

# Extracellular DNA: A Major Proinflammatory Component of *Pseudomonas aeruginosa* Biofilms

Juan I. Fuxman Bass,<sup>\*,†</sup> Daniela M. Russo,<sup>‡</sup> Maria L. Gabelloni,<sup>\*,†</sup> Jorge R. Geffner,<sup>\*,§</sup> Mirta Giordano,<sup>\*,§</sup> Mariana Catalano,<sup>§</sup> Ángeles Zorreguieta,<sup>‡</sup> and Analía S. Trevani<sup>\*,§</sup>

We previously demonstrated that extracellular bacterial DNA activates neutrophils through a CpG- and TLR9-independent mechanism. Biofilms are microbial communities enclosed in a polymeric matrix that play a critical role in the pathogenesis of many infectious diseases. Because extracellular DNA is a key component of biofilms of different bacterial species, the aim of this study was to determine whether it plays a role in the ability of biofilms to induce human neutrophil activation. We found that degradation of matrix extracellular DNA with DNase I markedly reduced the capacity of *Pseudomonas aeruginosa* biofilms to induce the release of the neutrophil proinflammatory cytokines IL-8 and IL-1 $\beta$  (>75%); reduced the upregulation of neutrophil activation markers CD18, CD11b, and CD66b ( $p < 0.001$ ); reduced the number of bacteria phagocytosed per neutrophil contacting the biofilm; and reduced the production of neutrophil extracellular traps. Consistent with these findings, we found that biofilms formed by the *lasI rhlI P. aeruginosa* mutant strain, exhibiting a very low content of matrix extracellular DNA, displayed a lower capacity to stimulate the release of proinflammatory cytokines by neutrophils, which was not decreased further by DNase I treatment. Together, our findings support that matrix extracellular DNA is a major proinflammatory component of *P. aeruginosa* biofilms. *The Journal of Immunology*, 2010, 184: 6386–6395.

Neutrophils are essential effectors in the first line of defense against invading microbial pathogens. They mediate the phagocytosis and destruction of bacteria through oxygen-dependent and -independent mechanisms. They also contribute to launch immune responses, and they can help to heal tissues as well as to destroy them (1, 2).

A great number of studies demonstrated that bacterial DNA mediates immune cell activation because of the presence of unmethylated-CpG motifs that trigger TLR9 activation (3–5). Other studies revealed the existence of cytosolic DNA sensors, such as DAI, AIM2, and the RNA polymerase III (6–9). By contrast, our previous work indicated that neutrophils are activated by bacterial DNA through a CpG- and TLR9-independent mechanism that does not require DNA internalization and that can be triggered by immobilized DNA (10, 11). These findings suggested that a surface molecule is involved in DNA recognition and neutrophil activation. More recently, we reported that neutrophil activation by bacterial DNA can only succeed with molecules of more than  $\approx 170$  nt and

can also be achieved by dsDNA (12). This mechanism entails a marked contrast to the canonical CpG-activation mechanism, which is activated in other cellular types by short single-stranded oligonucleotides. Thus, it is possible that this neutrophil surface molecule senses extracellular DNA from bacterial microenvironments and, in turn, triggers neutrophil activation.

Previous studies demonstrated that extracellular bacterial DNA plays an important role in the formation and composition of biofilms (13), which are structured microbial communities enclosed in a hydrated polymeric matrix adherent to an inert or living surface (14, 15). Biofilm represent a protective mode of growth that allows bacteria to survive in hostile environments and also disperse to colonize new niches (15). Biofilms are not simply passive assemblages of cells attached to surfaces; they are structurally and dynamically complex biological systems. They are crucial in the pathogenesis of many subacute and chronic bacterial infections, such as *Pseudomonas* infections in cystic fibrosis (CF) patient lungs, infectious kidney stones, bacterial endocarditis, as well as in medical devices- and foreign body-related infections (16–18). Recently, biofilms were also shown to be involved in chronic wound infections (19). The importance of these communities is best illustrated by observations suggesting that >60% of bacterial infections seem to involve them (20). Biofilms are difficult to eradicate because they are tolerant to biocides, antibiotics, and to the host immune responses. Particularly, biofilm-associated bacteria exhibit a type of antibiotic tolerance that is distinct from conventional antimicrobial resistance (21–26). In the last years it has become evident that, in addition to exopolysaccharides and proteins, DNA is a relevant component of the extracellular matrix of bacterial biofilms. In fact, extracellular DNA has been detected with *Pseudomonas aeruginosa* PAO-1 reference strain, a model organism in biofilm research, as well as in clinical *P. aeruginosa* isolates and in *Streptococcus* sp., *Staphylococcus* sp., and *Enterococcus* sp. biofilms (13, 27–34). Importantly, a significant amount of dsDNA has been found in biofilms formed in vivo by nontypeable *Haemophilus influenzae* (35).

\*Departamento de Inmunología, Instituto de Investigaciones Hematológicas and Instituto de Estudios Oncológicos “Fundación Maissa,” Academia Nacional de Medicina; <sup>†</sup>Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales and <sup>‡</sup>Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires; and <sup>§</sup>Fundación Instituto Leloir, Instituto de Investigaciones Bioquímicas-Buenos Aires CONICET, Buenos Aires, Argentina

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Address correspondence and reprint requests to Dr. Analía Trevani, Instituto de Investigaciones Hematológicas-Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina. E-mail addresses: analiatrevani@yahoo.com.ar and atrevani@fmed.uba.ar

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Abbreviations used in this paper: AFU, arbitrary fluorescence unit; CF, cystic fibrosis; CFP, cyan fluorescent protein; CLSM, confocal laser scanning microscopy; DHR-123, dihydrorhodamine 123; LB, Luria-Bertani; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PI, propidium iodide; rhDNase, recombinant human DNase I.

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Considering the crucial role that biofilms play in the pathogenesis of many bacterial infectious diseases, previous studies characterized neutrophil–biofilm interaction, and most have focused on the action exerted by quorum-sensing signals and matrix exopolysaccharides on neutrophil functions (36–40). Other studies demonstrated that the presence of neutrophils enhances initial *P. aeruginosa* biofilm development through the formation of polymers composed of actin and cellular DNA (41). However, no previous studies have analyzed whether extracellular bacterial DNA, beyond its role in the formation and composition of biofilms, might also play a role in the modulation of the host acute-immune response mediated by neutrophils. In this article, we provide evidence that matrix extracellular DNA is a critical component of the *P. aeruginosa* biofilms in triggering neutrophil activation.

## Materials and Methods

### Ethics statement

The experiments were reviewed and approved by the Academia Nacional de Medicina Review Board and Ethical Committee. All blood donors provided written informed consent for the collection of samples and subsequent analysis.

### Human neutrophil isolation

Blood samples were obtained from healthy donors by venipuncture of the forearm vein. Neutrophils were isolated from heparinized human blood by Ficoll-Hypaque gradient centrifugation (Ficoll, GE Healthcare, Munich, Germany; Hypaque, Winthrop Products, Buenos Aires, Argentina) and dextran (GE Healthcare) sedimentation, as previously described (11). Contaminating erythrocytes were removed by hypotonic lysis. After washing with saline, cells (>96% neutrophils on May–Grunwald–Giemsa-stained cytopreps) were suspended in complete medium: RPMI 1640 without phenol red (HyClone Laboratories, Logan, UT), supplemented with 5 mg/ml endotoxin-free human serum albumin (Laboratorio de Hemoderivados, Córdoba, Argentina). All neutrophil preparations were FACS analyzed after purification to guarantee that their forward scatter/side scatter parameters were compatible with those of nonactivated cells. To minimize neutrophil spontaneous activation, cells were used immediately after isolation.

