**Pseudomonas aeruginosa** proteolytically alters the interleukin 22-dependent lung mucosal defense

Antoine Guillon, Deborah Brea, Eric Morello, Aihua Tang, Youenn Jouan, Reuben Ramphal, Brice Korkmaz, Magdiel Perez-Cruz, François Trottein, Richard J. O’Callaghan, and Mustapha Si-Tahar

Institut National de la Santé et de la Recherche Médicale, Center d’Etude des Pathologies Respiratoires (CEPR), INSERM UMR 1100, Tours, France; Université François Rabelais de Tours, Tours, France; CHRU de Tours, Service de Réanimation Polyvalente, Tours, France; Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, MS, USA; Institut Pasteur de Lille, Center d’Infection et d’Immunité de Lille, Lille, France; Université Lille Nord de France, Lille, France; Centre National de la Recherche Scientifique, UMR 8204, Lille, France; Institut National de la Santé et de la Recherche Médicale, U1019, Lille, France

**ABSTRACT**

The IL-22 signaling pathway is critical for regulating mucosal defense and limiting bacterial dissemination. IL-22 is unusual among interleukins because it does not directly regulate the function of conventional immune cells, but instead targets cells at outer body barriers, such as respiratory epithelial cells. Consequently, IL-22 signaling participates in the maintenance of the lung mucosal barrier by controlling cell proliferation and tissue repair, and enhancing the production of specific chemokines and anti-microbial peptides. **Pseudomonas aeruginosa** is a major pathogen of ventilator-associated pneumonia and causes considerable lung tissue damage. A feature underlying the pathogenicity of this bacterium is its capacity to persist and develop in the host, particularly in the clinical context of nosocomial lung infections. We aimed to investigate the ability of **P. aeruginosa** to disrupt immune-epithelial cells cross-talk. We found that **P. aeruginosa** escapes the host mucosal defenses by degrading IL-22, leading to severe inhibition of IL-22-mediated immune responses. We demonstrated in vitro that, protease IV, a type 2 secretion system-dependent serine protease, is responsible for the degradation of IL-22 by **P. aeruginosa**. Moreover, the major anti-proteases molecules present in the lungs were unable to inhibit protease IV enzymatic activity. In addition, tracheal aspirates of patients infected by **P. aeruginosa** contain protease IV activity which further results in IL-22 degradation. This so far undescribed cleavage of IL-22 by a bacterial protease is likely to be an immune-evasion strategy that contributes to **P. aeruginosa**-triggered respiratory infections.

**Introduction**

Ventilator-associated pneumonia is the most frequent hospital-acquired infection in patients requiring mechanical ventilation and its estimated incidence is 10–20%. In addition to being an independent factor for mortality, ventilator-associated pneumonia is associated with longer intensive care unit and hospital stays, prolonged mechanical ventilation, and higher costs. **Pseudomonas aeruginosa** is a major pathogen of nosocomial pneumonia. During invasive mechanical ventilation, **P. aeruginosa** lung infection progresses rapidly, is difficult to eradicate and is life-threatening. Many patients require antibiotic treatment to treat such infections, but this is also a recognized risk factor for the emergence of multi-drug resistance bacteria. Rates of antibiotic resistance by **P. aeruginosa** are increasing worldwide. Another critical feature underlying the pathogenicity of this bacterium is its capacity to evade the host’s immune response and thus to persist in the host. Thus, accurately deciphering the mechanisms by which **P. aeruginosa** circumvents the host immune response is a necessary milestone for innovating therapeutic development.

The respiratory epithelium, a component of the lung immune system, has mechanisms that provide a first line of defense against invading pathogens. The interleukin (IL)-22 was recently shown to be a key mediator of mucosal innate immunity. IL-22 (a member of the IL-10 family) is likely to be an immune-evasion strategy that contributes to **P. aeruginosa**-triggered respiratory infections.
cytokine) is an important component of immune-epithelial cells cross-talk and plays a critical role in regulating the maintenance of the mucosal barrier. Consequently, IL-22 is crucial for limiting bacterial replication and dissemination, probably by stimulating epithelial cells at barrier surfaces to produce antimicrobial peptides, such as β-defensin-2 that plays a pivotal role in the control of *P. aeruginosa* infection. IL-22 also directly targets cells at outer-body barriers, such as respiratory epithelial cells, to induce expression of genes involved in tissue homeostasis and control the repair and turnover of lung epithelia cells damaged by infection.

