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Pseudomonas aeruginosa isolates from cystic fibrosis patients induce neutrophil extracellular traps with different morphologies that could correlate with their disease severity

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ABSTRACT

Cystic fibrosis (CF) is a disease characterized by bacterial chronic infection of the respiratory tract and inflammation, which leads to a progressive decrease in lung function. *Pseudomonas aeruginosa* is commonly isolated from the sputum of patients and their presence is associated with a predominant airway inflammation with neutrophils, causing chronic colonization and higher mortality rates. Neutrophil extracellular traps (NETs) have been observed in response against *Pseudomonas*, however, these cannot eliminate the pathogen from the respiratory tract, so one possibility is that the bacteria could promote their production to use them as a scaffold to colonize the lungs and as a nutrient source, however, their overproduction could also lead to increased damage to the lungs. In this work, we evaluated NETs formation by *Pseudomonas* clinical isolates obtained from CF patients and found that these induced NETs formation with globular or spread morphologies, of note, we found that there is a trend by which the spread forms were induced mainly by isolates obtained from patients with severe disease, whereas, the globular morphologies were observed for isolates obtained from patients with mild/moderate disease. Finally, we screened for bacterial molecules implicated in NETs formation and found that Exotoxin S, pyocin S2 and pyoverdine could participate in the process.

1. Introduction

Cystic fibrosis (CF) is a genetic disease caused by mutations of the cystic fibrosis transmembrane regulator (CFTR) gene, located on chromosome 7. This gene encodes a protein member of the ATP-binding cassette (ABC) transporter superfamily, which functions as a chloride channel that controls the transport of ions and water across epithelial tissues. The clinical manifestations of the CF are dominated by respiratory and gastrointestinal symptoms, such as chronic airway infection and inflammation, which lead to a progressive decrement in lung function, pancreatic insufficiency, malnutrition, and hepatobiliary symptoms, among others (Knowles and Durie, 2002). Lung disease is characterized by chronic bacterial infections, in which *Pseudomonas aeruginosa* is the major pathogen involved, overproducing thick mucus and neutrophil-predominant airway inflammation (Regamey et al., 2012).

Despite the neutrophilic infiltrate, neutrophils are not able to eliminate bacteria from the airway, but contribute to lung damage (Kelly et al., 2008). In response to infection, neutrophils migrate to the site of inflammation and, once activated, perform different functions, including phagocytosis and secretion of proteins and enzymes by degranulation (Cortjens et al., 2016). In addition, neutrophils have the ability to kill pathogens by releasing neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012). To release NETs, neutrophils undergo morphological changes leading to the extrusion of the nuclear content creating fiber-like structures, composed of DNA mixed with granular and nuclear proteins (Cortjens et al., 2017).

Although it has been proposed that the formation of NETs has

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antimicrobial activity in CF, evidence suggests that they contribute to lung inflammation and tissue damage (Lynch and Bruce, 2013). In CF patients, NETs are present in the sputum and their abundance contributes to mucus viscosity, due to a particularly high DNA content (Manzenreiter et al., 2012; Martinez-Aleman et al., 2017). *Pseudomonas aeruginosa* has been recognized as one of the most prominent pathogens in CF patients, causing chronic colonization and higher rates of morbidity and mortality. Additionally, it has been previously reported that these bacteria are also one of the main inducers of NETs release in CF, which could be correlated with increased damage to the lungs leading to decreased pulmonary function (Yoo et al., 2014). Information about the molecules of this pathogen related to NETs induction is scanty; however, this pathogen encodes several virulence factors that could be implicated in this process.

P. aeruginosa produces virulence factors that can be actively secreted into the extracellular medium, or that can be injected directly into the cells through type III secretion systems (TTSS) to promote bacterial establishment and proliferation by inhibiting the host immune response (Garai et al., 2019; Kuchma et al., 2005). It is well characterized that these bacteria produce diffusible pigments such as pyocyanin and pyoverdine, thus avoiding elimination by the immune system through their free radical scavenging activity in immune cells (Morel et al., 1992; Vinckx et al., 2010). Exoenzymes secreted by TTSS allow the bacteria to modify the host cells and prevent the immune response. In this regard, Exoenzyme S (ExoS) enhances the persistence and spread of *Pseudomonas* in the lungs of patients by inhibiting phagocytosis and bacterial destruction within the phagocytic cells, which has important effects in acute pneumonia (Rangel et al., 2015). The implication of these proteins in NETs formation can be proposed, since it is known that free radical formation and phagocytosis are essential steps in the formation of some types of NETs (Johnson et al., 2017; Van Avondt and Hartl, 2018).

CF patients are commonly coinfected with different bacterial species, and this may be correlated with their disease status due to increased tissue damage caused by the immune response or by bacterial toxic factors (Bhagirath et al., 2016). Additionally, an aspect that could also contribute to damage is intra- and interspecific bacterial competition in patient's lungs. Pyocins S1 and S2 are bacteriocins whose activity has been observed in *Pseudomonas* isolates from CF patients. It has been described that these proteins can promote cell death of other bacteria through their DNase activity and lipid modification (Ling et al., 2010; Sano et al., 1993). Likewise, it has also been shown that they are able to induce the cell death of mammalian cells (Abdi-Ali et al., 2004; Watanabe and Saito, 1980); therefore, the activity of these proteins could also contribute to increase the severity of the disease in patients. On the other hand, since the modification of chromatin is important for NETs formation, it is tempting to analyze the possible role of these proteins in the process.

In the present work, we evaluated the NETs formation induced by *Pseudomonas aeruginosa* strains isolated from CF patients and observed whether a correlation could be established between NETs formation and the clinical status of the patients. Furthermore, we identified some bacterial molecules that could participate in the process.

2. Methods

2.1. Patients

We sampled strains of *Pseudomonas aeruginosa* from sputum cultures and/or throat swab from 14 patients with CF, which were recruited at the University Hospital and the School of Medicine of the Autonomous University of Nuevo Leon in Monterrey, Mexico. Our study protocol was approved by the Ethics and Research Committees of the University Hospital (Registration no. NM11015 and MB16−00002). We obtained an appropriate written informed consent from the patients, or their caregivers. High-resolution chest computed tomography (HRCT) was performed and evaluated using the Bhalla score (Bhalla et al., 1991). Pulmonary function tests were performed by spirometry following the guidelines of the American Thoracic Society (Miller et al., 2005) Tests were conducted using the Platinum EliteSeries™, BreezeSuite™ version 7.2 SP3 plethysmograph (MGC Diagnostics, St. Paul, MN, USA). Results are expressed as the percentage of the predicted values for FEV1by sex, age and height. To stablish chronic colonization by *Pseudomonas aeruginosa*, the criteria established by Lee and cols. previously was followed (Lee et al., 2003), for this, a total of 6 bacterial cultures from sputum were carried out for each patient in one year and in for those cases in which *Pseudomonas aeruginosa* was isolated in at least 4 occasions the patient was classified as chronic. Pancreatic insufficiency/sufficiency was determined by Computed tomography (CT) scanning.

2.2. P. aeruginosa strains

To carry out this study, we used 14 clinical isolates from patients with CF isolated in the bacteriology laboratory located in the regional center for the control of infectious diseases of the Faculty of Medicine of the Autonomous University of Nuevo Leon, and 5 mutant strains by transposon insertion of *P. aeruginosa* PW1059 (PAORF PA0044; exoT), PW4736 (PAORF PA2191; exoY), PW7478 (PAORF PA3841; exoS), PW3083 (PAORF PA1150; pys2), PW5032 (PAORF PA2396; pvdF) and their parental strain PAO1 were obtained from the University of Washington, USA *Pseudomonas aeruginosa* mutant strain collection (Jacobs et al., 2003). All strains were reactivated in tubes with Luria Bertani liquid medium (LB) and incubated at 37 °C with gentle shaking at 200 rpm for 24 h (Labnet International, Inc. Model 222 DS Edison, NJ). We recorded tubes with presence or absence of pigment in the LB medium, since a green coloration suggestive of pyoverdine was primarily observed in most of the tubes, pigment production was monitored at 400 nm with a Multiskan Sky Microplate Spectrophotometer (Thermo scientific). Subsequently, each strain of *P. aeruginosa* was seeded in Petri dishes with Cetrimide Agar (BD Bioxon), incubated at 42 °C for 24 h (Thermo Fisher Scientific, Inc. Model 815, Waltham, MA), and stored at 4 °C until later use. To identify *Pseudomonas aeruginosa*, bacteria inoculum of each isolate was identified by using API 20NE (bioMérieux; France).