### Bacterial strains and growth conditions

*P. aeruginosa* was used as a model organism in this study. PAO-1 (wild-type) and PAO-JP2 (*lasI rhlI* double mutant) strains were kindly provided by Prof. Barbara Iglewski (Department of Microbiology and Immunology, University of Rochester, Rochester, NY), and GFP-tagged PAO-1 (wild-type) and cyan fluorescent protein (CFP)-tagged PAO-1 (wild-type) strains were provided by Prof. Tim Tolker-Nielsen (Centre for BioScience and Technology, Technical University of Denmark, Lyngby, Denmark). *P. aeruginosa* mucoid strains isolated from chronically infected CF patients were kindly provided by Dr. Daniela Centron (Universidad de Buenos Aires; strain 1208) and Dr. Marisa Almuzara (Hospital de Clínicas “José de San Martín”, Buenos Aires; strain F1432). Bacteria were routinely grown at 37°C in Luria-Bertani (LB) broth or on LB agar. For biofilm development, bacteria were grown in LB medium at 37°C for 18 h (OD at 600 nm ~1), and the culture was used as an inoculum at a 1:1000 dilution in AB minimal medium supplemented with 10 mM glucose (42). Then, bacteria were seeded on polystyrene 96-well flat-bottom tissue culture plates (Greiner Bio-one, Frickenhausen, Germany) or on Lab-Tek chambered coverglass slides containing a borosilicate glass base of 170 µm thickness (Nunc, Roskilde, Denmark) for confocal laser scanning microscopy (CLSM) studies and cultured for 48 h at 37°C. Medium was refreshed after 24 h of culture.

### Neutrophil binding of *P. aeruginosa* DNA

PAO-1 DNA obtained by cetyltrimethylammonium bromide DNA extraction was biotinylated with Photoprobe (long-arm) biotin (Vector Laboratories, Burlingame, CA) photocoupling, according to the manufacturer's instructions. Neutrophil DNA binding was evaluated as previously described (12).

### CLSM

A confocal laser-scanning microscope [LSM510-Axiovert 100M (Carl Zeiss, Göttingen, Germany) equipped with a Plan-Neofluar 100×/1.3 immersion oil objective, a C-Apochromat 63×/1.2 W corr objective, and

a C-Apochromat 40×/1.2 W corr objective lens] was used to examine the biofilm development on chambered coverglass slides and to visualize the interaction between neutrophils and bacterial biofilms. Some images were acquired using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with a Plapon 60×/1.42 objective. Images were acquired by sequentially scanning with optimal settings for each fluorophore used. The images were acquired by scanning different optical sections, and three-dimensional images were reconstructed using the Zeiss LSM Image Browser version 4.0.0.157 or the Olympus FV10-ASW software. The quantitative analysis of the optical sections of the stacks was performed with ImageJ (National Institutes of Health, Bethesda, MD).

### DNase I treatment of biofilms

To remove unbound bacteria, 2-d-old biofilms were gently washed with NaCl 0.9% and then with RPMI 1640 medium. Biofilms in RPMI 1640 were incubated in the presence of 90 Kunitz units/ml DNase I (Invitrogen, Carlsbad, CA), heat-denatured DNase, or medium (control) for 60 min at 37°C. Finally, unless otherwise stated, they were washed twice with RPMI 1640 and cocultured with neutrophils, as indicated in each experiment, or subjected to evaluation of biomass and structure integrity as follows.

Biofilm biomass after DNase I treatment was quantified by staining with crystal violet (0.1% w/v), as previously described (43), or by protein content. Extraction and quantification of DNA in biofilms was performed as previously described (44). Biofilm structure and extracellular DNA content after DNase I treatment were monitored on CFP-tagged PAO-1 biofilms cultured in chambered coverglass slides. The exopolysaccharide was stained with Con A-FITC (5 µg/ml) and DNA with propidium iodide (PI, 3 µM; Sigma-Aldrich, St. Louis, MO).

### Measurement of neutrophil cytokine production

Neutrophils (100 µl,  $2 \times 10^6$ /ml) were seeded as a drop in the center of control or DNase I-treated PAO-1 or JP-2 biofilm-containing wells and cocultured for 3 h at 37°C. Finally, culture supernatants were collected, and IL-8 (R&D Systems, Minneapolis, MN) and IL-1β (eBioscience, San Diego, CA) concentrations were measured by ELISA.

In some experiments, planktonic PAO-1 cells from midexponential-phase cultures, grown in AB minimal medium with 10 mM glucose with continuous shaking, were incubated in the presence of 90 Kunitz units/ml DNase I or medium (control) for 60 min at 37°C. Then, bacteria were washed twice and cocultured with neutrophils (100 µl,  $2 \times 10^6$ /ml) suspended in complete medium for 3 h at 37°C. Finally, the cultures were centrifuged, the supernatants were collected, and IL-8 and IL-1β concentrations were measured by ELISA.

In the experiments shown in Fig. 1, neutrophils suspended in complete medium ( $5 \times 10^6$  per ml) were seeded into 96-well flat-bottom plates, stimulated with 50 µg/ml soluble or immobilized PAO-1 genomic DNA (45), and cultured for 3 h at 37°C. Culture supernatants were collected, and IL-8 concentration was measured by ELISA.

### Neutrophil surface marker expression

Neutrophils (300 µl,  $2 \times 10^6$ /ml) were seeded as a drop in the center of control or DNase I-treated GFP- or CFP-tagged PAO-1 biofilms grown in chambered coverglass slides for 40 min at 37°C. Then, the supernatant was carefully removed, and neutrophils settled on the biofilms were stained with FITC-conjugated anti-human CD18 (BD Pharmingen, San Diego, CA), PE-conjugated anti-human CD11b (Immunotech, Marseille, France), or FITC-conjugated anti-human CD66b Abs (BD Pharmingen) for 20 min at 4°C. After a gentle washing step, where indicated, PI (3 µM) was added immediately before CLSM image acquisition to visualize extracellular DNA.

### Myeloperoxidase immobilization to biofilm

Neutrophils (300 µl,  $2 \times 10^6$ /ml) were seeded as a drop in the center of control or DNase I-treated PAO-1 CFP biofilms grown in chambered coverglass slides and incubated for 60 min at 28°C. Then, after carefully removing the supernatants, biofilms were stained with FITC-conjugated anti-human myeloperoxidase (MPO) Ab for 20 min at 4°C (Immunotech). After a washing step, PI (3 µM) was added immediately before CLSM image acquisition to visualize extracellular DNA.

### Phagocytosis

Neutrophils (300 µl,  $2 \times 10^6$ /ml) were seeded as a drop in the center of control or DNase I-treated PAO-1 GFP biofilms cultured in chambered coverglass slides and incubated for 40 min at 37°C. Neutrophil membranes were stained with PE-conjugated anti-CD11b Ab for 20 min at 4°C. Then,

cells were fixed, and phagocytosis was evaluated in the intact cocultures in an inverted confocal scanning microscope by counting the number of bacteria inside each neutrophil, in at least five fields per treatment per neutrophil donor.

In a different set of experiments, FITC-conjugated polybead polystyrene microspheres (Polysciences, Warrington, PA) were incubated with neutrophils, mock-treated, or DNase-treated PAO-1 biofilm cocultures for 40 min at 37°C, to evaluate the internalization of an inert substratum. The visualization was performed as described for evaluation of bacterial phagocytosis.

