

Eosinophil extracellular trap cell death–derived DNA traps: Their presence in secretions and functional attributes

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Background: Activated human eosinophils, as well as neutrophils, can release extracellular chromatin to form DNA traps through cytolytic extracellular trap cell death (ETosis). Although formations of neutrophil DNA traps are recognized in patients with various inflammatory conditions, neither the presence of ETosis-derived eosinophil DNA traps in human allergic diseases nor the characteristics of these DNA traps have been studied.

Objective: We investigated the presence of ETosis-derived DNA traps in eosinophil-rich sinus and ear secretions and the functional attributes of ETosis DNA traps.

Methods: Eosinophil-rich secretions obtained from patients with eosinophilic chronic rhinosinusitis and eosinophilic otitis media were studied microscopically. *In vitro* studies of ETosis and DNA trap formation used blood-derived eosinophils and neutrophils, and studies of the binding capacities of DNA traps used labeled bacteria and fluorescent microbeads. Stabilities of DNA traps were evaluated by using fluorescence microscopy.

Results: Abundant nuclear histone H1–bearing DNA traps formed *in vivo* in the eosinophilic secretions and contributed to their increased viscosity. *In vitro*, after brief shear flow, eosinophil ETosis-elicited DNA traps assembled to form stable aggregates. Eosinophil DNA traps entrapped bacteria and fungi

and, through hydrophobic interactions, microbeads. In comparison with neutrophil-derived DNA traps, eosinophil DNA traps ultrastructurally exhibited thicker fibers with globular structures and were less susceptible to leukocyte-derived proteolytic degradation, likely because of the lesser protease activities of eosinophils.

Conclusions: In human allergic diseases local cytotoxicity of eosinophils not only releases free eosinophil granules but also generates nuclear-derived DNA traps that are major extracellular structural components within eosinophil-rich secretions. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Eosinophilic chronic rhinosinusitis, eosinophils, extracellular trap cell death, extracellular DNA traps, neutrophils

A novel form of rapid cell death, namely extracellular trap cell death (ETosis),¹ was first recognized in neutrophils.² In contrast to other known cell death types, including apoptosis and necrosis, ETosis is characterized by its striking final morphology: release of web-like chromatin structures (DNA traps) through breakdown of nuclear and plasma membranes. Recently, we reported that eosinophil extracellular trap cell death (EETosis) mediates eosinophil cytotoxicity, which is well recognized in tissues in association with diverse eosinophil-associated diseases.³

Similar to neutrophil extracellular trap cell death (NETosis), EETosis is a rapid cell death that is actively mediated through production of reactive oxygen species by nicotinamide adenine dinucleotide phosphate oxidase. In eosinophils stimuli elicited loss of the bilobular form of the eosinophil nucleus followed by nuclear envelope disintegration with nuclear chromatin spilling into the cytoplasm. Finally, the eosinophil plasma membrane ruptures, allowing extrusion of the nuclear DNA traps and release of intact eosinophil granules. EETosis has several differences relative to NETosis. In the process of NETosis, neutrophil granule membranes are lost within the cell, and granule proteins are mixed intracellularly with nuclear chromatin, thereby binding granule proteins to nuclear DNA when it is released as DNA traps.^{4,5} In contrast, eosinophil granules are released as intact structures, and eosinophil granule proteins remain within granules that themselves could bind to DNA traps.³ Scanning electron microscopy revealed that neutrophil DNA traps consist of 5- to 10-nm smooth stretches (composed of stacked cylindrical nucleosome) and 25- to 50-nm globular domains,^{2,6} although eosinophil DNA traps consisted mostly of fibers with diameters of 25 to 35 nm.³

Neutrophil DNA traps, which contain antibacterial proteins, such as histones and granule enzymes (eg, elastase and myeloperoxidase), are considered to play a role in innate immunity by

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Abbreviations used

ECRS: Eosinophilic chronic rhinosinusitis
 EETosis: Eosinophil extracellular trap cell death
 EOM: Eosinophilic otitis media
 ETosis: Extracellular trap cell death
 NETosis: Neutrophil extracellular trap cell death
 PMA: Phorbol 12-myristate 13-acetate
 RT: Room temperature

trapping various microorganisms.^{2,7,8} On the other hand, neutrophil DNA traps could be pathogenic in themselves when they are produced in excess.^{9,10} For instance, neutrophil DNA traps provide a scaffold for cell localization, including RBC and platelet aggregation, and serve to promote coagulation and thrombosis.¹¹ With their sticky nature, neutrophil DNA traps contribute to sputum viscosity and pulmonary dysfunction in patients with cystic fibrosis, and therefore inhalation of DNase is currently used to degrade DNA aggregates.^{10,12}

To date, neutrophil DNA traps have been shown to be present in patients with various infectious diseases and pathologic conditions. However, it is still unknown whether EETosis-mediated DNA traps are found *in vivo* in human allergic diseases. Here we investigated the presence of eosinophilic DNA traps in 2 human diseases that specifically are noted by predominantly eosinophil-rich exudative secretions. The trapping capacities and differences relative to neutrophils were also investigated in this study to clarify the characteristics of EETosis-derived DNA traps that are important for understanding their functions after eosinophil cytotoxicity.

METHODS**Sample collection and cell preparations**

Written informed consent was obtained from donors under institutional review board–approved protocols. Sinus specimens were obtained from 3 surgical patients with eosinophilic chronic rhinosinusitis (ECRS), according to the diagnostic criteria.¹³ We studied this form of rhinosinusitis, which is prevalent in Japan, because its exudative mucin contains mostly eosinophils (>96%), with negligible neutrophils among nucleated cells. In addition, we studied ear secretions from patients with eosinophilic otitis media (EOM).¹⁴ For *in vitro* studies, eosinophils were purified from normal donor blood by means of negative selection, as previously described.¹⁵ Briefly, venous blood was collected in a 6% dextran saline solution, and RBCs were allowed to sediment. Buffy coat was centrifuged over Ficoll-Paque (GE Healthcare, Pittsburgh, Pa) to separate granulocyte pellets. Eosinophils were isolated by means of incubation with a depletion antibody cocktail (StemSep; STEMCELL Technologies, Vancouver, British Columbia, Canada), followed by passage over magnetized columns (Miltenyi Biotec, Auburn, Calif). The purity of isolated eosinophils was greater than 98% of nucleated cells, and viability was greater than 99%. Neutrophils (>90% neutrophils; viability, >98%) were obtained from buffy coats, followed by hypotonic lysis of RBCs. In some experiments eosinophils were isolated by using a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described.^{16,17}

Microscopic study for eosinophilic mucin

Eosinophilic mucin was fixed in 10% formalin and embedded in paraffin. For histone H1 staining, the deparaffinized sample slides were incubated with 3% BSA and 1% saponin containing PBS for 30 minutes. The slides were then incubated with primary mouse anti-human nuclear histone H1 mAb (AE-4, 2 µg/mL, 3 hours at room temperature [RT]; Abcam, Cambridge, United

Kingdom). Anti-human neutrophil elastase (G-2, 1:100, 1 hour at RT; Santa Cruz Biotechnology, Dallas, Tex) was used to study the presence of neutrophils. Alexa Fluor 488–conjugated antibody (goat anti-mouse IgG; 1:300, 30 minutes at RT; Invitrogen, Carlsbad, Calif) was used for secondary incubation. Control antibodies and Hoechst 33342 (Invitrogen) were used for each experiment. Fixed mucin was stained with SYTOX green (Invitrogen) to study the 3-dimensional structure of DNA traps. Images were obtained with a Carl Zeiss LSM510 confocal microscope (Zeiss, Oberkochen, Germany). Z-stack images were obtained by using Zeiss LSM software.