Lung tissue damage is a hallmark of *P. aeruginosa*-induced lung pathogenesis. *P. aeruginosa* produces many virulence factors, including secreted proteases that can degrade natural proteins, enabling bacteria to evade the host immune system and colonize host tissues. In view of the major role of IL-22 in innate defense mechanisms, we sought to determine the consequences of *P. aeruginosa*-secreted proteases on IL-22 signaling. We demonstrate that *P. aeruginosa*-derived protease IV is a new immuno-evasion factor that weakens mucosal innate defenses by proteolytically decreasing the IL-22-dependent response.

**Results**

**IL-22 is degraded by T2SS-dependent products of *P. aeruginosa***

*P. aeruginosa* produces numerous virulence factors that target several host cell types or molecular effectors, thus manipulating the host immune response to the bacterium’s advantage. Among these factors is a panel of extracellular proteases. We tested the integrity of IL-22 in the presence of the *P. aeruginosa* secretome since IL-22 is a key effector against *P. aeruginosa*-triggered lung alterations.

IL-22 was degraded by ~41%, ~68%, and ~79% after one, 2 and 6 hours exposure to *P. aeruginosa* PAK strain, respectively (Fig. 1A). *P. aeruginosa* releases proteins using 6 different secretion systems (T1SS - T6SS), with T2SS and T3SS being the most responsible for fatalities due to *P. aeruginosa* pneumonia. We used the T3SS-deficient *P. aeruginosa* strain PAK ΔpscF and the double T3SS and T2SS-deficient strain PAK ΔpscF ΔxcpQ to identify the secretion system involved in IL-22 cleavage. IL-22 was strongly degraded after incubation with the PAK ΔpscF secretome (by 70% one hour after challenge), and was barely altered after incubation with the PAK ΔpscF ΔxcpQ secretome (by 20% one hour after challenge) (Fig. 1A). We conclude that IL-22 is cleaved essentially by T2SS secretion products.

The proteins secreted by the T2SS system include a large number of proteolytic enzymes, such as protease IV, elastase B (LasB), alkaline protease (Alk; also secreted by T1SS), and possibly “*P. aeruginosa* small protease” (PASP). We tested the ability of each of these proteases to cleave IL-22. We initially verified the enzymatic activity of each purified protease by gelatin zymography (data not shown). We found that IL-22 was mostly degraded by protease IV (Fig. 1B). We further confirmed this result by comparing the impact on IL-22 integrity of the secretome of a protease IV-deficient *P. aeruginosa* strain (PAK ΔpscF Δpiv) with that of a LasB-deficient *P. aeruginosa* strain (PAK ΔpscF ΔLasB); as LasB is a major extracellular protease of *P. aeruginosa* known to cleave several soluble and matrix proteins. LasB-deficient *P. aeruginosa* efficiently cleaved IL-22 whereas protease IV-deficient *P. aeruginosa* was barely active (Fig. 1C). Thus, protease IV is responsible for the alteration of IL-22 (Fig. 1C).

**Degradation of IL-22 by protease IV inhibits lung epithelial-dependent immune responses**

The role of protease IV in ocular infections has been extensively studied. This *P. aeruginosa* protease also causes considerable damage in keratitis because it destroys host proteins, including fibrinogen and components of the immune system. By contrast, little is known regarding its impact on lung mucosa, although Protease IV was found to be expressed by *P. aeruginosa* lung isolates from chronically infected adult patients. 20 To gain insight into the functional impact of protease IV on *P. aeruginosa*-infected lungs were positive for Protease IV. The protease IV production of numerous *Pseudomonas* strains was also found in all ocular isolates. To gain insight into the functional impact of protease IV on *P. aeruginosa*-infected lungs, we first observed that as little as 0.1 μM protease IV was enough to degrade IL-22 (Fig. 2A), with complete degradation after exposure to 1 μM Protease IV for 10 min. No fragments were observed on the blots (despite the use of a polyclonal antibody). We performed mass spectrometry on IL-22 (0.15 μM) after exposure to protease IV (1 μM) and confirmed that IL-22 was cleaved into several small fragments within 3 minutes of incubation (see Fig S1). We next assessed the biological activity of either intact- or Protease IV-treated IL-22 on human bronchial epithelial cells.