#### Neutrophil oxidative burst monitoring

Neutrophils were incubated for 15 min in complete medium with 10  $\mu$ M dihydrorhodamine 123 (DHR-123; Invitrogen). After washing, 300  $\mu$ l ( $2 \times 10^6$ /ml) neutrophil suspension were added to control or DNase I-treated biofilms grown in chambered coverglass slides and cocultured for 30 min at 28°C. Finally, CLSM images were acquired, as mentioned above.

#### Evaluation of neutrophil extracellular traps development

Neutrophils (300  $\mu$ l,  $2 \times 10^6$ /ml) were seeded on top of control or DNase I-treated PAO-1 biofilms grown in chambered coverglass slides for 150 min at 37°C. Then, nucleosomes were visualized with a specific mouse anti-nucleosome mAb and an FITC-conjugated goat anti-mouse Ab (BD Pharmingen) and DNA, by adding PI (3  $\mu$ M) immediately before the acquisition of CLSM images. Residual DNase I activity in the coculture supernatants of neutrophil and enzyme-treated biofilms was monitored by incubating undiluted supernatants with 50  $\mu$ g/ml calf thymus DNA for 150 min at 37°C. Then, reaction products were analyzed in 1% agarose gels.

#### Statistical analysis

Statistical significance was determined using the nonparametric Friedman test for multiple comparisons, with the Dunn posttest or Wilcoxon matched-ranked test. Statistical significance was defined as  $p < 0.05$ .

## Results

### Treatment with DNase I markedly reduced the ability of bacterial biofilms to stimulate the release of IL-8 and IL-1 $\beta$ by human neutrophils

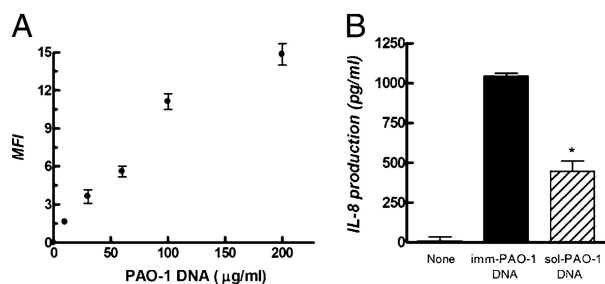
We first determined that, as we previously demonstrated for *Escherichia coli* DNA (12), purified double-stranded *P. aeruginosa* PAO-1 DNA used at concentrations compatible with those that a neutrophil might experience when it encounters a *P. aeruginosa* biofilm was able to bind and activate human neutrophils (Fig. 1, Supplemental Fig. 1). Immobilized PAO-1 DNA induced IL-8 synthesis by neutrophils >2-fold compared with that stimulated by soluble DNA (Fig. 1B). Then, to evaluate the contribution of

extracellular DNA of bacterial biofilm matrix to neutrophil activation, we used static biofilm assays with *P. aeruginosa* cultured for 48 h at 37°C. At day 2, the established biofilms were treated with DNase I (90 U/ml) or vehicle for 60 min at 37°C, washed twice, and cultured for three additional hours in RPMI 1640 medium, without phenol red. Under these conditions, treatment with DNase I did not significantly modify total biofilm biomass, as judged by crystal violet staining (Fig. 2A), or total protein content (Fig. 2B). To verify whether treatment with DNase I was able to digest the extracellular DNA present in the biofilm matrix that would be faced by neutrophils in coculture experiments, after DNase treatment, the matrix exopolysaccharides were stained with Con A-FITC (green; Fig. 2E, Fig. 2F) and extracellular DNA with PI (red; Fig. 2G, Fig. 2H), an intercalating agent that does not penetrate the membrane of live bacteria. CLSM images showed that the spatial distribution of extracellular DNA (red) in the CFP-*P. aeruginosa* PAO-1 biofilms (blue; Fig. 2C, Fig. 2D) was in accordance with that previously described, with DNA in the substratum and in the microcolonies, with the greatest concentrations on the surface of the microcolonies (42). The overlay of the images of the vertical projections acquired by CLSM from untreated and DNase I-treated biofilms (Fig. 2I–N) indicated that DNase I treatment eliminated the extracellular DNA contained in the superficial part of the biofilm structures, without affecting the biofilm architecture. Additional assays using LIVE/DEAD Bac-Light stain and CLSM indicated that 90 U/ml of DNase did not visibly affect bacterial viability (Supplemental Fig. 2).

To examine the influence of matrix DNA on the ability of neutrophils to produce and release proinflammatory cytokines, 2-d-old PAO-1 biofilms grown under static conditions were treated with DNase I, heat-inactivated DNase I, or vehicle for 60 min. Then, biofilms were washed, and neutrophils were allowed to settle on them for 3 h at 37°C. Finally, the supernatants were collected, and the IL-8 and IL-1 $\beta$  released from neutrophils was quantified by ELISA. The elimination of extracellular matrix DNA from biofilms reduced the amount of IL-8 and IL-1 $\beta$  released by neutrophils by >75% (Fig. 3A, 3B). This effect was not related to the elimination of a stimulating factor released from the biofilm by DNase I treatment, because similar results were obtained when we omitted the washing step after DNase I treatment (Fig. 3A, 3B). As expected, biofilm treatment with heat-inactivated DNase I did not affect cytokine release by neutrophils (Fig. 3A, 3B).

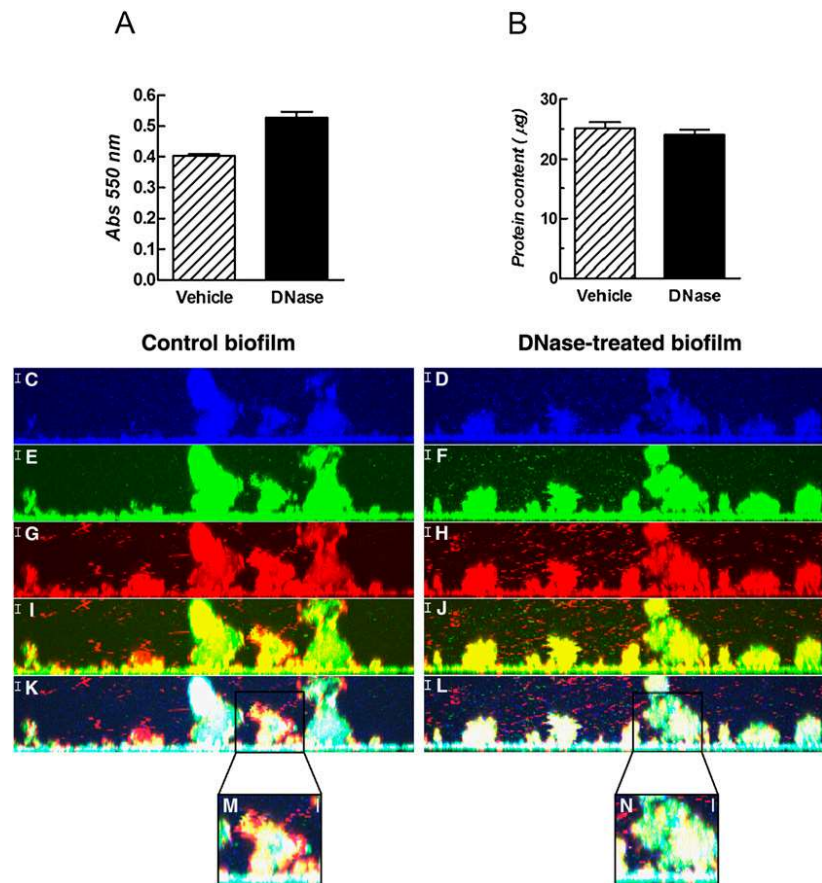
In contrast, when we treated with DNase I midlog-phase planktonic PAO-1 cultures, which contained a low basal level of extracellular DNA (42), no differences were observed in their ability to induce IL-8 or IL-1 $\beta$  release by the neutrophils coincubated with them (Fig. 3C, 3D), excluding the possibility that the reduced ability of DNase I-treated biofilms to trigger cytokines release was due to an effect of DNase I on bacteria themselves.

