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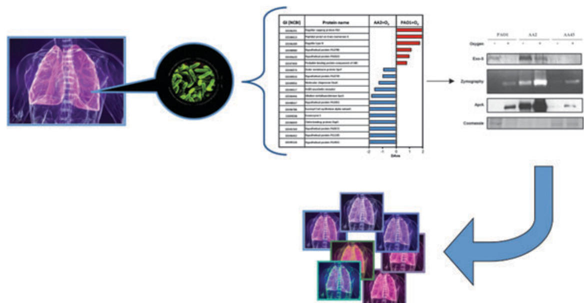

Q1 **MudPIT analysis of released proteins in *Pseudomonas aeruginosa* laboratory and clinical strains in relation to**
Q2 **pro-inflammatory effects**

Gabriella Bergamini, Dario Di Silvestre, Pierluigi Mauri, Cristina Cigana, Alessandra Bragonzi, Antonella De Palma, Louise Benazzi, Gerd Döring, Baroukh Maurice Assael, Paola Melotti* and Claudio Sorio*

In this work we applied a MudPIT approach combined with cell biology assays to identify putative virulence factors released by clinically relevant *Pseudomonas aeruginosa* strains.

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PAPER

MudPIT analysis of released proteins in *Pseudomonas aeruginosa* laboratory and clinical strains in relation to pro-inflammatory effects†

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Pseudomonas aeruginosa (Pa) is the most common virulent pathogen contributing to the pathogenesis of cystic fibrosis (CF). During bacterial lung colonization, the products of its metabolism are released in the extracellular space contributing to the pathogenic events associated with its presence. To gain insights on the mechanisms involved in the Pa pathogenesis we focused our attention on proteins released by Pa using a MudPIT approach combined with cell biology assays. Conditioned medium (CM) collected under aerobic and microaerobic conditions from Pa clinical strains (in early and late colonization), unlike the laboratory strain, induced expression of IL-8 mRNA in CF airway epithelial cells. We have identified proteins released by clinically relevant Pa strains, focusing on the pro-inflammatory effects as metalloproteases (MMPs). In fact, their expression pattern was associated with the highest pro-inflammatory activity measured in the early clinically isolated strain. The relation was further supported by the result of the analysis of a larger and independent set of Pa isolates derived from sporadically and chronically infected CF patients: 76% of sporadic samples expressed protease activity ($n = 44$), while only 27% scored positive in the chronically infected individuals ($n = 38$, $p < 0.0001$, Fisher's exact test). Finally, looking for a possible mechanism of action of bacterial MMPs, we found that CM from early clinical isolates can cleave CXCR1 on the surface of human neutrophils, suggesting a potential role for the bacterially released MMPs in the protection of the pathogen from the host's response.

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Insight, innovation, integration

We applied a recently developed shotgun proteomic approach (MudPIT) to protein-free conditioned medium (CM) obtained from a laboratory and two *P. aeruginosa* clinical strains to identify candidate molecules involved in *P. aeruginosa* virulence under comparable *in vivo* conditions. In our work we describe for the first time the effect of oxygen limitation on the profile of polypeptides released by laboratory and clinical isolates of *P. aeruginosa*. Bacterial MMPs were identified as putative biomarkers associated to Pa pro-inflammatory activity, and their activity was confirmed to be present in majority of sporadic strains isolated from different patients. This integrated approach is capable of providing quantitative and qualitative data that link the biology of *P. aeruginosa* strains to CF lung disease through the identification of released polypeptides capable of modulating host's response to this important pathogen.

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Introduction

Pseudomonas aeruginosa (Pa) is a common Gram-negative bacterium noted for becoming resistant to antibiotics and for causing opportunistic infections, ranging from acute, as in immunocompromised patients, to chronic, as in patients affected by cystic fibrosis (CF).[1] Pa chronic airway infection is the main cause of morbidity and mortality in CF patients.[2,3] CF lung disease is characterized by transient airway Pa infection and excessive neutrophils-dominated inflammation early in life followed by permanent chronic infection that causes persistent respiratory symptoms and decline in lung function.[4] Persistence of Pa in the CF airways involves several genotypic and phenotypic changes of bacterium ranging from loss of flagellar-dependent motility or antibiotic resistance to increased alginate production and occurrence of mucoid variants.[5–8]

In CF airways, bacteria may encounter a low-oxygen environment developed during infection or as a consequence of biofilm creation.[6,9] Under these conditions Pa strains often grow within the mucus plugs without direct contact with epithelium and they can utilize alternative external electron acceptors such as nitrate, nitrite or nitrous oxide that are present at high levels in CF airway mucus.[6] However, the products of their metabolism are released in the extracellular space contributing to the pathogenic events associated with the presence of Pa.[10]

In the present study we have evaluated the pro-inflammatory properties of the ultra-filtered (30 kDa) conditioned medium (CM), obtained from laboratory and clinical Pa strains grown under both aerobic and microaerobic conditions, on CF bronchial epithelial cells.

Several studies focusing on the Pa colonization in CF lung have been performed at the transcriptome level.[11–13] In contrast, a few works have been dedicated to proteomic analysis.[13–16] The identification of proteins that change their expression among clonal *P. aeruginosa* morphotypes represents a crucial aspect to understand which molecules are involved in Pa microevolution, as well as in the intrinsic adaptation ability of Pa. Therefore we investigated the polypeptides released by the laboratory strain PAO1 and clinical isolates cultured in both aerobiosis and microaerobiosis. By means of a semi-quantitative label-free approach[17] various sets of differentially represented proteins were obtained and some of them were confirmed by orthogonal methods, such as Western blot and zymography assay. In particular our attention focused on the expression and activity of some metalloproteases (MMPs) which were further assayed in a larger set (n = 82) of clinical isolated strains from various patients featuring sporadic or chronic colonization.[18] Finally it has been tested whether the bacterial MMPs could cleave the chemokine receptor CXCR1 on the surface of freshly isolated peripheral blood neutrophils (PMNs), a feature associated with the disabling of their bacterial killing capacity.[19]

Results

In this work we aimed to identify the bacterial proteins released by laboratory (PAO1) and Pa clinical strains, defined as early (AA2), and late (AA43) isolates.[20] to characterize candidate pro-inflammatory mediators, occurring during acute and/or chronic colonization. In addition the effect of oxygen limitation on the protein level of the Pa clinical strains, comparing their adaptation to microaerobiosis to that of PAO1, has been evaluated.