Visualization of DNA trap aggregates in culture condition

Cells (1×10^5 cells in 100 µL) were stimulated with A23187 (Sigma-Aldrich, St Louis, Mo), phorbol 12-myristate 13-acetate (PMA; Sigma), or immobilized human IgG (Sigma) in 96-well, flat-bottom tissue-culture plates in 0.1% BSA containing phenol red–free HEPES-buffered RPMI 1640. Eosinophils were heated at 60°C for 5 minutes and then cultured for 24 hours at 37°C to induce necrotic cell death. Apoptosis was induced by culturing for 48 hours in medium containing anti-Fas activating mAb (Millipore, Temecula, Calif).³ SYTOX green (1:10,000) was added to the medium. Without fixation, culture plates were shaken with an MS1 plate shaker (IKA Works, Wilmington, NC) for up to 120 seconds at 1000 rpm. The plates were viewed by using an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a Cooled Color Digital camera (Spot 1.3.0; Diagnostic Instruments, Sterling Heights, Mich) in conjunction with the image analysis software IP Lab (Scanalytics, Fairfax, Va). Hoechst 33342 (1:10,000) and acridine orange (3 µmol/L, Sigma) were used to stain DNA and lysosomal eosinophil granules, respectively. Stained aggregates were seeded on poly-L-lysine–coated glass slides, and images were obtained with a BX62 Olympus microscope (Olympus, Center Valley, Pa). To study the stability of DNA traps, neutrophils and eosinophils were stimulated with 2 µmol/L A23187 for 24 hours in the presence of SYTOX to induce ETosis in the culture plate and shaken to visualize aggregates. Five percent BSA-PBS, heat-inactivated FBS (Gibco, Grand Island, NY), DNase I (20 U/mL; New England Biolabs, Ipswich, Mass), proteinase K (20 mg/mL), trypsin (0.25%; Life Technologies, Grand Island, NY), elastase from human leukocytes (0.1 U/mL, Sigma), neutrophil lysate (1:5), and proteinase inhibitor cocktail (Complete Mini; Roche, Mannheim, Germany) were gently added to the medium and further incubated for the indicated time points at 37°C. For preparation of neutrophil lysates, neutrophils were re-suspended in PBS at 1×10^7 cells and subjected to 3 freeze-thaw and sonication cycles, and supernatants were obtained by means of centrifugation at 10,000g for 10 minutes.

Microorganism trapping assay

For scanning electron microscopy, purified eosinophils (2×10^5 cells in 0.1% BSA RPMI) were stimulated with 2 µmol/L A23187 for 3 hours on glass slides. After EETosis induction, culture medium was removed, and 1 McFarland saline suspensions of *Escherichia Coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), or *Candida albicans* (in-house) were gently added. Plates were shaken with an MS1 plate shaker for 5 minutes at 600 rpm. The samples were then prepared for scanning electron microscopic observation, as described previously,¹⁸ with slight modifications. Briefly, samples were fixed with 1.25% glutaraldehyde–1% paraformaldehyde, dehydrated in a graded series of ethanol, and then reduced to t-butyl alcohol. They were then freeze-dried, decorated with osmium by using an osmium coater (NEOC; Meiwafoods, Tokyo, Japan), and observed on a field emission scanning electron microscope (SU-8020; Hitachi High-Technologies, Tokyo, Japan). For fluorescence microscopic study, purified live eosinophils were loaded with Hoechst 33342 (1:10,000) for 10 minutes and washed twice. Hoechst-loaded cells (1×10^5 cells in 100 µL) were stimulated with indicated stimuli in 96-well flat-bottom tissue-culture plates. After EETosis induction, Alexa Fluor 594–labeled *E coli* and *S aureus* (10 µg in 20 µL of PBS/well, Invitrogen) were gently added. The plate was shaken with an MS1 plate shaker for 30 seconds at 1000 rpm and viewed by means of inverted microscopy.

