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Afa/Dr Diffusely Adhering *Escherichia coli* Strain C1845 Induces Neutrophil Extracellular Traps That Kill Bacteria and Damage Human Enterocyte-Like Cells

Viviana Marin-Esteban,^{a,c} Isabelle Turbica,^{a,b,c} Guillaume Dufour,^{b,c,d} Nicolas Semiramo,^{b,c} Aude Gleizes,^{a,b,c} Roseline Gorges,^{a,b,c} Isabelle Beau,^{b,c} Alain L. Servin,^{b,c} Vanessa Lievin-Le Moal,^{b,c} Catherine Sandré,^{a,b,c} and Sylvie Chollet-Martin^{a,b,c,e}

INSERM, UMR-S 996, Cytokines, Chemokines and Immunopathology, Châtenay-Malabry, France^a; INSERM, UMR-S 756, Physiopathologie des Cellules Épithéliales, Châtenay-Malabry, France^b; Université Paris-Sud, Faculté de Pharmacie, Châtenay-Malabry, France^c; AP-HP, Groupe Hospitalier, PNVS, Hôpital Bichat, Département d'Anesthésie et de Réanimation, Paris, France^d; and AP-HP, Groupe Hospitalier PNVS, Hôpital Bichat, Unité d'Immunologie Auto-Immunité et Hypersensibilités, Paris, France^e

We recently documented the neutrophil response to enterovirulent diffusely adherent *Escherichia coli* expressing Afa/Dr fimbriae (Afa/Dr DAEC), using the human myeloid cell line PLB-985 differentiated into fully mature neutrophils. Upon activation, particularly during infections, neutrophils release neutrophil extracellular traps (NETs), composed of a nuclear DNA backbone associated with antimicrobial peptides, histones, and proteases, which entrap and kill pathogens. Here, using fluorescence microscopy and field emission scanning electron microscopy, we observed NET production by PLB-985 cells infected with the Afa/Dr wild-type (WT) *E. coli* strain C1845. We found that these NETs were able to capture, immobilize, and kill WT C1845 bacteria. We also developed a coculture model of human enterocyte-like Caco-2/TC7 cells and PLB-985 cells previously treated with WT C1845 and found, for the first time, that the F-actin cytoskeleton of enterocyte-like cells is damaged in the presence of bacterium-induced NETs and that this deleterious effect is prevented by inhibition of protease release. These findings provide new insights into the neutrophil response to bacterial infection via the production of bactericidal NETs and suggest that NETs may damage the intestinal epithelium, particularly in situations such as inflammatory bowel diseases.

Escherichia coli causes a wide range of human diseases and particularly gastrointestinal infections. The pathogenic mechanisms of six diarrheagenic pathovars have been extensively studied (18). Diffusely adherent *E. coli* expressing Afa/Dr fimbriae (Afa/Dr DAEC) colonizes the small bowel, frequently causing childhood diarrhea (49). The pathogenicity of this pathovar is mainly due to Afa/Dr adhesin-host cell interactions. The enteric wild-type (WT) Afa/Dr DAEC strain C1845, which bears F1845 fimbriae, interacts with brush border-associated human decay-accelerating factor (hDAF) (40) and with carcinoembryonic antigen-related cell adhesion molecules (hCEACAMs) (2, 30). Interaction with hDAF triggers inflammatory responses, including interleukin-8 (IL-8) and vascular endothelial growth factor production by enterocytes (4, 12, 13), leading to transepithelial migration of polymorphonuclear neutrophils (PMNs) (4, 5). Recently, using the human myeloid cell line PLB-985 differentiated into fully mature PMNs, we observed interactions between PMNs and apical enterovirulent Afa/Dr DAEC bacteria. We found that these bacteria rapidly triggered the oxidative burst and degranulation of preformed mediators, followed by IL-1 β , tumor necrosis factor alpha, and IL-8 synthesis. The interaction between DAF and the bacterial virulence factor type 1 pili activated Erk1/2 and p38 mitogen-activated protein kinase, Src tyrosine kinase, and NF- κ B (47). We subsequently observed F1845 fimbria-mediated phosphatidyl serine (PS) externalization by differentiated PLB-985 cells through an apoptosis-independent mechanism that enabled macrophage engulfment of infected PLB-985 cells (48). Together, these results suggested that PMNs may participate in Afa/Dr DAEC pathogenesis and diarrhea through several mechanisms.

Upon activation, PMNs release web-like extracellular structures that ensnare and kill pathogens (9, 45), the neutrophil extra-

cellular traps (NETs), composed of decondensed chromatin decorated with granular and cytoplasmic proteins; DNase treatment is sufficient to dismantle NETs (10, 26, 41). This novel form of cell death, called netosis, seems to depend mainly on oxidative burst triggered by NADPH oxidase (NOX₂) activation (55, 57). Another key marker of netosis is posttranslational deimination of arginine residues to citrullines in histones, catalyzed by peptidyl arginine deaminase 4 (PAD4) (37). The qualitative and quantitative composition of NET proteins was recently studied by using a proteomic approach, leading to the identification of 24 proteins of nuclear (histones), granular (elastase, proteinase 3, myeloperoxidase [MPO], etc.), cytoplasmic (S100 proteins), or cytoskeletal (actin, etc.) origin (52). The protective effect of NETs has been observed in several animal models of infection and also in humans. However, there is also increasing evidence that NETs contribute to some inflammatory and autoimmune disorders, possibly by preventing potentially noxious mediators from diffusing away and allowing them to cause local tissue damage or thrombosis. Mast cells, monocytes, and eosinophils can also produce antimicrobial extracellular traps (50, 54, 58).

A large variety of proinflammatory mediators activate NET release, including bacterial lipopolysaccharide (LPS), IL-8, gran-

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Editor: S. M. Payne

Address correspondence to Sylvie Chollet-Martin, sylvie.chollet-martin@u-psud.fr.

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ulocyte-macrophage colony-stimulating factor plus C5a, and phorbol myristate acetate (PMA). NET formation can be triggered *in vitro* by direct exposure to pathogens such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella enterica* serovar Typhimurium, *Mycobacterium tuberculosis*, *Candida albicans* (hyphae and yeast cells), and the protozoan parasite *Leishmania amazonensis*. However, NET formation triggered by *Escherichia coli* is poorly documented (29, 34).