2.3. P. aeruginosa inoculum preparation

Culture tubes with *P. aeruginosa* bacterial growth were centrifuged at 1500 *g* for 15 min, the bacterial pellet was resuspended in 1 mL of RPMI medium (Life Technologies, Mainway Drive Burlington, ONT), measured in the microplate reader (Bio-Rad, Hercules, CA) at λ600 nm, and adjusted to 1 OD (optical density) (equivalent to 1×10^7 bacteria/mL) in the microplate reader (Bio-Rad. Hercules, CA). From this bacterial concentration, we prepared multiplicities of infection for each experiment by serial dilutions.

2.4. Determination of the biofilm formation index (BFI)

For this we followed a previous stablished protocol (Delissalde and Amabile-Cuevas, 2004) with few modifications. Briefly, clinical isolates of *Pseudomonas aeruginosa* were incubated in a BHI broth at 37 °C with shaking for 24 h. After this, they were adjusted to 1OD at 600 nm, then 100 µL of the culture was transferred to a 96 wells microplate and incubated at 37 °C for 24 h. After this, absorbance at 630 nm was determined with a Multiskan Sky microplate reader (Thermo), supernatant was removed and each well was washed with 200 µL of sterile distilled water, biofilm adhered to the surface was stained for 15 min with 200 µL crystal violet (0.1 % w / v), three more washes were performed and biofilm was solubilized with 200 µL of 95 % ethanol for 5 min at room temperature and absorbance was determined at 570 nm on the microplate reader. The Biofilm Formation Index (BFI) was determined with the formula: BFI = (AB– CW) / G, where AB is the optical density at 570 nm of the stained bacteria, CW is the optical

density at 570 nm of the medium culture (free of microorganisms) stained and G corresponds to the optical density at 630 nm of cell growth in unstained suspended culture. According to the IFB value, the biofilm is considered: strong if IFB is ≥1.10, moderate if it is between 0.70–1.09, weak between 0.35−0.69 and no biofilm if the BFI is ≤0.35.

2.5. Neutrophil isolation

Peripheral blood from healthy individuals was collected in tubes with EDTA (BD, Franklin Lakes, NJ). 7 mL of whole blood were laid on top of the equivalent amount of Histopaque 119 (Sigma-Aldrich, St. Louis, MO) in two conical 15 mL tubes. The tubes were centrifuged at 800 *g* for 20 min, with no brake. The layer containing the PMNs was collected using a Pasteur pipette, washed with 10 mL of PBS 1x, and centrifugated at 300 *g* for 10 min. The pellet from each tube was suspended in 2 mL of PBS 1 × . The neutrophils were further purified by a Percoll (Sigma-Aldrich, St. Louis, MO) gradient. In brief, five Percoll dilutions at 85, 80, 75, 70, and 65 % were prepared with PBS 1x from a 100 % stock solution (9 parts Percoll plus 1-part PBS 10x). Next, in 15 mL tubes, 2 mL of each dilution was consecutively placed from high to low concentration, and at the end, the PMNs were laid over the gradients. The gradients were centrifuged for 20 min at 800 *g* (no brake), and then, the interphase between the 70 % and 75 % dilutions was collected and washed with PBS 1x by centrifugation at 300 *g* for 10 min. Finally, the neutrophils were resuspended in 2 mL of RPMI medium, quantified in a Neubauer chamber, and their viability was determined by trypan blue exclusion.

2.6. NETs Induction and quantification by nuclear expansion

1×10^5 neutrophils (≥ 95 % viability) were seeded in 24-well plates in RPMI medium and allowed to adhere for 20 min at room temperature. Subsequently, 50 μL of *P. aeruginosa* (MOI 1:100, 1:10, 1:1 and 1:0.1), or supernatant obtained by filtration through 0.22 μM membranes at 3% or 30 % (final concentration in the well) for isolate 131752 and 30 % for all other isolates were added. As controls, non-stimulated neutrophils, neutrophils incubated with non-pathogenic *Escherichia coli* (MOI 1:10), and neutrophils stimulated with 40 nM of PMA (Sigma, Saint Louis, MO) were used. The cells were incubated at 37 °C for 3 h, and then 10 μL of the Sytox® Green dye 0.5 mM (Molecular probes, Life Techonologies, Eugene, OR) was added to each well and incubated at room temperature in darkness for 15 min. After, NETs were quantified by fluorescence intensity in a fluorometer (Thermo Scientific, Inc., Model 5210450. Waltham, MA), and by nuclear expansion as reported previously (Papayannopoulos et al., 2010). In brief, phase contrast and fluorescence images were acquired with a fluorescence microscope using the FITC filter (Ex = 480 and Em = 535) with 20x objective lenses (Zeiss Axioplan, West Germany). On these, a total of 600 cells per treatment were analyzed using ImageJ software, and the distribution of the number of cells across the range of nuclear area was obtained using the frequency function of Microsoft Excel. The Sytox-positive counts were divided by the total number of cells, determined from corresponding phase contrast images (Sytox/Total cells), and plotted as the percentage of Sytox-positive cells within each area range. By using this method, a cell ongoing NETs formation has decondensed nucleus exceeding the normal average area of the nucleus of an unstimulated neutrophil, which is ±80 μm^2, assuming a circular shape, and using the formula π x r^2.

2.7. NETs categorization

To distinguish the different nuclear morphologies, we based our analysis on the nuclear changes that occurs during NETs formation as described by Hakkim et. al. (Hakkim et al., 2011), coupled with nuclear area quantification described previously (Gonzalez et al., 2014; Papayannopoulos et al., 2010). Briefly, we classify nuclear morphology by arbitrarily subdividing the NETs extension as “diffused” for those cells in which the nuclear area expanded above 80 μm2 (normal size of neutrophil nucleus) but below 160 μm^2, and “spread” for the cells in which the nuclear expansion was greater than 160 μm^2. For the stacked analysis the nuclear expansion data showed in Figs. 1D and Fig. 2E were joined to group the clinical isolates of *P. aeruginosa* according to the type of NETs induced by both, the bacteria and their secreted products.

2.8. Fluorescence quantification

To quantitate the NETs induced after incubation of neutrophils with the *Pseudomonas aeruginosa* mutant strains, we took images with the 40x objective for each condition of at least 10 different fields (randomly selected) and calculated the Corrected Total Field fluorescence (CTFF) for each treatment using the ImageJ software (protocol established by the QBI Advanced Microscopy Facility, The University of Queensland, Australia with some modifications). To do this, first a rectangular ROI (region of interest) of 200 × 200 (Width x Height) was established and the parameters analyzed were the area size, the mean fluorescence and the integrated density (which represents the total amount of fluorescence in the box) for each treatment. The background was established by measuring an area without fluorescence. The results obtained were pasted into a Excel spreadsheet and the CTFF was calculated with the formula: CTFF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings). Finally, the CTFF of each treatment was graphed using GraphPad Prism 8 software.

2.9. Statistical analysis

Analysis of variance (ANOVA) was carried out to determine the significant differences in the induction of the NETs by *P. aeruginosa* when using the fluorometry technique. When fluorescence intensity was carried out, one-way ANOVA with multiple comparisons was performed for each molecule analyzed. To determine the statistical significance among the different NETs morphologies (globular and spread) non-parametric Wilcoxon signed-rank test were carried out. Analyses were performed in GraphPad Prism 8 software.

