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Staphylococcus aureus degrades neutrophil extracellular traps to promote immune cell death

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Abstract

Bacterial invasion of host tissues triggers polymorphonuclear leukocytes to release DNA (NETs, neutrophil extracellular traps), thereby immobilizing microbes for subsequent clearance by innate defenses including macrophage phagocytosis. We report here that *Staphylococcus aureus* escapes these defenses by converting NETs to deoxyadenosine, which triggers the caspase-3 mediated death of immune cells. Conversion of NETs to deoxyadenosine requires two enzymes, nuclease and adenosine synthase, that are secreted by *S. aureus* and necessary for the exclusion of macrophages from staphylococcal abscesses. Thus, the pathogenesis of *S. aureus* infections has evolved to anticipate host defenses and to repurpose them for the destruction of the immune system.

Staphylococcus aureus is a leading cause of skin and soft tissue infections, bacteremia, sepsis and endocarditis (1). The vast spectrum of human morbidity and mortality has been attributed to the unique ability of staphylococci to evade innate and adaptive immune responses (2). The hallmark of *S. aureus* infection is the formation of abscesses, which comprise a bacterial community surrounded by fibrin deposits and a cuff of host immune cells (3) (Fig. 1 and fig. S1). Such lesions evolve by the infiltration of neutrophils, which release their DNA, also known as neutrophil extracellular traps (NETs) that immobilize the pathogen and enhance the bactericidal activity of antimicrobial peptides (4). *S. aureus* produces a spectrum of virulence factors that counter neutrophil defenses, and include proteins that kill neutrophils (2) or block their extravasation (5), chemotaxis (6), opsonization and phagocytosis (7), and inhibit reactive oxygen-mediated killing (8). Earlier work has reported that staphylococcal nuclease, secreted by *S. aureus*, degrades NETs (9). Thus, staphylococci persist and replicate within abscesses and seed new lesions at different sites or disseminate to other hosts via the purulent exudate (3).

Although the interactions between *S. aureus* and neutrophils have been studied in detail, comparatively little is known about the contributions of macrophages towards the establishment of abscesses and the potential clearance of staphylococcal infections. Here, we combined immuno-histochemical staining of abscesses and the genetic analysis of *S. aureus* to explore the fate of macrophages during staphylococcal infections in mice. Immuno-histochemical examination of renal tissues isolated 5 days after intravenous injection of *S.*

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aureus into BALB/c mice revealed bacteria surrounded by infiltrates of immune cells, primarily composed of Ly-6G positive neutrophils (Fig. 1A-D). Macrophages, identified by F4/80-specific staining, were observed to accumulate only at the periphery of abscesses and were absent from the cuff of neutrophils surrounding bacteria (Fig. 1E,H). We used a collection of *S. aureus* mutants with insertional lesions in virulence genes (10) to search for variants with phenotypic defects in which macrophages entered abscesses and the frequency of lesions was reduced (Table S1). Two mutants, with mutations in staphylococcal nuclease (*nuc*) or in adenosine synthase A (*adsA*) were selected. Abscesses that had been caused by the *nuc* mutant harbored staphylococci surrounded by a cuff of neutrophils and with infiltrates of F4/80-positive macrophages at the periphery of the immune cell cuff, but not in the central part of the abscess (Fig. 1F,I). In contrast, abscesses caused by the *adsA* mutant were characterized by diffuse infiltrates of F4/80-positive macrophages throughout the neutrophil cuff (Fig. 1G,J). Expression of plasmid encoded *nuc* and *adsA* in the mutant strains restored the wild-type phenotype (figs. S1 and S2). As a control, abscesses caused by *S. aureus* variants with mutations in the genes for clumping factor A (*clfA*) or coagulase (*coa*) (3), did not affect the frequency or structure of abscesses (fig. S1). Taken together, these results indicate that macrophages are excluded from *S. aureus*-induced abscesses by mechanisms requiring staphylococcal secretion of nuclease and AdsA.

