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Staphylococcus aureus directly activates eosinophils via platelet-activating factor receptor

Koa Hosoki, Akiko Nakamura,† Mizuho Nagao,* Yukiko Hiraguchi,* Hisashi Tanida,* Reiko Tokuda,* Hideo Wada,† Tsutomu Nobori,† Shigeru Suga,* and Takao Fujisawa*,[1]*

*Institute for Clinical Research, Mie National Hospital, Mie, Japan; and †Central Clinical Laboratories, Mie University Hospital, Mie, Japan

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ABSTRACT

Colonization by SA is associated with exacerbation of AD. Eosinophilic inflammation is a cardinal pathological feature of AD, but little is known about possible direct interaction between SA and eosinophils. PAFR appears to be involved in phagocytosis of Gram-positive bacteria by leukocytes. The objective of this study was to investigate whether SA directly induces eosinophil effector functions via PAFR in the context of AD pathogenesis. Peripheral blood eosinophils were cultured with heat-killed SA, and EDN release, superoxide generation, and adhesion to fibronectin-coated plates were measured. Cytokines, released in the supernatants, were quantified by multiplex bead immunoassays. FISH-labeled SA was incubated with eosinophils and visualized by confocal laser-scanning microscopy. PAFR-blocking peptide and PAFR antagonists were tested for inhibitory effects on SA-induced reactions. SA induced EDN release and superoxide generation by eosinophils in a dose-dependent manner. IL-5 significantly enhanced SA-induced EDN release. IL-5 and IL-17A significantly enhanced SA-induced superoxide generation. SA enhanced eosinophil adhesion to fibronectin, which was blocked by anti-CD49d, and induced eosinophil secretion of various cytokines/chemokines (IL-2R, IL-9, TNFR, IL-1β, IL-17A, IP-10, TNF-α, PDGF-bb, VEGF, and FGF-basic). After incubation of eosinophils with SA, FISH-labeled SA was visualized in the eosinophils' cytoplasm, indicating phagocytosis. A PAFR-blocking peptide and two PAFR antagonists completely inhibited those reactions. In conclusion, SA directly induced eosinophil activation via PAFR. Blockade of PAFR may be a novel, therapeutic approach for AD colonized by SA. ***J. Leukoc. Biol.*** **92: 333–341; 2012.**

Introduction

SA has been implicated in the pathogenesis of AD. Over 80% of AD patients are colonized by SA, even on nonlesional skin [1, 2], and a strong correlation between the numbers of SA present on the skin and the severity of AD was reported [3, 4]. SA promotes inflammation and sensitization [5] in AD via its products, such as enterotoxins and enzymes, through interactions with T cells and keratinocytes [6]. Eosinophil infiltration is also an important feature of the pathology of AD and correlates with disease severity [7, 8]. During exacerbation of AD, eosinophils are accumulated and activated in the skin to release/deposit toxic granule proteins that cause tissue damage [7]. Thus, SA and eosinophils play important roles in the pathogenesis of AD. A direct relationship between the two, however, has not been demonstrated. The close proximity of SA and eosinophils in skin lesions [9] and the antiapoptotic effect of SA exotoxin on eosinophils [10] have merely suggested a possible link.

PAF is an endogenous phospholipid that has been implicated as a mediator of allergic and inflammatory processes [11]. PAFR, a GPCR, is expressed on various cell types, including eosinophils [12–14]. PAF is involved in eosinophil degranulation, superoxide production, enhanced adhesion, and chemotaxis [15–18]. PAFR expression on eosinophils was reported to be associated with asthma [19]. In addition, bacteria can bind to PAFR via their cell wall phosphorylcholine, which mimics endogenous PAF. Through this mechanism, which is an innate invasion system [20, 21], bacteria effectively invade susceptible host cells, such as neurons and cardiac muscle cells, and can cause severe organ damage. To defend against this assault, host inflammatory cells that also express PAFR respond as innate immune effectors. As eosinophils can function in host defense and sometimes cause inflammation [22], PAFR on eosinophils may be the connection between SA and eosinophils in the context of skin inflammation in AD.

Abbreviations: AD=atopic dermatitis, EDN=eosinophil-derived neurotoxin, FISH=fluorescence in situ hybridization, IL-1RA=IFN receptor antagonist, IP-10=IFN-inducible protein 10, MIG=monokine induced by IFN-γ, PAF=platelet-activating factor, SA=*Staphylococcus aureus*

1. Correspondence: Institute for Clinical Research, Mie National Hospital, 357 Osatokubota, Tsu City, Mie, 514-0125, Japan. E-mail: fujisawa@mie-m.hosp.go.jp

We hypothesized that eosinophils interact directly with SA through PAFR and exert proinflammatory functions that lead to exacerbation of AD in the context of dysregulated skin immune systems in AD.

MATERIALS AND METHODS

Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). Anti-CD16 and anti-CD14 antibody-coated magnetic beads were purchased from Miltenyi Biotec (Auburn, CA, USA). HBSS, HEPES buffer solution, and FBS were obtained from Life Technologies BRL (Grand Island, NY, USA). Human rIFN-γ, human rIL-17A, and human rIL-5 were from PeproTech (Rocky Hill, NJ, USA). Horse heart ferricytochrome C (type VI), SOD, gelatin, human albumin, RPMI 1640, EGTA, and ABT-491were purchased from Sigma-Aldrich (St. Louis, MO, USA). PTX was obtained from Calbiochem (San Diego, CA, USA). Mouse anti-human TLR2 mAb (clone TL2.1; IgG2aκ) and mouse anti-human TLR4 mAb (clone HTA125; IgG2aκ) were obtained from Imgenex (San Diego, CA, USA). Mouse anti-human dectin-1 mAb (clone 259,931; IgG2B) was obtained from R&D Systems (Minneapolis, MN, USA). Mouse anti-human CD18 mAb (clone TS1/18; IgG1κ) was from BioLegend (San Diego, CA, USA). Mouse anti-CD36 mAb (clone 185-1G2; IgG2) and FITC-conjugated mouse anti-human CD18 mAb (clone 3EB) were obtained from Abcam (Cambridge, UK). Anti-human CD49d mAb was purchased from Ancell (Bayport, MN, USA). FITC-conjugated mouse anti-human CD49d mAb, mouse IgG1 mAb, and mouse anti-CD35 mAb (clone J3D3; IgG1κ) were obtained from Beckman Coulter (Fullerton, CA, USA). A purified goat anti-PAFR-blocking peptide, sc-8742P, and Ginkgolide B were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Two scrambled peptides of PAFR-blocking peptide were obtained from American Peptide (Sunnyvale, CA, USA) and used as controls.