Effects of Pa conditioned medium on IL-8 mRNA expression in a CF airway epithelial cell line

Looking for potential virulence factors contributing to the typical inflammatory response, the induction of a pro-inflammatory marker (IL-8) expressed by a CF epithelial airway cell line (16HBE 14o- AS3) in response to 30 kDa cut-off ultra-filtered CM derived from Pa strains was measured. Following exposure to CM from AA2 grown under both aerobic and microaerobic conditions, the IL-8 mRNA expression was induced approximately 5 and 4 times, respectively (Fig. 1). When 16HBE 14o- AS3 cells were exposed to CM of AA43 the IL-8 mRNA expression increased about 4 and 2.5 times, in aerobiosis and microaerobiosis, respectively. In contrast, the exposure of CF cells to CM derived from the laboratory strain PAO1 did not induce statistically significant regulation.

To evaluate whether polypeptides were involved in regulation of IL8 expression, we incubated CM from AA2 overnight at 37 °C in the presence or absence of trypsin. Loss of metalloprotease activity under both experimental conditions was detected and was followed by a decreased expression of IL-8 mRNA of about 60% by the CF epithelial cells treated with CM from AA2 incubated at 37 °C (data not shown).

Identification of released proteins by Pa laboratory and clinical strains

Replicate MudPIT analyses (n = 7) of CM from laboratory and clinical strains permitted the identification of 363 distinct proteins, about 50% of them identified at least by 2 different

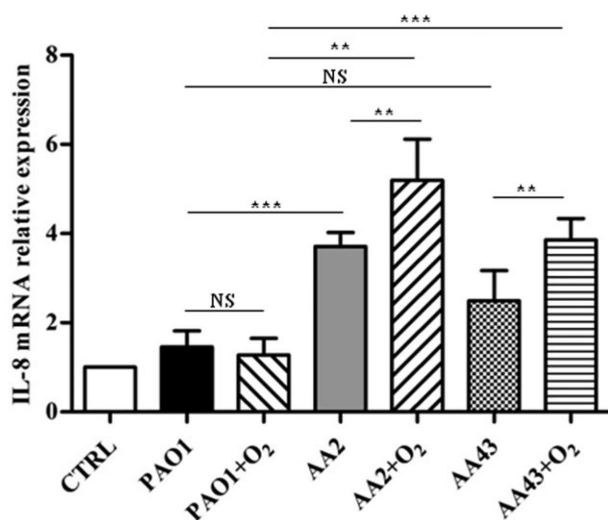

Fig. 1 IL-8 mRNA expression. Relative expression of IL-8 mRNA based on real-time PCR analysis of 16HBE 14o-AS3 cells exposed to 10% of CM of PAO1, AA2 and AA43 grown under aerobic or microaerobic conditions (means +/– SD) (n = 5; ** $p < 0.01$, *** $p < 0.001$). CTRL, corresponding to the basal IL-8 mRNA expression of 16HBE 14o-AS3 cells exposed to 10% MVBM alone, was set to 1.

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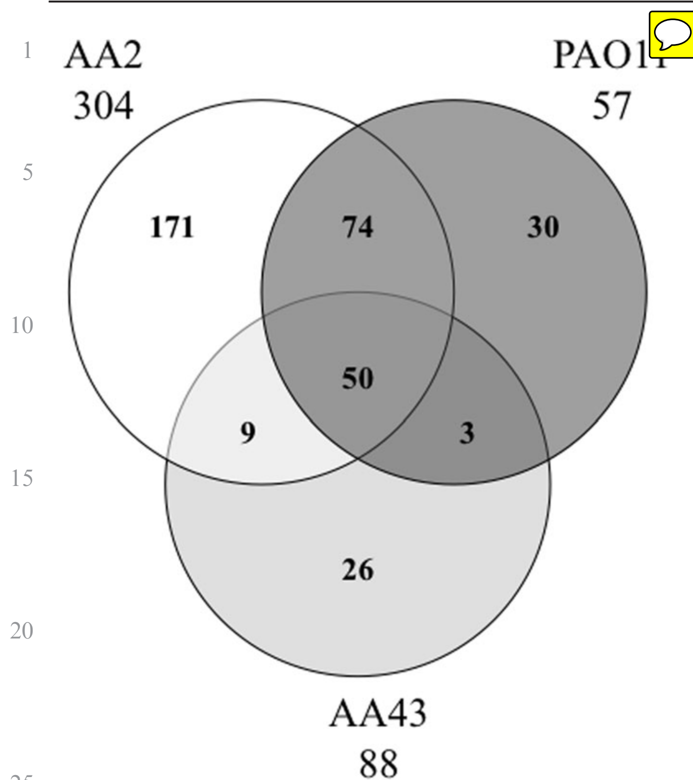

Fig. 2 Venn diagram of the proteins identified by MudPIT experiments. Distribution of the total proteins identified in the CMs of PAO1, AA2 and AA43 grown under aerobic and microaerobic conditions (B).

peptides (see ESI†—Table S1). Specifically, 157, 304 and 88 total proteins were characterized from CM of PAO1, AA2 and AA43, respectively (Fig. 2). Using MAProMa software each sample list was plotted on a 2D map, using theoretical MW and pI of identified proteins. As an example, Fig. S1 (see ESI†) shows a typical 2D map obtained for AA2 strain. The protein lists corresponding to the different conditions and strains (see ESI†—Table S1) were processed by unsupervised hierarchical clustering indicating a grouping affected more by strain type than by culture conditions ($+/-$ O_2). In addition, isolated late strain (AA43) grouped closer to the laboratory one (PAO1) than to the early isolated strain (AA2) (Fig. 3).