3. Results

3.1. Characteristics of the CF patient cohort

The clinical data of the patients and detailed information on the status of their disease are shown in Table 1. Clinical analysis showed that some of the patients had respiratory problems and chest X-rays corresponding to an advanced disease. It should be noted that the age of the patient could be a determining factor in our cohort, since almost all patients aging 12 years and older had a moderate to severe status according to their Bhalla score (a score that considerate multiple factors in the patient disease status like the severity and extend of bronchiectasis, peribronchial thickening, mucous plugs and presence of abscesses or sacculations, extent of emphysema, number of bullae, collapse or consolidation in the lungs). Chronic colonization with *P. aeruginosa* was stablished for each patient isolate following Lee‘s criteria, and found that half of our isolates corresponded to chronic colonization, of note, we found that mucous or rough morphologies not correlated with chronic colonization since almost all our isolated presented mucous phenotypes except but one.

Additionally, analysis of pancreatic insufficiency demonstrated that in our cohort not all of the patients presented pancreatic problem independently of its age or the mutation it carries.

3.2. Pseudomonas aeruginosa isolates from CF patients possess high capacity to induce NETs

We sought to analyze the capacity to induce NETs of *P. aeruginosa* clinical isolates of CF patients. We next assayed if these bacteria

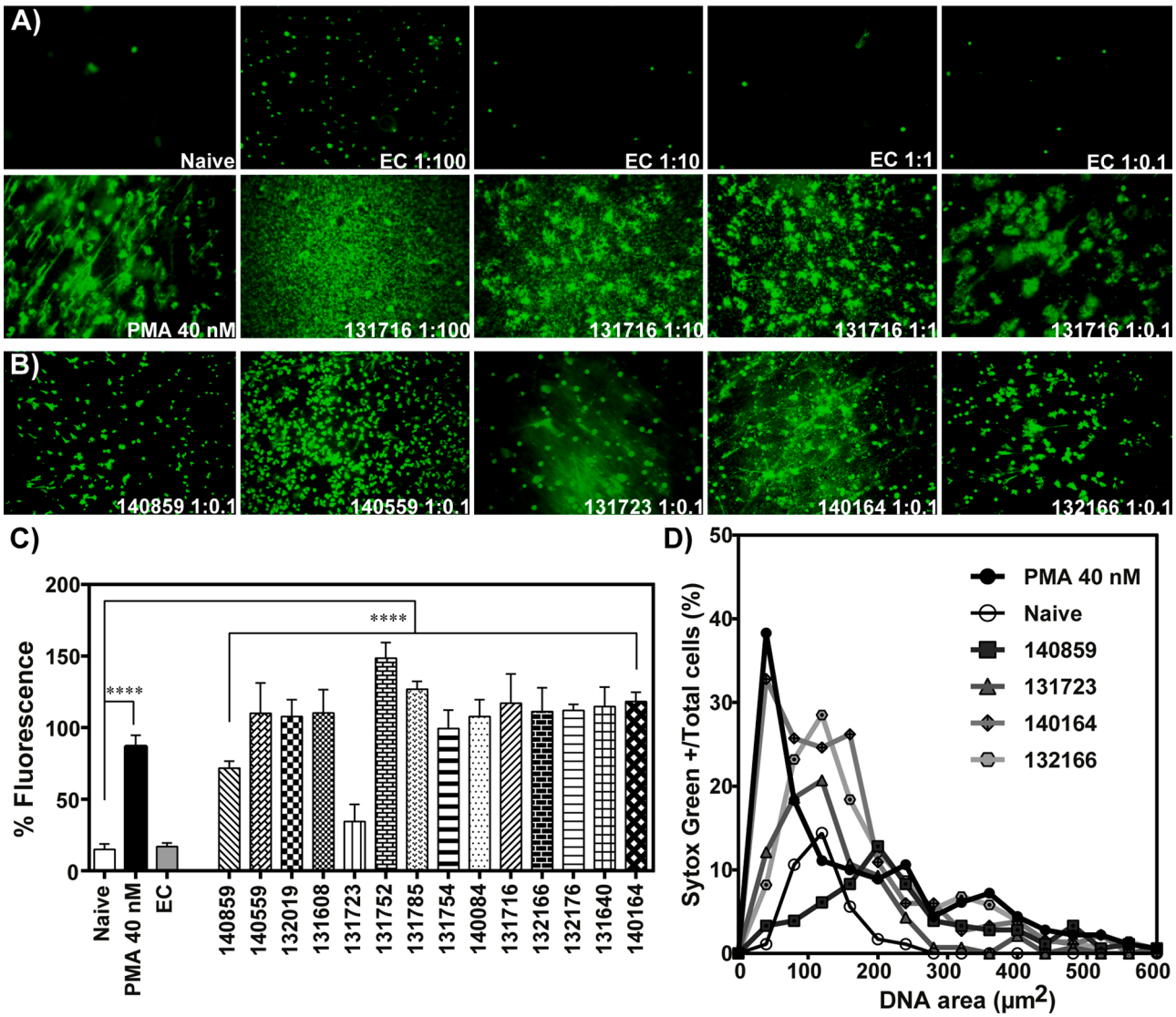

Fig. 1. *Pseudomonas aeruginosa* isolates induces extensive NETs. A) Fluorescence microscopy images of non-treated neutrophils (Naive) or treated with bacteria at different multiplicities of infection (MOI) and stained with Sytox to detect extracellular DNA after 3 h of treatment are shown. The treatments were: PMA (40 nM), non-pathogenic *Escherichia coli* (EC), and *Pseudomonas* clinical isolate (131716). B) Neutrophils were incubated with different *Pseudomonas* isolates at a MOI of 0.1 for 3 h and NETs morphology was detected by Sytox staining. C) NETs induced by each of the 14 CF isolates (MOI 1:0.1), PMA, E. coli or left untreated were quantified by fluorometry. The mean and the standard deviation are shown, the results are representative of three independent experiments and each condition was tested in triplicate. **** $p \leq 0.0001$. D) Nuclear area quantification of non-treated (Naive) neutrophils, PMA- treated or incubated with *Pseudomonas aeruginosa* isolates (MOI 1:0.1) is shown. The nuclear area of neutrophils after stimulation was calculated as indicated in the Materials and Methods section, and the value corresponding to a given nuclear area range was plotted against the percentage of Sytox positive cells. Results are representative of three independent experiments and each condition was tested in triplicate.

presented increased capacity to induce formation of extracellular traps from NETs. In order to do this, we tested the multiplicity of infection (MOI) necessary to induce NETs of an isolate coming from a patient suffering of a chronic infection; we found that this strain possess high capacity to induce NETs, a capacity not displayed by and intestinal isolate of *Escherichia coli*. Importantly, we observed that *Pseudomonas* isolates possess a very high capacity to promote NETs formation (MOI 1:0.1; neutrophil: bacteria ratio), which is comparable to that induced with the positive PMA control (Fig. 1A). For this, we used the 0.1 MOI in the following experiments.

As demonstrated by fluorescence microscopy, this bacterium has a varied capacity to induce NETs, since some isolates showed high inducing capacity, while others proved to be poor inducers (Fig. 1B). Quantification of the extruded DNA by fluorometry corroborated our previous result, as most of our isolates promoted increased DNA extrusion when compared to PMA (Fig. 1C). Microscopy images showed different NETs morphology for many isolates, with some of the strains promoting DNA extrusion with a spread morphology (isolate 131716), while others induced a more globular morphology (isolates 140859, 140559, 131723, 140164, 132166) (Fig. 1B). To quantify the different morphologies, we analyzed the NETs by measuring the nuclear expansion. We observed that some of them promoted expansion of the nucleus in a spread form similar to that observed after treatment with PMA, while other isolates induced a more compact form (Fig. 1D). Taken together, we found that *P. aeruginosa* possess a strong capacity to induce NETs with a varied nuclear morphology.