Preparation of eosinophils

Eosinophils were purified from healthy adult volunteers by Percoll density gradient centrifugation and negative selection with immunomagnetic beads (MACS anti-CD16 microbeads) [23]. The purity of eosinophils was >98%. In some experiments, eosinophils were purified further by using anti-CD16 and anti-CD14 immunomagnetic beads. All volunteers gave informed consent, and the study was approved by the Ethics Committee of Mie National Hospital (Japan).

Preparation of bacterial strain

SA (JCM No. 2151, ATCC No. 6538) was obtained from RIKEN BioResource Center (Tsukuba, Japan). It was incubated at 35°C under anaerobic conditions for ~48 h and centrifuged for 20 min at 3000 g. The bacteria were then washed and suspended in PBS and killed by heating at 121°C for 15 min. The processed bacteria were stored at −80°C until use in experiments. The supernatant obtained after the 48-h culture was similarly stored and also used for experiments.

EDN assay

Freshly isolated eosinophils at 2×10^6 cells/ml in RPMI with 10% FBS were incubated with SA at 1×10^6, 1×10^7, and 1×10^8 CFU/ml in 96-well tissue-culture plates in the presence and absence of IL-5 at 10 ng/ml, IFN-γ at 10 ng/ml, or IL-17A at 50 ng/ml for 4 h at 37°C and 5% CO_2. The cell-free supernatants were collected and stored at −20°C until assay for EDN using an EDN ELISA kit (Medical & Biological Laboratories, Nagoya, Japan), according to the manufacturer's directions. To examine the calcium dependency of eosinophil degranulation, cells were preincubated with 1 mM EGTA for 15 min at 37°C in 5% CO_2 and then stimulated with SA. Preincubation was also performed with PTX at 100 ng/ml for 2 h at 37°C in 5% CO_2 before incubation with SA.

Superoxide anion generation assay

Superoxide generation by eosinophils was measured by SOD-inhibitable reduction of cytochrome c using a modification of a method reported elsewhere [24]. In brief, freshly purified eosinophils at 1.25×10^6 cells/ml were preincubated with SA at 1×10^8 CFU/ml in 96-well tissue-culture plates for 30 min at 37°C in 5% CO_2 and then resuspended in HBSS containing 10 mmol/L HEPES, 0.03% gelatin, and 100 μmol/L cytochrome c. The eosinophil and SA suspension were dispensed into the wells of 96-well tissue-culture plates, followed by addition of IL-5, IFN-γ, or IL-17A. The absorbance at 550 nm was measured for over 4 h using a microplate autoreader (Wallac 1420 ARVO SX, PerkinElmer, Waltham, MA, USA). Superoxide anion generation was calculated with an extinction coefficient of 21.1×10^3 cm^{-1} mol^{-1} L for reduced cytochrome c at 550 nm and was expressed in nanomoles of superoxide produced/10^5 cells. Each reaction was carried out in duplicate and compared with an identical control reaction system that contained 20 μg/mL SOD.

Expression of CD49d on eosinophils

Eosinophils at 2×10^6 cells/ml were incubated with SA at 1×10^8 CFU/ml in RPMI 1640 with 10% FCS for 5 min at 37°C in 5% CO_2. After incubation, FITC-conjugated anti-human CD49d or isotype control antibodies were added and incubated for 15 min at 37°C in 5% CO_2. Then, CD49d expression on the eosinophils was analyzed using an FC500 flow cytometer (Beckman Coulter).

Adhesion assay

Eosinophil adhesion to fibronectin was measured using CultreCoat (Trevigen, Gaithersburg, MD, USA), according to the manufacturer's instructions. Briefly, isolated eosinophils labeled with calcein-AM fluorescent dye (Molecular Probes, Life Technologies, Grand Island, NY, USA) and resuspended in RPMI/10% FBS at 5×10^5/well were incubated with SA at 1×10^8 CFU/ml and dispersed onto fibronectin-coated plates in the presence and absence of anti-human CD49d mAb (Ancell) at 10 μg/ml. The plates were incubated for 20 min at 37°C and then rinsed twice with PBS to remove unbound cells. Adherent cells were quantified with a fluorescence plate reader (Wallac 1420 ARVO SX, PerkinElmer) at 485–520 nm.

Cytokine and chemokine assays

Eosinophils at 2×10^6 cells/ml were incubated with SA at 1×10^8 CFU/ml in RPMI 1640 with 10% FCS for 24 h at 37°C in 5% CO_2. The supernatants were assayed for a panel of cytokines and chemokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-7, IL-8, IL-10, IL-12p40/70, IL-13, IL-15, IL-17A, IL-1RA, IL-2R, IFN-α, IFN-γ, TNF-α, G-CSF, GM-CSF, eotaxin, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4, MIG/CXCL9, HGF, EGF, PDGF-bb, FGF-basic, and VEGF. The assays were performed using multiplex bead immunoassays (Luminex, Austin, TX, USA), according to the manufacturer's instructions, using kits (Cat. Nos. 33,995-60003, 33,995-00004, 33,995-01041; BioSource, Invitrogen, Life Technologies, Carlsbad, CA, USA). TGF-β1 was measured with an ELISA kit (Quantikine, R&D Systems), according to the manufacturer's instructions. The cytokines were quantified (pg/ml) by using the standards supplied with the kits.

Apoptosis assay

Eosinophils at 2×10^6 cells/ml were incubated with SA at 1×10^8 CFU/ml in RPMI 1640 with 10% FCS for 24 h at 37°C in 5% CO_2. The cells were then stained with annexin V and PI by using an apoptosis detection kit (Calbiochem), according to the manufacturer's instructions. Early apoptotic eosinophils (annexin V+/PI−) were detected by flow cytometry.