We wondered whether secreted virulence factors of *S. aureus* affect the viability of mouse or human macrophages. Incubation of *S. aureus*-conditioned culture medium with mouse or human macrophages (see below) or the human macrophage cell line U937 did not increase trypan blue staining, which serves as an indicator of loss of cell viability (Fig. 2A). To test whether a host product contributed, first, we incubated bacterial cultures with human blood neutrophils and then added macrophages. Incubation of *S. aureus* culture media with macrophages alone did not cause an increase in cytotoxicity (Fig. 2A). However, treatment of neutrophils with phorbol 12-myristol 13-acetate (PMA), an inducer of NET formation (4), caused staphylococci to generate a product that, when transferred to U937 cells, triggered loss of macrophage viability (8% ±1.6 (SEM) trypan blue positive cells for *S. aureus* plus neutrophil NETs compared with 1.5% ±0.6 (SEM) for neutrophil NETs alone; $P<0.05$) (Fig. 2A). This cytotoxic activity was reduced when *S. aureus* NET samples were derived from *nuc* or *adsA* variant strains (Fig. 2A). Nuclease is a DNA cleavage enzyme with endo- and exonuclease activity (11) and AdsA has been reported to function as a 5'-nucleotidase (12). Nuclease is secreted via its N-terminal signal peptide into the extracellular medium. AdsA is anchored to the envelope and subsequently released into the extracellular medium (13).

To identify the toxic product generated by staphylococci, we treated NETs with staphylococcal culture medium and analyzed the products with reversed-phase high performance liquid chromatography (rpHPLC) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) (Fig. 2C). A single ion (*m/z* 251.074) was identified in NET samples treated with staphylococci that was absent from mock treated NETs. The mass was consistent with 2'-deoxyadenosine (dAdo), a deoxyribonucleoside base of DNA (Fig. 2C). NETs treated with either the *nuc* or *adsA* mutants, which stimulated less macrophage cytotoxicity, had less dAdo. NETs that had not been exposed to staphylococci did not have dAdo. The extracellular medium of

staphylococcal cultures also lacked dAdo (Fig. 2C). These data indicate that staphylococcal nuclease and AdsA are both required to release dAdo from neutrophil NETs and this compound may be responsible for the observed cytotoxic effect on macrophages. To test this model, we incubated human HL60 neutrophils, THP-1 monocytes and U937 macrophages with purified dAdo or with adenosine, another immune-suppressive product that staphylococcal AdsA generates from ATP released by damaged host tissues (13). Purified dAdo, but not adenosine induced cytotoxicity of U937 macrophages and THP-1 monocytes whereas HL60 neutrophils were insignificantly affected (Fig. 2D).

Treatment of human or animal cells with dAdo causes accumulation of intracellular dATP, which quenches DNA synthesis and triggers apoptosis (14). Rapidly dividing cells of the hematopoietic lineage are particularly susceptible to dAdo-induced apoptosis (14). Under physiological conditions, accumulation of dAdo and adenosine is prevented by adenosine deaminases (ADA), enzymes of the purine salvage pathway that deaminate dAdo and adenosine to generate inosine or deoxyinosine, respectively (15). Inability to degrade Ado or dAdo, as occurs in individuals with heritable defects in ADA expression, causes severe combined immunodeficiency (15). ADA activity is observed both within cells and in plasma. Two isoenzymes, designated ADA1 and ADA2, are expressed in mammalian tissues. ADA activity in plasma is predominantly derived from macrophage ADA2 and, depending on cell or tissue type, intracellular activities are attributable to ADA1 and/or ADA2 (16). Conditioned media collected from HL60, THP-1 and U937 cell overnight cultures cleaved [^{14}C] adenosine to generate [^{14}C] inosine, as observed by thin-layer chromatography (fig. S3). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an effective inhibitor of ADA1 (17), quenched adenosine deaminase activity of HL60 and THP-1 cells, but not of U937 macrophages. Similarly, the activity of purified recombinant ADA1 was blocked by EHNA, whereas the activity of U937 cells was only slightly reduced (fig. S3). Treatment with 2′-deoxycoformycin (dCF), a potent ADA1/2 inhibitor, blocked the adenosine deaminase activity of U937, HL60 and THP-1 cells (fig. S3). dCF treatment also increased the macrophage cytotoxicity of neutrophil-NETs incubated with *S. aureus* (Fig. 2B). Cytotoxic activity was abolished in the *S. aureus adsA* mutant and reduced in the *nuc* variant. Together these data indicate that the phosphodiesterase activity of nuclease, which predominantly cleaves DNA and RNA into 3'phosphomononucleotides and dinucleotides (11), degraded neutrophil NETs into products that AdsA subsequently converted into dAdo. In agreement with this model, rpHPLC analysis of 2'-deoxyadenosine-3'-monophosphate and 2'-deoxyadenosine-5'-monophosphate treated with purified AdsA, revealed the generation of dAdo, indicating that AdsA also functions as a 3'-nucleotidase in addition to its reported 5'-nucleotidase function (fig. S4)(12).