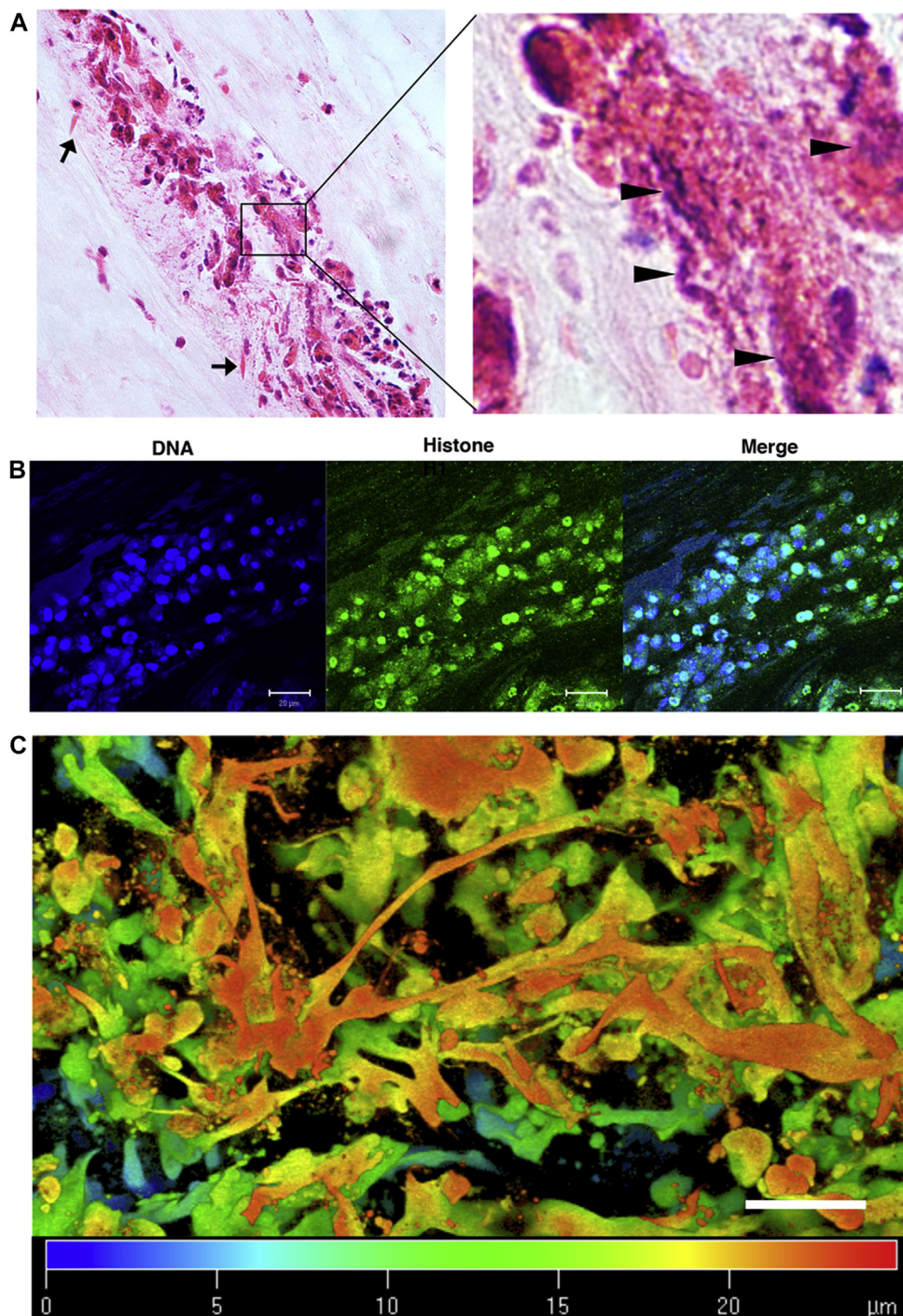


FIG 1. Presence of abundant nuclear DNA traps and free granules in sinus exudative secretions from patients with ECRS. **A**, Infiltrated eosinophils and their free granules are abundantly present in surgically obtained specimens from the ethmoidal sinus of a patient with ECRS. Mucin contains massive infiltration of eosinophils (>96% of nucleated cells) with negligible numbers of other cells, including neutrophils. Light microscopic images were obtained from hematoxylin and eosin–stained sections of eosinophilic mucin (*left panel*, $\times 20$ objective). Arrows show Charcot-Leyden crystals, a characteristic of eosinophil infiltration. The *right panel* is the boxed area seen at higher magnification ($\times 100$ objective). Arrowheads show lytic eosinophils with decondensed nuclei, and some show a filamentous structure. **B**, Section of eosinophilic mucin was stained for the linker histone H1 (green) and DNA (Hoechst 33342; blue) and analyzed by using a laser scanning confocal microscope. Scale bar = 20 μm . **C**, Pseudocolor representation of DNA traps evaluated throughout the depths of eosinophilic mucin. Fixed eosinophilic mucin was stained for DNA by using SYTOX green. Depth-level serial Z-stack images were pseudocolored according to the indicated color depth scale and projected to 3 dimensions by using Zeiss LSM software. White scale bar = 20 μm for the x-axis/y-axis. Experiments were repeated with samples from 3 patients with ECRS, with similar results.