Confocal microscopy

It is well known that human eosinophils express CD18 on their surface [25]. We incubated eosinophils with FITC-conjugated anti-human CD18

mAb for 1 h at 37°C in 5% CO_2. FISH of SA using a 16S rRNA-targeted oligonucleotide kit was performed, according to the manufacturer's directions (Ribo Technologies BV, Groningen, The Netherlands). Then, the CD18-FITC-stained eosinophils and the FISH-labeled SA were incubated for 1 h at 37°C and 5% CO_2. Interaction of the eosinophils and the SA was visualized by confocal laser-scanning microscopy with FluoView software (FV1000, Olympus, Tokyo, Japan). SA was visualized in red due to the fluorescence of Cy3, and eosinophils were in green.

Blocking assays

Eosinophils were preincubated with a PAFR-blocking peptide or control scrambled peptides at 50 μg/ml for 20 min at 37°C in 5% CO_2. After incubation, confocal imaging, EDN assay, superoxide production assay, and adhesion assay were performed using the same protocols as described above. For cytokine assay, TNF-α was measured as a representative SA-induced cytokine using a human TNF-α ELISA kit (Quantikine, R&D Systems). The effects of blocking antibodies against a panel of receptors (anti-dectin-1 mAb at 3 and 10 μg/ml; anti-CD18 mAb at 40 μg/ml; anti-CD35 mAb at 20 μg/ml; CD36 mAb at 5, 10, and 40 μg/ml; anti-TLR2 mAb at 10 and 20 μg/ml; and anti-TLR4 mAb at 10 and 20 μg/ml) and PAFR antagonists (ABT-491 at 100 μM and Ginkgolide B at 100 μM) were tested in a similar manner.

Statistical analysis

Data were expressed as the means ± SEM. Statistical comparisons were performed using one-way ANOVA, and the significance of individual differences was evaluated by Bonferroni's multiple comparison test. The software package GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used for all data analyses and preparation of graphs.

RESULTS

SA induces EDN release from eosinophils

First, experiments were conducted to test whether SA induces eosinophil degranulation. We found that SA significantly induced degranulation, as measured by EDN release from eosinophils, in a dose-dependent manner (**Fig. 1A**). Based on those results, 10^8 CFU/ml was selected for the subsequent experiments. IL-5 (Fig. 1B) enhanced the degranulation of eosinophils cocultured with SA, but IL-17A and IFN-γ did not (data not shown).

Neither the SA culture supernatant nor plain latex beads induced EDN (data not shown), indicating that a direct and specific interaction with SA is necessary for eosinophil degranulation. Pretreatment with EGTA significantly inhibited SA-induced degranulation, indicating that SA-induced EDN release was an active process dependent on intracellular Ca^{2+} signaling (Fig. 1C). Finally, we found that PTX partially inhibited SA-induced degranulation, indicating that SA activates eosinophils via GPCRs (Fig. 1D).

SA induces eosinophil superoxide production

Our second set of experiments showed that SA significantly induced superoxide production by eosinophils (**Fig. 2A**). Moreover, IL-5 and IL-17A significantly enhanced the superoxide generation (Fig. 2B and D). Although IFN-γ also appeared to enhance the reaction, the difference did not reach statistical significance (Fig. 2C). The SA culture supernatant did not induce superoxide generation (data not shown).

Eosinophils stimulated with SA exhibit enhanced adhesion to fibronectin via CD49d up-regulation

Eosinophils cocultured with SA expressed significantly higher levels of CD49d than unstimulated eosinophils (**Fig. 3A** and **B**). Hence, adhesion of the stimulated cells to fibronectin was also markedly enhanced (Fig. 3C), but this was completely inhibited by CD49d-blocking antibody (Fig. 3D).

SA induces release of various cytokines and chemokines from eosinophils

Supernatants from SA-stimulated eosinophils were tested for a panel of cytokines and chemokines by using multiplex bead immunoassays (Luminex). To help visualize the profiles of release of cytokines/chemokines from eosinophils, we plotted the net amounts of release and fold-changes of release with SA

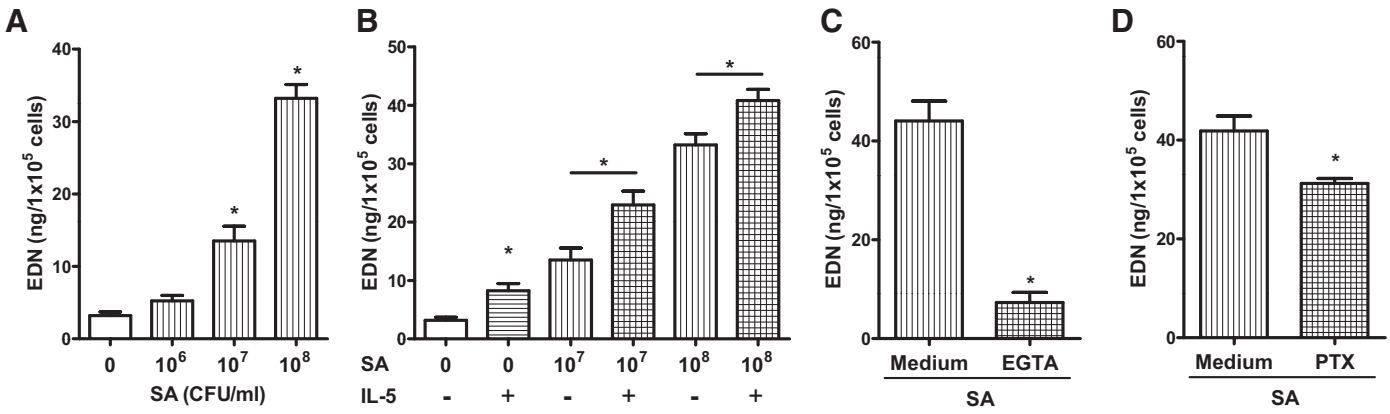

Figure 1. EDN release from eosinophils is induced by SA. Eosinophils were incubated with various concentrations of SA for 4 h, and EDN levels in the supernatants were measured by ELISA. (A) SA induced EDN release from eosinophils in a dose-dependent manner. (B) IL-5 significantly enhanced SA-induced EDN release. (C) EGTA inhibited SA-induced EDN release. (D) PTX inhibited SA-induced EDN release. Each bar shows the mean ± SEM of six independent experiments. *$P < 0.05$.