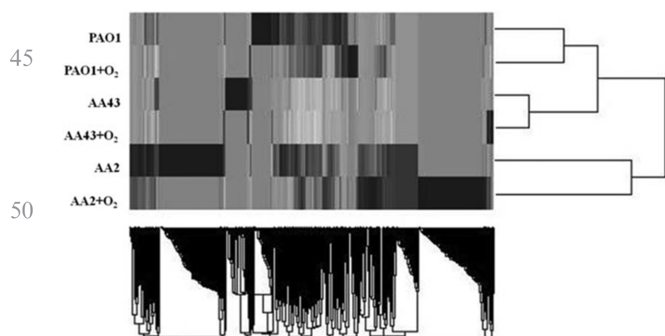

Fig. 3 Unsupervised hierarchical clustering of protein lists identified in CM of PAO1, AA2 and AA43 grown under aerobic and microaerobic conditions. Clustering was performed using *Ward* method distance[47] and the SEQUEST Score values of the proteins identified with high confidence for each analyzed experimental condition (see ESI†—Table S1).

Differential analysis between the different Pa strains and oxygen conditions

In previous works the correlation between Score or Spectral Count values, obtained from the SEQUEST algorithm, and the relative amount of the proteins was demonstrated.[21,22] In this context, using the DAve and DCI algorithms[17] of MAProMa software and taking into account the hierarchical clustering results, the relative protein abundance by means of a label-free quantitative approach was evaluated. In this way, 18 proteins showed differential regulation upon comparing PAO1 and AA2 grown under aerobic conditions: 12 proteins were up- and 6 down-regulated in the CM of the clinical strain (Fig. 4A). While 22 proteins resulted to be differentially regulated upon comparing PAO1 and AA2 grown under microaerobic conditions (Fig. 4B). Some of these proteins presented the same trend comparing laboratory and early isolated strains regardless of the growth conditions. Specifically, flagellar capping protein FliD (GI 15596291) and flagellin type B (GI 15596289) were found up-regulated in the laboratory strain PAO1; while exoenzyme S (GI 15599036) and molecular chaperone DnaK (GI 15599955) showed up-regulation in the early isolated clinical strain AA2.

The role of oxygen limitation, a condition associated with acute and chronic flogosis and/or as a consequence of biofilm creation,[6,9] was also evaluated by comparing the proteins released by each strain grown under aerobic and microaerobic conditions. Of note, a probable binding protein component of ABC (GI 15597400) presented a similar regulation from aerobiosis to microaerobiosis for both laboratory and clinical strains (see ESI†—Fig. S2).

To gain more insights on the role of candidate virulence factors and at the same time to further validate our approach, two pro-inflammatory candidates, the exoenzyme S and the alkaline metalloprotease AprA, characterized by up-regulation in the AA2 CM compared to the PAO1 one, were selected (Fig. 4). On the other hand, alkaline metalloprotease AprA and exoenzyme S resulted to be up- and down-regulated, respectively, when AA2 strain was grown under aerobic conditions (see ESI†—Fig. S2).

Western blot analysis confirmed that, in aerobiosis, exoenzyme S was undetectable in PAO1 but it was present in AA2 strain. In microaerobiosis, it was identified in the PAO1 strain and found up-regulated in AA2; while it was undetectable in AA43 CM (Fig. 5, lane A). Concerning alkaline metalloprotease AprA, functional assay (zymography) and Western blot analysis confirmed that this protein was more expressed in AA2 strain (specifically up-regulated in aerobiosis). In contrast, its level in PAO1, like in AA43, was undetectable in microaerobiosis and moderately increased under aerobiosis conditions (Fig. 5, lanes B and C).

Metalloprotease activity in a large set of sporadic and chronic isolated clinical Pa strains

Several proteases, including metalloproteases (MMPs), were identified by the MudPIT analyses. Using high stringency thresholds for DAve and DCI algorithms (0, 4 and 400, respectively), only AprA showed differential regulation. However, other proteases presented a similar behaviour;