We sought to reconstitute dAdo production from neutrophil NETs using purified nuclease and AdsA. Treatment of NETs with nuclease generated a spectrum of cleavage products, mostly mononucleotides such as 2'-deoxyadenosine-3'-phosphate, whereas treatment with nuclease and AdsA produced dAdo (Fig. 3A). When added to U937 macrophages, we observed a corresponding increase in cytotoxicity from nuclease and AdsA treated NETs (Fig. 3B). Cytotoxicity was increased even further when NETs were added to U937 cells in the presence of dCF, which prevents cleavage of dAdo to deoxyinosine (Fig. 3B).

Neutrophil NETs consist of DNA and associated granule proteins including calprotectin, elastase, myeloperoxidase and antimicrobial peptides (4). Following neutrophil degranulation and NET formation, granule proteins contribute to tissue damage and trigger inflammatory responses (18). We sought to discern whether dAdo from NET DNA was sufficient to induce macrophage apoptosis, or whether granule proteins were also required. Purified thymus DNA devoid of granular enzymes was incubated with nuclease and AdsA. The reaction products were analyzed by rpHPLC, which showed dAdo was produced (Fig. 3C). Thymus DNA digestion products were cytotoxic for U937 macrophages and primary human monocytes but not for neutrophils (Fig. 3 and fig. S5). Cell death in U937 cells and human monocytes was associated with annexin V staining, indicating that treatment with nuclease and AdsA digested DNA products induces apoptosis (fig. S6)(19). These data indicate that staphylococcal nuclease- and AdsA-derived NET cytotoxicity selectively targets monoctyes and macrophages. This phenotype is attributable to the production of dAdo and is not dependent on the concomitant release of neutrophil granular enzymes.

dAdo treatment of U937 macrophages triggered the conversion of pro-caspase 3 to active caspase-3, a potent inducer of apoptosis (Fig. 3E)(20). While thymus DNA alone did not activate caspase-3, nuclease and AdsA digestion of DNA triggered U937 macrophage conversion of procaspase-3 to active caspase-3, which was also detected when ADA activity was blocked with dCF (Fig. 3E). Inhibition of caspase-3 with Z-DEVD-FMK peptide significantly reduced dAdo induced cell death (fig. S7). Caspase-3 activation was observed in *S. aureus* abscesses with immuno-histochemical staining (fig. S8). Less caspase-3 staining was observed in abscesses from the *nuc* mutant strain and was not detectable in lesions caused by the *adsA* mutant strain (fig. S8).