Allesen-Holm et al. (42) reported that *P. aeruginosa lasI rhII* mutant biofilms exhibited a significant reduction in matrix extracellular DNA compared with wild-type biofilms. In accordance with these investigators' findings, we found that 2-d-old wild-type biofilms contained  $8.7 \pm 2.8$   $\mu$ g DNA/mg total protein content, whereas *lasI rhII* mutant biofilms contained  $0.46 \pm 0.03$   $\mu$ g DNA/mg total protein content. Although we detected similar biomass levels in the wild-type and *lasI rhII* mutant biofilms (Supplemental Fig. 3), the latter induced reduced levels of IL-8 and IL-1 $\beta$  when cocultured with neutrophils compared with those elicited by the wild-type biofilm (Fig. 3E, 3F). Moreover, treatment with DNase I did not result in a diminished ability of the *lasI rhII* biofilms to stimulate the release of cytokines by neutrophils (Fig. 3E, 3F), further supporting a role for extracellular DNA in triggering neutrophil proinflammatory responses.



**FIGURE 1.** Capacity of PAO-1 DNA to bind and activate human neutrophils. *A*, Neutrophils ( $5 \times 10^6$ /ml) were incubated in the presence of 0, 10, 30, 60, 100, or 200  $\mu$ g/ml of biotinylated PAO-1 DNA for 30 min at 4°C. After washing, binding was revealed by the addition of avidin-FITC by flow cytometry. Background fluorescence levels ( $\sim$ MFI 4) determined in cells incubated with only avidin-FITC were subtracted from total binding before plotting the values. Data represent the mean  $\pm$  SEM of three experiments. *B*, Neutrophil activation by PAO-1 dsDNA. Neutrophils ( $5 \times 10^6$ /ml) were incubated in the presence of vehicle or soluble or immobilized double stranded PAO-1 DNA (50  $\mu$ g/ml) for 3 h at 37°C. Then, IL-8 release was evaluated in supernatants by ELISA. Data represent the mean  $\pm$  SEM of four experiments. \* $p < 0.05$  versus cells stimulated with immobilized PAO-1 DNA.

**FIGURE 2.** Treatment of the biofilm with DNase I did not affect total biomass or biofilm architecture, but it digested extracellular matrix DNA exposed to neutrophils. Two-day-old biofilms were treated or not with DNase I (90 U/ml) for 60 min at 37°C, washed twice, and cultured for three additional hours in RPMI 1640 medium, without phenol red. Total biomass was quantified by staining with crystal violet (A) and by protein content (B). Data represent the mean  $\pm$  SEM of two experiments performed in triplicate (A) and an experiment performed in quadruplicate (B). C–N, Two-day-old CFP-tagged PAO-1 biofilms (blue) were incubated with medium (left panels) or DNase I (90 U/ml; right panels) for 60 min at 37°C. Then, the exopolysaccharide was stained with Con A-FITC (5  $\mu$ g/ml; green) and DNA with PI (3  $\mu$ M; red), and the biofilm architectures were constructed by vertically projected images obtained with a C-Apochromat 40 $\times$ /1.2 W corr objective lens by CLSM. C and D, CFP PAO-1 emission fluorescence (blue). E and F, Exopolysaccharide distribution (Con A-FITC; green). G and H, Dead bacteria and extracellular matrix DNA location (PI; red). I and J, Overlays of the images of E and G and F and H, respectively. K and L, Overlays of the images of C, E, and G and D, F, and H, respectively. M and N, Magnification of images shown in K and L, respectively. C–N, Original magnification  $\times$ 400. Vertical bars, 5  $\mu$ m.



*Treatment with DNase I markedly reduced the ability of bacterial biofilm to stimulate the upregulation of neutrophil activation markers*

We then evaluated the impact of matrix extracellular DNA digestion on the ability of neutrophils to increase the expression of the Mac-1 integrin components: the CD11b and CD18 subunits. This integrin is expressed in the membrane of secretory vesicles and those of gelatinase and specific granules, and it becomes part of the plasma membrane upon neutrophil degranulation. Neutrophils were allowed to settle on untreated or DNase I-treated biofilms, and the expression of CD18 was evaluated by CLSM. As shown in Fig. 4A–C, the expression of CD18 was markedly higher in neutrophils settled on untreated biofilms compared with that in neutrophils settled on DNase I-treated biofilms. Moreover, when neutrophils were cocultured with GFP-tagged PAO-1 biofilms pretreated or not with DNase I, the expression of CD11b was significantly higher in neutrophils exposed to biofilms not treated with DNase I (Fig. 4D–F). Similar findings were obtained when the expression of CD66b, a specific granule marker, was analyzed (Fig. 4G–I). Together, these results support that degradation of extracellular DNA compromises the capacity of bacterial biofilms to trigger neutrophil activation.

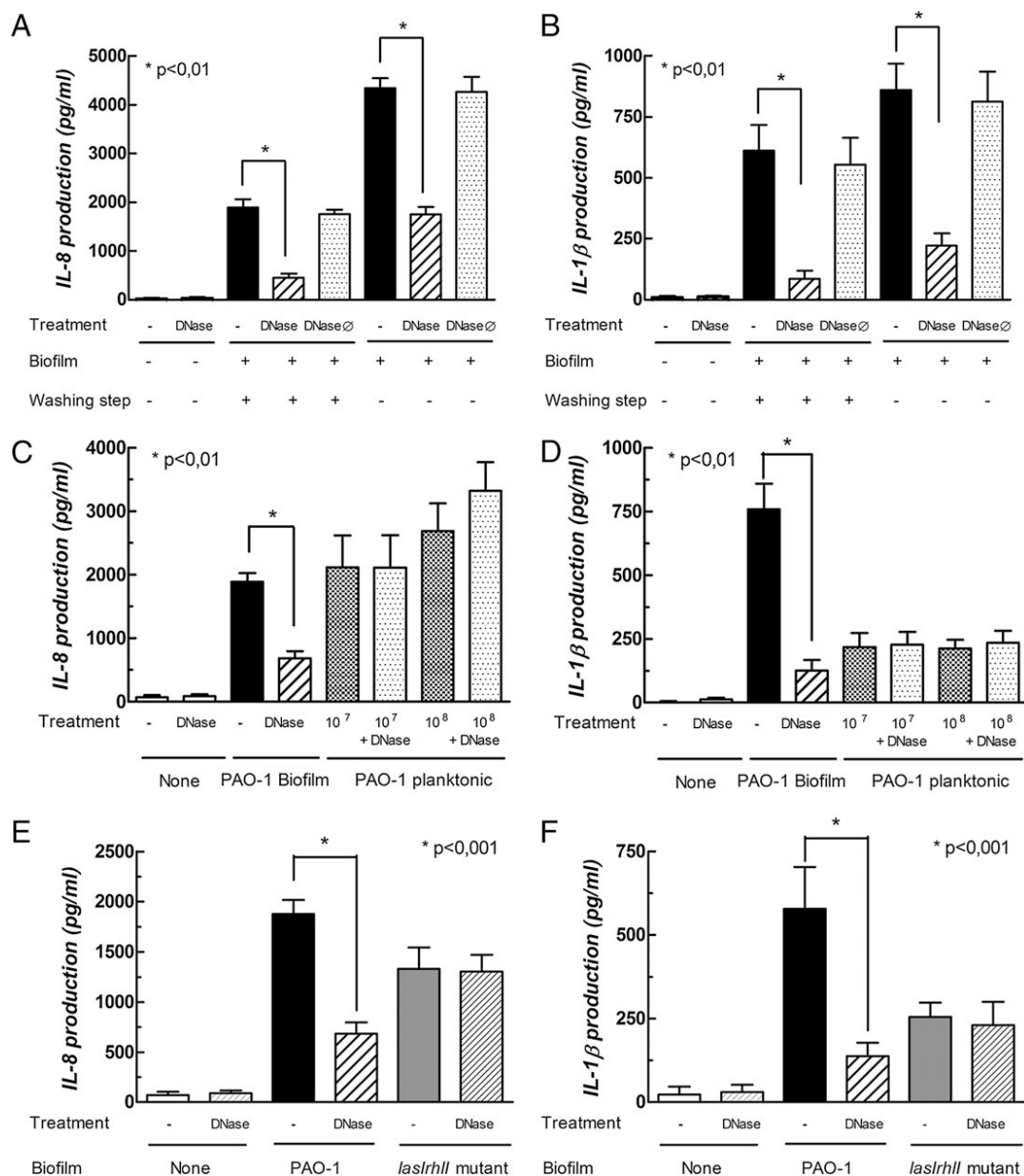
*Treatment with DNase I markedly reduced the ability of biofilms to immobilize MPO*