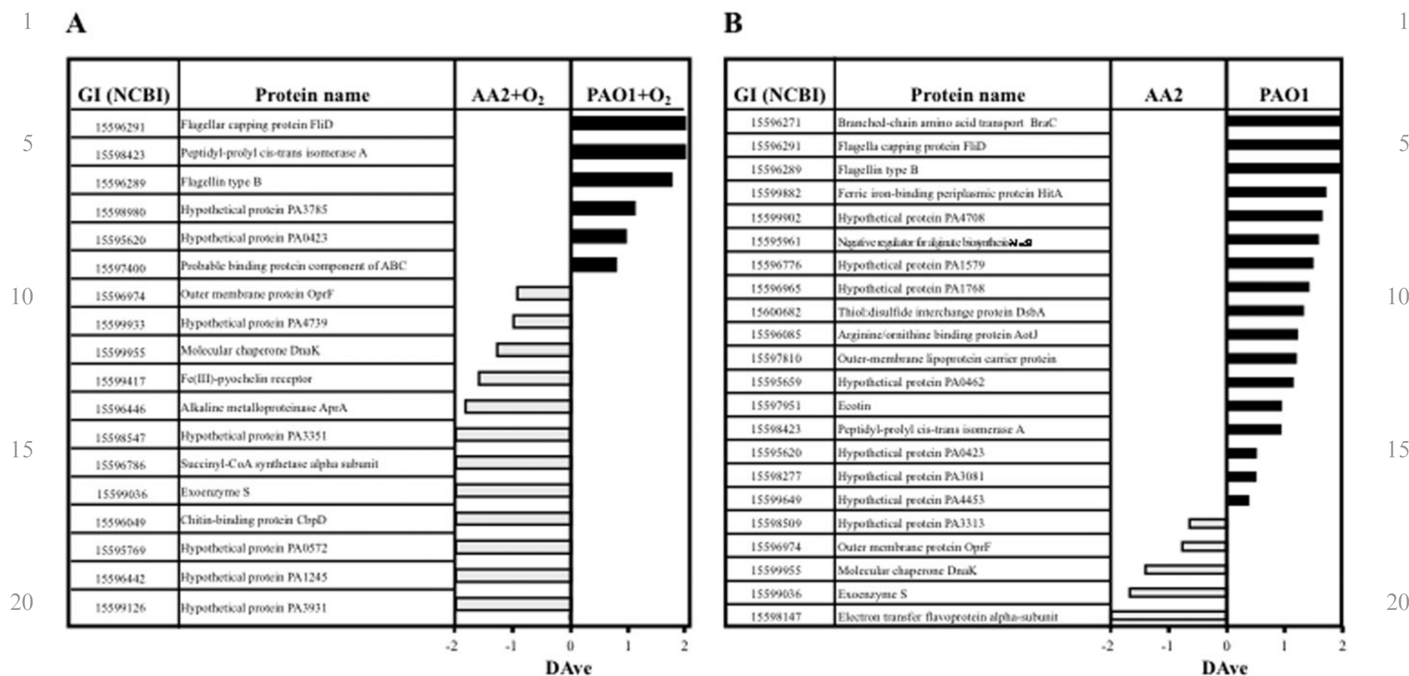

Fig. 4 Differentially regulated proteins identified in PAO1 *versus* AA2, under aerobic (A) and microaerobic conditions (B). Positive DAve values (black bars) correspond to up-regulation in PAO1 *versus* AA2, negative DAve values (grey bars) indicate down-regulation in PAO1 *versus* AA2.

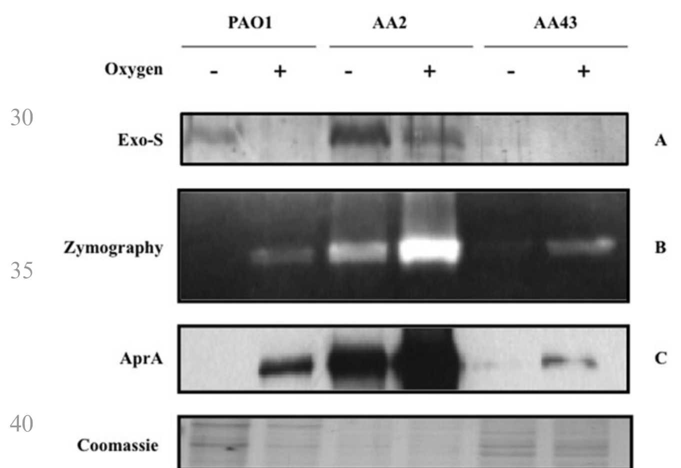

Fig. 5 Target validation. Western blotting analysis of exoenzyme S in CM of PAO1, AA2 and AA43 strains grown in microaerobiosis in comparison to aerobiosis (lane A). Zymography assay (lane B) and Western blot analysis (lane C) of alkaline metalloproteinase AprA in CM of PAO1, AA2 and AA43 strains grown in microaerobiosis in comparison to aerobiosis. Coomassie stain of the CM utilized for the assay is shown.

in particular, their abundance increased under aerobic conditions (see ESI†—Table S2). Of note, a protease inhibitor, the ecotin (GI 15597951), was readily identified to be up-regulated in the CM of PAO1 (Fig. 4B and ESI†—Table S2).

For evaluating the impact of the MMPs, their activity and the specific expression of AprA were assayed in a large set of clinical strains isolated from two groups of CF patients selected on the basis of the type of colonization ($n = 82$). In particular, MMP activity was detected in 76% (29 of 38) and 27% (12 of 44) of sporadic and chronic strains, respectively

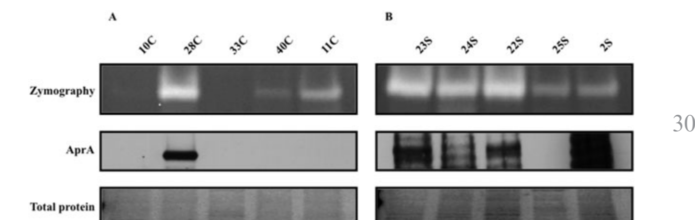

Fig. 6 Target validation in a large set of clinical isolated Pa strains. Zymography and Western blot analysis of alkaline metalloproteinase AprA in CM of clinical isolated Pa chronic (A) and sporadic (B) strains. Pa strains were isolated from patients identified as chronically and sporadically infected (see methods for description of clinical criteria).

($p < 0.0001$, Fisher's exact test; see ESI†—Tables S3 and S4). In order to evaluate whether the MMP activity detected was associated with alkaline metalloprotease AprA, we investigated the presence of this protein in a subset of both chronic and sporadic strains selected for a positive MMP activity. As AprA was not detected in all of the strains positive for MMP activity, the presence of other gelatinases is the most likely explanation (Fig. 6A and B). These findings confirm the MudPIT data concerning the presence in CM of other proteases (see ESI†—Table S2).