Here, we present evidence that *S. aureus* infection of host tissues is associated with the production of dAdo from neutrophil NETs that are cleaved by two secreted bacterial products, nuclease and AdsA. Production of dAdo by AdsA is sufficient to induce apoptosis of macrophages via the activation of caspase-3. This mechanism restricts the survival of macrophages entering the immune-cell cuff that surrounds staphylococcal abscess communities, thereby antagonizing phagocytosis of the invading pathogen and promoting the establishment of persistent infections. Nuclease facilitates production of dAdo through the degradation of neutrophil-NET DNA into deoxyadenosine monophosphate, a substrate for AdsA, thereby supporting macrophage exclusion at the periphery of the immune cell cuff. These insights may aid in the design of therapies that enhance macrophage survival and staphylococcal clearance from infected tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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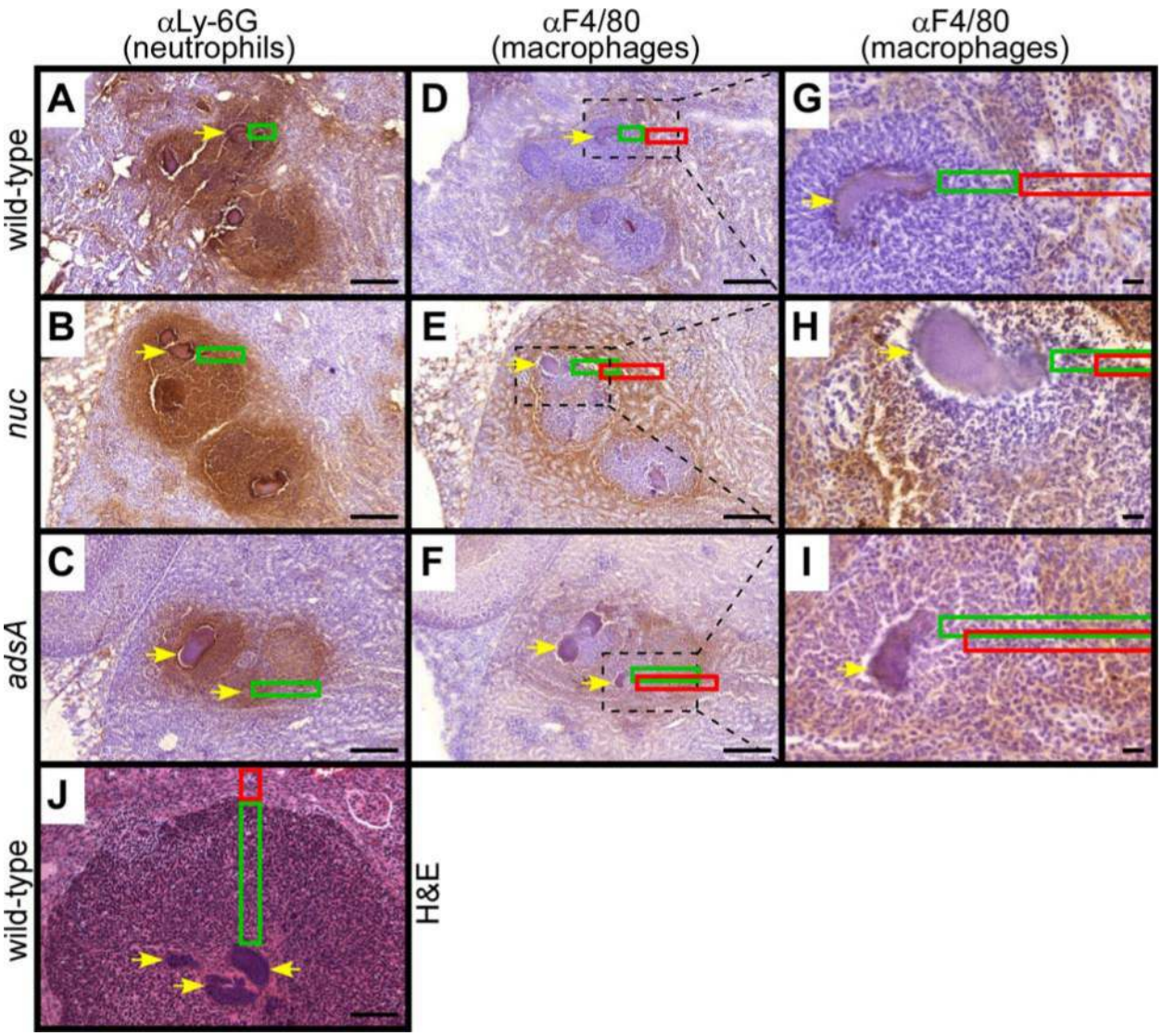

Fig. 1. Macrophages are excluded from staphylococcal abscesses

BALB/c mice were infected by intravenous injection with *S. aureus* wild-type or variants with mutations in genes for nuclease (*nuc*) or adenosine synthase A (*adsA*). Animals were euthanized 5 days post infection and kidneys removed for histopathology. (**A** and **J**) Wild-type staphylococci are distinguishable as central nidus (yellow arrowheads) surrounded by a fibrin capsule, a zone of infiltrating neutrophils (green box - immune cell cuff) and macrophages at the periphery of these lesions (red box, panel **J** - hematoxylin-eosin stained tissue). Cryo-sections of renal tissues were examined by immuno-histochemistry with αLy-6G antibodies (neutrophils stained as brown pigment, **A**-**C**) or αF4/80 antibodies (macrophages, **D**-**I**) and counterstained with hematoxylin. Panels **G**-**I** represent magnifications of the area framed by the black box in panels **D**-**F**. Macrophages (red box) are excluded from the neutrophil cuff of abscesses formed by wild-type *S. aureus* (green box) but not from those formed by the *nuc* or *adsA* mutants. Images are representative of similar data from kidneys of each cohort (*N*=4) of infected mice. Black bars represent 200 μm and 20 μm (panels G-I) lengths.