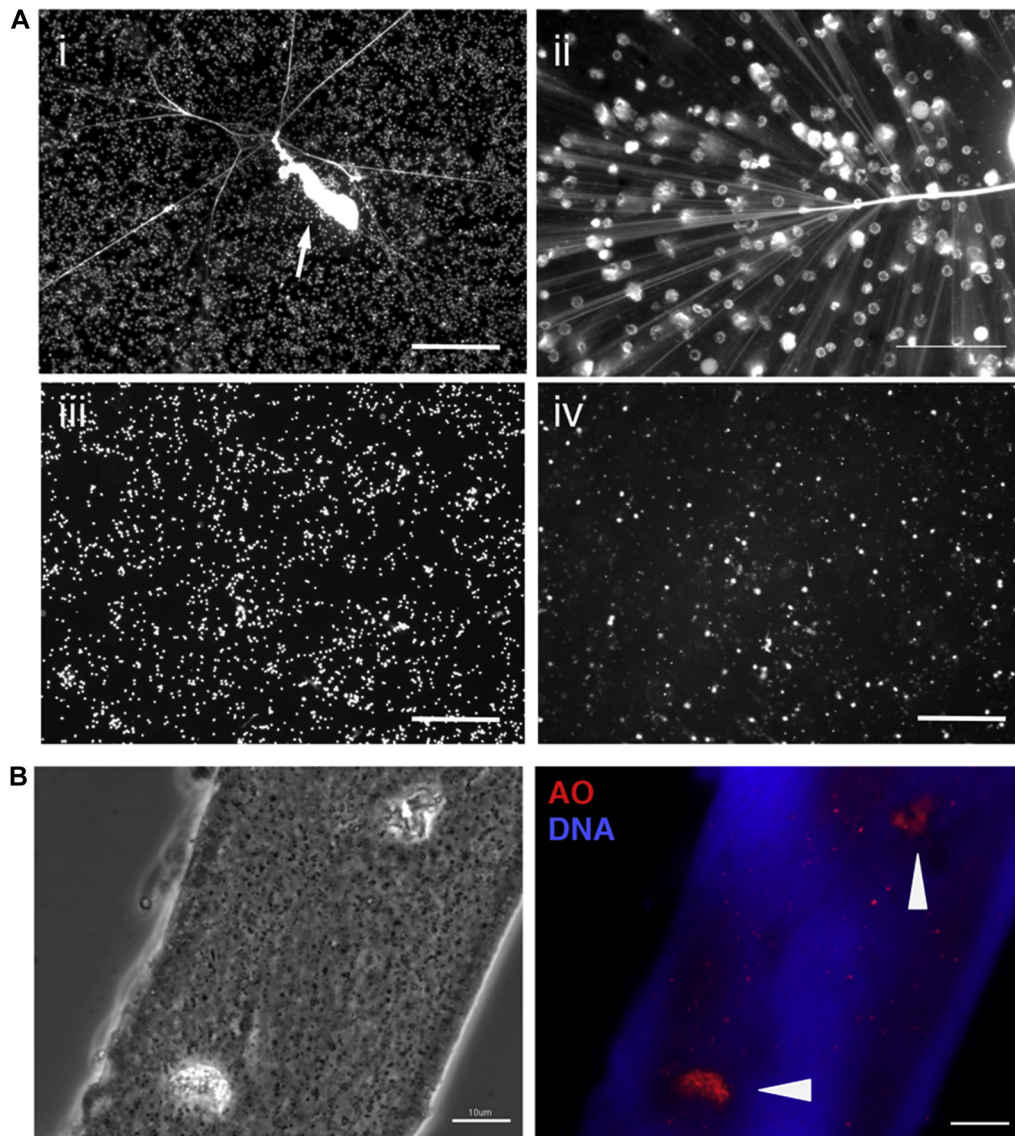


FIG 2. Detection of EETosis-mediated DNA trap aggregation in culture conditions. **A**, Purified eosinophils were stimulated with A23187 (2 $\mu\text{mol/L}$) for 24 hours in SYTOX (cell-impermeable DNA-specific dye)-containing medium and studied with inverted fluorescence microscopy ($\times 4$ objective). With brief shear flow stress, DNA traps were clearly visualized and formed aggregates in the middle of the culture plate (*i*, arrow). Stretched DNA traps emergent from adherent dead cells aggregated to form larger bundles (*ii*). Neither heat-induced necrotic cells (*iii*) nor apoptotic cells induced by anti-Fas-activating mAb (*iv*) produced DNA traps and formed aggregates. Small dots are nuclei of dead cells. Scale bars = 200 μm (Fig 2, *i*, *iii*, and *iv*) and 100 μm (Fig 2, *ii*). Data are representative of more than 3 experiments from independent donors with similar results. **B**, Microscopic image of shear flow-induced DNA trap aggregates (*left*, phase contrast image; *right*, fluorescence image). Scale bar = 10 μm . Aggregates consisted of DNA (blue: Hoechst 33342) and cell debris, including acridine orange (AO)-stained intact free granules (red). Arrowheads designate clusters of free eosinophil granules.

Microbead trapping assay

Fluorescent 1- μm microspheres (Molecular Probes, Eugene, Ore), both hydrophobic (sulfate microspheres [F8851]) and hydrophilic (positively charged; amine microspheres [F8765]) were used. For quantification of trapped beads, cells (1×10^5 cells in 100 μL) underwent induction of EETosis with A23187 for 24 hours in RPMI medium. Twenty microliters of DNase I (150 U/mL) or vehicle was added to each well and incubated for 90 minutes at 37°C. Medium was aspirated carefully and changed to the indicated buffer: HBSS (with calcium and magnesium), HBSS with 1%

BSA, 10% FBS, 0.3% Tween 20 (Sigma), 5 mmol/L EDTA, or 600 mmol/L NaCl. After adding the 20 μL of microsphere suspension (diluted to 1:10 with PBS), the plate was shaken with an MS1 plate shaker for 3 minutes at 800 rpm. Immediately after shaking, 10 μL of buffer was collected from each well and resuspended in 100 μL of PBS in a 96-well flat-bottom culture plate, followed by measurement of the fluorescence intensity of the beads with a GloMax-Multi detection system (Promega, Madison, Wis). The fluorescence intensity of DNase-treated wells was considered as 100% microbead recovery.

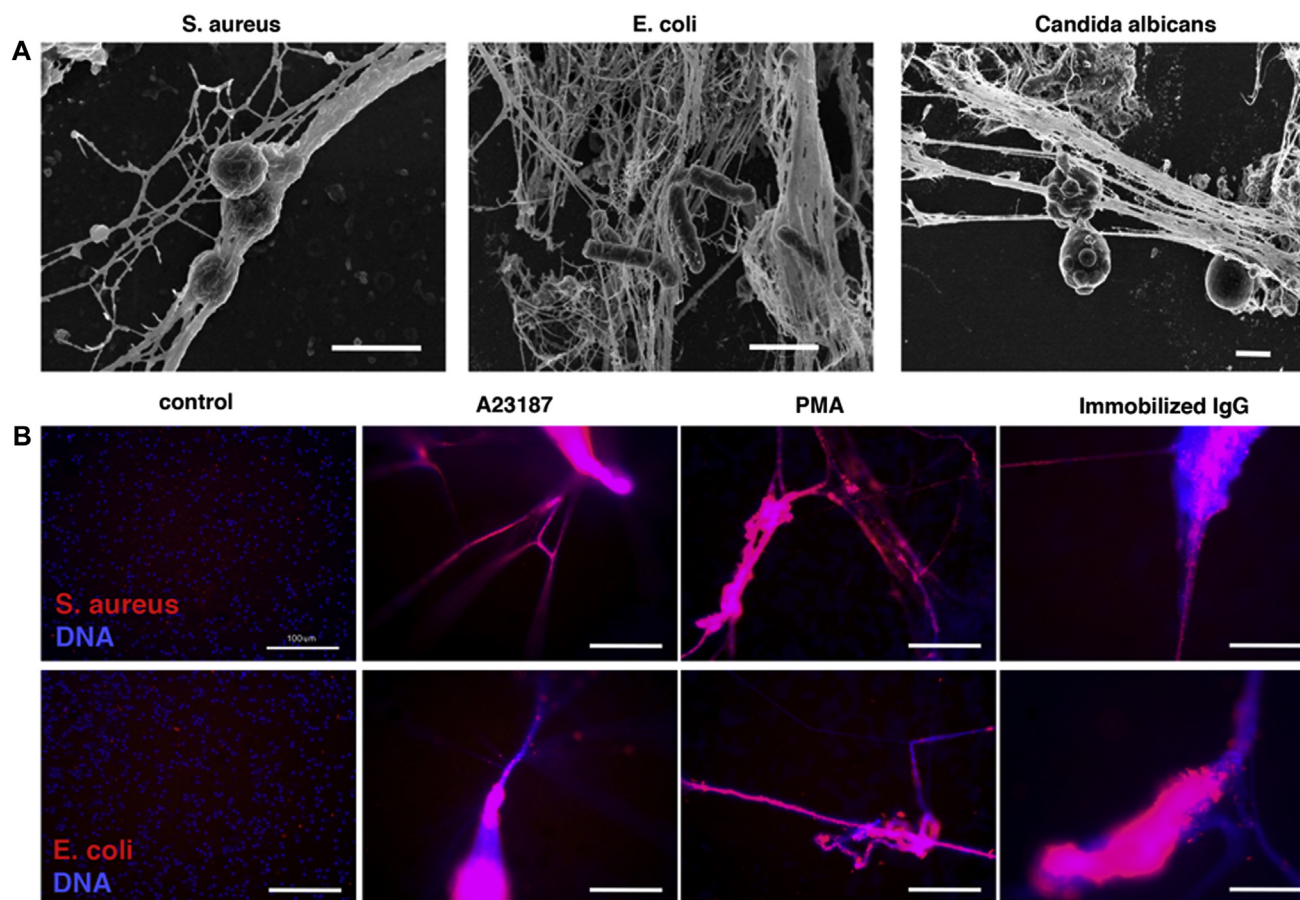


FIG 3. Eosinophil extracellular DNA traps are able to capture microorganisms. **A**, Microorganisms trapped by eosinophil DNA traps were observed by using a scanning electron microscope. **B**, DNA dye (Hoechst 33342: blue)-loaded eosinophils were stimulated with 2 μ mol/L A23187, 10 ng/mL PMA, or immobilized IgG (1 mg/mL) for 24 hours to induce EETosis. Thereafter, inactivated bacteria (Alexa Fluor 594-labeled *S aureus* or *E coli*) were added to the culture medium, and DNA aggregates were induced with brief shear flow. *Control*, Nonstimulated control cells did not produce DNA traps. Fluorescence-labeled bacteria not bound to DNA were out of focus. Extracellular DNA released from EETosis cells bound abundant bacteria. Data are representative of more than 3 experiments from independent donors with similar results. Scale bar = 100 μ m.