3.3. Secreted factors of P. aeruginosa clinical isolates participate in NETs formation

We first isolated and characterized the bacterial phenotype; for this we separated our clinical isolates by analyzing if they were able to

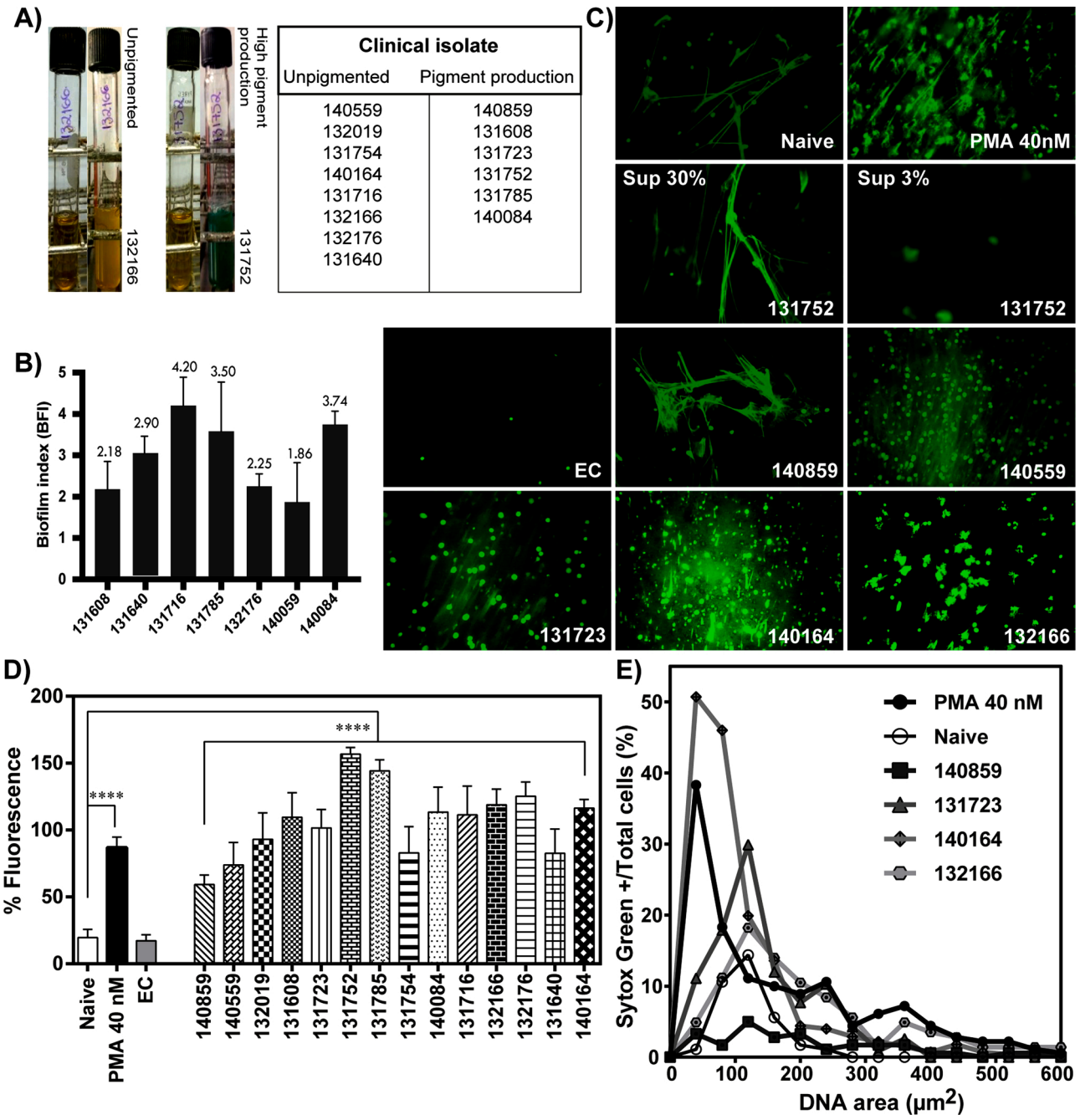

Fig. 2. Biofilm formation by *Pseudomonas aeruginosa* isolates and NET induction by its secreted factors. A) *Pseudomonas* CF isolates demonstrated variable pigment production when cultured in liquid medium, all the isolates were categorized according to their pigment production as shown in table. B) Biofilm formation of representative *Pseudomonas aeruginosa* isolates was determined and expressed as Biofilm Formation Index (BFI). BFI is strong if ≥1.10, moderate if 0.70-1.09, weak if 0.35-0.69 or negative if ≤0.35 (as indicated in materials and methods) C) Neutrophils were incubated with filtered supernatant of *Pseudomonas* isolates at different concentrations (30 % final concentration in the well unless indicated differently), after 3 h extracellular DNA was stained with Sytox and visualized by fluorescence microscopy. D) NETs induced by supernatant of each isolate, PMA, supernatant of *E. coli* (EC) or left untreated were quantified by fluorometry. The mean and the standard deviation are shown, the results are representative of three independent experiments and each condition was tested in triplicate. **** $p \leq 0.0001$. E) Nuclear area quantification was determined as described before and plotted against the percentage of Sytox positive cells. Results are representative of three independent experiments and each condition was tested in triplicate.

change the color of their growth medium, since this a simple way to know which isolates were able to secrete metabolites (i.e. pyocyanin or pyoverdine, among others). We found great variability among our strains, as some showed high and fast secretion, whereas others showed poor or even null pigment production (Fig. 2A). In an attempt to analyze if pigment production was related to the chronicity of the infection, we analyzed the bacterial phenotype in culture and found that the majority of our isolates presented a mucous colonial phenotype however, not all of them were obtained from patients with chronic infection (Table 1). After, to analyze if the mucous phenotype could be correlated with exopolysaccharide production to form biofilms we assayed the capacity of some isolates to form biofilms *in vitro* and found that all the isolates were strong biofilm inducers although in different levels (Fig. 2B), however, biofilm formation was independent of their capacity to produce diffusible pigments.

Next, we incubated neutrophils with two concentrations (3% and 30

Table 1
Patient clinical data.

Registry No.	Mutation	Sex	Age (years)	*P.a* Mucoid	*P.a* Chronic	Bhalla score	FEV1 %	Pancreatic insufficiency	*Staphylococcus aureus* coinfection
140859	F508del/F508del	Men	11	yes	no	7	104	yes	yes
140559	F508del/x	Women	5	no	no	6	84	yes	yes
132019	F508del / G542X	Men	1	yes	no	2	ND	yes	no
131608	G542x/x	Women	15	yes	yes	11	87	no	yes
131723	ND	Women	1	yes	no	1	ND	yes	yes
131752	F508del/R334	Men	1	yes	no	1	ND	yes	yes
131785	F508del /F508del	Women	13	yes	yes	8	66	yes	yes
131754	2789 + 5G > A/ 2789 + 5G > A	Women	20	yes	yes	14	25	no	yes
140084	F508del /X	Women	2	yes	no	1	ND	yes	yes
140164	F508del /3849 + 10 K	Women	13	yes	yes	8	78	no	no
131716	F508del/F508del	Men	12	yes	yes	14	28	yes	yes
132166	F508del /G85E	Men	5	yes	yes	2	95	yes	no
132176	1148 T/X	Men	12	yes	no	7	80	yes	yes
131640	S549 N/S549N	Men	20	yes	yes	14	33	yes	no

Pa mucoid: Mucoid *Pseudomonas aeruginosa.*
Pa Chronic: Chronic Pseudomonas *aeruginosa* using Lee's Criteria (Lee et al., 2003).
Bhalla score: 1–5 Medium, 6–9 Moderate, >9 Severe (Bhalla et al., 1991).
FEV1 %: Percentage of predicted Forced expiratory volume in 1 s.
ND: No Data.

%) of bacterial supernatant from a pigment producer isolate (131752). We found that both tested concentrations induced NETs, we observed that the highest concentration induced more robustly, for this reason we selected the 30 % concentration for the next experiments (Fig. 2C). To analyze if this was a conserved trait among the pigment-producers strains we extended the analysis to include additional strains (140859, 131723) and found that the NETs morphologies is not dependent on pigment production as a mixture of globular and extended morphologies were observed. When supernatant of some poor or not-pigment producer isolates (132166,140164 and 132150) were incubated with neutrophils, they could also induce robust NETs formation with mixed morphologies (Fig. 2C). Moreover, when extruded DNA was quantified by fluorometry, most of the strains were characterized as strong NETs inducers, as these produced more NETs than PMA (Fig. 2D) and these results were confirmed by nuclear expansion quantification (Fig. 2E).