During neutrophil stimulation, the granules fuse with the nascent phagosome and release their contents inside it, thereby exposing the ingested microbes to an array of toxic agents with seemingly redundant activities. However, during this process, the granule content can also be released to the extracellular milieu, as was shown when neutrophils were cocultured with *P. aeruginosa* biofilms (36). Our above-mentioned results regarding Mac-1 and CD66b expression

indicated that degradation of the biofilm matrix DNA reduced the release of specific and, probably also, gelatinase granules, stimulated by contact of the neutrophils with the biofilm. To determine the effect of the digestion of the biofilm matrix DNA on azurophilic granule release, we evaluated the activity of MPO in culture supernatants. Unexpectedly, we found a greater MPO activity in the supernatants from DNase I-treated biofilms compared with untreated biofilms (data not shown). This finding seemed to contrast with the results described above, showing that DNase I-treated biofilms displayed a lower ability to trigger neutrophil activation. Considering the highly cationic nature of MPO, we speculated that this result might merely reflect that MPO released by neutrophils became attached to the matrix DNA on the surface of untreated biofilms. After revealing the MPO bound to the biofilm surface by CLSM, we found that untreated biofilms (Fig. 5A; control) exhibited a great amount of MPO that colocalized with the DNA of the extracellular matrix, whereas DNase I-treated biofilms were nearly devoid of DNA and MPO (Fig. 5A; DNase).

*Treatment with DNase I markedly reduced the ability of bacterial biofilm to stimulate neutrophil phagocytosis but not the respiratory burst*

Phagocytosis is a crucial host-defensive process that characterizes neutrophil function. Jesaitis et al. (36) showed by transmission electron microscopy of transverse sections of biofilms exposed to neutrophils that these cells phagocytose bacteria on their biofilm-adjacent surfaces. Thus, we evaluated whether digestion of extracellular DNA from the biofilm matrix affected this response. By CLSM, we observed that neutrophils in contact with DNase I-treated biofilms displayed a 35% reduction in the mean number of bacteria inside each cell, relative to neutrophils exposed to



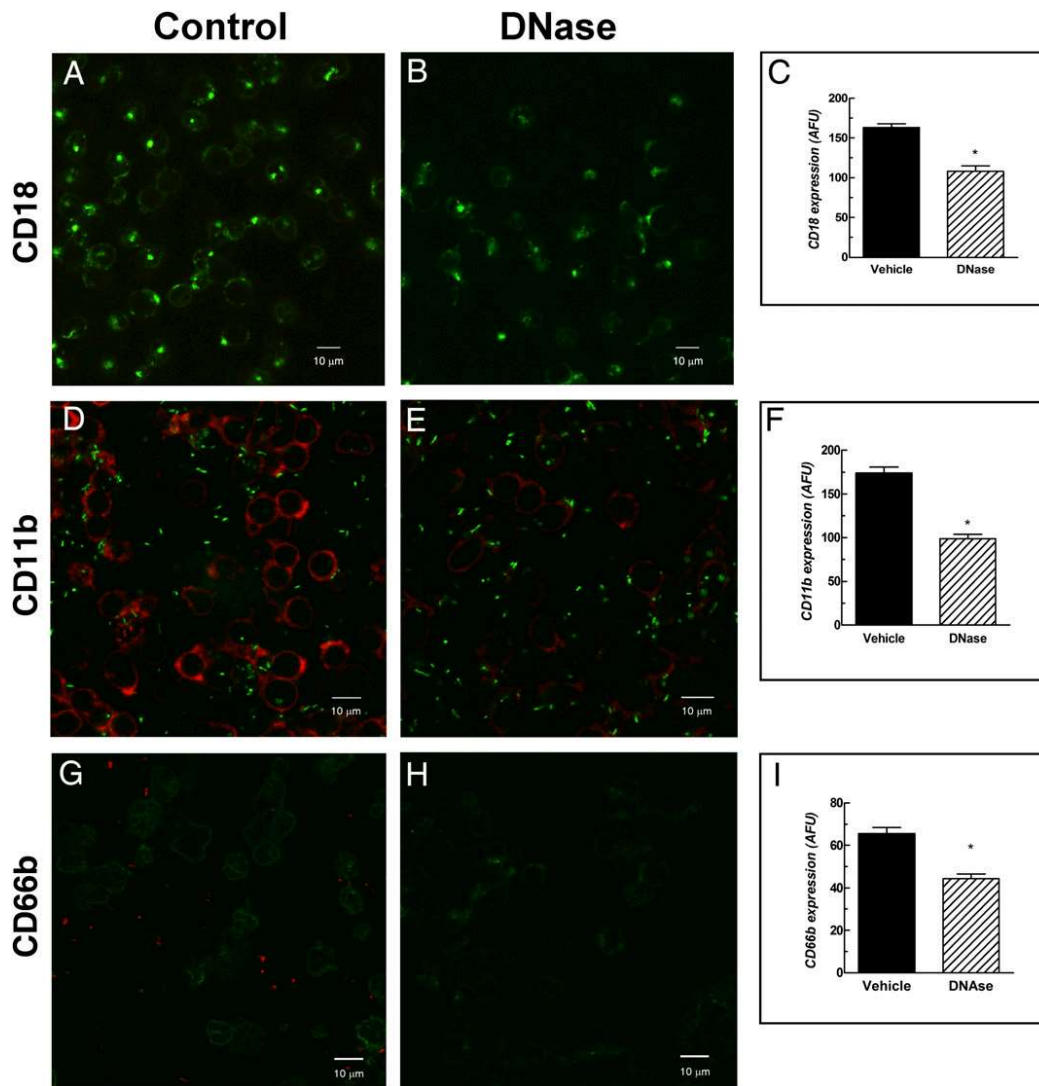
**FIGURE 3.** Treatment of wild-type biofilms with DNase I markedly reduced neutrophil cytokine release. Two-day-old PAO-1 (A, B) and PAO-JP2 (*las/rhlI* double mutant; E, F) biofilms and midexponential-phase cultures of planktonic PAO-1 cells (C, D) were treated with vehicle, DNase I (90 U/ml), or heat-inactivated DNase I (DNase Ø; 90 U/ml) for 60 min at 37°C and washed twice or left unwashed. Neutrophils ( $2 \times 10^6$ /ml) were seeded on the biofilms, on the planktonic bacteria-containing wells, or on empty wells (negative controls) and cocultured for 3 h at 37°C. Finally, culture supernatants were collected, and IL-8 (A, C, E) and IL-1 $\beta$  (B, D, F) concentrations were quantified by ELISA. Data represent the mean  $\pm$  SEM of six to nine experiments performed in triplicate.