Measurement of protease activity

Protease activity was measured with an Amplitude Universal Protease Activity Assay Kit (AAT Bioquest, Sunnyvale, Calif), according to the manufacturer's instructions. Fluorescence intensities were measured with FLUOstar Omega (BMG Labtech, Offenburg, Germany).

Transmission electron microscopy

Eosinophils and neutrophils were seeded on Aclar film (Nishin EM, Tokyo, Japan), stimulated with 10 ng/mL PMA for 3 hours, and immediately fixed in a mixture of freshly prepared aldehydes (1.25% glutaraldehyde and 1% paraformaldehyde) in 0.1 mol/L cacodylate buffer. After incubation for 2 hours in 0.1 mol/L cacodylate buffer containing 1% OsO₄, the specimens were dehydrated in an ethanol series, passed through propylene oxide, and embedded in epoxy resin. Ultrathin sections (80 nm) were collected on copper grids and stained for 20 minutes in 4% uranyl acetate and 0.5% lead citrate. The specimens were viewed with a Hitachi H-7650 transmission electron microscope at 100 KV.

Statistical analysis

Results were expressed as means $\pm$ SDs. Differences between groups were evaluated by using the 2-tailed Student *t* test, with the level of statistical significance taken as a *P* value of less than .05.

RESULTS

Presence of EETosis-derived extracellular DNA traps in eosinophilic exudative secretions

Neutrophil DNA traps that consist of filamentous chromatin structures are found in a variety of secretions, such as abscesses and sputum, and contribute to their viscosity. ECRS is characterized by massive infiltration of eosinophils (within negligible potentially confounding neutrophils) in tissues and the sinus cavity, which is filled with highly viscous fluid called "eosinophilic mucin."^{13,19,20} Therefore we searched for EETosis cells and DNA traps in eosinophilic mucin from patients with ECRS. Present in eosinophilic mucin from patients with ECRS, in addition to intact eosinophils, were significant numbers of eosinophils that showed the defining nuclear characteristics of EETosis: decondensed nuclei, loss of lobulation, and/or disrupted nuclei. Clusters of free intact eosinophil granules were found near EETosis cells (Fig 1, A). To ascertain the presence of nuclear-derived genomic DNA, we stained the eosinophilic mucin with a DNA dye and an anti-histone H1 antibody and an isotype-matched control antibody. We observed colocalizations of DNA and histone H1 showing fibrous structures with morphologic DNA trap characteristics (Fig 1, B). Staining of mucin from patients with ECRS

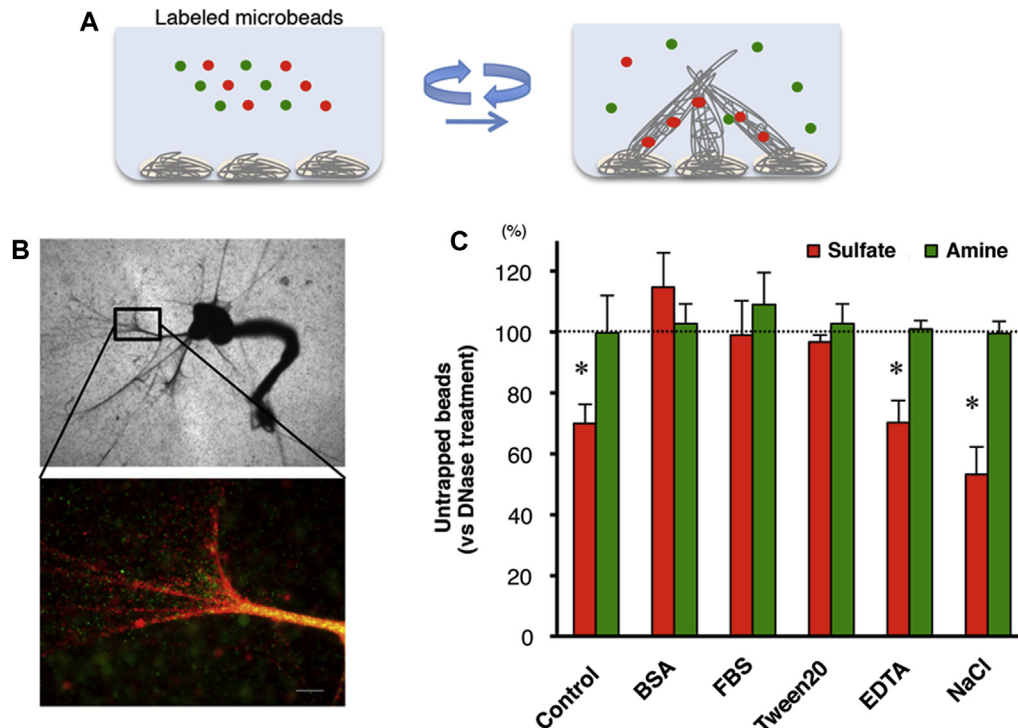


FIG 4. Eosinophil extracellular DNA entraps microbeads by means of nonelectrostatic forces. **A**, Two differently colored 1- μ m fluorescent microbeads (red, sulfate-modified beads; green, amine-modified beads) were used to study the mechanism of interactions between DNA traps and particles. Eosinophils were stimulated with A23187 (2 μ mol/L for 24 hours) to induce EETosis, followed by addition of sulfate- and amine-modified beads. Aggregation was induced on a plate shaker and viewed by means of inverted microscopy. **B**, Sulfate beads preferentially bind DNA traps to form large aggregates. The upper panel shows a bright-field image ($\times 4$ objective). A fluorescence image of the boxed area is shown in the lower panel. Scale bar = 20 μ m. **C**, Quantification of bead trapping in different conditions. The assays measure unbound beads, and therefore decreases are a measure of beads adherent to DNA traps. Medium conditions were as follows: HBSS (control), 1% BSA, 10% FBS, 0.3% Tween-20, 5 mmol/L EDTA, or 600 mmol/L NaCl. Immediately after shaking, medium was collected from each well, and the fluorescence intensity of beads was measured. Fluorescence intensities of DNase-treated wells were considered as 100% recovery. Data are expressed as means $\pm$ SDs ($n = 3$, independent donors). * $P < .05$ versus DNase-treated well.