Here we examined the possible role of netosis in Afa/Dr DAEC pathogenesis by using neutrophil-like differentiated PLB-985 cells. We found that the enterovirulent Afa/Dr wild-type *E. coli* strain C1845 induced NET release and that these NETs captured and killed wild-type C1845 bacteria. Moreover, using a coculture model of PLB-985 cells and enterocyte-like Caco-2 cells, we obtained evidence that NETs may contribute to epithelial injury through local direct contacts between NET proteases and intestinal epithelial cells. These findings may have relevance to the situation in the intestinal lumen after bacterium-induced neutrophil transepithelial migration, in patients with inflammatory bowel diseases, for example.

MATERIALS AND METHODS

Reagents and antibodies. *N,N*-Dimethyl formamide (DMF) was from Carlo Erba (Rodano, Italy); all-*trans*-retinoic acid (ATRA), cytochalasin D (CytD), PMA, hydrogen peroxide, pentoxifylline, DNase I, poly-L-lysine, and Hoechst 33342 bisbenzimidazole were from Sigma-Aldrich (Chimie SARL, L'Isle d'Abeau Chesnes, France); and Sytox green was from Molecular Probes (Eugene, OR). Monoclonal antibodies against MPO (clone 1B10; BD Biosciences, San Jose, CA) and β 2-integrin (anti-CD11b; Dako, Glostrup, Denmark) and rabbit polyclonal antibodies against histone H3 (Abcam, ab1791) were used, together with fluorescein isothiocyanate-conjugated (FITC; BD Biosciences) or Alexa Fluor 488-conjugated (Molecular Probes) secondary antibodies. Rhodamine-labeled phalloidin was from Cytoskeleton Inc. (Denver, CO).

PLB-985 cell culture and differentiation. The human myeloid leukemia cell line PLB-985 was cultured as previously described (47, 48). Briefly, cells were cultured in RPMI 1640/glutamine medium supplemented with 10% de complemented fetal calf serum (FCS), 50 units/ml penicillin, and 50 μ g/ml streptomycin (Cambrex Bio-Sciences, Verviers, Belgium) at 37°C in humidified air containing 5% CO₂. For granulocytic differentiation, exponentially growing cells were cultured in RPMI 1640 medium supplemented with 0.5% DMF, 2 μ M ATRA, and 5% FCS. This medium was renewed on day 3 of the 6-day differentiation period.

In some experiments we used X-CGD PLB-985 cells (a generous gift from M. J. Stasia and M. Dinauer), which do not express the *gp91-phox* gene (61) and serve as a model of X-linked chronic granulomatous disease (X-GCD). These cells were differentiated in RPMI 1640 medium supplemented with 0.5% DMF and 10% FCS (61).

On day 6, granulocytic differentiation was checked by morphological analysis of cytocentrifuged cells stained with May-Grünwald-Giemsa and also in terms of β 2-integrin CD11b expression at the cell surface and superoxide anion production, as previously described (47).

Isolation of human blood polymorphonuclear neutrophils. Blood samples were obtained from informed healthy volunteers. As previously described (28), leukocytes were isolated by sedimentation on a separating medium containing 9% Dextran T500 (Pharmacia, Uppsala, Sweden) and 38% Radioslectan contrast agent (Schering, Lys-lez-Lannoy, France). After red cell sedimentation, polymorphonuclear leukocytes were separated by centrifugation on a Ficoll density gradient (Eurobio, Les Ulis, France). Contaminating erythrocytes were removed by hypotonic lysis, and polymorphonuclear leukocytes were resuspended in Hanks balanced salt solution (HBSS; Ca²⁺ Mg²⁺; Life Technologies, San Diego, CA).

Epithelial cell culture and differentiation. The Caco-2/TC7 clone (Caco-2/TC7) (43), established from the parental Caco-2 cell line (15),

was routinely grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 25 mM glucose, 15% FCS, and 1% nonessential amino acids (Life Technologies) at 37°C in air–10% CO₂. Cells were maintained by weekly passage, using 0.02% trypsin in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) containing 3 mM EDTA.

Bacterial strains and growth conditions. We used the wild-type Afa/Dr DAEC strain C1845 (WT C1845) (7), as well as three other enterovirulent *E. coli* strains as controls: LF82 (19), CFA-I (25), and CFA-III (20). For each experiment, exponentially growing bacteria were subcultured in Luria-Bertani (LB) broth at 37°C for 18 h and then washed, counted in a Salubini chamber, and adjusted to the appropriate number of CFU per ml.

NET immunofluorescence. Differentiated PLB-985 cells (3×10^5) were seeded on 0.001% poly-L-lysine-coated glass coverslips and allowed to settle for 1 h. They were then exposed to bacteria at a multiplicity of infection (MOI) of 100 bacteria per cell or to 25 nM PMA for 1 to 4 h. The cells were then fixed with 3% paraformaldehyde (PFA). For protein immunostaining, nonspecific binding sites were blocked and the cells were incubated at +4°C with monoclonal antibodies directed against MPO, β 2-integrin CD11b, or histone 3 (H3). Primary antibodies were detected with FITC- or Alexa Fluor 488-labeled secondary antibodies. Isotype-matched antibodies were used as controls. DNA was stained with 0.5 μ g/ml Hoechst 33342 bisbenzimidazole (Hoescht). The coverslips were mounted in fluorescent mounting medium (DakoCytomation), and the cells were examined with a conventional epifluorescence microscope (AxioObserver Z1_Colibri [Zeiss, Germany] or Eclipse 80i [Nikon, Japan]).

Field emission scanning electron microscopy (FESEM) of NETs. Differentiated PLB-985 cells (3×10^5) were seeded on poly-L-lysine-coated glass coverslips and allowed to settle for 1 h. Bacteria (MOI, 20) were then added for 3 h. The samples were fixed overnight at 4°C with 2.5% glutaraldehyde in sodium cacodylate buffer (0.2 M, pH 7.4). After washing, the samples were dehydrated in a graded ethanol series (from 50 to 100%) and air dried. The samples were finally mounted on stubs with conductive silver paint (Agar Scientific Ltd., Stansted, United Kingdom) and coated with gold-palladium (180 s, 10 mA) in a sputter coater (SC7640 Polaron; Quorum Technologies, Elexience, France). Specimens were studied by field emission-gun scanning electron microscopy (FESEM; Hitachi S4500, Tokyo, Japan) with a microscope operated at 5 kV at the MIMA2 microscopy platform (<http://voxel.jouy.inra.fr/mima2>).