untreated biofilms (Fig. 5B). Interestingly, recent studies performed by William Nauseef's group showed bleaching of phagocytosed *Staphylococcus aureus*-expressing cytosolic GFP, mediated by HOCl produced as a consequence of neutrophil NADPH oxidase activation (46). Thus, we performed additional experiments to evaluate bacterial phagocytosis by neutrophils pretreated with diphenylene iodonium, a potent inhibitor of flavoproteins, including the phagocyte NADPH oxidase (47), to avoid fluorescence bleaching due to NADPH oxidase activation. Results indicated that the mean number of bacteria taken up per neutrophil was greater when neutrophils were pretreated with diphenylene iodonium, indicating that some fluorescence bleaching was taking place inside the phagosomes. But even more relevant, under these conditions, the greater phagocytic capacity of neutrophils cocultured with mock-treated biofilms was more clearly

evidenced (bacteria/PMN:  $2.7 \pm 0.4$  versus  $1.52 \pm 0.3$ ; mock-versus DNase-treated biofilm).

We performed additional experiments by incorporating FITC-conjugated polystyrene microspheres into neutrophil-biofilm cocultures and evaluated their internalization by confocal microscopy. Neutrophils in contact with mock-treated biofilms also internalized a greater number of fluorescent microspheres than did those cocultured with DNase-treated biofilms (Fig. 5C), ruling out the possibility that the smaller number of bacteria observed inside neutrophils in contact with DNase I-treated biofilms was due to differential intracellular killing.

When we monitored the oxidative burst of neutrophils exposed to biofilms by using the H<sub>2</sub>O<sub>2</sub>-sensitive compound DHR-123, we did not find differences between the oxidative burst mediated by neutrophils exposed to DNase I-treated or untreated biofilms (Fig. 5D).



**FIGURE 4.** Treatment of the biofilms with DNase significantly reduced neutrophil upregulation of activation markers. Two-day-old PAO-1 biofilms (A, B, G, H) or GFP-tagged PAO-1 biofilms (D, E; green) were treated or not with DNase I (90 U/ml; 60 min at 37°C). Then, they were washed twice and cocultured with neutrophils for 40 min at 37°C. The expression of CD18 was evaluated by staining with FITC-conjugated specific mAb (A, B; green); the expression of CD11b was visualized with a PE-conjugated specific mAb (D, E; red); and the expression of CD66b was monitored with an FITC-conjugated mAb (G, H; green). In G and H, PI (3  $\mu$ M; red) was added before CLSM image acquisition to visualize extracellular DNA. C, F, and I, Quantitative analysis of fluorescence of 7–10 horizontal optical sections acquired for at least two independent experiments for each marker. Results are expressed as arbitrary fluorescence unit (AFU). \* $p < 0.001$ . A C-Apochromat 63 $\times$ /1.2 W corr objective (A, B) and a Plapon 60 $\times$ /1.42 immersion oil objective (D, E, G, H) were used.

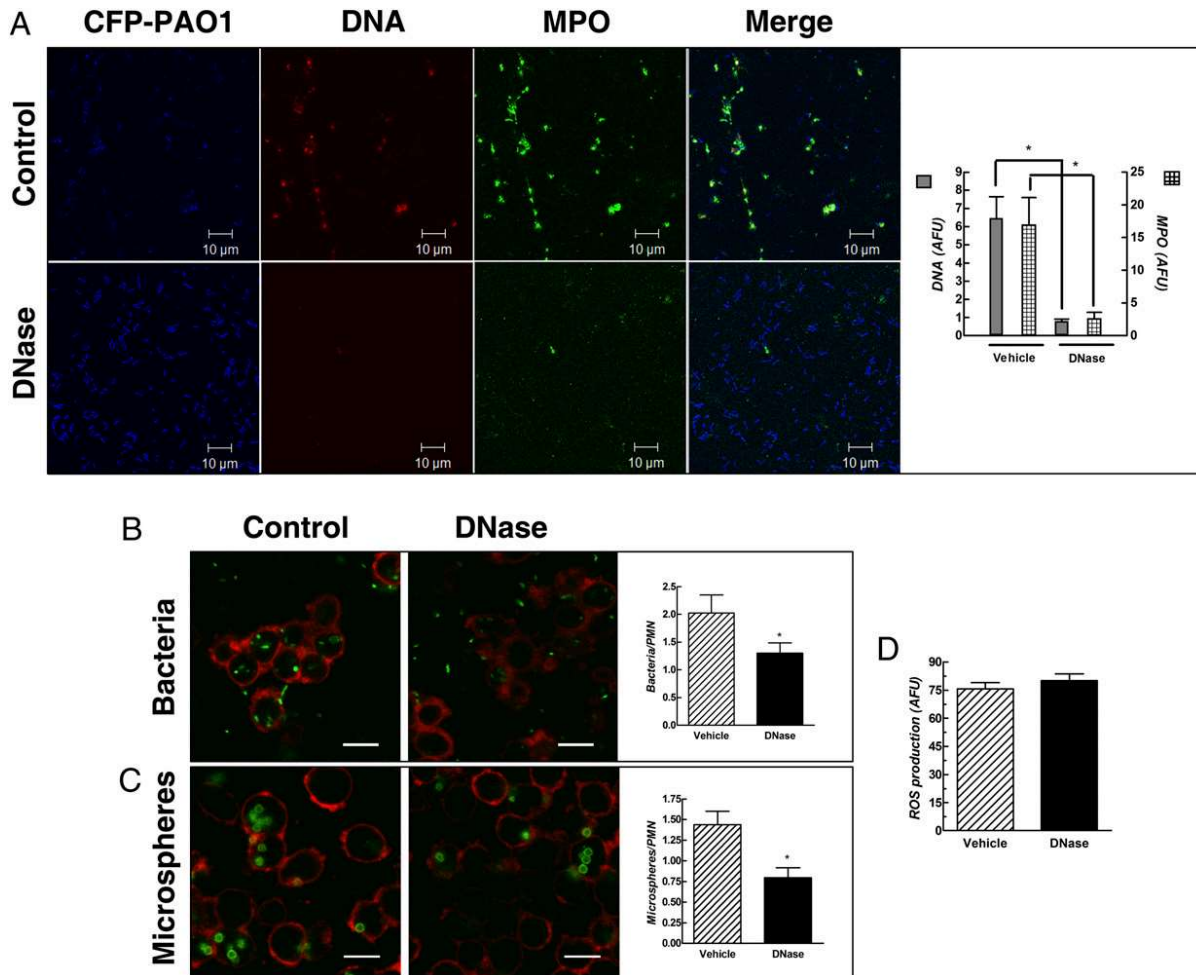
*Bacterial biofilms strongly stimulated neutrophil extracellular trap formation; treatment of biofilm with DNase reduced neutrophil extracellular trap release by neutrophils*

We observed that cocultures of neutrophils with biofilms for >2 h led to the unexpected appearance on the biofilm surface of a dense network of an extracellular filamentous material that strongly stained with PI and was present in greater amounts in untreated biofilms. Because these DNA fibers were visualized in a focal plane lying on the biofilm, and their aspect completely differed from the extracellular DNA observed in biofilms cultured in the absence of neutrophils, we suspected that they might be neutrophil extracellular traps (NETs) released from biofilm-stimulated neutrophils. NETs are extracellular structures composed of chromatin and specific proteins from the neutrophilic granules attached (48). To confirm our presumption, 2-d-old PAO-1 biofilms were treated or not with DNase I, washed twice, and exposed to neutrophils for 150 min. Then, putative NETs were carefully stained with an anti-nucleosome Ab and PI. Nucleosomes colocalized with DNA stain

(yellow in the merge panels), indicating the human origin of this network (Fig. 6). Neutrophils exposed to DNase I-treated biofilms released fewer NETs than did those stimulated by untreated biofilms (Fig. 6). To rule out the possibility that DNase I-pretreated biofilms exhibited a reduced amount of NETs because of some residual DNase I that could persist in the culture, after the 150 min of coculture with neutrophils, the supernatant was collected, and DNase I activity was assessed. No significant DNase activity was detected (data not shown).