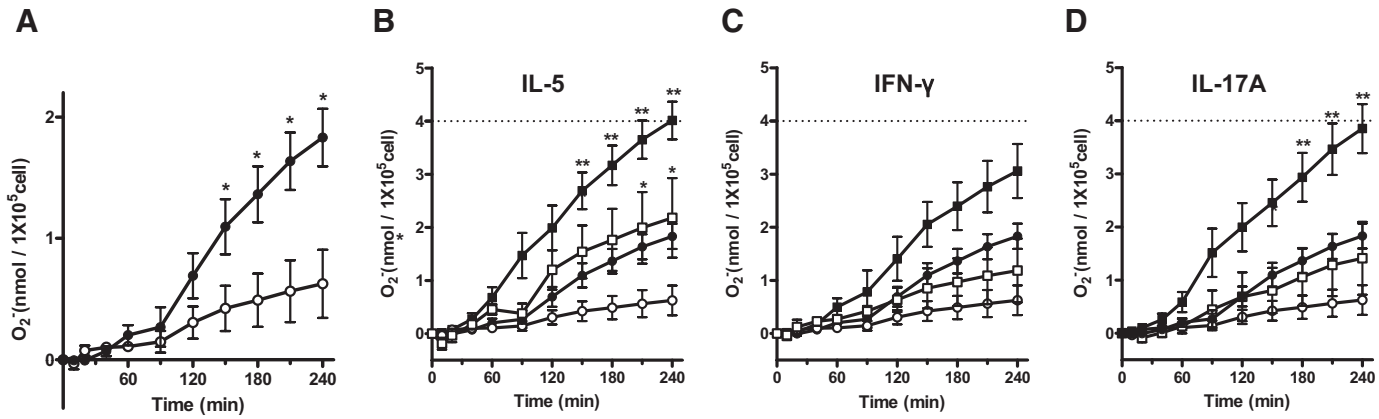

Figure 2. Superoxide generation is induced by SA. Superoxide (O_2^-) production was measured by SOD-inhibitable reduction of cytochrome c, as described in Materials and Methods. The kinetics of superoxide production were measured by repeated readings with a microplate autoreader. (A) Superoxide generation by eosinophils cocultured with SA for the times indicated. (B and D) IL-5 and IL-17A significantly enhanced SA-induced superoxide generation, but (C) IFN-γ did not. Open circles show untreated eosinophils, closed circles show eosinophils cultured with SA, open squares show eosinophils stimulated with the indicated cytokines, and closed squares show eosinophils cultured with SA and the indicated cytokines. Data are presented as means ± SEM for six experiments. *$P < 0.05$ compared with untreated control; **$P < 0.05$ compared with cytokine-stimulated samples.

stimulation compared with the vehicle control for each corresponding cytokine/chemokine (**Fig. 4**). Those plotted in the upper-right quadrant, indicative of strongly released cytokines/chemokines, are TNF-α, TNFR, IL-17A, IL-2R, IL-1β, IL-9, IP-10, PDGF-bb, VEGF, and FGF-basic.

SA reversed antiapoptotic activities of IL-5 and IFN-γ

After incubation of eosinophils in the presence and absence of SA, the percentage of early apoptotic eosinophils (annexin V+/PI−) was determined by flow cytometry. As has been documented by others [26, 27], IL-5 and IFN-γ—but not IL-17A—suppressed early eosinophil apoptosis (**Fig. 5A**). SA reversed the antiapoptotic effects of IL-5 and IFN-γ (Fig. 5B).

Eosinophils phagocytize SA

We morphologically examined the interaction of eosinophils with SA by confocal laser microscopy. After incubation

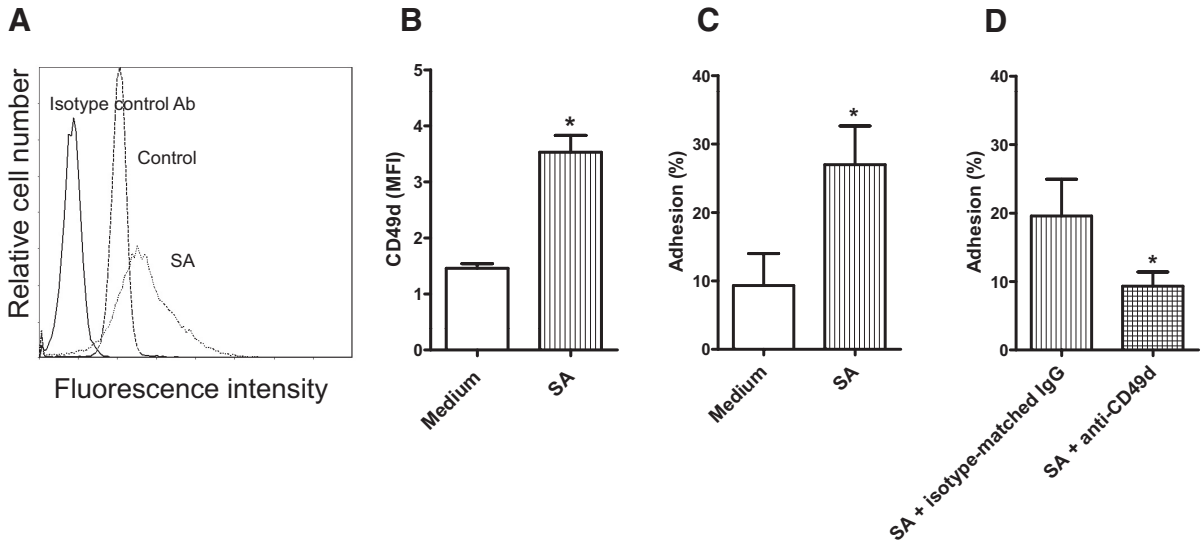

Figure 3. SA induces eosinophil adhesion to fibronectin via CD49d expression. (A) A representative histogram illustrates the differences in surface expression of CD49d for cells stimulated with SA, medium, and isotype control antibody. (B) Expression of CD49d on eosinophils was enhanced significantly after incubation with SA. MFI, Mean fluorescence intensity. (C) Eosinophil adhesion to fibronectin-coated plates was enhanced significantly in the presence of SA. (D) SA-enhanced adhesion was inhibited by addition of anti-CD49d but not by isotype-matched IgG. Eosinophils were labeled with calcein-AM, and adhesion (%) was expressed as fluorescence of adherent cells/fluorescence of total cells dispersed in a well × 100. *$P < 0.05$.