NET quantification. To follow the kinetics of NET formation, differentiated PLB-985 cells, differentiated X-CGD PLB-985 cells, and fresh human PMNs (2.5×10^5 cells) were seeded in 96-well black plates in the presence of 0.5% FCS and 5 μ M Sytox green, a non-cell-permeant DNA binding dye. The cells were stimulated with increasing concentrations of PMA or hydrogen peroxide at 37°C in the dark. Released DNA was quantified by reading Sytox green fluorescence at various time points. In some endpoint experiments, cells were treated with 90 U/ml DNase I in order to disrupt NETs. To test the effect of bacterial challenge on NET production, cells were incubated with bacteria (MOI, 60) for 4 h at 37°C. Sytox green fluorescence was measured in a microplate fluorescence reader (GENios spectrophotometer; Tecan, Salzburg, Austria) at an excitation wavelength of 485 nm and an emission wavelength of 527 nm. All samples were tested in duplicate. In parallel, 4',6-diamidino-2-phenylindole-labeled samples were also observed under the fluorescence microscope to check for the absence of apoptotic or necrotic cells.

PLB-985 NET killing of *Escherichia coli* C1845. Differentiated PLB-985 cells (1×10^6) were allowed to settle for 30 min in 24-well tissue culture plates and then stimulated with 20 nM PMA for 4 h to induce NETs. The cells were then treated for 15 min either with DNase I (90 U/ml) to dismantle NETs or with CytD (10 μ g/ml) to inhibit phagocytosis, before adding bacteria (MOI, 0.1). The plates were centrifuged at $700 \times g$ for 10 min and then incubated for 45 min at 37°C.

After incubation, PMA-stimulated cells and supernatants were recovered by thorough scraping; each well was rinsed with 500 μ l of cold HBSS containing 0.5% FCS pooled with the supernatant. For nonstimulated

cells, supernatants were carefully recovered and the cells were lysed in the wells with 500 μ l of ice-cold distilled water for 5 min and then scraped and pooled with the supernatant. Samples were serially diluted (10^{-1} , 10^{-2} , 10^{-3}), plated on LB agar, and incubated for 24 h at 37°C to determine the numbers of CFU. Experiments were carried out in triplicate and repeated at least five times.

The percentage of bacterial killing by netosis was determined as previously reported (24), considering the number of bacteria recovered after challenge of PMA-stimulated cells versus the number recovered after challenge of PMA-DNase-treated cells, as follows: $[(df \times CFU \text{ with DNase} - df \times CFU \text{ with no DNase})/df \times CFU \text{ DNase}] \times 100$, where df is the dilution factor and CFU represents numbers of CFU.

The percentage of phagocytosis-dependent intracellular bacterial killing was similarly determined, considering the number of bacteria recovered after challenge of CytD-treated and untreated cells, as follows: $[(df \times CFU \text{ with CytD} - df \times CFU \text{ with no CytD})/df \times CFU \text{ CytD}] \times 100$.

Neutrophil-epithelial cell interaction model. Overnight bacterial cultures were incubated in PBS with gentamicin (200 μ g/ml) for 1 h at room temperature, and bacterial density was adjusted to 5×10^8 /ml in PBS containing gentamicin. Treated bacteria were washed with PBS and centrifuged, and bacterial killing was quantified by plating on LB agar. For infection assays, Caco-2/TC7 cells (2.5×10^4) were seeded on glass coverslips in 24-well plates and cultured for 3 days. After a washing step, 10^6 differentiated PLB-985 cells and/or 10^8 bacteria (MOI, 100) in HBSS were added per well. In some experiments, prior to bacterial challenge, PLB-985 cells were treated with 5 mM pentoxifylline, a phosphodiesterase inhibitor that prevents degranulation. The plates were incubated at 37°C in air–10% CO₂ for 4 h, and the cell preparations were washed and fixed with 3% PFA. After nonspecific binding site blockade and permeabilization with 0.2% Triton X-100, F actin was stained with rhodamine-labeled phalloidin. PLB-985 cells were identified by immunolabeling of the β 2-integrin CD11b. Alternatively, for NET observation, PLB-985 cells were treated with gentamicin-killed C1845 bacteria (MOI, 100) and incubated with Caco-2/TC7 monolayers. The cells were then fixed with PFA, with no washing steps. The coverslips were mounted and NETs were examined as described above. F actin was examined by epifluorescence microscopy with a confocal laser scanning microscope (Zeiss LSM 510) or with an Axiovert 100 M microscope. To quantify NET production in this coculture model, 4×10^3 Caco-2/TC7 cells were seeded in 96-well plates and cultured for 3 days. PLB-985 cells (2×10^5) were treated with C1845 bacteria (MOI, 100) and immediately added to Caco-2/TC7 cells. Sytox green (5 μ M) was also added. The plates were incubated for 4 h in the dark, and fluorescence was measured as described above.

Statistical analysis. NET production and bacterial killing were expressed as the mean \pm standard error of the mean (SEM). Data were analyzed with either the Wilcoxon matched-pairs signed test or analysis of variance-Fisher's test. P values below 0.05 were considered to denote significant differences.

RESULTS

Differentiated PLB-985 cells are a suitable model for studying NETs. PMA is known to induce NET production (46). We studied the capacity of differentiated PLB-985 cells to generate NETs by immunofluorescence microscopy. Differentiated PLB-985 cells seeded on coverslips were activated with PMA for 4 h, resulting in the release of nuclear DNA into the extracellular medium, i.e., NET formation. Figure 1A shows a range of NET aspects. To better characterize the molecular composition of the observed NETs, we labeled them for cell granule-derived proteins. MPO colocalized along the NETs (Fig. 1B, left), and β 2-integrin CD11b/CD18 also associated with the NETs with a pointed pattern (Fig. 1B, right). PMA-activated PLB-985 cells were then observed with the FESEM method (Fig. 1C): activated PLB-985 cells released fiber-