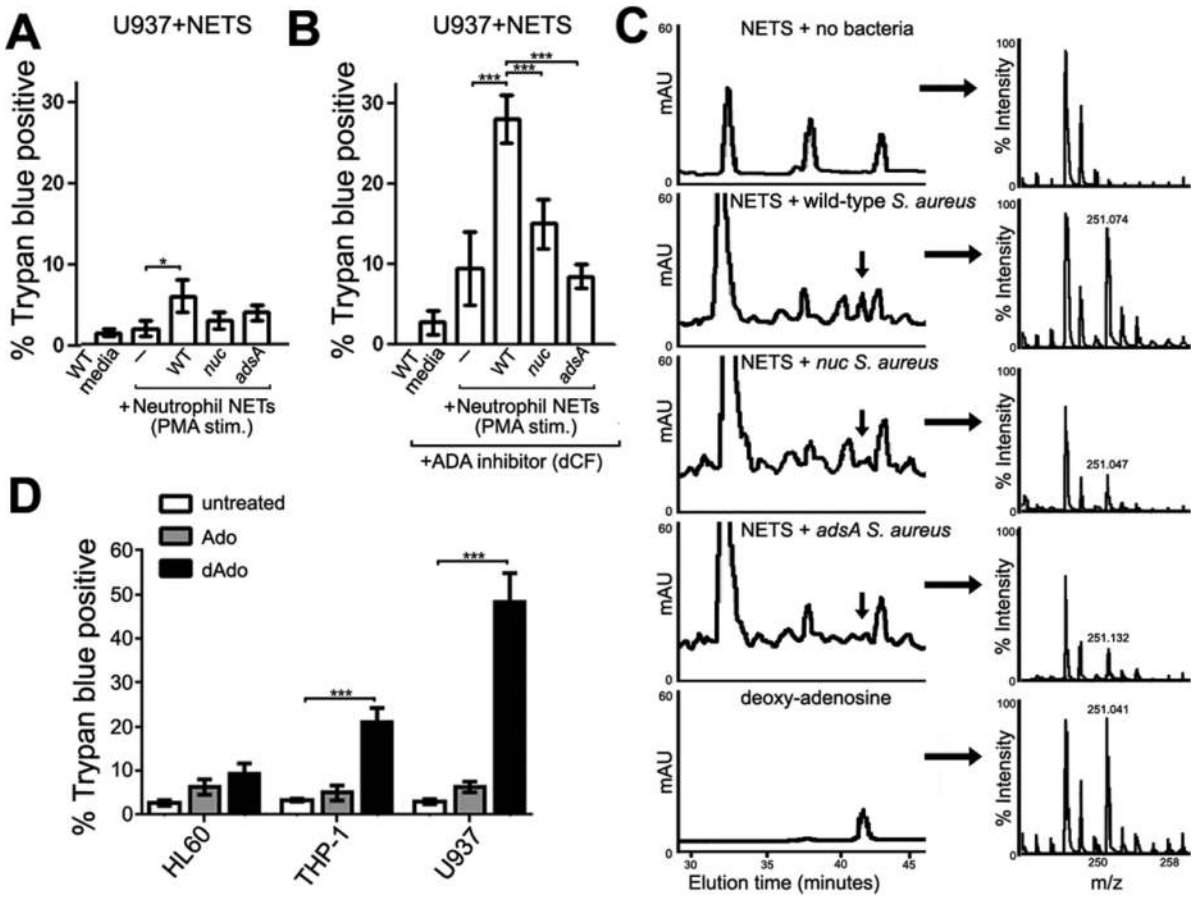

Fig. 2. *S. aureus* generates deoxyadenosine from neutrophil NETs to induce macrophage cytotoxicity