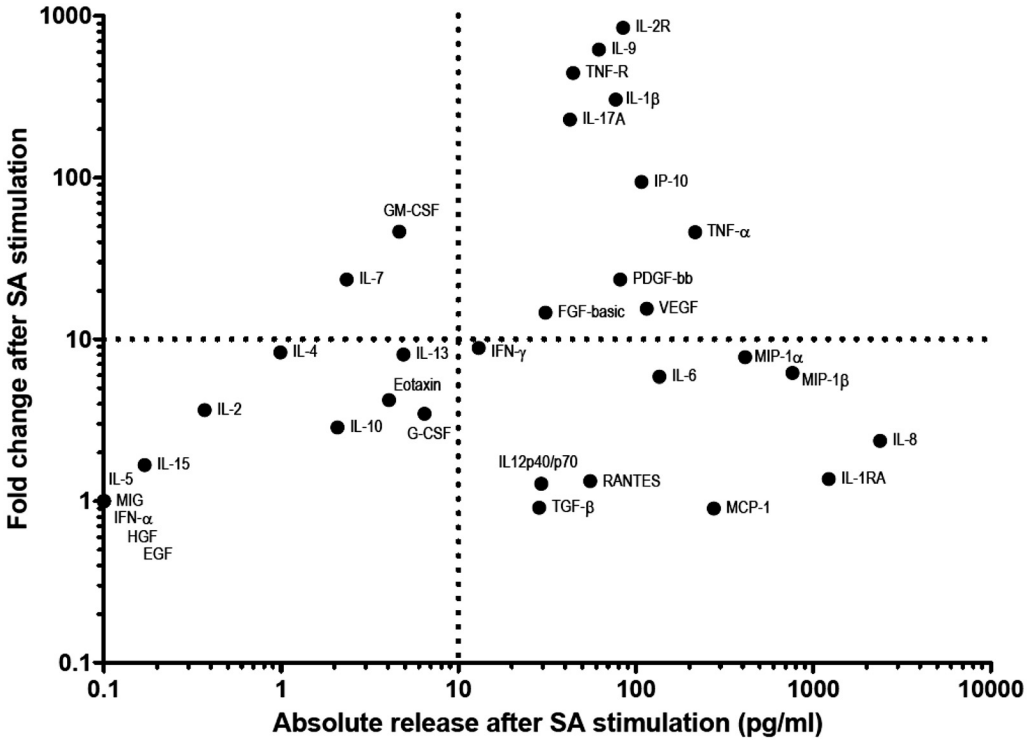

Figure 4. SA induces release of cytokines and chemokines from eosinophils. Eosinophils, purified using anti-CD16 and anti-CD14 immunomagnetic beads (1×10^5 cells/well), were incubated with SA at 1×10^8 CFU/ml in RPMI 1640 with 10% FCS for 24 h at 37°C in 5% CO_2. The supernatants were assayed for a panel of cytokines and chemokines. Vertical axis shows the fold change after SA stimulation compared with no stimulation. Horizontal axis shows the absolute release after SA stimulation (pg/ml). Each dot shows the mean of six independent experiments. HGF, Hepatocyte growth factor.

of eosinophils with SA for 1 h, Cy3-labeled SA was observed in the eosinophils' cytoplasm, indicating ingestion of SA by eosinophils (**Fig. 6A**).

Eosinophils phagocytize SA via PAFR, and blocking of PAFR inhibits eosinophil effector functions induced by SA

In contrast to the confocal microscopic findings described above, eosinophils treated with a PAFR-blocking peptide did not appear to ingest SA (Fig. 6B). This finding and partial blockade of SA-induced degranulation by PTX prompted us to confirm the hypothesis that SA activates eosinophils by binding to PAFR, a GPCR, on eosinophils. As expected, pretreatment with a PAFR-blocking peptide significantly inhibited SA-induced eosinophil degranulation (**Fig. 7A**), superoxide production (Fig. 7B), TNF-α production (Fig. 7C), and adhesion to fibronectin (Fig. 7D), whereas control peptides had no effect. Moreover, two PAFR antagonists, ABT-491 at 100 μM and Ginkgolide B at 100 μM, also completely inhibited the eosinophil functions (**Fig. 8A–D**).

However, eosinophil apoptosis induced by SA was not suppressed by a PAFR-blocking peptide (data not shown). None of anti-dectin-1 mAb at 3 and 10 μg/ml; anti-CD18 mAb at 40 μg/ml; anti-CD35 mAb at 20 μg/ml; anti-CD36 mAb at 5, 10, and 40 μg/ml; anti-TLR2 mAb at 10 and 20 μg/ml; or anti-TLR4 mAb at 10 and 20 μg/ml had any effect on SA-induced effector functions (data not shown).