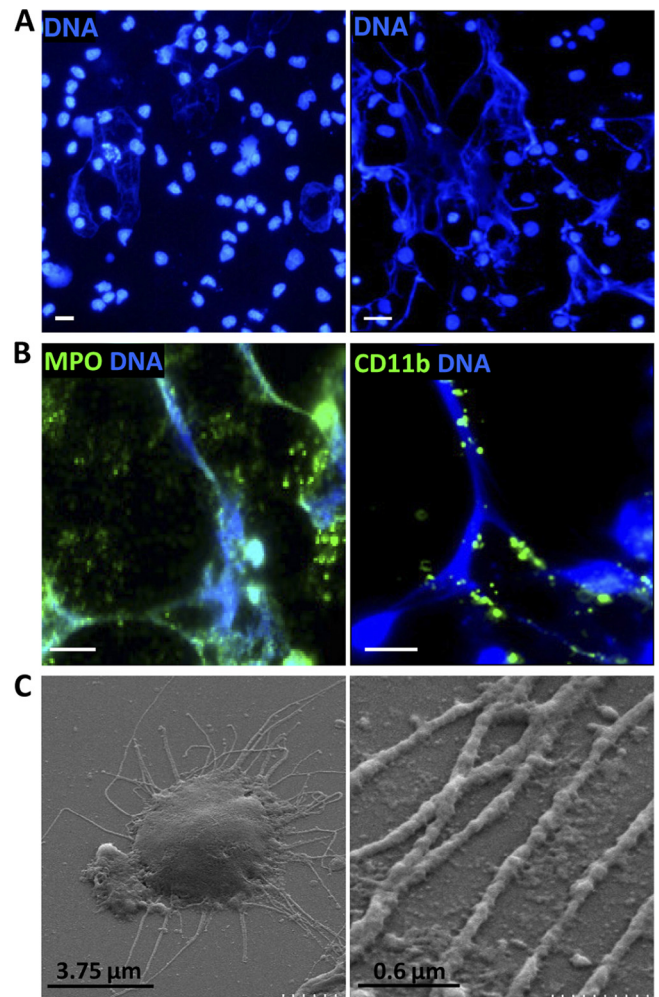


FIG 1 Characterization of PLB-985-derived NETs by immunofluorescence and FESEM. Cells were seeded on poly-L-lysine-coated coverslips and stimulated for 4 h with PMA in 0.5% FCS-supplemented HBSS medium. (A) Hoechst-stained DNA from cells stimulated with PMA exhibiting a web-like aspect (left) or a fiber-like aspect (right); (B) colocalization of Hoechst-stained DNA with MPO (left) or with β 2-integrin (CD11b/CD18) (right); (C) FESEM images of NETs. Bars on immunofluorescence pictures, 10 μ m.

like structures corresponding to NETs (Fig. 1C, left) and consisting of smooth fibers and globular domains (Fig. 1C, right).

To evaluate the intensity of NET production by differentiated PLB-985 cells, we quantified extracellular DNA by fluorimetry with Sytox green, a cell-impermeant fluorescent dye. Figure 2A to C show the time course of extracellular DNA release by PLB-985 cells, X-CGD PLB-985 cells, and fresh blood neutrophils in response to increasing concentrations of PMA. In the absence of PMA, the Sytox green signal remained at the basal level throughout the experiment, regardless of the model cells (Fig. 2A to C). In the presence of PMA, extracellular DNA release by blood PMNs started to rise as early as 180 min, gradually increasing for up to 300 min in a PMA concentration-dependent manner (Fig. 2A). Differentiated PLB-985 cells exhibited a similar concentration- and time-dependent response to PMA (Fig. 2B). As reactive oxygen species (ROS) are needed to induce netosis in blood PMNs (9), we evaluated the response of *gp91-phox*-deficient X-CGD PLB-985 cells to PMA: no extracellular DNA was detected, what-

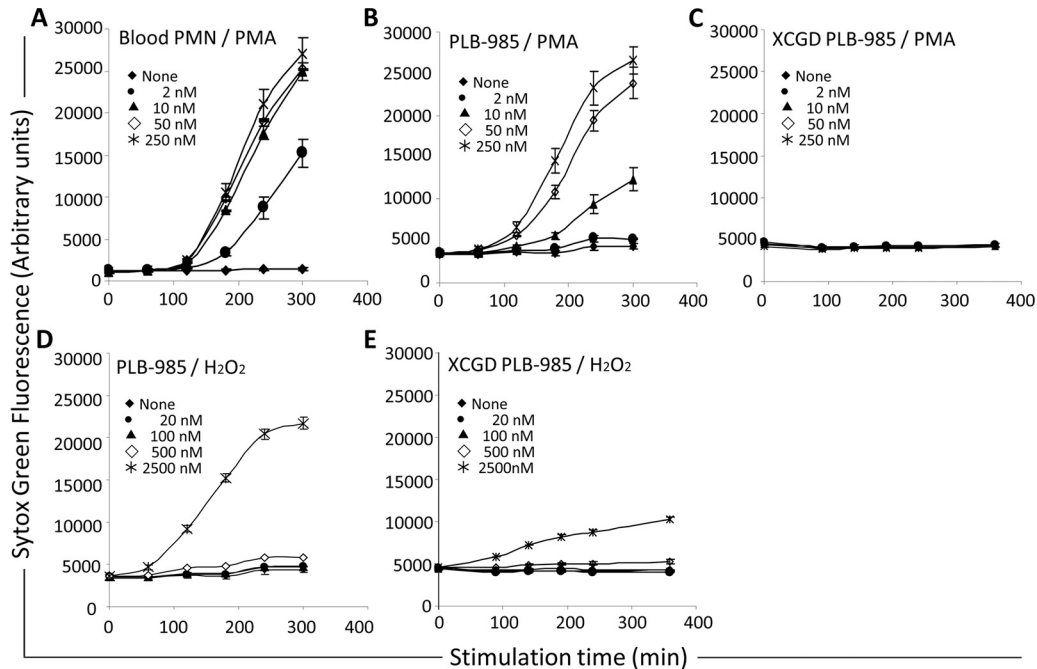


FIG 2 Differentiated PLB-985 cells produce NETs in response to PMA via a ROS-dependent mechanism. Extracellular DNA was detected using Sytox green, a non-cell-permeant DNA dye. The time course and concentration effect of PMA were examined on freshly isolated blood PMNs (A), differentiated PLB-985 cells (B), and differentiated X-CGD PLB-985 cells deficient in *gp91-phox* NOX₂ subunits (C). Cells were incubated with 5 μ M Sytox green and with PMA at the indicated concentrations (0 to 250 nM). In some experiments, wild-type (D) and X-CGD PLB-985 (E) cells were also directly stimulated with hydrogen peroxide (0 to 2,500 nM). Sytox green fluorescence was recorded in arbitrary units (AU) at increasing incubation times (from 0 to 300 min). The data are means \pm SEMs and are representative of at least 3 separate experiments.

ever the PMA concentration or stimulation time (Fig. 2C). When a high concentration of hydrogen peroxide (2,500 nM) was added directly to X-CGD PLB-985 cells, circumventing their NADPH oxidase deficiency, weak, time-dependent extracellular DNA release (Fig. 2E) lower than that observed with normal PLB 985 cells (Fig. 2D) was observed. Finally, the addition of 45 U of DNase after PMA stimulation of PLB-985 cells induced an 82% decrease in extracellular DNA release, confirming the capacity of these cells to produce NETs. Together, these results indicate that differentiated PLB-985 cells provide a neutrophil-like model suitable for studying NETs and netosis.

