol.* **48,** 410–418.
59. Wuyts, A., Van Osselaer, N., Haelens, A., Samson, I., Herdewijn, P., Ben-Baruch, A., Oppenheim, J. J., Proost, P., Van Damme, J. (1997) Characterization of synthetic human granulocyte chemotactic protein 2: usage of chemokine receptors CXCR1 and CXCR2 and in vivo inflammatory properties. *Biochemistry* **36,** 2716–2723.
60. Bazzoni, F., Cassatella, M. A., Rossi, F., Ceska, M., Dewald, B., Baggiolini, M. (1991) Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J. Exp. Med.* **173,** 771–774.
61. Cassatella, M. A. (1995) The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* **16,** 21–26.
62. Tecchio, C., Micheletti, A., Cassatella, M. A. (2014) Neutrophil-derived cytokines: facts beyond expression. *Front. Immunol.* **5,** 508.
63. Scapini, P., Lapinet-Vera, J. A., Gasperini, S., Calzetti, F., Bazzoni, F., Cassatella, M. A. (2000) The neutrophil as a cellular source of chemokines. *Immunol. Rev.* **177,** 195–203.
64. Shen, C., Sun, X. G., Liu, N., Mu, Y., Hong, C. C., Wei, W., Zheng, F. (2015) Increased serum amyloid A and its association with autoantibodies, acute phase reactants and disease activity in patients with rheumatoid arthritis. *Mol. Med. Rep.* **11,** 1528–1534.
65. De Seny, D., Cobraiville, G., Charlier, E., Neuville, S., Esser, N., Malaise, D., Malaise, O., Calvo, F. Q., Relic, B., Malaise, M. G. (2013) Acute-phase serum amyloid A in osteoarthritis: regulatory mechanism and proinflammatory properties. *PLoS One* **8,** e66769.
66. Duff, G. W. (1994) Cytokines and acute phase proteins in rheumatoid arthritis. *Scand. J. Rheumatol. Suppl.* **100,** 9–19.
67. Urbanowska, T., Mangialaio, S., Hartmann, C., Legay, F. (2003) Development of protein microarray technology to monitor biomarkers of rheumatoid arthritis disease. *Cell Biol. Toxicol.* **19,** 189–202.

KEY WORDS:
chemotaxis · G protein-coupled receptor · chemoattractants

Serum amyloid A1α induces paracrine IL-8/CXCL8 via TLR2 and directly synergizes with this chemokine via CXCR2 and formyl peptide receptor 2 to recruit neutrophils

Mieke De Buck, Nele Berghmans, Noëmie Pörtner, et al.

J Leukoc Biol 2015 98: 1049-1060 originally published online August 21, 2015
Access the most recent version at doi:10.1189/jlb.3A0315-085R