did not detect neutrophil elastase (data not shown). Confocal Z-stacks and 3-dimensional reconstruction for SYTOX DNA staining showed the presence of abundant DNA traps that spread and aggregated (Fig 1, C). Furthermore, DNase I treatment of sinus secretions significantly inhibited the thread-forming ability (see Videos E1 and E2 in this article's Online Repository at www.jacionline.org). Similarly, DNA traps were observed in ear secretions from patients with EOM (see Fig E1 in this article's Online Repository at www.jacionline.org).¹⁴ Control isotype antibody for the anti-histone H1 antibody yielded no staining in patients with ERCS (data not shown) and those with EOM (see Fig E1). These results indicated that EETosis-derived eosinophil DNA traps are major components of eosinophil-rich exudative secretions in human "allergic" diseases and contribute to their viscosity.

Detection of EETosis-mediated DNA traps in culture conditions

To investigate the characteristics of EETosis-mediated DNA traps *in vitro*, we next aimed to mimic the DNA trap aggregate using purified human eosinophils. EETosis was induced by calcium ionophore, PMA, or immobilized IgG in SYTOX

(cell-impermeable DNA-specific dye)-containing medium and studied with fluorescence microscopy. Consistent with our previous report,³ EETosis cells demonstrated SYTOX-positive nuclei and loss of nuclear lobular formation. Filamentous DNA traps were released around the originating cells and difficult to visualize in static culture conditions (see Fig E2, A, in this article's Online Repository at www.jacionline.org). We developed a simple method to visualize extracellular DNA traps by inducing larger DNA aggregates with brief experimental shear flow (Fig 2, A, and see Fig E2, B). In this system EETosis eosinophils remained adherent to the culture plate, and DNA traps released from EETosis cells formed large aggregates (Fig 2, Ai and Aii). The aggregates consisted of accumulated DNA traps and cell debris, including acridine orange-stained, approximately 1- μ m size structures, and their clusters (Fig 2, B, arrowheads), which is indicative of intact cell-free eosinophil granules. To ascertain the difference among necrosis, apoptosis, and EETosis, we induced necrosis and apoptosis by brief heating and anti-Fas antibodies, respectively. DNA trap aggregates were never observed in necrotic (Fig 2, Aiii) and apoptotic (Fig 2, Aiv) cells. Using the extracellular histone staining that can recognize EETosis cells,³ we confirmed the DNA traps retained histone H1 (see Fig E3 in this article's Online Repository at www.jacionline.org).

Although EETosis is a rapid cell death and develops within 30 and 120 minutes of stimulation, we incubated eosinophils for a longer time period to determine nuclear shape change as a fate of cell death. After 24 hours of stimulation, approximately 95% of cells underwent cell death (SYTOX-positive) in our settings. In the presence of a nicotinamide adenine dinucleotide phosphate oxidase inhibitor, diphenyleneiodonium chloride, or an extracellular calcium chelator, EDTA, there was no significant release of extracellular DNA (see Fig E2, A) and no formation of DNA aggregates (see Fig E2, B). In contrast, a pancaspase inhibitor did not inhibit the nuclear decondensation and aggregate formation (see Fig E2). These results confirmed that EETosis is dependent on reactive oxygen production and calcium but independent of caspase-mediated processes.

EETosis-mediated DNA traps entrap microorganisms by passive contact

Neutrophil DNA traps are known to entrap microorganisms. EETosis-induced eosinophils were incubated with *S aureus*, *E coli*, *Candida albicans*, or *Aspergillus* species to show the association between DNA traps and gram-positive and gram-negative bacteria or fungi. As shown in Fig 3, A, these microorganisms (*Aspergillus* species, data not shown) were captured by filamentous DNA structures. To confirm the interaction between DNA traps and bacteria, we stimulated DNA dye-loaded eosinophils with different stimuli for 24 hours to induce EETosis. Thereafter, Alexa Fluor 594-labeled *S aureus* or *E coli* was added to the culture medium and incubated under shear flow conditions. As shown in Fig 3, B, large DNA-bacteria aggregates were observed after 30 seconds of shaking with EETosis cells but not in nonstimulated cells. No significant differences were observed in terms of the EETosis induced with different stimuli or among microbial species. These results indicate that EETosis-mediated DNA traps are able to ensnare microorganisms by means of passive contact.

Eosinophil extracellular DNA traps provide adhesive surfaces mediated by nonelectrostatic forces

Although a charge-mediated binding has been proposed for the trapping capacity of DNA structures,⁹ the detailed mechanisms are poorly understood. To study the mechanism of the adhesive property of eosinophil-derived DNA traps, we used 2 different 1- μ m fluorescent microbeads, hydrophobic sulfate-modified beads, and hydrophilic, positively charged amine-modified beads (Fig 4, A). Notably, sulfate beads were preferentially bound to DNA traps to form aggregates with brief shear flow (Fig 4, B). We quantified the trapping capacity by measuring the fluorescence intensity of unbound microbeads (ie, those not entrapped bound to eosinophil DNA traps) in comparison with DNase-treated EETosis cells (Fig 4, C). Approximately 30% of total sulfate beads were trapped to DNA in our system, although amine beads were not. Different medium conditions were tested to study the binding mechanism. Sulfate bead trapping was inhibited by the presence of albumin (BSA) or serum (FBS) and by a nonionic detergent (Tween-20) that inhibits hydrophobic interactions. Cationic ions have been reported to affect nucleosome structure, and high salt conditions inhibit electrostatic interactions,^{21,22} but neither EDTA nor medium containing high salt levels affected the trapping of beads. These results suggest that nonelectrostatic binding, especially

through hydrophobic interactions, plays a predominant role in the binding capacity of eosinophil-derived nuclear DNA traps.

Lesser eosinophil protease activities contribute to the stability of eosinophil DNA traps

Human neutrophils undergo ETosis (NETosis) in response to calcium ionophore or PMA.²³ Experiments were conducted with purified neutrophils to test whether NETosis can form similar aggregates in our system. NETosis-mediated DNA trap aggregates were elicited by A23187 or PMA (see Fig E4, A, in this article's Online Repository at www.jacionline.org). Neutrophil DNA traps formed larger aggregates compared with eosinophils. Detachment of scattered cells from culture plates was observed after experimental shear flow (see Fig E4, B). Aggregated neutrophil DNA trap structures showed a white-yellow gel-like appearance (see Fig E4, C). These results indicated the different characteristics of eosinophil- and neutrophil-derived DNA traps.