Upon infection by the Afa/Dr DAEC WT strain C1845, PLB-985 cells produce NETs that colocalize with the bacteria and can kill them. PLB-985 cells were challenged with WT C1845 bacteria for 4 h. Phase-contrast microscopy showed bacteria close to the DNA lattices (Fig. 3A). FESEM confirmed that the bacteria were associated with NETs (Fig. 3B). Moreover, Fig. 3C shows that NETs of PLB-985 cells induced by WT C1845 bacteria were in contact with infecting bacteria, suggesting that NETs could capture the bacteria. Extracellular DNA release in the supernatants was quantified by using Sytox green, as described above. As shown in Fig. 3D, WT strain C1845 significantly stimulated extracellular DNA release from infected PLB-985 cells; interestingly, three other enterovirulent *E. coli* strains (LF82, CFA-I, and CFA-III) also induced DNA release (Fig. 3E).

We then evaluated the efficiency with which PLB-985 cells kill WT C1845 bacteria through NET release (Fig. 4). Bacterial killing dependent on netosis, considered as the DNase-sensitive mechanism on activated cells, was calculated as previously reported (24) from CFU values obtained with PMA-stimulated cells treated and

not treated with DNase. Netosis accounted for $18.0\% \pm 4.1\%$ of WT C1845 bacterial killing by PLB-985 cells (Fig. 4A). Phagocytosis-dependent bacterial killing by the cytochalasin D-sensitive mechanism on unstimulated cells accounted for $34.3\% \pm 14.8\%$ of bacterial killing (Fig. 4B). These results show that netosis, though less efficient than phagocytosis, is actively involved in the antibacterial response of PLB-985 cells against enterovirulent WT C1845 bacteria.

NETs from C1845-treated PLB-985 cells damage intestinal epithelial cells. In proinflammatory situations generated by enterovirulent bacteria, subepithelial neutrophils are activated and transmigrate across the epithelial cell barrier. This phenomenon occurs upon infection of colonic cell monolayers by Afa/Dr DAEC WT strain C1845 (4, 5). The structural and functional impact of the presence of transmigrated neutrophils in the luminal compartment of polarized cells lining the epithelial intestinal barrier has rarely been investigated. We thus examined whether NETs induced after WT C1845 treatment have an impact on cultured human enterocyte-like Caco-2/TC7 cells. As WT C1845 infection can directly inflict brush border structural and functional damage on enterocyte-like Caco-2 cells (3, 42), we used killed WT C1845 to pretreat PLB-985 cells before coculture with Caco-2/TC7 cells. We checked that gentamicin-killed bacteria expressed F1845 fimbriae (data not shown) and bound to PLB-985 cells in the same way as live WT C1845 cells. Indeed, 20 ± 5 live bacteria and 22 ± 6 gentamicin-killed bacteria adhered per PLB 985 cell, as shown by microscopy. We then examined whether WT C1845-induced NETs produced by PLB-985 cells affected enterocyte-like Caco-2/TC7 cytoskeleton integrity. Caco-2/TC7 cells were cocultured for 4 h with PLB-985 cells alone or with PLB-985 cells treated with