Binding of IL-22 to its receptor induces downstream signaling that requires phosphorylation of the transcription factor STAT3 on Tyr705. Phosphorylated STAT3 then mediates the biological effects of IL-22 on epithelial cells, which both facilitate host defenses and limit lung
injury by regulating the expression of genes that encode regenerative and mitogenic molecules. Bronchial epithelial cells incubated with intact IL-22 had a higher level of phosphorylated STAT3 than resting cells, as expected. In contrast, bronchial epithelial cells incubated with protease IV-treated IL-22 had a substantially lower level of phosphorylated STAT3 than those incubated with intact IL-22, similar to that of resting cells (Fig. 2B). IL-22-induced STAT3 activation regulates the expression of several immunity genes including those encoding antimicrobial peptides such as β-defensin 2, S100A7, S100A12, and lipocalin 2.25–28 

Intact IL-22 induced β-defensin 2 expression (as assessed by RT-qPCR), whereas protease IV-treated IL-22 did not (Fig. 2C). Furthermore, viability of bronchial epithelial cells exposed to a protease IV-deficient P. aeruginosa strain (PAK ΔpscF Δpiv) was higher than viability of cells exposed to an intact, control P. aeruginosa strain (PAK ΔpscF; Fig. 2D).

Altogether, our data demonstrate that protease IV is a virulent factor that can damage the lung epithelium and can also degrade IL-22, thus weakening tissue repair and the anti-microbial defense against P. aeruginosa in the airway epithelium.

Figure 1. The degradation of IL-22 is dependent on P. aeruginosa T2SS and protease IV. (A) IL-22 (50 ng) was incubated with secretomes from a wild-type P. aeruginosa PAK strain, a T3SS-deficient strain (PAKΔpscF), a double T3SS- and T2SS-deficient strain (PAKΔpscΔxcpQ), or Luria broth as a control. (B) IL-22 samples were incubated with the following purified P. aeruginosa proteases: elastase B (LasB), PA small protease (PASP), protease IV (P-IV), or with alkaline protease (Alk). (C) IL-22 was also incubated with secretomes from PAK ΔpscΔLasB (a T3SS and elastase B-deficient strain) or PAK ΔpscΔpiv (a T3SS and protease IV-deficient strain). Proteins were extracted after incubation for various times as indicated in panels (A, C) or after 30 min (B) and analyzed by protein gel blotting using a specific anti-IL-22 antibody. Results are representative of 3 independent experiments.
The lungs contain a battery of antiprotease molecules to counterbalance the often harmful effects of pulmonary proteases. The major serine protease inhibitors (the so-called serpins) present in the lungs include α1-antitrypsin (α1AT), elafin, secretory leukocyte protease inhibitor (SLPI), and α1-antichymotrypsin (α1ACT). We tested whether protease IV enzymatic activity was compatible with the lung environment, rich in protease inhibitors. None of the tested lung antiproteases were able to significantly diminish the proteolytic activity of protease IV (Fig. 3A). The absence of an inhibitory effect was not due to a proteolysis of those antiproteases molecules as protease IV cleaved none of them (Fig. 3B). Remarkably, protease IV was also still active in the presence of plasma, although it contains several potent serpins such as α2-macroglobulin, antithrombin III, C1-inhibitor and α2-antiplasmin in addition to α1AT and α1ACT (Fig. 3). Consistent with a previous report, we were only able to inactivate protease IV using AEBSF and TLCK, 2 irreversible pharmacological inhibitors of trypsin and trypsin-like serine proteases.