*Impact of treatment with DNase I on the ability of biofilms formed by mucoid P. aeruginosa strains isolated from CF patients to stimulate cytokine release by neutrophils*

*P. aeruginosa* has enormous genetic and metabolic flexibility that allows it to adapt to the milieu and persist within the airways of CF patients. The genotypes and phenotypes of the strains present in late stages of the disease differ substantially from those that initially colonize the lungs (49). In fact, the conversion of *P. aeruginosa*



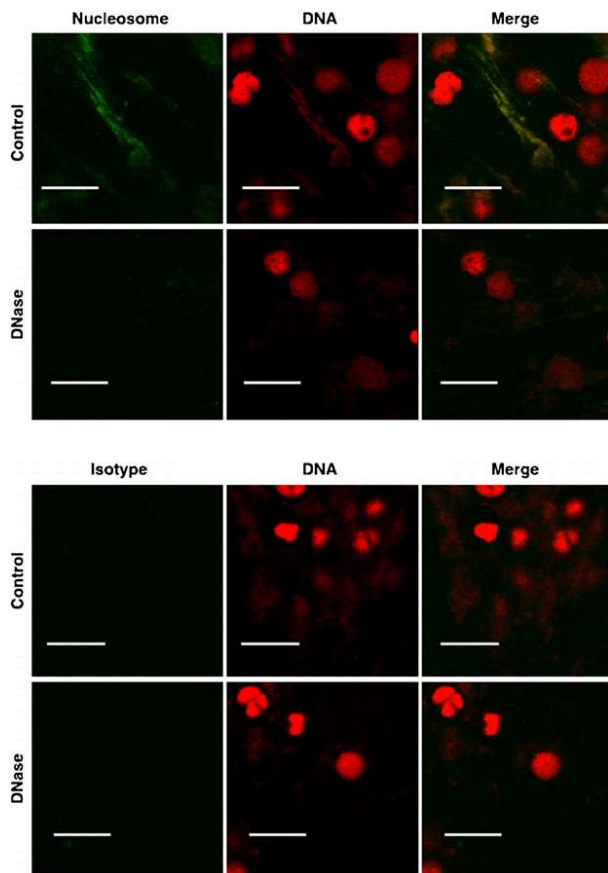
**FIGURE 5.** A, Treatment of the biofilms with DNase I reduced the immobilization of the MPO released by neutrophils. Two-day-old CFP-tagged PAO-1 biofilms (blue) were treated or not with DNase I (90 U/ml) for 60 min at 37°C, washed twice, and cocultured with neutrophils ( $2 \times 10^6$ /ml) for 60 min at 28°C. After carefully removing culture supernatants, the extracellular MPO immobilized to the surface of the biofilm was revealed with an FITC-conjugated anti-human MPO mAb (green), and the extracellular DNA was revealed by the addition of PI (3  $\mu$ M; red) before CLSM image acquisition. The images show horizontal optical sections located over the substratum from the vehicle-treated biofilms (control) and the DNase I-treated biofilm. A Plan-Neofluar 100 $\times$ /1.3 immersion oil objective was used. Bar graphs depict the quantitative analysis of fluorescence of five horizontal-plane images acquired from four independent experiments. Phagocytosis of bacteria (B) and internalization of FITC-polystyrene microspheres (C) by neutrophils cocultured with mock- or DNase I-treated GFP-tagged PAO-1 (B) or PAO-1 (C) biofilms. Cell periphery was stained with a PE-conjugated anti-CD11b mAb. The images show horizontal optical sections of neutrophils deposited over the biofilms. A Plapon 60 $\times$ /1.42 immersion oil objective was used. Bar graphs depict the quantitative analysis of fluorescence of five microscopic fields per treatment per neutrophil donor from six different neutrophil donors. D, Oxidative burst of DHR-123-stained neutrophils settled on mock- or DNase I-treated PAO-1 biofilms. The oxidative burst was visualized by CLSM by the green fluorescence emitted when DHR-123 was oxidized to rhodamine-123 as a result of the production of H<sub>2</sub>O<sub>2</sub>. Data represent the mean  $\pm$  SEM of the fluorescence quantified from eight horizontal-plane images acquired from a representative experiment of six. \* $p < 0.001$ .

from a nonmucoid to a mucoid phenotype marks the transition to a more persistent state, characterized by antibiotic resistance and accelerated pulmonary decline (50–52). Thus, we performed additional experiments to determine the relevance of the matrix DNA in biofilms formed by two mucoid *P. aeruginosa* strains isolated from CF patients' lungs. We found that biofilms formed by these strains differed in their DNA content and in the efficiency of DNase I treatment (Fig. 7A). In biofilms formed by the #1208 strain, which exhibited a greater amount of extracellular DNA, treatment with DNase I was more efficient in reducing the amount of extracellular DNA present in the biofilm, and it significantly reduced the ability of the biofilm to stimulate the release of IL-8 and IL-1 $\beta$  by neutrophils cocultured with it. By contrast, biofilms formed by the F1432 strain displayed a lower extracellular DNA content, and DNase I treatment did not significantly reduce the amount of the extracellular DNA in the biofilm nor the cytokines released by neutrophils in contact with these biofilms (Fig. 7).

## Discussion

In this study, we reported that extracellular DNA from the *P. aeruginosa* biofilm matrix plays a critical role in triggering neutrophil proinflammatory responses. We found that degradation of extracellular DNA markedly reduced the release of proinflammatory cytokines by neutrophils settled on the biofilm, as well as the up-regulation of activation markers and bacterial phagocytosis.

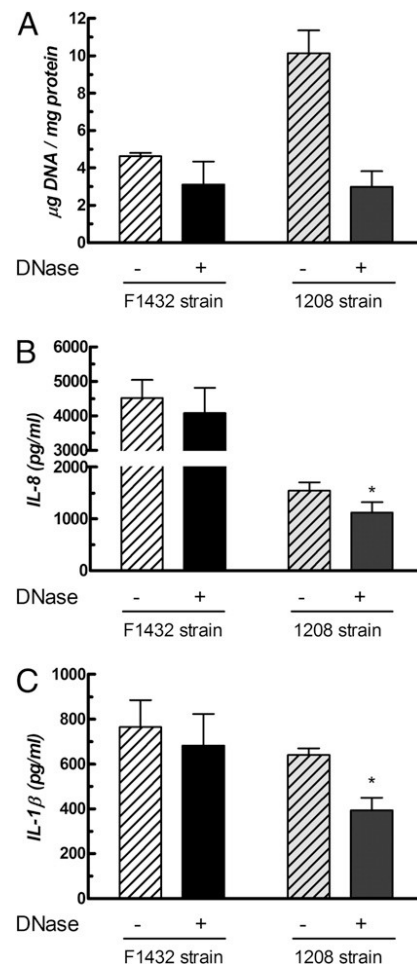
Quorum sensing is the mechanism that allows bacteria to perceive the density of the surrounding bacterial population and to coordinately respond to this information by regulating various genes (53). *P. aeruginosa* uses the quorum-sensing systems lasRI and rhIRI to control the expression of a wide range of virulence factors (54–56). Moreover, quorum-sensing molecules can also stimulate eukaryotic cells (53). We found that biofilms formed by the *lasI rhII* mutant strain, which were shown to be also deficient in the release of extracellular DNA (42), stimulated lower levels of cytokine production. Although our findings do not exclude the