In an attempt to compare the stability of NETosis- and EETosis-mediated DNA traps, the aggregates were incubated in culture conditions for longer periods. Once the aggregates were formed, EETosis-mediated DNA traps were very stable for days, keeping their shape for more than 7 days (data not shown). In contrast, neutrophil DNA traps showed spontaneous dissociation and were more susceptible to increased concentrations of albumin or FBS or treatment with DNase, indicating their lesser stability (Fig 5, A).

Because neutrophil elastase has been reported to enhance DNA trap solubilization by cleaving histones,²⁴ we hypothesized that lesser eosinophil protease activities might play a role in the stability of eosinophil DNA traps. Incubation with proteinase K, trypsin, neutrophil elastase, or neutrophil lysates solubilized eosinophil DNA traps (Fig 5, B). However, eosinophil lysates did not induce solubilization of eosinophil DNA traps (data not shown). The effect of neutrophil lysates was not observed in the presence of proteinase inhibitors (Fig 5, B). Indeed, neutrophils contained significantly greater proteinase activities than eosinophils (Fig 5, C). Transmission electron microscopy showed globular components of eosinophil DNA traps, suggesting the presence of intact chromatin (Fig 5, D). Taken together, eosinophil DNA traps are more stable than neutrophil DNA traps at least in part because of lesser exposures to endogenous leukocyte-derived proteinases that can cleave the protein nucleosome components of nuclear-derived DNA traps.

DISCUSSION

Eosinophilic secretions in patients with ERCS and those with EOM were chosen to study the *in vivo* presence of eosinophil-derived DNA traps in lesional sites. In line with previous reports,^{14,20} these tenacious eosinophilic secretions displayed a highly viscous consistency. Within the secretions, tightly clustered eosinophils often showed their nuclei to be smudged and elongated, sometimes forming collections of nuclear debris and exhibiting cytolytic release of cell-free extracellular eosinophil granules. Here we demonstrated that eosinophil DNA traps represent major extracellular structural components scaffolding eosinophilic secretions and increasing their viscosity. Activated eosinophils after luminal entry can undergo EETosis to produce nuclear-derived DNA traps that contribute to the pathogenesis of eosinophilic diseases. Regulating excess EETosis could be a novel therapeutic target.

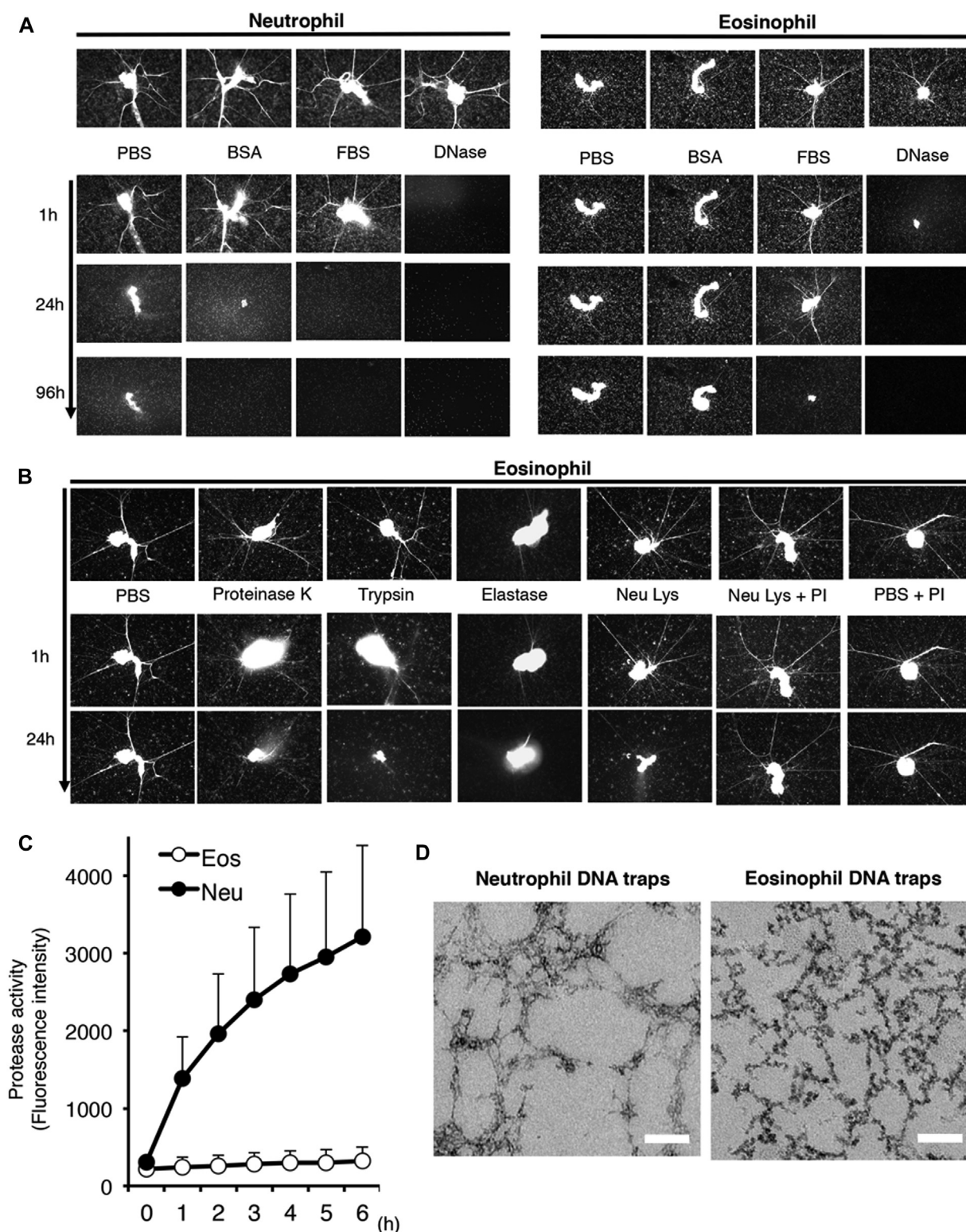


FIG 5. Eosinophil DNA traps showed higher stability because of lesser leukocyte protease activity. **A**, Eosinophil DNA traps formed stable aggregates. NETosis and EETosis were induced by stimulation with A23187 in SYTOX-containing medium, followed by DNA trap aggregate formation, and then the indicated agents (PBS, 1% BSA, 10% FBS, and 20 U/mL DNase I) were added to the culture medium and studied by using fluorescence microscopy at indicated time points. Data are representative of more than 3 experiments from independent donors with similar results. **B**, Eosinophil DNA traps were degraded by proteases. Indicated proteases were gently added to the culture medium and studied by using fluorescence microscopy at indicated time points. Vehicle, proteinase K, trypsin, elastase, neutrophil lysate (*Neu Lys*), and proteinase inhibitors (*PI*) were added to the medium and further incubated for the indicated time points at 37°C. Data are