3.4. *P. aeruginosa clinical isolates and their secreted products induce NETs with varied morphology*

Analyses of nuclear expansion quantification demonstrated that our isolates or their supernatant possess different capacity to induce NETs with a heterogeneous morphology (Fig. 1D and 2E). The analysis revealed that all the isolates were able to induce globular NETs although in different extent, however, their capacities to induce spread NETs was more variable. In an attempt to observe if the NETs morphology could be correlated with the disease status of the patient, we grouped the isolates according to their capacity to induce NETs measured by nuclear expansion (Fig. 1D and 2E) and the Bhalla score of the patient from which they were obtained (Fig. 3). By doing this we corroborated that both the bacteria and their secreted factors induced NETs, however, we observed that when NETs were induced with bacteria there is a trend in which the higher the Bhalla score increases the amount of expanded NETs and, conversely, the lower the score the more globular NETs are induced (Fig. 3, top panel). On the other hand, when the ability of bacterial supernatants to induce NETs with varied morphologies was analyzed, no identifiable trend was found (Fig. 3, bottom panel). However, in this case the percentage of total NETs induced did demonstrate a trend to increase according to the severity of the disease.

3.5. *Pyoverdine, leucine aminopeptidase (ExoS), and pyocin S2 from Pseudomonas participate in NETs formation*

Since we found that secreted factors could contribute to NETs induction in our strains and according to literature we decided to analyze if virulence factors pyoverdine and pyocin S2 or exotoxins ExoS, ExoT and ExoY of *Pseudomonas* could be participating in NETs induction. In order to analyze whether these proteins were implicated in NETs formation, we performed an experiment using mutant bacteria to each of these factors (Fig. 4A). We found that incubation of neutrophils with defective strains for pyoverdine, pyocin S2 or ExoS showed a decreased NETs formation when compared to that observed for the parental strain PAO1 or PMA, as was determined by fluorescence microscopy (Fig. 4B), and fluorescence quantification (Fig. 4C). Finally, we also tested mutant bacteria for ExoT and ExoY but no NETs reduction was observed (Fig. 4B and C).

4. Discussion

Decreased respiratory capacity due increased mucus production in lungs of CF patients has been recognized as one of the main problems associated with mutation in the CFTR channel. However, recent research described that high amounts of extracellular DNA also have an important role in worsening patients' health (Marcos et al., 2015; Rada, 2017b). Neutrophils are the main cells recruited to patients' airways, however, they are incapable to eliminate bacteria and, instead, through NETs formation, their granular components are released leading to increased tissue damage, perhaps functioning as scaffold for the establishment of polymicrobial infections (Rahman and Gadjeva, 2014). In this regard, one important aspect to be considered is NETs morphology, which is still poorly understood, but which may be highly relevant, as the extent of tissue damage is possibly dependent on this feature. The observation of extended DNA fibers decorated with lytic granules has been commonly referred to as NETs, however, there are reports in which it is possible to observe "globular" or "extended" NETs morphologies that seem to be related to the stimulus used (Campos-Garcia et al., 2019; Kenny et al., 2017). In the present article, we observed that *P. aeruginosa* isolates or their secreted factors induce NET with globular or expanded morphologies. Likewise, the proportion of each of these morphologies seems to have a trend that could be correlated with the disease state of the patient. Therefore, we think that the characterization of the capacity of bacterial isolates to induce NETs and their different morphologies may be a useful tool to determine the disease status of patients, as it has been described that in response to infection, the neutrophil infiltration occurs before the onset of symptoms (Yonker et al., 2015). However, if these morphologies can be presented individually or if the globular forms precede the extended forms and these are subsequently extended

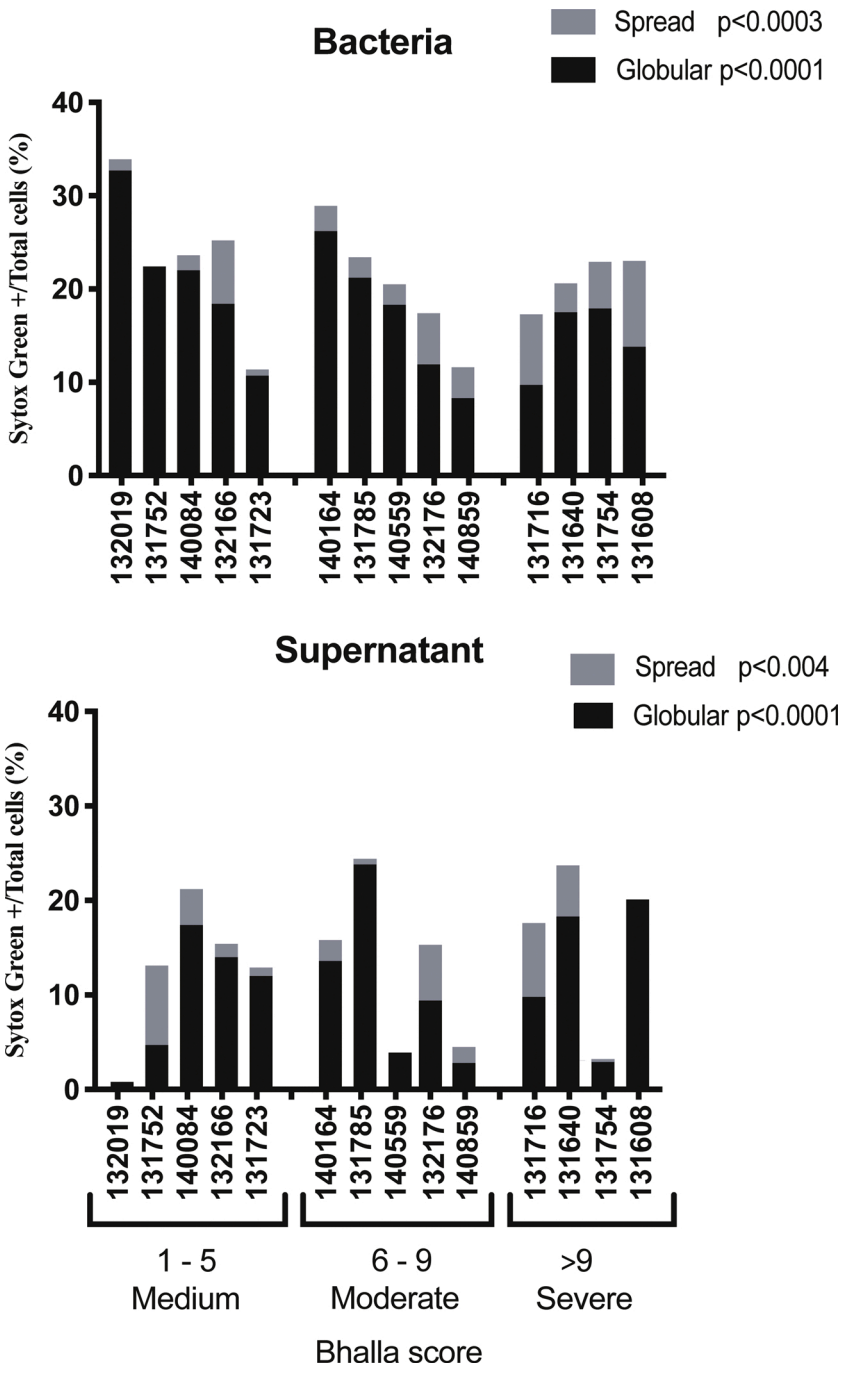

Fig. 3. Nuclear expansion morphologies induced by *Pseudomonas aeruginosa* isolates or their secreted factors. a) Nuclear area expansion of neutrophils treated with bacteria or their secretion products was quantified. For this, when the neutrophil nuclei possessed areas higher than 80 μm^2 (average size of normal nucleus in neutrophils) but smaller than 160 μm^2 were classified as globular nucleus and plotted against the percentage of Sytox positive cells b) Spread NETs were designated for those cases in which bacteria or its supernatants induced nuclear expansion with areas higher than 160 μm^2 but smaller than 320 μm^2, the results were plotted against the percentage of Sytox positive cells. In both graphs the isolates where classified according to the Bhalla score of the patients and stacked analyses shown the amount of spread NETs induced by the bacteria or their supernatant. Non-parametric Wilcoxon analyses were performed to determine the significance among the different morphologies for the isolates. The results are representative of three independent experiments for each condition.

by the effect of flow, has yet to be studied.