**Degradation of IL-22 in human respiratory fluids containing P. aeruginosa protease IV**

The airways of mechanically-ventilated patients are at increased risk of colonization by bacterial pathogens. A significant fraction (10–30%) of patients with...
Figure 3. Assessment of the activity of physiological and synthetic protease inhibitors on protease IV. (A) Protease IV was incubated 30 min with or without the different inhibitors and its proteolytic activity measured using the substrate N-p-Tosyl-Gly-Pro-Lys-pNa. Data (mean ± SEM) are represented as the percentage of inhibition relative to protease alone. (B) SDS-PAGE of α1AT, α1ACT, SLPI and elafin showing the absence of their proteolysis by the protease IV. α1AT (10^{-7} M), α1ACT (10^{-7} M), elafin (3.10^{-7} M) and SLPI (3.10^{-7} M) were incubated with protease IV (10^{-7} M) during 30 min at 37°C and then separated on 12% gradient gel and detected by silver staining.
ventilator-associated trachea-bronchitis progress to ventilator-associated pneumonia. The most common microorganism in such infections is *P. aeruginosa* (30%). We examined whether respiratory fluids of *P. aeruginosa*-infected ventilated patients may contain active protease IV in a concentration sufficient to inhibit the IL-22 signaling pathway. We used 2 types of synthetic peptide substrates to, not only quantify, but also distinguish the distinct protease activities present in these respiratory fluids. Such tracheal aspirates may contain not only *P. aeruginosa* Protease IV but also serine proteases released by polymorphonuclear neutrophils, i.e. elastase, cathepsin G, and protease. The 2 substrates were Abz-HPVPVYAFSPQ-EDDnp and N-p-Tosyl-Gly-Pro-Lys-pNa. Abz-HPVPVYAFSPQ-EDDnp was cleaved by all neutrophil serine proteases but not protease IV (Fig. 4A), whereas N-p-Tosyl-Gly-Pro-Lys-pNa was cleaved only by protease IV (Fig. 4A). Thus, the substrate N-p-Tosyl-Gly-Pro-Lys-pNa is specific for protease IV activity, among pseudomonal and neutrophil serine proteases. We tested N-p-Tosyl-Gly-Pro-Lys-pNa with the secretomes produced by the above-mentioned *P. aeruginosa* mutant strains and confirmed that the T2SS-deficient- or Protease IV-deficient *P. aeruginosa* strains did not cleave N-p-Tosyl-Gly-Pro-Lys-pNa, whereas the other mutant strains did (data not shown). We collected tracheal aspirates from 2 groups of ventilated patients with tracheo-bronchitis, one positive for *P. aeruginosa* infection, the other negative (Table 1). We first measured the concentration of MPO, a potent inflammatory marker associated with neutrophil recruitment and activation, in the respiratory fluids. We found that MPO levels were not related to the presence or absence of *P. aeruginosa* in these samples (Fig. 4B). In

![Figure 4](image_url)

**Figure 4.** Degradation of IL-22 by human respiratory fluids infected by *P. aeruginosa*. (A) *P. aeruginosa* and neutrophil proteases were assayed by incubating each protease with specific chromogenic or fluorogenic peptide substrates, i.e., N-p-Tosyl-Gly-Pro-Lys-pNa and Abz-HPVPVYAFSPQ-EDDnp, respectively, at 37°C for 30 min. Cleavage of the peptide substrates was assessed by measuring the increase of either fluorescence emission or optical density. Gelatin zymography was used to verify the enzymatic activities of all *P. aeruginosa* proteases. The activity of each enzyme was graded on a scale of (−) for “no detectable activity” to (+++) for “maximal activity.” (B) MPO concentration and N-p-Tosyl-Gly-Pro-Lys-pNa cleavage measured in tracheal aspirates from non-infected patients (PA−, n = 10) or patients infected by *P. aeruginosa* (PA+, n = 6). (C) IL-22 (10 ng) was incubated with human respiratory fluids for 15 minutes and quantified by ELISA. Results are representative of 2 independent experiments (A) or expressed as the mean ± SEM (B, C). *: P < 0.05; ns: not significant. LasB: elastase B, P-IV: protease IV, PASP: *P. aeruginosa* small protease, Alk: alkaline protease, SN-PA: supernatant of *P. aeruginosa*, SN-PMN: supernatant of neutrophils, NE: neutrophil elastase, CG: cathepsin G, PR-3: proteinase 3, ND: not determined.
contrast, proteolysis of N-p-Tosyl-Gly-Pro-Lys-pNa was clearly associated with the tracheal aspirates positive for *P. aeruginosa*, indicating the presence of protease IV only in these fluid samples (Fig. 4B). IL-22 was preferentially degraded by the Protease IV-positive fluids when we assessed the stability of IL-22 in the 2 types of tracheal aspirates (Fig. 4C). Comparison of Protease IV activity and IL-22 degradation showed a positive and significant correlation (p < 0.05). We thus demonstrate that respiratory fluids of ventilated patients with *P. aeruginosa* tracheo-bronchitis contain active protease IV which efficiently cleaves IL-22.