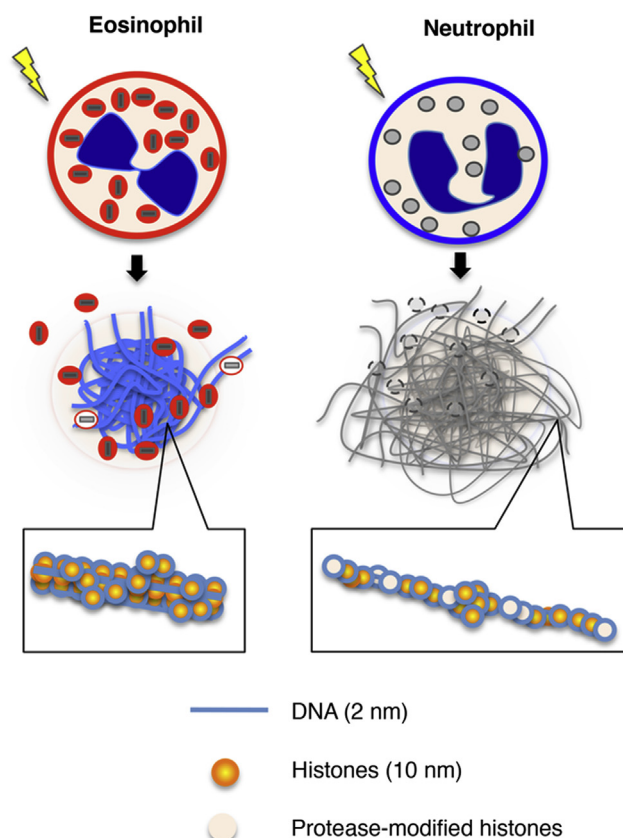


FIG 6. ETosis-mediated eosinophil and neutrophil DNA traps. Proposed differential characteristics between eosinophil and neutrophil DNA traps are shown. Both activated eosinophils and neutrophils undergo nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ETosis to release DNA traps that can spread and provide adhesive surfaces. In contrast to granule lysis and release of abundant protease in neutrophils, eosinophils contain much less protease, and granules are intact after ETosis. Eosinophils release globular chromatin fibers that contribute to the stability and viscosity of secretions.

Blood-derived eosinophils were used to study the characteristics of ETosis-mediated DNA traps. The process of ETosis is mediated through reactive oxygen species production and calcium signaling. ETosis releases nuclear contents as filamentous chromatin structures, and neither apoptosis nor necrosis induce DNA traps.^{3,4} We developed a simple method to visualize extracellular DNA traps by inducing formation of large DNA aggregates with brief experimental shear flow. The aggregates contained cell debris, including intact granules, mimicking the *in vivo* clinical findings. Another advantage of our simple plate-based system is the assessment of the characteristics of ETosis-derived DNA traps without fixation that could possibly affect their nature.

In response to specific stimuli, live eosinophils can release mitochondrial DNA in a noncytolytic catapult-like manner.²⁵

However, in general, small amounts of mitochondrial DNA (approximately 1% of the total DNA) are present in cells.²⁶ As evidenced by histone staining, the bulk of extracellular DNA traps in this study, found both *in vivo* and *in vitro*, were derived from the nucleus. In contrast to active projection of mitochondrial DNA, ETosis-mediated DNA release was a result of cell death; therefore spreading DNA traps in the fluid phase was through a passive process. Released eosinophil nuclear DNA traps rapidly assembled together and ensnared small particles, such as intact eosinophil granules and microorganisms. Hydrophobic interactions played a major role in adhesive capacity and DNA trap folding to make stable aggregates. These characteristics of extracellular DNA traps are important for ensnaring microorganisms to prevent their dissemination, but excessive formation might be counterproductive, preventing proper clearance and increasing the viscosity of secretions, as observed in samples from patients with ERCS and those with EOM.

Our study highlights the different characteristics between eosinophil and neutrophil DNA traps. In NETosis the granule membranes disappear intracellularly, followed by translocations of neutrophil elastase and myeloperoxidase to the neutrophil nucleus.^{4,5} This intracellular degranulation process appears to be essential for binding of these proteins to nuclear chromatin before neutrophils rupture. In contrast, eosinophil granules are released as intact structures, and eosinophil granule proteins remain largely within released granules.³ In neutrophil DNA traps nuclear core histones were the most abundant proteins and accounted for 70% of all net-associated proteins.⁶ A long strand of DNA is wrapped around core histones to form a nucleosome structure like “beads on a string.”²⁷ Neutrophil elastase promotes DNA traps’ chromatin decondensation in sputum from patients with cystic fibrosis by means of proteolytic processing of histones.²⁴ Eosinophil DNA traps from clinical and experimental sources were uniformly stained by using an mAb against the nuclear linker histone H1, which plays a key role in formation of 30-nm chromatin fibers.²⁸ Notably, the ultrastructural morphology of neutrophil DNA traps showed thinner and less globular fibers in contrast to the ultrastructure of eosinophil DNA traps. Taken together, we propose that eosinophil DNA traps are well conserved, with nucleosomes that are less susceptible to the limited proteolytic degradatory capacities of human eosinophils (Fig 6).

Eosinophil DNA traps provide adhesive surfaces that can entrap intact eosinophil granules and microorganisms. In concert with our studies that demonstrated that cell-free eosinophil granules can continue to function as independent extracellular secretion-competent structures,^{3,15,29} our current findings shed light on the roles of residual eosinophil “cell debris,” including entrapped intact eosinophil granules, which have been overlooked both experimentally and clinically. Cytolytic release from eosinophils of nuclear DNA traps and free extracellular granules can exert long-lasting effects, even after lysed eosinophils are no longer recognizable.

representative of more than 3 experiments from independent donors with similar results. **C**, Neutrophils contain abundant proteases. Neutrophils and eosinophils were lysed, and protease activity was measured with a fluorescence plate reader for the indicated time points. Data are expressed as means $\pm$ SDs ($n = 4$, independent donors). **D**, Analysis of ultrathin sections of DNA traps using transmission electron microscopy indicates thicker and more globular structures of eosinophil DNA traps compared with neutrophil DNA traps. Scale bar = 200 nm.

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Key messages

- EETosis-derived DNA traps are abundantly present in eosinophilic secretions and contribute to an increase in viscosity.
- Eosinophil DNA traps provide long-lasting adhesive surfaces for postmortem functions.

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