P. aeruginosa can actively colonize patient's lungs and establish chronic infections (Bhagirath et al., 2016; Murray et al., 2007; Rada, 2017a); it has been demonstrated however that neutrophils from patients are able to recognize the bacteria and extrude NETs, although some clinical isolates are able to acquire resistance to NETs mediated killing (Young et al., 2011). Resistance mechanisms have not been fully elucidated; however, it has been proposed that nuclease secretion or the acquisition of mucoid phenotypes can help individually or synergistically for this purpose (Wilton et al., 2018; Young et al., 2011). Therefore, it could be possible for clinical isolates to promote the formation of NETs to be used as scaffolding to establish infection in the lungs, while preventing their antimicrobial function. In our work, most isolates presented a mucous phenotype, and all were able to induce NETs formation; however, the amount and morphologies of the induced NETs were different. We think that the reason behind this could reside in the virulence factors expressed in our clinical isolates.

Growth in liquid media showed that some isolates produced pigments while others did not, among the pigments that have been determined to be secreted into the environment by this bacterium are pyocyanin and pyoverdine. It was previously shown that mutant bacteria for pyocyanin mildly decreased NET formation (Rada et al., 2013), but so far, pyoverdine had not been related to NETs. Here we showed a decreased NETs induction after incubation of neutrophils with mutant bacteria for pyoverdine. We think that the possible role of these molecules in NETs induction reside in their capacity to regulate free radical production in neutrophils, as it has been shown that pyocyanin can increase ROS production through NADPH oxidase activation and pyoverdine levels are increased as a response in oxidative stress conditions (Rada et al., 2013; Vinckx et al., 2008). On the other hand, pyoverdine

A)

Strain name	Gene abreviation	Putative ORF function
PW3083	pys2	pyocin S2
PW5032	pvdF	pyoverdine synthetase F
PW7478	exoS	exoenzyme S
PW1059	exoT	exoenzyme Y
PW4736	exoY	exoenzyme S
PAO1		Parental strain

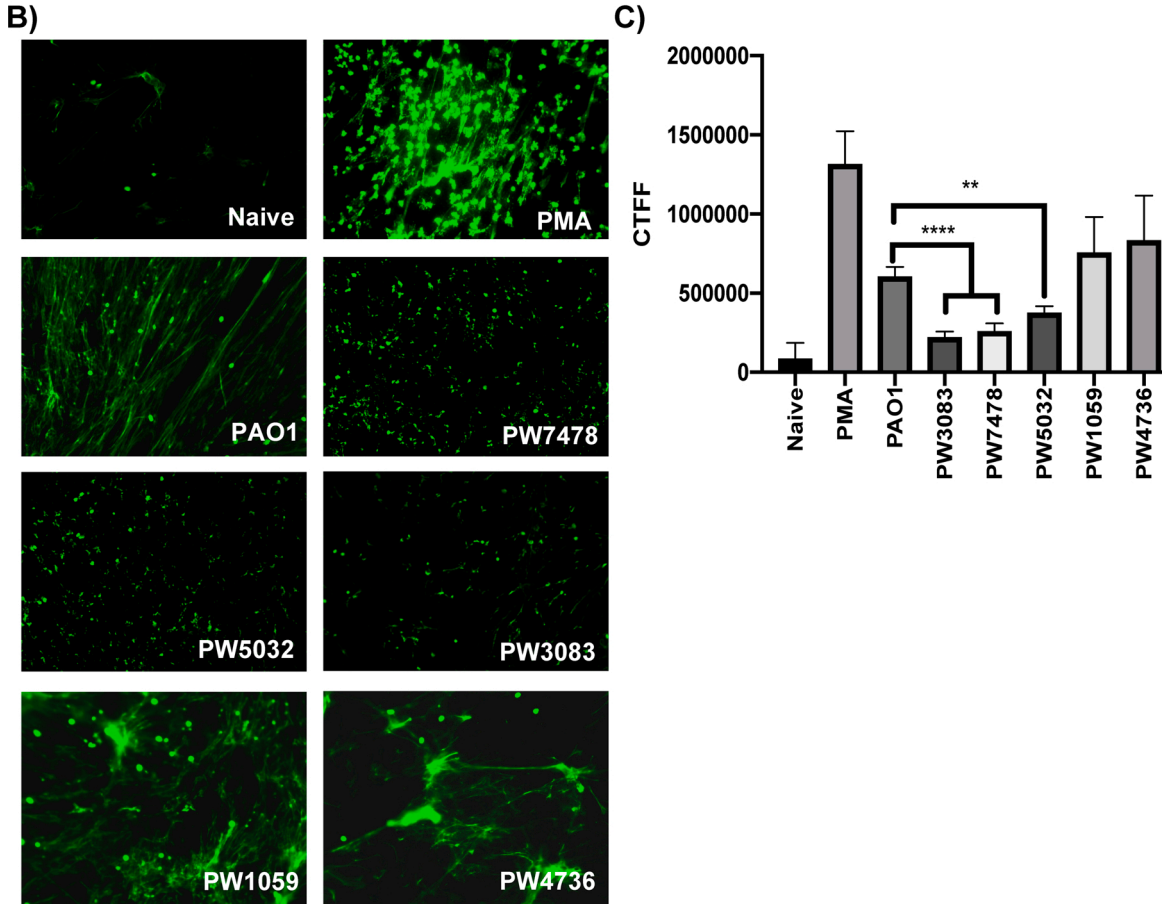

Fig. 4. ExoS, Pyocin S2 and pyoverdine of *Pseudomonas aeruginosa* participate in NETs formation. A) Transposon insertion mutant bacteria and their predicted inhibited function used in this study B) Fluorescence microscopy images of non-treated neutrophils (Naive), PMA-treated, parental *Pseudomonas* strain PAO1 or mutant bacteria treated (MOI 1:10) were stained with Sytox to detect extracellular DNA after 3 h of treatment. C) NETs induced were quantified by fluorescence intensity. The corrected total field fluorescence (CFFT) for each treatment was quantified as described in materials and methods. The mean and the standard deviation of at least 10 different fields for each treatment are shown. ** $p \leq 0.009$, **** $p \leq 0.0001$.

may help the bacteria to resist its elimination by the immune response, as it possesses a free radical scavenging ability (Morel et al., 1992). Nevertheless, other not-producing pigment isolates were also able to induce robust NETs formation, indicating that these bacterial pigments are not the only molecules involved in this process.

ExoS is an exoenzyme secreted by the type III secretion system that inhibits phagocytosis when interacts with phagocytic cells (Garai et al., 2019; Rocha et al., 2003). It has been reported that phagocytosis defects or when this cannot be carried out then NETs can be formed (Branzk et al., 2014; Manfredi et al., 2018). In our work, this was supported by the use of an ExoS mutant, as we observed that, when neutrophils were incubated with this strain, NETs induction decreased perhaps as a possible effect of increased bacterial phagocytosis and elimination. On the other hand, It has also been described that *Pseudomonas* biofilm formation

promotes ExoS deregulation (Hogardt et al., 2004), since all of our isolates were able to form biofilms this could be inconsistent with our results. A possible explanation for this difference is that in the mentioned work they used the *Pseudomonas* strain PAO1, and we used clinical strains where it is known that these strains can possess hypermutable phenotypes that allow their maintenance and establishment in the patient's lungs (Hogardt and Heesemann, 2013). Furthermore, we did not analyze the phenotype of our isolates, but it could be possible that some may possess an ExoS / ExoU phenotype whose presence has been linked as high-risk clones among clinical strains (Horna et al., 2019). In addition, another possibility is that biofilm formation could promote ExoS dysregulation, but would promote increased production of pyoverdine (Kang and Kirienko, 2017; Kang et al., 2017; Visaggio

et al., 2015) and pyocins (Oluyombo et al., 2019; Smith et al., 2012) in *Pseudomonas* isolates that we found to also participate in NET formation.