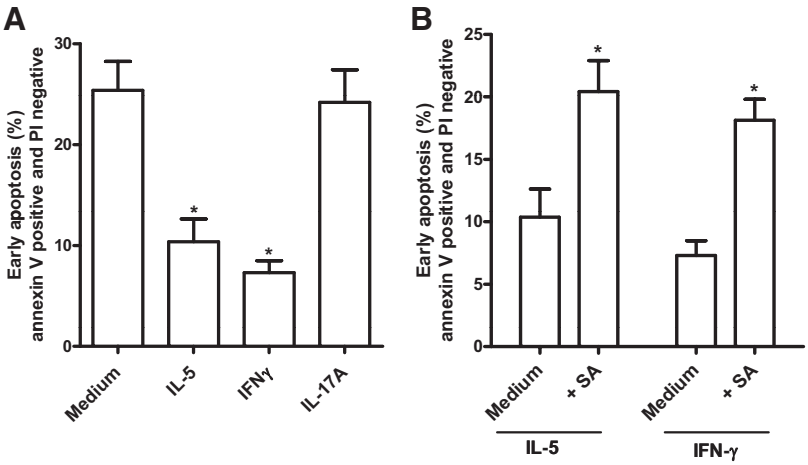

Figure 5. SA induces eosinophil apoptosis. Eosinophils were incubated with SA (1×10^8 CFU/ml) for 24 h. Apoptosis was measured as described in Materials and Methods. The bar graphs show the percentages of early apoptosis eosinophils as annexin-V-positive and PI-negative cells. (A) IL-5 and IFN-γ—but not IL-17A—reduced the percentage of naturally apoptotic cells. (B) Addition of SA, together with IL-5 or IFN-γ, appeared to reverse the antiapoptotic effects of these cytokines. Data represent four independent experiments and are shown as means ± SEM. $^*P < 0.05$ compared with medium alone.

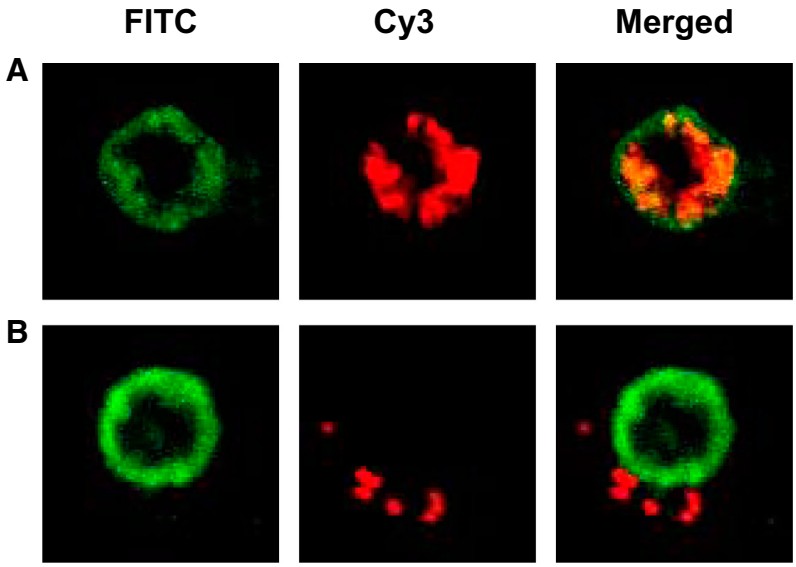

Figure 6. Confocal microscopic images of eosinophils cocultured with SA. Cy3-labeled SA was incubated with FITC-conjugated, CD18-stained eosinophils for 1 h at 37°C and 5% CO_2 and visualized by confocal laser-scanning microscopy. The green color represents surface CD18 on the eosinophil, and the red color indicates SA. (A) The merged image shows the presence of SA in the cytoplasm of eosinophils cultured with SA (yellow color). (B) On the other hand, following treatment with a PAFR-blocking peptide, SA does not appear to be present in the cytoplasm and is seen only outside of the cells.

DISCUSSION

Colocalization of SA and eosinophils in inflamed skin is a common feature of AD [9]. Although SA and its products can activate T cells and keratinocytes [6], and eosinophil recruitment has been regarded as merely a secondary phenomenon, our observation that eosinophils interact directly with SA and exhibit a variety of proinflammatory functions may be an important, additional novel mechanism by which SA promotes the skin inflammation of AD. It is especially noteworthy that eosinophils used their PAFRs to interact with SA, which is, to the best of our knowledge, a novel finding in this field.

First, we demonstrated that SA directly induced eosinophil degranulation in a dose-dependent manner. Eosinophils' toxic granules have been implicated in the tissue damage seen in AD [8]. Even when AD skin lesions appeared to be devoid of eosinophil infiltration, massive deposition of eosinophil major basic protein was observed at the same sites [7]. Electron microscopic findings implicated cytolytic degranulation as the mechanism by which eosinophils degranulate in AD skin [28].