**Discussion**

We demonstrate that the protease IV secreted by *P. aeruginosa* degrades IL-22 and that none of the major antiproteases present in the lung environment inhibit protease IV activity. IL-22 cleavage by *P. aeruginosa* leads to the inhibition of IL-22-mediated immunity, markedly altering the host mucosal defense and possibly conferring multiple survival advantages to this opportunistic bacterium. Hence, we have characterized a new *P. aeruginosa* immuno-evasion mechanism, which may contribute to the deterioration of lung function of patients infected by *P. aeruginosa*, including mechanically-ventilated individuals.

*P. aeruginosa* protease IV is known to be a corneal virulence factor, able to degrade several proteins important for innate immunity. Protease IV in the lungs has been poorly studied. *In vitro* observations suggest that *P. aeruginosa* protease IV degrades surfactant proteins, reducing pulmonary surfactant-based host defense and biophysical functions. Our findings demonstrate that protease IV not only degrades a final effector of innate immunity, but also inactivates a key component of immune-epithelial cell cross-talk. Indeed, IL-22 is the only known cytokine secreted by immune cells that acts exclusively on non-immune epithelial cells. Consequently, IL-22 signaling is critical for the preservation of the lung mucosal barrier by increasing cell survival, cell proliferation, cell migration, and tissue repair, and by triggering the production of specific chemokines and anti-microbial peptides. A study of lung infection with *Klebsiella pneumoniae* demonstrated a large, significant increase in mortality in IL22−/− mice due to increased bacterial adhesion and invasion. Furthermore, intranasal instillation of recombinant mouse IL-22 before introduction of a sublethal dose of *P. aeruginosa* increased survival in neutropenic mice.

Animal models are important for the translation of experimental findings from the bench to bedside. We originally intended to compare the in *vivo* response of mice infected either by *P. aeruginosa* or a protease IV-deficient *P. aeruginosa* strain. A model, by definition, is not a perfect replication of the clinical condition, although it is designed for translation to human disease. The human disease herein is ventilator-associated pneumonia due to *P. aeruginosa*. In humans, these infections generally develop over several days and steadily progress from initial respiratory tract colonization to tracheo-bronchitis (defined by fever, increased purulent-appearing endotracheal aspirate, and hyperleukocytosis), and ultimately pneumonia (defined by the previous signs, plus confirmation of lung consolidation by chest X-ray). The biological properties of protease IV may be clinically relevant at early stages of lung infection by *P. aeruginosa*, to counteract innate defense mechanisms. Animal models poorly mimic this progressive spread of lung infection, but rather model acute lung infection or artificial *P. aeruginosa*-biofilm with alginate beads. We chose not to study animal models without a clear translational perspective. In contrast, the strength of our work has been the comparison of our *in vitro* observations with results obtained using respiratory fluids of patients newly infected by *P. aeruginosa*. Indeed, we confirmed the increased instability of IL-22 in these fluids. Direct visualization of IL-22 cleavage fragments in respiratory fluids would have strengthened the relevance of the study, but the complete degradation of IL-22 in *situ* made this impossible.

The treatment of the early stages of nosocomial lung infections is an ongoing matter of debate. Antibiotic treatment of patients newly colonized by *P. aeruginosa* may potentially reduce the incidence of pneumonia, but there is no clear clinical evidence of this. Furthermore, not all cases of respiratory tract colonization convert to pneumonia. Treating all patients with respiratory tract colonization would increase the emergence of multi-drug-resistant bacteria in these critically ill patients. This conundrum highlights the need to improve the therapeutic arsenal against respiratory infections to reduce the need for antibiotherapy. Our findings may lead to the design of new drugs that target host innate immunity to reinstate at least part of the immune defenses altered by pseudomonal proteases. Protease IV appears to be a promising antimicrobial therapeutic

**Table 1. Bacterial Strains used in this study were as follows.**

| Strain | Relevant characteristics | Reference |
|--------|--------------------------|-----------|
| PAK    | Wild-type clinical isolate | D. Bradley 16 |
| PAK ΔpscF | In-frame partial deletion of pscF from bp 78–213 in PAK strain | 16 |
| PAK ΔpscF-ΔxcpQ | In-frame deletion of xcpQ in PAK ΔpscF | This study |
| PAK ΔpscF-ΔlasB | In-frame deletion of lasB in PAK ΔpscF | This study |
| PAK ΔpscF-Δpiv | In-frame deletion of piv in PAK ΔpscF | This study |

Bacterial Strains used in this study were as follows.
target for the protection of IL-22 signaling to restore or prevent \textit{P. aeruginosa}-triggered alterations of lung mucosal defenses.