An observation attracting our attention was the diminished NETs formation induced by pyocin S2 mutant strains. Pyocin S2 has been characterized as a bacteriocin; however, during infection, it has been shown that this molecule may enter mammalian cells after being recognized by transferrin receptors in the membrane and then, through a hormone-like mechanism, it reaches the nucleus where it exerts its cytotoxic effect (Abdi-Ali et al., 2004; Farkas-Himsley and Musclow, 1986). During NETs formation, DNA decondensation by neutrophil's elastase activity and in some cases, through PAD4 activity is a primordial step (Papayannopoulos et al., 2010; Saha et al., 2019). We think that *Pseudomonas* isolates may contribute to this step through pyocin S2 DNAse activity, which promotes extensive NETs formation in the patients by increasing the tissue damage, leading to diminished respiratory capacity. Although our work suggests the participation of pyoverdine, exoS as well as pyocinS2 in the formation of NETs, it is not possible for us at this point to correlate the activity of any of these individually with the morphology of NETs. We are currently working to investigate if there is a synergistical effect between these molecules, or if they have an independent effect in order to known if the NETs morphologies correlate with their activities.

An important perspective in our work is to analyze the killing capacity of the different morphologies of NETs induced by our isolates. We think that NETs morphology could play an important role in their killing activities, as the expanded phenotypes could be more effective in entrapping and preventing the dispersion of bacteria, but they are less effective in killing, since the local concentration of antimicrobial molecules contained in them is more disperse. In contrast, NETs with a small expanded nucleus or with globular morphologies may be less effective to entrap bacteria but could have an increased killing activity. Another possibility is that the expanded NETs phenotype could produce a greater area of damage in patients, decreasing their respiratory capacity in comparison to that induced by the globular morphologies.

NETs formation should be a process regulated by the neutrophils, although it could be beneficial in the antimicrobial response, it can also be detrimental for the organism, due to the large number of harmful components released to the extracellular space. The recent description of bacterial proteins as potent NETs inducers is interesting, as it offers a new insight into the immunopathology caused by these diseases. It is noteworthy that, although NETs were described more than a decade ago, many aspects still remain to be discovered due to the difficulties associated to their study. However, new pathways implied in their formation are constantly being discovered, which provide additional insights in the process that could lead to the development of new therapeutic measures or of new strategies that may allow to know the state of the diseases in which these structures have an important role.

Author contributions

The following authors contributed to this paper: Wrote the manuscript: MAS, BAE, JVRJ, SGA; Performed the experiments: MAS, BAE, SGA; helped with manuscript preparation: JVRJ, SGA and helped with commentaries and writing of the manuscript: GGM.

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Declaration of Competing Interest

The authors report no declarations of interest.

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References

Abdi-Ali, A., Worobec, E.A., Deezagi, A., Malekzadeh, F., 2004. Cytotoxic effects of pyocin S2 produced by Pseudomonas aeruginosa on the growth of three human cell lines. Can. J. Microbiol. 50, 375–381.
Bhagirath, A.Y., Li, Y., Somayajula, D., Dadashi, M., Badr, S., Duan, K., 2016. Cystic fibrosis lung environment and Pseudomonas aeruginosa infection. BMC Pulm. Med. 16, 174.
Bhalla, M., Turcios, N., Aponte, V., Jenkins, M., Leitman, B.S., McCauley, D.I., Naidich, D.P., 1991. Cystic fibrosis: scoring system with thin-section CT. Radiology 179, 783–788.
Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G., Brown, G.D., Papayannopoulos, V., 2014. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat. Immunol. 15, 1017–1025.
Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. Science 303, 1532–1535.
Brinkmann, V., Zychlinsky, A., 2012. Neutrophil extracellular traps: is immunity the second function of chromatin? J. Cell Biol. 198, 773–783.
Campos-Garcia, L., Jimenez-Valdes, R.J., Hernandez-Bello, R., Palma-Nicolas, J., Gonzalez, G.M., Sanchez-Gonzalez, A., 2019. Candida Albicans and non-albicans isolates from bloodstream have different capacities to induce neutrophil extracellular traps. J. Fungi Basel (Basel) 5.
Cortjens, B., de Boer, O.J., de Jong, R., Antonis, A.F., Sabogal Pineros, Y.S., Lutter, R., van Woensel, J.B., Bem, R.A., 2016. Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. J. Pathol. 238, 401–411.
Cortjens, B., van Woensel, J.B., Bem, R.A., 2017. Neutrophil Extracellular Traps in Respiratory Disease: guided anti-microbial traps or toxic webs? Paediatr. Respir. Rev. 21, 54–61.
Delissalde, F., Amabile-Cuevas, C.F., 2004. Comparison of antibiotic susceptibility and plasmid content, between biofilm producing and non-producing clinical isolates of Pseudomonas aeruginosa. Int. J. Antimicrob. Agents 24, 405–408.
Farkas-Himsley, H., Musclow, C.E., 1986. Bacteriocin receptors on malignant mammalian cells: are they transferrin receptors? Cell. Mol. Biol. 32, 607–617.
Garai, P., Berry, L., Moussouni, M., Bleves, S., Blanc-Potard, A.B., 2019. Killing from the inside: intracellular role of T3SS in the fate of Pseudomonas aeruginosa within macrophages revealed by mgtC and oprF mutants. PLoS Pathog. 15, e1007812.
Gonzalez, A.S., Bardoel, B.W., Harbort, C.J., Zychlinsky, A., 2014. Induction and quantification of neutrophil extracellular traps. Methods Mol. Biol. 1124, 307–318.
Hakkim, A., Fuchs, T.A., Martinez, N.E., Hess, S., Prinz, H., Zychlinsky, A., Waldmann, H., 2011. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat. Chem. Biol. 7, 75–77.
Hogardt, M., Heesemann, J., 2013. Microevolution of Pseudomonas aeruginosa to a chronic pathogen of the cystic fibrosis lung. Curr. Top. Microbiol. Immunol. 358, 91–118.
Hogardt, M., Roeder, M., Schreff, A.M., Eberl, L., Heesemann, J., 2004. Expression of Pseudomonas aeruginosa exoS is controlled by quorum sensing and RpoS. Microbiology 150, 843–851.
Horna, G., Amaro, C., Palacios, A., Guerra, H., Ruiz, J., 2019. High frequency of the exoU+/exoS+ genotype associated with multidrug-resistant "high-risk clones" of Pseudomonas aeruginosa clinical isolates from Peruvian hospitals. Sci. Rep. 9, 10874.
Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenthner, D., Bovee, D., Olson, M. V., Manoil, C., 2003. Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U.S.A 100, 14339–14344.
Johnson, C.J., Kernien, J.F., Hoyer, A.R., Nett, J.E., 2017. Mechanisms involved in the triggering of neutrophil extracellular traps (NETs) by Candida glabrata during planktonic and biofilm growth. Sci. Rep. 7, 13065.
Kang, D., Kirienko, N.V., 2017. High-throughput genetic screen reveals that early attachment and biofilm formation are necessary for full pyoverdine production by Pseudomonas aeruginosa. Front. Microbiol. 8, 1707.
Kang, D., Turner, K.E., Kirienko, N.V., 2017. PqsA promotes pyoverdine production via biofilm formation. Pathogens 7.
Kelly, E., Greene, C.M., McElvaney, N.G., 2008. Targeting neutrophil elastase in cystic fibrosis. Expert Opin. Ther. Targets 12, 145–157.
Kenny, E.F., Herzig, A., Kruger, R., Muth, A., Mondal, S., Thompson, P.R., Brinkmann, V., Bernuth, H.V., Zychlinsky, A., 2017. Diverse stimuli engage different neutrophil extracellular trap pathways. Elife 6.
Knowles, M.R., Durie, P.R., 2002. What is cystic fibrosis? N. Engl. J. Med. 347, 439–442.
Kuchma, S.L., Connolly, J.P., O'Toole, G.A., 2005. A three-component regulatory system regulates biofilm maturation and type III secretion in Pseudomonas aeruginosa. J. Bacteriol. 187, 1441–1454.