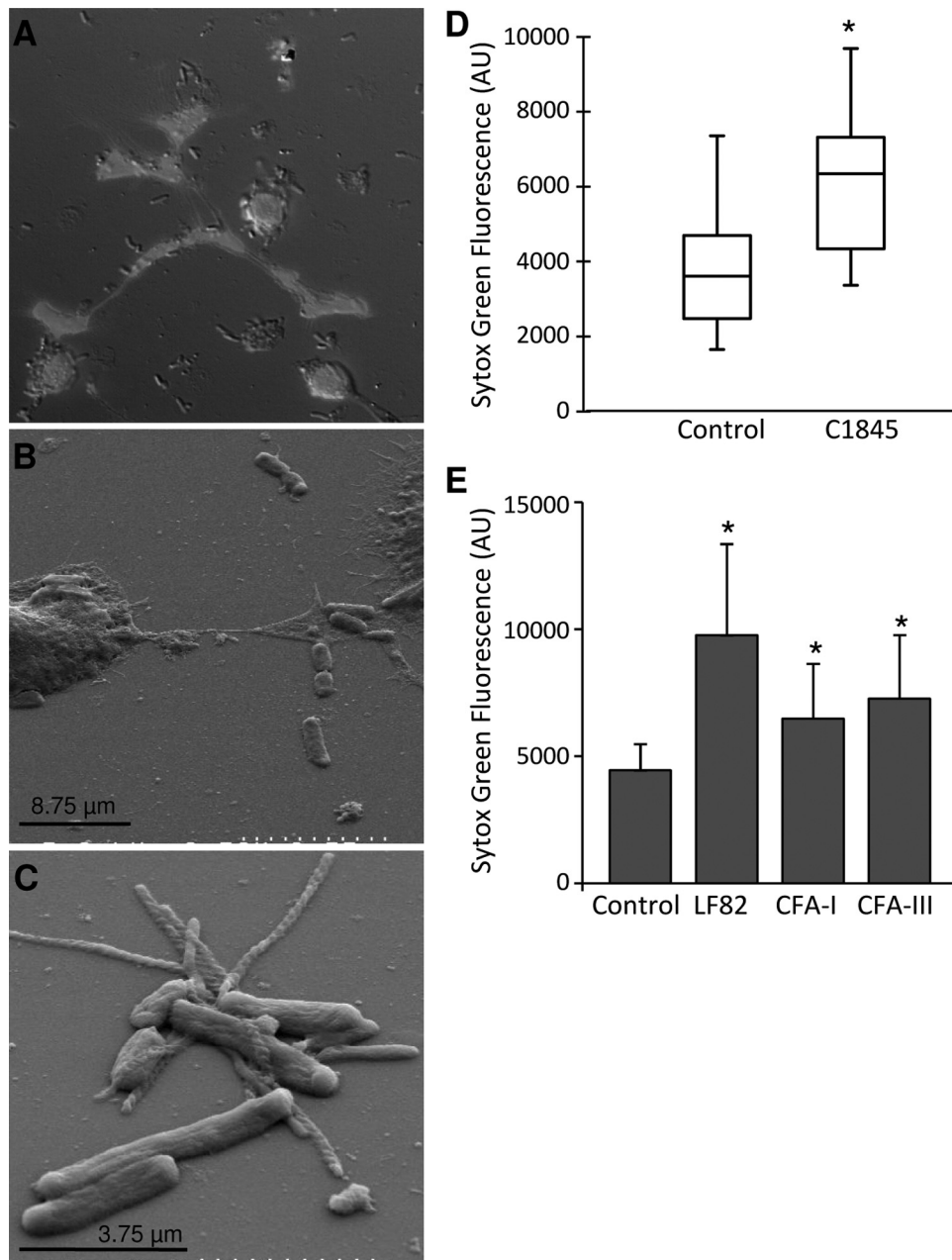


FIG 3 NET induction by enterovirulent Afa/Dr DAEC. PLB-985 cells were seeded on poly-L-lysine-coated glass slides and incubated for 4 h with WT C1845 at an MOI of 10 in 0.5% FCS-supplemented HBSS medium at 37°C. (A) Hoechst-stained DNA and phase-contrast micrograph of C1845 bacteria; (B and C) FESEM images showing C1845 bacterial cells associated with NETs; (D and E) for quantitative experiments, PLB-985 cells were incubated for 4 h with or without WT C1845, LF-82, CFA-I, or CFA-III (MOI, 60 bacteria per cell). Extracellular DNA was quantified with Sytox green as described in the text. Data from six different experiments are represented. In panel D, the boxes extend from the first to the third quartiles, the median is indicated by a horizontal line, and maximal and minimal values are represented at the end of the whiskers. In panel E, the data are means \pm SEMs. *, $P < 0.05$ compared with noninfected control cells.

gentamicin-killed WT C1845. In the absence of PLB-985 cells, Caco-2/TC7 cells exhibited a typical F-actin network (Fig. 5A), which was not affected by coinubation with untreated PLB-985 cells (Fig. 5B) or with gentamicin-killed WT C1845 (Fig. 5C). In contrast, Caco-2/TC7 cells incubated with PLB-985 cells pretreated with gentamicin-killed WT C1845 exhibited a markedly disorganized F-actin cytoskeleton (Fig. 5D); the presence of PLB-985 cells at the surface of damaged Caco-2/TC7 cells was confirmed by anti-CD11b staining (Fig. 5E).

We then investigated the mechanism by which WT C845-treated PLB-985 cells damaged Caco-2/TC7 cells. As NETs are known to carry granule-derived proteins at their surface, including proteases, we postulated that these proteins might be responsible, at least in part, for the observed Caco-2/TC7 cell injury. Preincubation of PLB-985 cells with pentoxifylline, a degranulation inhibitor, prior to contact with gentamicin-killed WT C1845 abrogated the deleterious effect of WT C1845-treated PLB-985 cells on the Caco-2/TC7 cell cytoskeleton (Fig. 5F), suggesting that

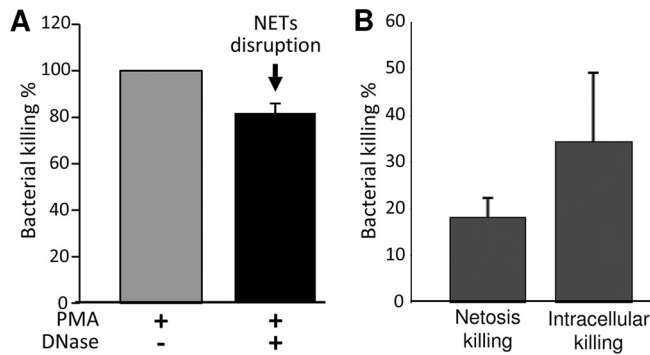


FIG 4 Extracellular bacterial killing is associated with NET formation independently of phagocytosis-mediated killing. PLB-985 cells were seeded in 24-well tissue culture plates and prestimulated for 4 h with 20 nM PMA. Before bacterial challenge, PMA-stimulated cells were treated with DNase (to dismantle NETs), whereas unstimulated cells were treated with cytochalasin D (phagocytosis inhibitor). After coinoculation of PLB-985 cells and C1845 bacteria (MOI, 0.1) for 45 min, the number of surviving bacteria was evaluated as the number of CFU recovered after 24 h of culture. (A) Percent bacterial killing by netosis was calculated from the CFU values obtained with PMA-stimulated cells subsequently treated with DNase. (B) Percent bacterial killing by phagocytosis was calculated from the CFU values obtained with unstimulated cells treated with cytochalasin D and was compared to those obtained after netosis killing. The graph indicates mean bacterial killing \pm SEM in five separate experiments.

the observed epithelial cell damage was partly due to protease. We thus searched for NETs in our coculture model. We observed β 2-integrin-positive PLB-985-derived NETs (Hoechst positive) containing trapped bacteria in the very close vicinity of Caco-2/TC7 cells (Fig. 5G). To confirm this observation, extracellular DNA was quantified by using Sytox green. As shown in Fig. 5H, the amount of extracellular DNA associated with WT C1845-treated PLB-985 cells cocultured with Caco-2/TC7 cells was higher than that observed with control Caco-2/TC7 cells or Caco-2/TC7 cells treated with gentamicin-killed WT C1845 alone. Collectively, these results suggest that PLB-985-derived NETs might directly contribute to epithelial cell damage via NET-bound proteases.

DISCUSSION

As we have previously shown that enterovirulent Afa/Dr DAEC can modulate numerous PMN functions (47, 48), here we examined NET production by PMNs after Afa/Dr DAEC WT C1845 challenge. We first established a model of netosis by using the PLB-985 cell line differentiated into fully mature neutrophils. The use of *gp91-phox*-deficient PLB-985 cells confirmed the major role of the oxidative burst in NET formation in our model. We then showed that WT C1845 bacteria could induce NET formation by activated differentiated PLB-985 cells and that the same bacteria could be trapped and killed by the NETs that they induced. Finally, coculture experiments pointed to a deleterious effect of NET-bound granule-derived proteins on cultured human enterocyte-like Caco-2/TC7 cells.