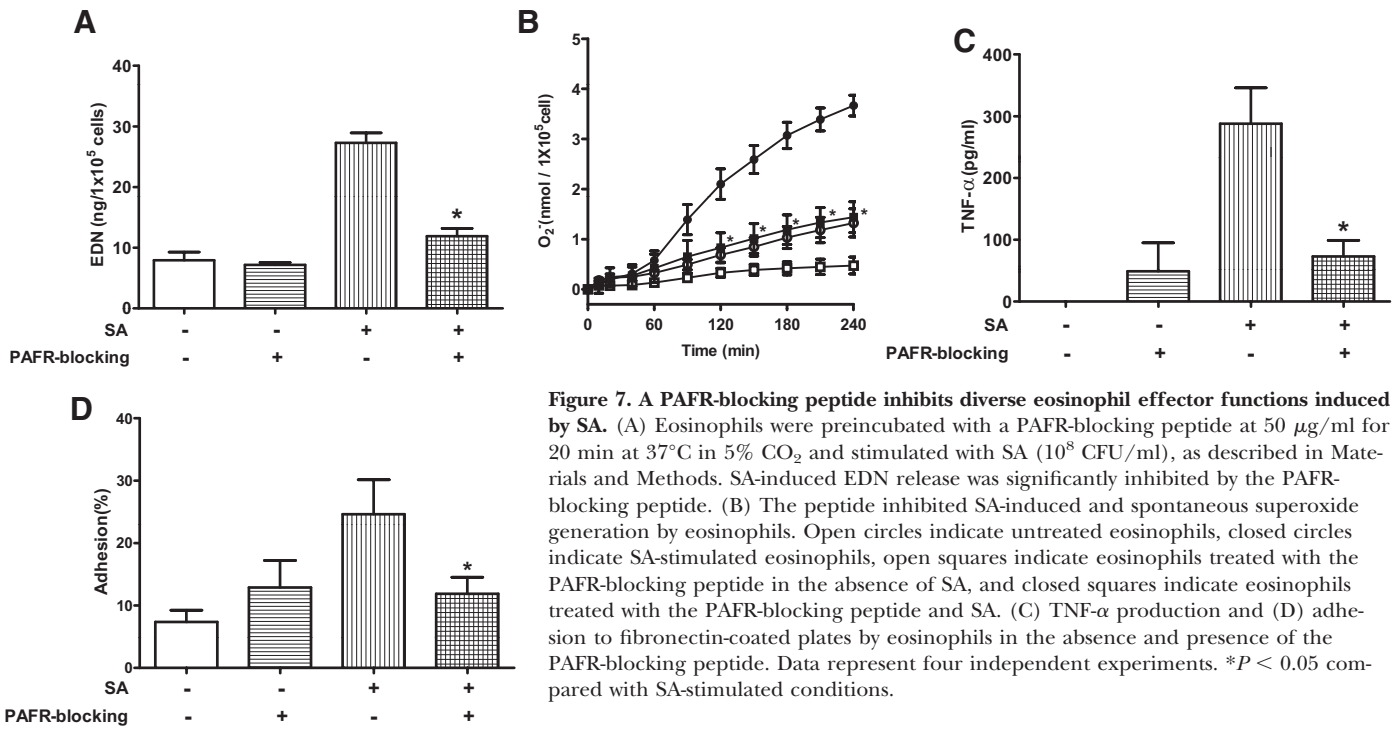

Figure 7. A PAFR-blocking peptide inhibits diverse eosinophil effector functions induced by SA. (A) Eosinophils were preincubated with a PAFR-blocking peptide at 50 μg/ml for 20 min at 37°C in 5% CO_2 and stimulated with SA (10^8 CFU/ml), as described in Materials and Methods. SA-induced EDN release was significantly inhibited by the PAFR-blocking peptide. (B) The peptide inhibited SA-induced and spontaneous superoxide generation by eosinophils. Open circles indicate untreated eosinophils, closed circles indicate SA-stimulated eosinophils, open squares indicate eosinophils treated with the PAFR-blocking peptide in the absence of SA, and closed squares indicate eosinophils treated with the PAFR-blocking peptide and SA. (C) TNF-α production and (D) adhesion to fibronectin-coated plates by eosinophils in the absence and presence of the PAFR-blocking peptide. Data represent four independent experiments. *$P < 0.05$ compared with SA-stimulated conditions.

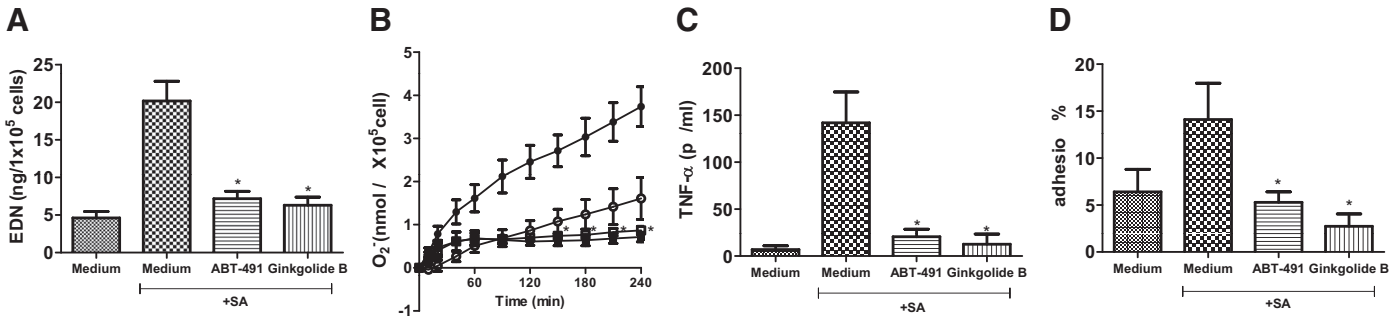

Figure 8. PAFR antagonists, ABT-491 and Ginkgolide B, inhibited SA-induced eosinophil functions. (A) Eosinophils were preincubated with ABT-491 at 100 μM, Ginkgolide B at 100 μM or medium for 20 minutes at 37°C in 5% CO_2 and stimulated with SA (10^8 CFU/ml), as described in Methods. SA-induced EDN release was significantly inhibited by the antagonists. (B) The antagonists inhibited SA-induced superoxide generation by eosinophils. Open circles indicate untreated (medium only) eosinophils, closed circles indicate SA-stimulated eosinophils, open squares indicate SA-stimulated eosinophils treated with ABT-491 and closed squares indicate SA-stimulated eosinophils treated with Ginkgolide B. (C) TNFα production and (D) adhesion to fibronectin-coated plates by eosinophils in the absence and presence of the PAFR antagonists. Data represent four independent experiments. *$P < .05$ compared with SA-stimulated conditions.

In our study, EGTA completely blocked SA-induced degranulation, whereas IL-5 enhanced it. More importantly, a PAFR-blocking peptide and PAF antagonists inhibited the degranulation. These findings strongly indicate that SA initiates an active process of eosinophil degranulation via PAFR. It was reported that PAFR coupled with several species of Gα proteins—namely Gαo, Gαi, and Gαq/11—the former two proteins are sensitive, and Gαq/11 is insensitive to PTX. As eosinophil degranulation is transduced by p38 and Erk, [29] and p38 is activated by PAFR-coupled Gαq/11 and Erk by PAFR-coupled Gαq/11 and Gαo [30], our finding that the inhibition of PTX of SA-induced degranulation was partial may explain the involvement of the above-mentioned signaling molecules. In addition, the seemingly conflicting finding that SA abrogated the antiapoptotic activity of IL-5 for eosinophils may mean that SA initially induces active degranulation and later leads to cytolytic release of EDN, as reported by others [28].