Metalloproteases cleave CXCR1 on the surface of neutrophils (PMNs)

In CF individuals it has been reported that neutrophil-derived proteases could mediate the cleavage of CXCR1 on their surface, thus disabling their bacterial-killing capacity.[19] To test whether also Pa-derived proteases could contribute to cleave CXCR1, peripheral blood neutrophils purified from healthy donors were treated for 2 hours at 37 °C with CM obtained from laboratory (PAO1) and clinical strains

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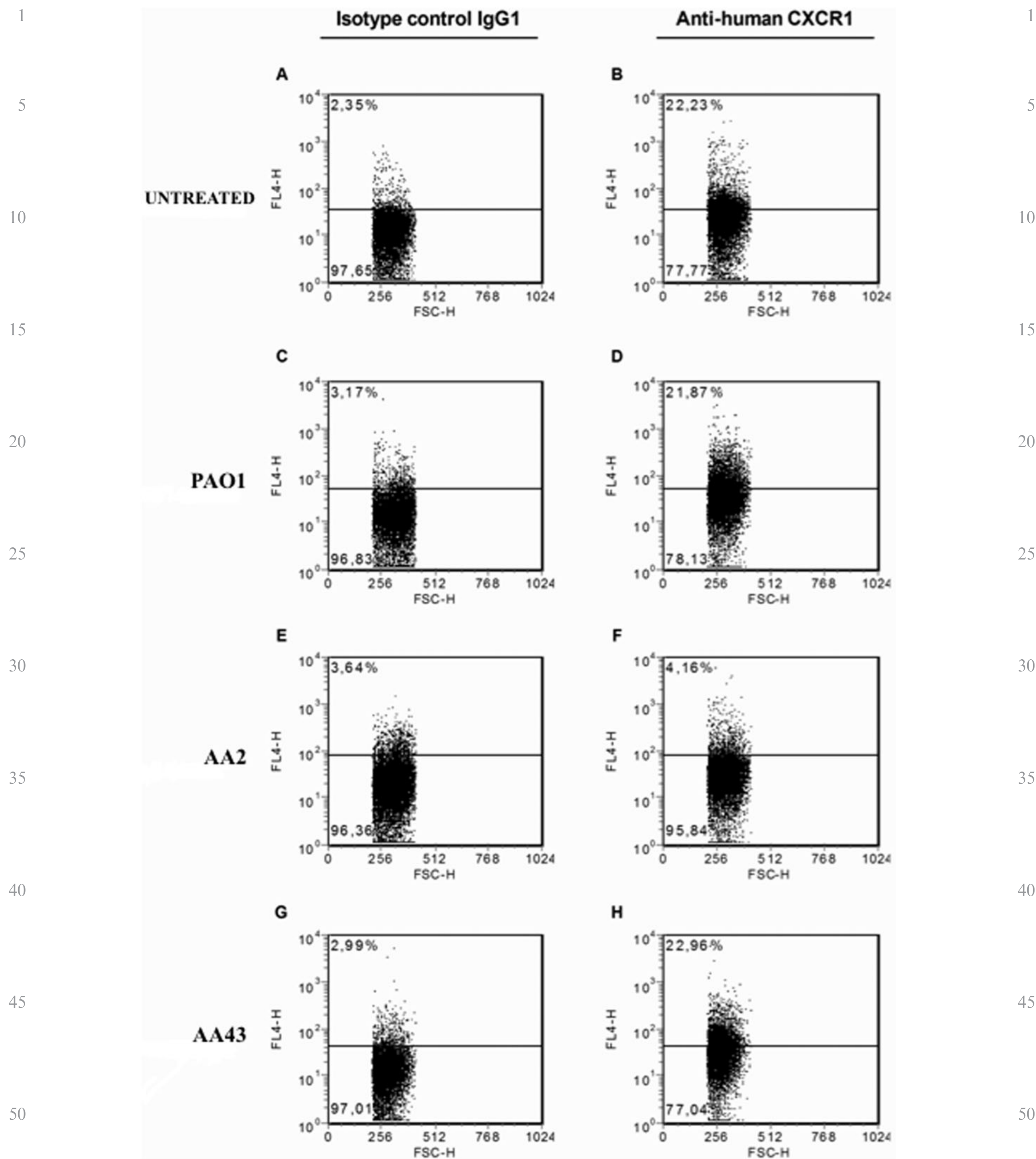

Fig. 7 Flow cytometric analysis of CXCR1 expression on peripheral blood neutrophils (PMNs). Purified peripheral blood PMNs, isolated from the buffy coat of healthy donors, were incubated for 2 hours at 37 °C in media alone (A–B) or with 10% of CM 5X from PAO1 (C–D), AA2 (E–F) and AA43 (G–H) cultured under aerobic conditions. Cells were labeled with an Alexa Fluor® 647 anti-human CXCR1 to visualize cell surface receptors and an antibody isotype IgG1 control. Data are represented as dot plots with the *x*-axis indicating Forward Light Scatter and the *y*-axis indicating CXCR1 fluorescence intensity measured on a log10 scale.

(AA2 and AA43) grown under aerobic conditions. A strong decrease of CXCR1 expression (80% reduction of MFI) followed the treatment with CM derived from AA2 clinical strain (Fig. 7). In contrast, CXCR1 surface expression on PMNs incubated with PAO1 and AA43 CM was almost unaffected, in line with the reduced MMP activity detected in these strains.

Discussion

Inflammation in cystic fibrosis is a hallmark of the disease and the causative event associated with the negative outcome of the patients who almost invariably suffer and eventually die, if not subjected to organ transplantation, because of inflammation-driven chronic pulmonary insufficiency. The expression of the cytokine IL-8 in human bronchial epithelial cells exposed to CM derived from Pa strains indicated that clinical strains isolated during the early phase of lung colonization (AA2) had a pro-inflammatory capability higher than induced by the Pa strain isolated during chronic colonization (AA43) (Fig. 1).