The neutrophil response to enterovirulent *E. coli in vitro* may partly mimic what happens in the intestinal lumen after bacterium-induced transepithelial neutrophil migration. We chose to use differentiated PLB-985 cells, as we have already shown that they are suitable for studying the effect of Afa/Dr DAEC WT strain C1845 on neutrophil adherence, oxidative burst, degranulation,

cytokine production, and apoptosis (47, 48). Other recent studies used promyelocytic leukemia HL-60 cells to study NET formation (39, 56). This is the first reported use of human neutrophil-like differentiated PLB-985 cells to study NET production, including after bacterial infection. We found that PLB-985 cells formed NETs upon activation by various stimuli, including PMA and H_2O_2 , in a time- and concentration-dependent manner, and we obtained very similar results with blood neutrophils in control experiments. NET formation was confirmed by quantifying extracellular DNA. The structure and composition of PLB-985-derived NETs were analyzed by immunofluorescence and FESEM. As in previous reports, NETs consisted of smooth fibers and globular domains of DNA, histone H3 (data not shown), and various embedded proteins. In particular, MPO and β 2-integrin, proteins that are well-known markers of neutrophil-derived NETs (26, 52), were present at the surface of PLB-985-derived NETs. We also examined the role of the oxidative burst and ROS production in our model, as several *in vitro* and *in vivo* models of oxidative burst deficiency are associated with impaired NET formation (26, 39, 60). In particular, Bianchi et al. showed that *gp91-phox* gene therapy restored the ability of blood neutrophils to produce NETs in a patient with chronic granulomatous disease (CGD) (6). We thus used the X-CGD PLB-985 cell line, which lacks functional NOX_2 (21, 48). No extracellular DNA was detected after PMA stimulation of these cells, while exogenous H_2O_2 restored slight NET formation. These findings confirm that NOX_2 and ROS are important for NET induction in the PLB-985 cell line. Our findings are in keeping with those of Munafo et al., who found that microorganism-derived DNase I could inhibit the neutrophil oxidative burst, leading to deficient killing (39). Together, these experiments show that differentiated PLB-985 cells are a suitable model for studying netosis.

Neutrophils actively contribute to pathogen eradication, notably, through netosis, a process that has been extensively reviewed with respect to its fundamental (10) and clinical (38, 51) implications. The bacterium-induced release of neutrophil DNA during netosis is different from that observed during apoptosis and necrosis. In particular, netosis occurs more quickly than apoptosis, does not require caspases, and is not accompanied by DNA fragmentation (26, 38). *Mycobacterium tuberculosis* can induce both neutrophil netosis and apoptosis (44), and our previous findings suggest that Afa/Dr DAEC strain WT C1845 may trigger various neutrophil death programs with different kinetics (48). Various bacteria have been shown to stimulate NET formation, as shown here with WT C1845 and PLB-985 cells, and also with three other enteropathogenic *E. coli* strains (LF-82, CFA-I, and CFA-III). These results corroborate reported descriptions of NET induction by *E. coli* (29, 34). *Shigella flexneri*, an enteric pathogen that, like WT C1845, induces intestinal inflammation, can also induce NET formation (23). Recently, an amplification loop involving PMN proinflammatory cytokine release and NET formation triggered by biofilm extracellular DNA was described in a model of *Pseudomonas aeruginosa* infection (27). These results support our previous finding that WT C1845 can induce autoamplified IL-8 production (47).

We found that NETs were able to attach to the bacteria that induced their formation. This interaction may simply rely on electrostatic interactions between NETs and negatively charged surfaces (10, 26, 53). Although a more specific interaction has not been ruled out, candidate molecular pairs include the β 2-integrin