Lee, T.W., Brownlee, K.G., Conway, S.P., Denton, M., Littlewood, J.M., 2003. Evaluation of a new definition for chronic Pseudomonas aeruginosa infection in cystic fibrosis patients. J. Cyst. Fibros. 2, 29–34.
Ling, H., Saeidi, N., Rasouliha, B.H., Chang, M.W., 2010. A predicted S-type pyocin shows a bactericidal activity against clinical Pseudomonas aeruginosa isolates through membrane damage. FEBS Lett. 584, 3354–3358.
Lynch, S.V., Bruce, K.D., 2013. The cystic fibrosis airway microbiome. Cold Spring Harb. Perspect. Med. 3, a009738.
Manfredi, A.A., Ramirez, G.A., Rovere-Querini, P., Maugeri, N., 2018. The neutrophil's choice: phagocytose vs make neutrophil extracellular traps. Front. Immunol. 9, 288.
Manzenreiter, R., Kienberger, F., Marcos, V., Schilcher, K., Krautgartner, W.D., Obermayer, A., Huml, M., Stoiber, W., Hector, A., Griese, M., Hannig, M., Studnicka, M., Vitkov, L., Hartl, D., 2012. Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. J. Cyst. Fibros. 11, 84–92.
Marcos, V., Zhou-Suckow, Z., Onder Yildirim, A., Bohla, A., Hector, A., Vitkov, L., Krautgartner, W.D., Stoiber, W., Griese, M., Eickelberg, O., Mall, M.A., Hartl, D., 2015. Free DNA in cystic fibrosis airway fluids correlates with airflow obstruction. Mediators Inflamm. 2015, 408935.
Martinez-Aleman, S.R., Campos-Garcia, L., Palma-Nicolas, J.P., Hernandez-Bello, R., Gonzalez, G.M., Sanchez-Gonzalez, A., 2017. Understanding the Entanglement: Neutrophil Extracellular Traps (NETs) in Cystic Fibrosis. Front. Cell. Infect. Microbiol. 7, 104.
Miller, M.R., Hankinson, J., Brusasco, V., Burgos, F., Casaburi, R., Coates, A., Crapo, R., Enright, P., van der Grinten, C.P., Gustafsson, P., Jensen, R., Johnson, D.C., MacIntyre, N., McKay, R., Navajas, D., Pedersen, O.F., Pellegrino, R., Viegi, G., Wanger, J., Force, A.E.T., 2005. Standardisation of spirometry. Eur. Respir. J. 26, 319–338.
Morel, I., Cillard, J., Lescoat, G., Sergent, O., Pasdeloup, N., Ocaktan, A.Z., Abdallah, M. A., Brissot, P., Cillard, P., 1992. Antioxidant and free radical scavenging activities of the iron chelators pyoverdin and hydroxypyrid-4-ones in iron-loaded hepatocyte cultures: comparison of their mechanism of protection with that of desferrioxamine. Free Radic. Biol. Med. 13, 499–508.
Murray, T.S., Egan, M., Kazmierczak, B.I., 2007. Pseudomonas aeruginosa chronic colonization in cystic fibrosis patients. Curr. Opin. Pediatr. 19, 83–88.
Oluyombo, O., Penfold, C.N., Diggle, S.P., 2019. Competition in biofilms between cystic fibrosis isolates of Pseudomonas aeruginosa is shaped by R-Pyocins. mBio 10.
Papayannopoulos, V., Metzler, K.D., Hakkim, A., Zychlinsky, A., 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J. Cell Biol. 191, 677–691.
Rada, B., 2017a. Interactions between neutrophils and Pseudomonas aeruginosa in cystic fibrosis. Pathogens 6.
Rada, B., 2017b. Neutrophil extracellular trap release driven by bacterial motility: relevance to cystic fibrosis lung disease. Commun. Integr. Biol. 10, e1296610.
Rada, B., Jendrysik, M.A., Pang, L., Hayes, C.P., Yoo, D.G., Park, J.J., Moskowitz, S.M., Malech, H.L., Leto, T.L., 2013. Pyocyanin-enhanced neutrophil extracellular trap formation requires the NADPH oxidase. PLoS One 8, e54205.
Rahman, S., Gadjeva, M., 2014. Does NETosis Contribute to the Bacterial Pathoadaptation in Cystic Fibrosis? Front. Immunol. 5, 378.
Rangel, S.M., Diaz, M.H., Knoten, C.A., Zhang, A., Hauser, A.R., 2015. The role of ExoS in dissemination of Pseudomonas aeruginosa during pneumonia. PLoS Pathog. 11, e1004945.
Regamey, N., Tsartsali, L., Hilliard, T.N., Fuchs, O., Tan, H.L., Zhu, J., Qiu, Y.S., Alton, E. W., Jeffery, P.K., Bush, A., Davies, J.C., 2012. Distinct patterns of inflammation in the airway lumen and bronchial mucosa of children with cystic fibrosis. Thorax 67, 164–170.
Rocha, C.L., Coburn, J., Rucks, E.A., Olson, J.C., 2003. Characterization of Pseudomonas aeruginosa exoenzyme S as a bifunctional enzyme in J774A.1 macrophages. Infect. Immun. 71, 5296–5305.
Saha, P., Yeoh, B.S., Xiao, X., Golonka, R.M., Singh, V., Wang, Y., Vijay-Kumar, M., 2019. PAD4-dependent NETs generation are indispensable for intestinal clearance of Citrobacter rodentium. Mucosal Immunol. 12, 761–771.
Sano, Y., Matsui, H., Kobayashi, M., Kageyama, M., 1993. Molecular structures and functions of pyocins S1 and S2 in Pseudomonas aeruginosa. J. Bacteriol. 175, 2907–2916.
Smith, K., Martin, L., Rinaldi, A., Rajendran, R., Ramage, G., Walker, D., 2012. Activity of pyocin S2 against Pseudomonas aeruginosa biofilms. Antimicrob. Agents Chemother. 56, 1599–1601.
Van Avondt, K., Hartl, D., 2018. Mechanisms and disease relevance of neutrophil extracellular trap formation. Eur. J. Clin. Invest. 48 (Suppl 2), e12919.
Vinckx, T., Matthijs, S., Cornelis, P., 2008. Loss of the oxidative stress regulator OxyR in Pseudomonas aeruginosa PAO1 impairs growth under iron-limited conditions. FEMS Microbiol. Lett. 288, 258–265.
Vinckx, T., Wei, Q., Matthijs, S., Cornelis, P., 2010. The Pseudomonas aeruginosa oxidative stress regulator OxyR influences production of pyocyanin and rhamnolipids: protective role of pyocyanin. Microbiology 156, 678–686.
Visaggio, D., Pasqua, M., Bonchi, C., Kaever, V., Visca, P., Imperi, F., 2015. Cell aggregation promotes pyoverdine-dependent iron uptake and virulence in Pseudomonas aeruginosa. Front. Microbiol. 6, 902.
Watanabe, T., Saito, H., 1980. Cytotoxicity of pyocin S2 to tumor and normal cells and its interaction with cell surfaces. Biochim. Biophys. Acta 633, 77–86.
Wilton, M., Halverson, T.W.R., Charron-Mazenod, L., Parkins, M.D., Lewenza, S., 2018. Secreted phosphatase and deoxyribonuclease are required by Pseudomonas aeruginosa to defend against neutrophil extracellular traps. Infect. Immun. 86.
Yonker, L.M., Cigana, C., Hurley, B.P., Bragonzi, A., 2015. Host-pathogen interplay in the respiratory environment of cystic fibrosis. J. Cyst. Fibros. 14, 431–439.
Yoo, D.G., Floyd, M., Winn, M., Moskowitz, S.M., Rada, B., 2014. NET formation induced by Pseudomonas aeruginosa cystic fibrosis isolates measured as release of myeloperoxidase-DNA and neutrophil elastase-DNA complexes. Immunol. Lett. 160, 186–194.
Young, R.L., Malcolm, K.C., Kret, J.E., Caceres, S.M., Poch, K.R., Nichols, D.P., Taylor-Cousar, J.L., Saavedra, M.T., Randell, S.H., Vasil, M.L., Burns, J.L., Moskowitz, S.M., Nick, J.A., 2011. Neutrophil extracellular trap (NET)-mediated killing of Pseudomonas aeruginosa: evidence of acquired resistance within the CF airway, independent of CFTR. PLoS One 6, e23637.