30 kDa cut-off ultra-filtered CM and the observed decrease of inflammation, after incubation at 37 °C (see the Results section), suggest that the pro-inflammatory effect is for a major part associated to proteins. In this context it is interesting to note that AA2 released more or less twice as many proteins as PAO1 and AA43 (Fig. 2). The use of CM obtained from cells grown at the same optical density, during the exponential growing phase, and the correlation with the same number of colony-forming units per millilitre (see the Materials and Methods section) excluded the possibility that the differential protein expression was due to a different degree of cell lysis/death.[23] Furthermore, these findings are in agreement with those reported by Wehmhöner and colleagues who investigated on Pa released proteins, by means of 2-D gel electrophoresis, reporting the interclonal and intraclonal diversity of the secretomes of cultured *P. aeruginosa*.[14] The hypermutability of the early isolated clinical strains is used to perform a more complex protein synthesis program necessary for their adaptation to the airways of CF patients.[24] However, Pa strains isolated during chronic colonization decrease their pathogenicity in order to survive and successfully colonize the host.[25] In fact, unsupervised cluster analysis of the identified protein profiles indicates that chronic strain (AA43) grouped closer to PAO1 than to the sporadic one (AA2). In this context, the comparison between laboratory and sporadic strains evidenced 34 total proteins to be differentially expressed (Fig. 4). In particular, our data concerning two of these proteins, exoenzyme S (GI 15599036) and alkaline metalloproteinase AprA (GI 15596446), are confirmed also by literature data and represent interesting candidates to act as pro-inflammatory molecules.[15,26]

Another polypeptide identified in our analysis is exoenzyme S, a virulence factor delivered to target cells by means of a type III contact-dependent mechanism or acting as a soluble extracellular factor. It causes cytotoxicity by inducing apoptosis of T cells as well as activating monocytes which lead to the production of pro-inflammatory cytokines and chemokines.[27] In addition, an interaction between its C-terminal domain and 14-3-3 proteins of eukaryotic cells has been reported.[28,29]

In our MudPIT analyses, exoenzyme S was detected in PAO1 and, at higher levels in AA2 strain grown under microaerobiosis. However, aerobiosis induced a down-regulation of the protein level, a result in contrast with the increased expression of IL-8 measured in epithelial cells (Fig. 1 and 5, lane A). Concerning the alkaline metalloproteinase AprA, MudPIT data indicating its up-regulation in sporadic strain and aerobiosis conditions were confirmed by zymography and Western blot analysis. These findings suggested that the proteolytic activity was mainly due to AprA and it was seen to be associated with the IL-8 expression level (Fig. 1 and 5). Although the laboratory strain PAO1 expresses detectable amounts of AprA the lower induction of pro-inflammatory IL-8 mRNA expression by the PAO1 CM could be explained by the presence of high levels of ecotin (Fig. 4B and ESI†—Table S2), a dimeric periplasmic protein acting as a potent inhibitor of many trypsin-like serine proteases.[30]

The investigation of 82 CF patients, characterized by detailed genetic and clinical records (see ESI†—Tables S3 and S4), shows the high percentage of correlation among sporadic Pa infection and MMP activity, although the anti-AprA antisera did not react with all the samples showing proteolytic activity. This observation is consistent with the heterogeneity of MMPs responsible for proteolytic activity and is in keeping with the reported variability of Pa secretomes.[14] Moreover, it suggests that the relative amount of MMPs, as well as their inhibitors, must be taken into account when the biological effects of the CM are evaluated. In fact, other proteases were identified in the sporadic strain CM grown under aerobic conditions (see ESI†—Table S2). Some of these proteins, such as LasA protease, elastase LasB and Protease IV, have been previously described as released, playing an important role in the pathogenesis of Pa infection through the activation of protease-activated receptor 2 (PAR-2), modulating host inflammatory and immune response.[31] They are known for their strong proteolytic activities responsible for the rupture of the tight-junction of epithelium leading to tissue invasion and spreading of bacteria.[32] In addition, also aprX (synonym of PA1245) and aprE were found to be specifically identified in AA2 grown under aerobic conditions (Fig. 4A and ESI†—Table S1); their genes belong to the same transcriptional unit and they are implicated in the secretion of alkaline protease.[33]

The identified proteases could contribute to the degradation of biologically active proteins involved in host inflammation, as it has been reported in *Serratia marcescens* and *P. aeruginosa* where MMPs mimic the action of the endogenous shedding proteinase.[34] Recently, studies have implicated the activity of free airway elastase in the cleavage of CXCR1 in the airways of CF individuals.[19] In the same way, other studies showed that the proteolytic loss of CD35 (complement receptor-1) on neutrophils impaired the opsonophagocytosis and killing of *P. aeruginosa* in cystic fibrosis lung disease.[35,36] The selective cleavage of CXCR1 by the CM from AA2 strain indicates that microbial proteases may likely be involved in the modification of phagocytes' activity within the host organism. It is possible to speculate that the effect of released MMPs could be more relevant in the early phases of infection, when neutrophils encounter for the first time the microorganism and the need to reduce cell aggressiveness toward the microorganism is

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critical for its survival. Later, when inflammatory cells become predominant over bacteria the infection becomes chronic and bacteria switch to a less virulent phenotype.

Hierarchical clustering indicates a major difference in protein profiles due mainly to the strain type than the oxygen concentration. On the other hand, IL-8 mRNA relative expression shows a strong induction of the pro-inflammatory activity by clinical strains (AA2 and AA43) in comparison to the laboratory one (Fig. 1). The hypothetical protein PA3313, an outer membrane-bound periplasmic space protein, involved in phosphate transmembrane transporter activity was the only protein found increased following the shift from microaerobiosis to aerobiosis in AA2 and AA43 strains, while it was unchanged in PAO1. Its role as a putative Pa virulence factor deserves additional investigations.

Conclusion

The differential patterns of proteins released by clinical and laboratory strains represent an effective strategy to dissect the role and synergy of these proteins in the pathogenesis of CF lung disease. Bacterial MMPs were identified as putative biomarkers associated to Pa pro-inflammatory activity, and their activity was confirmed to be present in majority of sporadic strains isolated from different patients. MMP activity and Pa virulence appear related, an observation further supported by the functional effect, like the CXCR1 cleavage on the surface of neutrophils we have reported. Future evaluation of MMP activity in an even larger series of clinical isolates collected for specific clinical parameters will provide more insights on the correlation with lung function in patients colonized by Pa strains. In addition, the investigation of the complex mechanisms of Pa adaptation to the microaerobic environment of CF lung will help in elucidating the pathogenesis of chronic infection, as well as in finding effective anti-bacterial drugs targeting molecules responsible for the lung colonization. In this context, the MudPIT proteomic methodology coupled to a protein interaction network-based approach will be a powerful strategy to investigate the biological pathways involved in pathogenesis and adaptation of *P. aeruginosa* in CF patients and to identify clinically relevant targets.