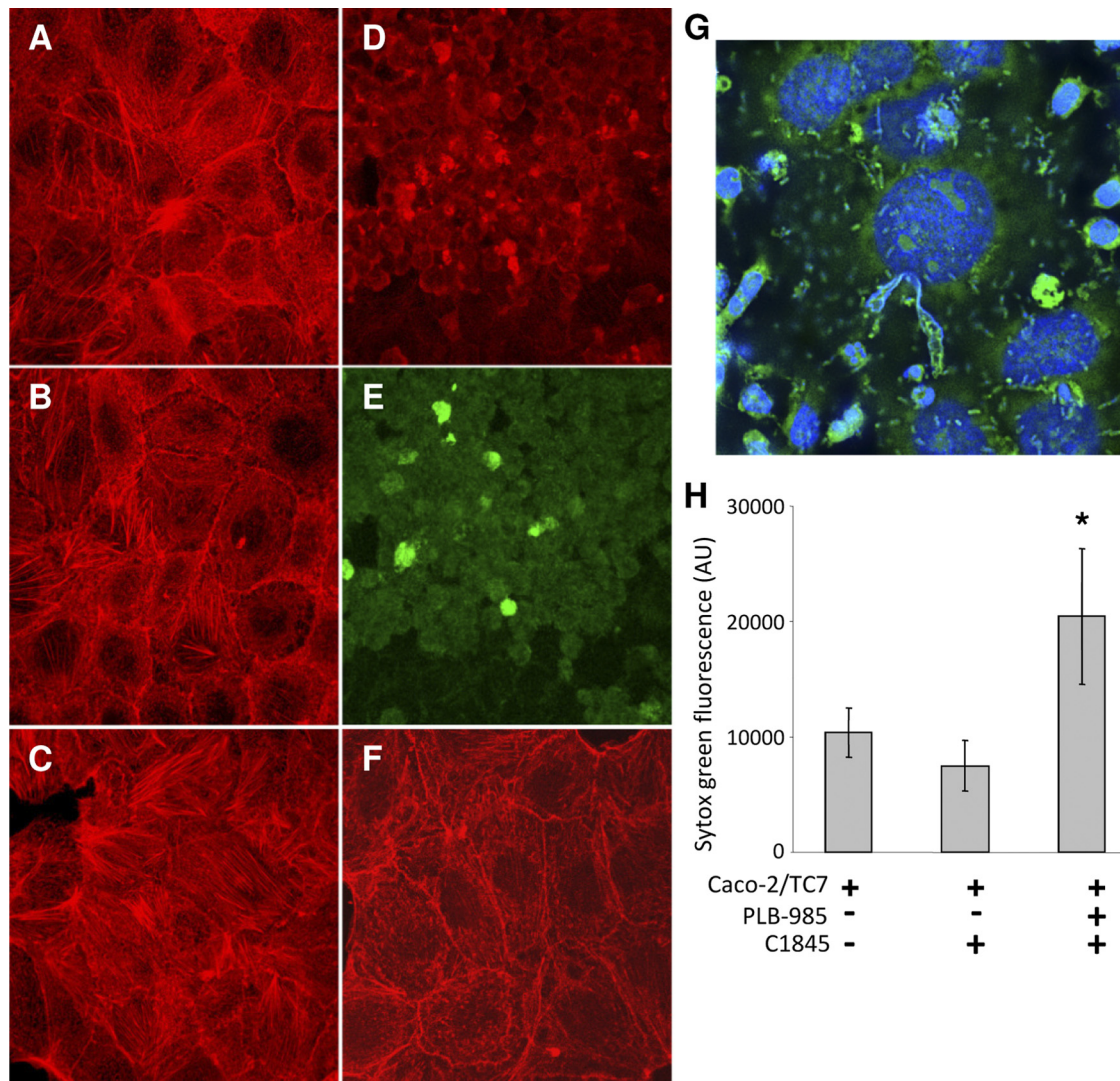


FIG 5 C1845-induced NETs affect F-actin cytoskeleton organization in Caco-2/TC7 enterocyte-like cells. PLB-985 cells were infected with gentamicin-killed C1845 prior to incubation with Caco-2/TC7 cell monolayers for 4 h. (A to G) After washing, the cells were labeled for F actin or β 2-integrin. Preparations were examined by epifluorescence microscopy with a confocal laser scanning microscope. Optical sectioning was used to collect 20 *en face* images 0.1 nm apart. Horizontal views were obtained by integrating images obtained with a step position of 1, using LSM510 (version 2.5) software (Zeiss, Germany). (A) F-actin staining of Caco-2/TC7 cells alone; (B) F-actin staining of Caco-2/TC7 cells cocultured with PLB-985 alone; (C) F-actin staining of Caco-2/TC7 cells cultured with killed C1845 cells; (D and E) Caco-2/TC7 cells cocultured with killed C1845-treated PLB-985 cells and stained for F-actin (red) and β 2-integrin (green), respectively; (F) F-actin staining of Caco-2/TC7 cells cocultured with PLB-985 cells treated with pentoxifylline prior to contact with killed WT C1845 cells; (G) evidence of bacteria trapped by extracellular DNA (Hoechst blue staining), which colocalized with β 2-integrin (green). The images are representative of three different experiments. Magnifications (A to G), $\times 63$. (H) The coculture model was adapted to the 96-well plate format, and extracellular DNA was quantified by fluorimetry using Sytox green; results are means \pm SEMs of at least three experiments. *, $P < 0.05$ compared with the other two conditions.

associated with NETs and the F1845 adhesin of WT C1845. A relatively specific interaction between antimicrobial peptides on NETs and *Staphylococcus epidermidis* δ -toxin was recently documented (17).

As expected, WT C1845 bacteria were killed intracellularly after phagocytosis by PLB-985 cells. Interestingly, DNase partially inhibited the killing of extracellular bacteria by PLB-985 cells, showing the contribution of netosis. Some bacteria, such as *Mycobacterium tuberculosis*, cannot be killed by NETs (44), but a variety of other pathogens are very sensitive to killing by high local concentrations of antimicrobial components such as proteases and histones from NETs (9, 26, 52). In addition to elastase, MPO,

and defensins, other mediators recently implicated in netosis include the LL-37 cathelicidin against *S. flexneri* (23) and *Staphylococcus aureus* (33) and calprotectin against *Candida albicans* (52). Nucleus-derived histones also play a role in pathogen killing by NETs, notably, during *Leishmania amazonensis* infection (31). It is thought that NET killing of *E. coli* might be partly driven by histone H2B, one of the most abundant proteins in NETs (34, 52). However, some bacteria have developed strategies to escape NET trapping and killing. In particular, *Streptococcus pyogenes*, *S. aureus*, and *Streptococcus pneumoniae* produce DNases (1, 53). Buchanan et al. demonstrated that Sda1 DNase is an important virulence factor that impairs netosis and thus renders group A

streptococci (GAS) resistant to neutrophil-mediated killing (11). In addition, GAS protein M1 and sialic acid residues promote survival within NETs by interfering with cathelicidin LL-37 and Siglec-5/9 lectins, respectively (14, 36). Interestingly, we found here that only 20% of WT C1845 bacteria were killed by netosis, whereas 50% of DH5 α pF1845 recombinants were killed by netosis under the same experimental conditions (data not shown), suggesting that Afa/Dr DAEC WT C1845 develops mechanisms of resistance against NET-dependent bactericidal activity.

Our results also show for the first time that WT strain C1845-induced NETs might also have a deleterious effect on host intestinal cells. Indeed, we found that WT C1845-induced NETs disrupted the F-actin cytoskeleton of culture human enterocyte-like Caco-2/TC7 cells and that a degranulation inhibitor prevented this effect. The mechanism of this deleterious effect requires further investigation but could be mediated by histones, proteases, or ROS production. Proteases expressed on NETs and, particularly, elastase, cathepsin G, or metalloproteases might also be involved and have been implicated in the pathophysiology of inflammatory bowel diseases. NET-related endothelial damage via extracellular histone toxicity during sepsis (16, 59), via proteinase 3 during small-vessel vasculitis (35), and in an *in vitro* coculture model of neutrophils and endothelial cells (32) has recently been documented. Very recently, proteases from extracellular traps have also been implicated in asthma (22), keratinocyte damage (50), and lupus nephritis (8). These recent clinical reports strengthen the relevance of our *in vitro* observations.

In conclusion, during the course of Afa/Dr DAEC intestinal infection, NETs could represent a double-edged sword, entrapping and killing bacteria but also damaging the epithelium. It remains to be determined whether other enterovirulent *E. coli* strains that promote proinflammatory responses (17) induce NET formation that might lead to structural and/or functional damage of enterocytes and mucus-secreting cells lining the intestinal epithelium. Our preliminary results with *E. coli* strains WT C1845, LF-82, CFA-I, and CFA-II support this hypothesis.

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