(**A**) Death of U937 macrophages was measured as trypan blue uptake. Human neutrophils were induced with phorbol 12-myristate 13-acetate (PMA) to form NETs that were then exposed to staphylococcal cultures and used to treat human U937 macrophages. Pairwise comparisons of mock/PBS (-) with the extracellular medium from wild-type (WT), *nuc* or *adsA* mutant *S. aureus* cultures were conducted. Data are the mean ±SD (*N*=3). Statistical significance was examined with the two-tailed Student's t-test;* $P<0.05$. (**B**) U937 macrophages were treated with the adenosine deaminase inhibitor 2′-deoxycoformycin (dCF) and with NETs that were mock treated or incubated with staphylococcal culture medium (*N*=3);*** $P<0.005$. (**C**) NET cleavage reactions (A) were subjected reversed-phase high performance liquid chromatography. Absorbance at 260 nm was monitored (left panels) and eluate was analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) (right panels), which identified *m/z* 251.074 as deoxyadenosine (dAdo, arrow). (**D**) Differentiated human HL60 neutrophil, THP-1 monocyte and U937 macrophage cell lines were incubated overnight with 10 μM adenosine (Ado) or dAdo and cell death monitored. Data are the mean ±SD (*N*=3). Statistical significance was examined with the two-tailed Student's t-test; *** $P<0.005$.

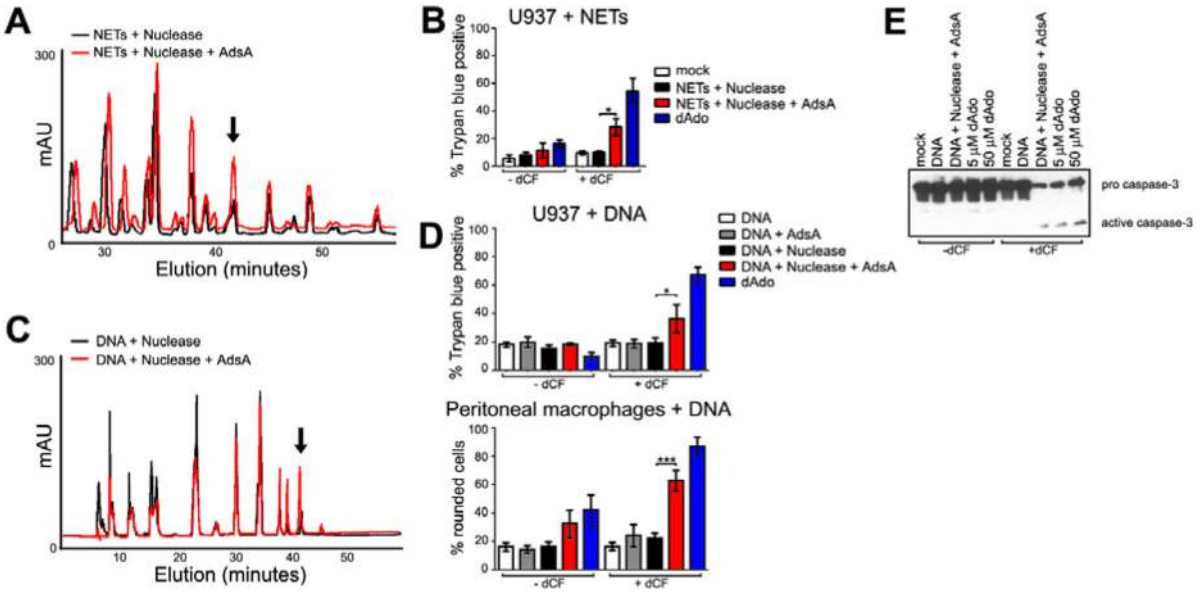

Fig. 3. Staphyloccocal nuclease and AdsA derived deoxyadenosine activates caspase 3- mediated apoptosis of macrophages

(**A**) PMA induced neutrophil NETs were incubated with purified nuclease alone or with AdsA and analyzed by rpHPLC. Arrow denotes dAdo. (**B**) U937 macrophages were incubated with NETs in the presence or absence of dCF (adenosine deaminase inhibitor) followed by trypan blue staining. Data are the mean ±SD (*N*=6). Statistical significance was examined with the two-tailed Student's t-test;* *P*<0.05. (**C**) Thymus DNA was treated with purified nuclease alone or with AdsA and digestion products were subjected to rpHPLC. (**D**) U937 macrophages or mouse macrophages were treated with thymus DNA samples that had been incubated with nuclease alone or with AdsA in the presence or absence of dCF followed by trypan blue staining. Digestions of DNA with either purified nuclease or AdsA alone and undigested thymus DNA were included as controls. Data are the mean ±SD (*N*=3). Statistical significance was examined with the two-tailed Student's t-test;* P<0.05; *** P<0.005. (**E**) U937 macrophages were incubated with thymus DNA pre-treated with nuclease alone or with AdsA. Cell lysates were subjected to immunoblotting with α-caspase-3 antibodies (*N*=3).