Experimental

Materials and methods

Collection of bacterial conditioned medium. *P. aeruginosa* PAO1 laboratory strain and two sequential *P. aeruginosa* isolates from a CF patient were chosen from the strains collection of the Cystic Fibrosis (CF) clinic in Hannover (kindly provided by B. Tummler, Medizinische Hochschule, Hannover, Germany). *P. aeruginosa* strain AA2 was collected at the onset of chronic colonization from the second positive culture and it was indicated as early isolate while the strain AA43 was defined as late and it was collected 7.5 years after colonization. Each strain was inoculated onto Difco ™ Tryptic Soy Agar (TSA: Becton, Dickinson and Company, Le Pont de Claix, France) plates and allowed to grow at 37 °C overnight. They were then inoculated into modified Vogel-Bonner medium (MVBM)[37] and incubated overnight with continuous agitation. The day after, Pa cells were diluted in MVBM to the concentration of 1×10^8 cfu ml^{-1} (OD of 0.1 at 600 nm). The cultures were incubated at 37 °C for 16 hours with continuous agitation in aerobiosis while anaerobiosis condition was established using an anaerobic jar by adding a sachet containing ascorbic acid as active component (Oxoid, Basingstoke, UK).

By adding MVBM the cultures were normalized to an optical density of 0.2 OD at 600 nm. Their conditioned medium (CM) was collected by centrifugation (7000 × *g*, 30 min, 4 °C) and filtered through a 0.22 μm filter to remove any remaining bacteria. Afterwards they were concentrated about 17-fold through centrifugation (800 × *g*, 10 min, 25 °C) with Amicon® Ultra-15 30K NMWL centrifugal filter devices (Millipore Corporation, Bedford, MA, USA) and precoated with 10mg ml^{-1} bovine serum albumin (BSA, Sigma-Aldrich). Finally, the samples were ultra-centrifuged at 70 000 × *g* for 1 h at 4 °C and each CM was subjected to gel filtration by means of PD-10 Desalting Columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) followed by filtration through a 0.22 μm filter and stored at −80 °C.

Pa strains isolated from a total of 82 CF patients featuring sporadic or chronic colonization, followed up at the Cystic Fibrosis Center of Verona, were also cultured. Chronic colonization was defined as the isolation of at least 3 isolates within 6 months (minimum 30 days interval) while sporadic colonization referred to the isolation of Pa in the bronchial tree in the presence or absence of direct or indirect signs of inflammation.[38] This study was conducted according to the principles expressed in the Declaration of Helsinki. The Institutional Review Board of Azienda Ospedaliera Universitaria Integrata di Verona approved the study as project 1612. All patients provided written informed consent for the collection of samples and subsequent analysis. The day after inoculation onto TSA plates, these Pa clinical isolates were treated exactly as described with the exception of concentration step.

Epithelial cell cultures. An epithelial respiratory cell line (16HBE14o- AS3) with CF phenotype, lacking CFTR expression following transfection with an antisense CFTR sequence phenotype (a kind gift from P. Davis, Case Western Reserve University, Cleveland, OH, USA),[39] was grown in Eagle's MEM (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (Cambrex Bio Science), 1% L-glutamine (Cambrex Bio Science) and 0.4% G418 sulfate (Calbiochem, CN biosciences, La Jolla, CA, USA).

The cell line was cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded in a concentration of 4.5×10^5 cells cm^{-2} and, after 24 hours, were exposed for 4 hours to 10% of CM derived from clinical and the laboratory Pa strains. Under these conditions, as determined by the Tripan Blue exclusion test, the cell viability was $>95\%$.

Protease digestion of conditioned medium. The CM derived from the sporadic strain (AA2) grown in aerobiosis was incubated at 37 °C overnight with and without trypsin (Sigma-Aldrich) of concentrations ranging from 200 ng ml^{-1} to 3.125 ng ml^{-1}. It was then treated, for 1 hour at room temperature, with 250 μM of

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trypsin inhibitor from Glycine max (Sigma-Aldrich Inc., St Louis, MO, USA). Finally, it was subjected to a gelatin/zymography assay to evaluate the extent of protease activity and select the appropriate concentration of trypsin (25 ng ml^{-1}) to degrade the polypeptides in AA2 CM.

RNA isolation, reverse transcription and quantification. The 16HBE14o- AS3 cells were lysed. Total RNA was extracted with the Total RNA Isolation kit (Roche, Germany) and converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, USA). The reaction was then incubated at 25 °C for 10 min and at 37 °C for 2 hours. Relative quantification of gene expression was performed by real-time quantitative PCR analysis as previously described.[40] Results were expressed as mean ± standard deviation (SD).

Proteomics analysis. A total of 200 μL of each CM sample was concentrated to 20 μL and brought to pH 7.9 by the addition of ammonium bicarbonate, obtaining a protein concentration of about 1 μg μL^{-1}. One μg aliquot of sequencing grade modified trypsin (Promega Inc., Madison, WI, USA) was added and the formic acid (Sigma-Aldrich Inc., St Louis, MO, USA) and the sample were desalted and concentrated using PepClean C-18 Spin Columns (PIERCE Biotechnology Inc., Rockford, IL, USA). The eluent was dried in a vacuum system and reconstituted in 5% acetonitrile, 0.1% formic acid.

Trypsin-digested samples were analyzed by two dimensional micro-chromatography coupled to ion trap mass spectrometry (2DC-MS/MS, also referred to as Multidimensional Protein Identification Technology, MudPIT),[41] by Thermo Electron Corporation (San José, CA, USA). To identify the maximum number of proteins and to verify the reproducibility of our approach multiple biological and technical replicate analyses, per condition, were performed (n = 7).