**FIGURE 6.** Treatment of the biofilms with DNase I reduced the formation of NETs by neutrophils settled on them. Two-day-old PAO-1 biofilms were treated or not with DNase I (90 U/ml) for 60 min at 37°C, washed twice, and cocultured with neutrophils ( $2 \times 10^6$ /ml) for 150 min at 37°C. The CLSM images show horizontal optical sections of fibrous material that had arisen over the neutrophil–biofilm cocultures stained with an anti-nucleosome mAb (green) and PI (3  $\mu$ M; DNA staining, red). Overlay of these images shows the colocalization of DNA and nucleosomes (yellow). Scale bars, 20  $\mu$ m.

possibility that this effect is mediated by a deficiency in another factor regulated by the Las and the Rhl systems, the fact that treatment of the *lasI rhlI* mutant biofilm with DNase I did not modify its ability to stimulate neutrophil IL-8 and IL-1 $\beta$  release is consistent with our conclusion that extracellular DNA plays a significant role in neutrophil activation by bacterial biofilms. Previous studies indicated that biofilms formed by the *lasR rhlR*-deficient strain triggered higher respiratory-burst activation than did wild-type biofilms (39). Although DNA content in biofilms formed with this strain was not determined, the possibility that a reduction in extracellular matrix DNA was the cause of its greater reactive oxygen species-producing capacity is excluded, because quorum sensing signals themselves were found to be responsible for these effects.

Studies showed that large polyanionic DNA polymers present in the purulent secretions in CF patients electrostatically bind and sequester cationic granule proteins, such as neutrophil elastase and MPO (57, 58). In line with these findings, we found that MPO released by neutrophils became immobilized to matrix extracellular DNA. Previous works demonstrated that MPO, independently of its catalytic activity, mediates neutrophil activation and delays its apoptosis by associating with the CD11b/CD18 (Mac-1) integrin (59, 60). Thus, DNA-attached MPO might promote proinflammatory responses by catalyzing the production of



**FIGURE 7.** Effect of DNase treatment on the proinflammatory capacity of biofilms formed by mucoid *P. aeruginosa* strains isolated from chronically infected CF patients. Two-day-old biofilms formed by the mucoid strains #1208 and F1432 were treated or not with DNase I (90 U/ml) for 60 min at 37°C and washed twice. Then, biofilms were cultured for three additional hours in RPMI 1640 medium, without phenol red. DNA and total protein contents were determined, and the DNA/protein ratio was plotted (A). Alternatively, biofilms were cocultured with neutrophils ( $2 \times 10^6$ /ml) for three additional hours at 37°C, and IL-8 (B) and IL-1 $\beta$  (C) released to supernatants was quantified by ELISA. Data represent the mean  $\pm$  SEM of four experiments performed in triplicate. \* $p < 0.05$  versus vehicle-treated biofilms.

hypochlorous acid and downstream-chlorinated amine compounds, as well as by enhancing neutrophil activation status and survival. The high local concentration of MPO on the biofilm and the augmented neutrophil Mac-1 expression, by increasing inflammation, might contribute to bacterial killing as well as to damage to the surrounding tissues, as previously proposed (61).

Lung *P. aeruginosa* infection in CF patients is the hallmark infection in which convincing evidence for biofilms in pathogenesis has been provided. CF is a hereditary disease in which the primary defect results in dysfunctional electrolyte secretion and absorption, particularly in the pancreas, lung, liver, and gastrointestinal tract. A vicious cycle ensues shortly after birth, characterized by thick mucus obstructing small airways and, thus, providing a niche for bacterial growth; infiltration of lung tissue by neutrophils; discharge of their contents, including the viscous polyanion DNA; and increasingly viscous mucus (62). Pulmonary colonization of the lower respiratory tract of CF patients begins in infancy or early childhood; by adolescence and early adulthood, most CF patients (up to 80%) have become colonized with *P. aeruginosa* (15). The



bacteria form biofilms in the host, which make them tolerant to the highest deliverable doses of antibiotics, and bacteria cannot be eradicated (15, 39). Remarkably, the current treatment regimen for CF patients includes inhalation of nebulized recombinant human DNase I (rhDNase) used as a mucolytic agent, because it breaks down polymerized DNA, which is a major contributor to increased sputum viscosity in CF airway secretions (62, 63). Although rhDNase treatment was also shown to be beneficial in young children who are not supposed to be colonized by *Pseudomonas* biofilms (62), if rhDNase treatment were able to remove matrix DNA from biofilms in those patients in which the biofilm has been installed, it might also contribute to reduce the inflammation. However, as judged by results obtained with mucoid biofilms formed with strains isolated from chronically colonized CF patients, this capacity might be dependent on the DNA content of the corresponding strain.

Our findings also showed that bacterial biofilms induced neutrophils to release abundant amounts of NETs, suggesting that the presence of biofilms in CF patients chronically infected with *P. aeruginosa* might exacerbate the viscosity of the purulent sputum by triggering NETs release.

Previous studies determined that bacterial DNA is a significant component of the extracellular matrix in static and flow cell-grown PAO-1 biofilms (64). In fact, the extracellular matrix of PAO-1 biofilms grown under static conditions consisted primarily of DNA, which was ~5 times more abundant than protein and 20 times more abundant than carbohydrates (64). Studies performed in the 1970s showed that slimes from 18 of 20 clinical *P. aeruginosa* isolates consisted primarily of DNA (28, 29). Moreover, extracellular DNA was detected in the matrix of biofilms formed by *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Haemophilus* species (30–34, 44). Different hypotheses have been proposed to explain the presence of extracellular DNA in biofilm matrix, such as a quorum sensing-regulated induction of a prophage that causes cell lysis (42, 65), lysis of membrane vesicles released by *P. aeruginosa* that were shown to contain DNA, and lysis of a small subpopulation of the *P. aeruginosa* bacteria induced by the bacteriolytic activity of the vesicles themselves (42). Additional studies showed that other species release DNA by lysis of a fraction of the population or through the use of type IV secretion systems (66, 67). Natural transformable bacteria release DNA to extracellular medium to allow the exchange of genetic material, and transformation was shown to occur with high efficiency in biofilms (68, 69). Together, these findings reveal a widespread distribution of extracellular DNA in bacterial biofilms and evolutionarily conserved mechanisms that assure its presence in them. The biofilm mode of growth constitutes a strategy for microbial survival that facilitates the transmission of pathogens by providing a stable protective environment and by acting as a reservoir for the dissemination of a great number of microorganisms to new surfaces (15). Thus, it is possible to speculate that throughout evolution, a mechanism arose to recognize bacterial biofilms by detecting DNA from its extracellular matrix to enhance the immune response against these persistent bacterial infections. In some cases, such as in *P. aeruginosa* infections in the lungs of CF patients or in chronic wound infections, extracellular DNA might contribute to trigger an oversized inflammatory response, causing bystander tissue damage.

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## Disclosures

The authors have no financial conflicts of interest.

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