Superoxide generation is another important proinflammatory function of eosinophils [31], and we found that SA induced superoxide production by eosinophils. Th cells were suggested to be differentially involved in the pathogenesis of AD: namely, Th2 cells in the initiation and acute phases, Th1 cells in the chronic phase, and Th17 cells in the acute and exacerbation phases [32–34]. Accordingly, we examined the effects of IL-5, IFN-γ, and IL-17A—representative cytokines of Th2, Th1, and Th17, respectively—on SA-induced superoxide generation. Eosinophils express receptors, not only for IL-5 and IFN-γ [25] but also for IL-17A [35]. IL-5 and IL-17A significantly enhanced superoxide generation. Although the degree of enhancement by IFN-γ did not reach statistical significance, it appeared to have the same effect. Recently, staphylococcal α-toxin was reported to induce IL-17A production by monocytes and Th17 cells [36]. Taken together, these results suggest that in the cytokine milieu of AD skin, SA potently promotes inflammation through eosinophil degranulation and superoxide generation.

Increased fibronectin deposition in AD skin was reported [37]. SA strains express "microbial surface components recognizing adhesive matrix molecules" that recognize human ECM proteins, such as fibronectin, fibrinogen, and collagen, implicating these proteins as potential ligands for binding of SA to AD skin [38]. Eosinophils express adhesion molecule CD49d, which can bind to fibronectin [39–41]. Here, we demonstrated that SA enhanced the adhesion of eosinophils to fibronectin through up-regulation of CD49d expression. In the context of AD pathogenesis, fibronectin might serve as a scaffold for eosinophils and SA, allowing eosinophils to efficiently contact and capture SA.

We found that SA induced release of a variety of cytokines and chemokines from eosinophils. To visualize the profile of secretion, we plotted them on the basis of the absolute amount released and the relative changes compared with vehicle controls (Fig. 4). Those with a high amount and strong response (tentatively defined as >10 pg/ml and greater than tenfold, respectively)—factors assumed to be important in the pathogenesis of AD—were IL-2R, IL-9, TNFR, IL-1β, IL-17A, IP-10, TNF-α, PDGF-bb, VEGF, and FGF-basic. Serum levels of soluble IL-2R were elevated, and they correlated with the clinical severity and response to treatment of AD patients [42, 43]. Although the source(s) of IL-2R in serum are unknown, the significant correlations between the serum levels of IL-2R and eosinophil cationic protein that were observed in previous reports [42, 43] and the results of the present study suggest the possibility of SA-eosinophil involvement. The serum levels of soluble TNFR [44] and IL-1β [45] were also shown to correlate with disease activity. IL-9 is a critical cytokine in the pathogenesis of asthma, and it is produced by not only T cells and Th9 cells [46] but also by eosinophils [47]. Although the serum level of IL-9 is not elevated in AD [48], IL-9 gene polymorphism was reported to be associated with the AD phenotype [49].

IL-17A-positive cells (Th17 cells) were increased in the peripheral blood and infiltrated the skin in AD, especially in the acute phase [33]. IL-17A protein was also detected in the acute phase of AD skin [34]. Our observation that SA potently induced IL-17A secretion by eosinophils may represent a novel

pathway in the exacerbation phase of AD. Serum IP-10 was not elevated in AD [50]. IP-10 attracts activated T cells, mainly in the context of Th1-type diseases, such as psoriasis [51]. Considering the biphasic nature of inflammation in AD [32], however, IP-10, produced by not only keratinocytes but also by eosinophils, may participate in the chronic phase of inflammation in AD. TNF-α is a multifunctional, proinflammatory cytokine and reflects the inflammatory Th2 nature of AD [52]. SA also induced several growth factors, including PDGF-bb, FGF-basic, and VEGF, in eosinophils. They may be involved in tissue remodeling in AD and thus warrant further investigation.

PAFR can act as a PAMP recognition molecules for phosphorylcholine expressed on the cell wall of Gram-positive bacteria, leading to uptake of the bacteria by host cells [20]. The present results demonstrated that eosinophils phagocytize SA, an action that was completely inhibited by a PAFR-blocking peptide and two PAFR antagonists. Those agents also inhibited other SA-eosinophil effector functions, such as degranulation, superoxide generation, adhesion, and cytokine release—with apoptosis as the only exception—indicating those functions to be dependent on PAFR. Although eosinophils abundantly express PAFR and are believed to be activated by PAF from mast cells and other inflammatory cells in allergic inflammation, the importance of PAF in allergic diseases has almost been forgotten, as clinical trials of PAF antagonists failed to improve chronic and allergen-induced asthma [53, 54] approximately two decades ago. However, our current results, demonstrating that the PAFR-blockade peptide effectively inhibited various SA-induced eosinophil functions, suggest the potential of a novel treatment approach using topical PAFR antagonists for AD with SA colonization.

As criticism of our interpretation of the study results, it might be argued that direct contact of eosinophils with SA may not occur in vivo and that our experimental conditions are unrealistic. However, eosinophil granule proteins are deposited in the upper dermis [55], where colonization by SA occurs, and colocalization of SA enterotoxin and eosinophils was seen in AD dermis [9]. Therefore, we believe that direct contact between SA and eosinophils in the dermis can indeed occur in AD. Another criticism may be the use of heat-killed SA, not live SA, in our experiments. We performed heat-processing to stabilize SA, as SA proliferates rapidly in culture, making it impossible to standardize the E:T ratio. We believe that heat-killed SA is sufficient for the purpose of the experiments, as cell integrity was well-preserved after heat processing, and the bacterial culture supernatants before processing had no effect on degranulation or superoxide generation. The possibility of contamination of the SA preparation with endotoxin was also excluded, as antibody to TLR4, a receptor for endotoxin, did not inhibit the SA-induced reactions.

In conclusion, we demonstrated that SA induced a multitude of effector functions of eosinophils via PAFR. Our results may explain, at least in part, the pathophysiological roles of SA and eosinophils in AD, and they suggest that PAFR may be a novel, therapeutic target for AD colonized by SA.

AUTHORSHIP

K.H. designed the study and performed most of the experiments. A.N., H.W., and T.N. cultured *S. aureus* and prepared it for experiments. M.N., Y.H., H.T., and R.T. performed eosinophil purification and cytokine assays. S.S. contributed to the design of the study. T.F. supervised the overall process of the study and contributed to the analysis and interpretation of the results.

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