Briefly, 10 μl of the digested peptide mixture was loaded onto a strong cation exchange column (Biobasic-SCX column, 0.32 i.d. 100 mm, 5 μm, Thermo Electron Corporation, Bellefonte, PA, USA), eluted stepwise with salt injections of increasing molarity (0, 20, 40, 80, 120, 200, 400, 600, 700 mM) and then captured in turn onto two peptide traps (Zorbax 300 SB-C18, 0.3 i.d. × 5 mm, 5 μm, Agilent Technologies, Palo Alto, CA) for concentration and desalting prior to separation on a reversed-phase C_{18} column (Biobasic-18, 0.180 i.d. 100 mm, 5 μm, Thermo Electron Corporation). Additional chromatographic and mass spectrometry details are reported by De Palma *et al.*[41]

The experimental mass spectra produced by MudPIT analyses were correlated to *in silico* peptide sequences of *P. aeruginosa* protein database (5561 entries) retrieved from NCBI in 2010 (http://www.ncbi.nlm.nih.gov/); data processing was performed by means of Bioworks 3.3.1, based on the SEQUEST algorithm.[42] The validity of peptide/spectrum matches was assessed using SEQUEST defined parameter thresholds. Specifically, matching between spectra was only retained if they had a minimum Xcorr of 1.5 for +1, 2.0 for +2 and 2.5 for +3 charge state. In addition, the thresholds of peptide/protein probability were determined to be $\leq 10^{-3}$ while the protein consensus score value ≥ 10.[43] The percentage of the false positives identification was estimated processing the raw mass spectra by means of the reverse database of *P. aeruginosa* and the procedure revealed a false positives rate less than 5% (data not shown).[44]

Protein lists were compared and the differential expression evaluation was performed by the MAProMA software as previously reported.[21,22] Unsupervised cluster analysis[45] was performed using a free of charge software such as Rapid Miner (http://rapid-i.com/). In addition, in-house R-scripts, based on *XlsReadWrite, clue, clValid* library, were employed (http://cran.r-project.org).

Gelatin/zymography for metalloprotease activity. The CM samples were treated as follows: 5 μl of 5X SDS sample buffer (5% SDS, 0.5 M Tris-HCl pH 6.8, 25% glycerol) were added to 20 μl of CM. The sample was run on a SDS-PAGE gel containing 1 mg ml^{-1} gelatin (Sigma-Aldrich). The gel was washed twice (20 min per cycle) with 2.5% Triton X-100 at room temperature, then incubated in 200 ml of activation buffer (10 mM Tris-HCl, 1.25% Triton X-100, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) overnight at 37 °C and finally stained with Coomassie Brilliant Blue G-250 in 20% methanol/10% phosphoric acid/10% ammonium sulfate and destained in water.

Western blot analysis of exoenzyme S and alkaline metalloprotease AprA. Proteins were precipitated from 12 ml of CM by drop-wise addition of 10% (final concentration) trichloroacetic acid with stirring at 4 °C. The sample was then centrifuged at 3000 × g for 30 min and washed 3 times with an excess of an acetone : methanol (8 : 1) mixture. The pellet was air-dried, resuspended in SDS sample buffer and subjected to SDS-PAGE and Western blotting. The sample proteins (20 μl per lane) were electrophoresed on SDS-PAGE using 10% acrylamide gel and transferred onto a nitrocellulose membrane (HybondTM ECLTM, Amersham, NJ, USA), using a mini trans-blot apparatus (Bio-Rad, California, USA) following the manufacturer's instructions. To verify equal loading, proteins were separated in 10% polyacrylamide gel containing SDS and stained with Coomassie Brilliant Blue G-250 in 20% methanol/10% phosphoric acid/10% ammonium sulfate, destained in water and examined for staining intensity. Non-specific binding on the membrane was blocked with 5% dry non-fat milk or 5% bovine serum albumin (BSA, Sigma-Aldrich) in TBS-T buffer (0.2% Tween 20 in Tris-buffered saline, pH 7.5) for 1 h at room temperature. The membrane was incubated with a 1 : 1000 dilution of chicken polyclonal antibody raised against exoenzyme S (Abcam plc. Cambridge, UK) or with a 1 : 500 dilution of purified rabbit IgG against alkaline metalloprotease AprA,[18] in TBS-T with 1% BSA overnight at 4 °C. The blot was washed four times in TBS-T and then incubated for 1 h at room temperature with a goat anti-chicken IgG secondary antibody conjugated to biotin (SouthernBiotech, Birmingham, AL, USA) diluted 1 : 25 000 or a donkey anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Amersham, NJ, USA) diluted 1 : 15 000 in TBS-T with 1% BSA. After washing bound HRP was detected using the ECL detection system (Millipore Corporation, Bedford, MA, USA).

Flow cytometry analysis of conditioned medium-treated neutrophils. Peripheral blood neutrophils (PMNs) were purified from the buffy coat of healthy donors as previously described.[46]

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After dextran sedimentation (Destran T500, Pharmacia, Uppsala, Sweden) and hypotonic lysis of contaminating erythrocytes, the PMN pellet was washed in PBS counted and resuspended in Hank's balanced salt solution (HBSS) with 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ (Gibco Laboratories). The neutrophils were incubated at 37 °C for 2 hours with 10% of CM 5X from clinical (AA2 and AA43) and laboratory (PAO1) strains grown in aerobiosis. Fresh and fixed neutrophils underwent 15 min Fc blocking. They were then incubated with an Alexa Fluor® 647 anti-human CD 181 (CXCR1) antibody (BioLegend, San Diego, CA, USA) for 15 min at room temperature, washed three times with PBS and finally analyzed by four-color flow cytometry (FACSCalibur, Becton-Dickinson); twenty thousand neutrophils were analyzed per sample. Gating of neutrophils was based on light scatter properties and positive expression for CD45, CD16 and CD15. Data treatment and analysis was performed with Cell Quest analysis software (Becton-Dickinson).

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