In conclusion, protease IV plays an important role in the pathogenesis of \textit{P. aeruginosa}-induced microbial keratitis, but little is known of its role in lung infection. Our findings suggest that protease IV reduces the innate immunity of the host by disrupting signaling from immune cells to epithelial cells. Altogether, these \textit{in vitro} and \textit{ex vivo} observations, combined with previous results,\textsuperscript{22,34} suggest that \textit{P. aeruginosa} protease IV may play a significant role in both colonization of the lung and the progression of associated lung tissue damage.

**Materials and methods**

**Materials**

Human recombinant IL-22 was obtained from ImmunoTools GmbH and the anti-IL-22 antibody from Abcam. Antibodies against phospho-STAT3 (Tyr705) and STAT3 were purchased from Cell Signaling Technology. The human bronchial epithelial cell line BEAS-2B was supplied by the American Type Cell Collection. Purified \textit{Pseudomonas} proteases including elastase B, alkaline protease, protease IV, and PA small protease were produced by the group of Dr. Richard O’Callaghan (University of Mississippi Medical Center, Jackson, MS, USA).\textsuperscript{38} The substrates used to measure protease activities were Abz-HPVPVYAFSPQ-EDDnp obtained from GeneCust Europe and N-p-Tosy-L-Gly-Pro-Lys-pNa purchased from Sigma-Aldrich. IL-22 and myeloperoxidase (MPO) concentrations were determined using DuoSet ELISA assay kits (R&D Systems). The materials and methods for RT-qPCR and mass spectrometry have been previously described.\textsuperscript{39–41} α1AT and ACT were purchased from Preparatis. Pepstatin, Tosyl-L-lysyl-chloromethane hydrochloride, and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride were obtained from Enzo Life Sciences. Aprotinin and SLPI were purchased from Abcam. Elafin was produced as previously described.\textsuperscript{42}

**Bacterial strains**

The \textit{P. aeruginosa} PAK strain used for mutant constructions was grown in Luria broth at 37°C with shaking at 180 rpm or on 1.5% L-agar plates with or without gentamicin (Gm). The plates used for sucrose selection contained 10% sucrose. Gm selection of \textit{Escherichia coli} was achieved using a final Gm concentration of 15 µg/mL and Gm selection of \textit{P. aeruginosa} was performed using 50 µg/mL Gm. Deletion mutants of \textit{lasB} and \textit{piv} were constructed in the PAK\textit{ΔpscF} strain using the pEX vector and a sucrose-selectable marker.\textsuperscript{43} Each gene was deleted in-frame by cloning 1-kb DNA fragments upstream and downstream of the region to be deleted into the pEX18Gm vector. The pEX deletion plasmid was verified by sequence analysis and then inserted into the PAK\textit{ΔpscF} mutant strain by electroporation. The gentamicin single crossovers were resolved by late log phase growth in LB and plating on LB agar and sucrose plates. Sucrose-resistant colonies were then screened for gentamicin sensitivity and the double crossover or deletion strains confirmed by PCR using gene-specific primers.

\textit{P. aeruginosa} proteases were assayed in the supernatants of bacteria grown at 37°C with shaking (250 rpm) in M9 minimal medium (2 mM MgSO\textsubscript{4}, 0.1 mM CaCl\textsubscript{2}, 0.4% glucose, 12.8 g/L Na\textsubscript{2}HPO\textsubscript{4}.7H\textsubscript{2}O, 3 g/L KH\textsubscript{2}PO\textsubscript{4}, 0.5 g/L NaCl, and 1g/L NH\textsubscript{4}Cl) until they reached the stationary growth phase (OD\textsubscript{600nm} ∼1.2). Cultures were centrifuged (40 mL, 30 min, 8000 × g, 4°C) and the resulting supernatants filtered and concentrated 50-fold by ultrafiltration using a 10 kDa cut-off PES membrane (Vivaspin\textsuperscript{®}; Sartorius).

**Analysis of proteolytic degradation of IL-22**

Recombinant IL-22 (50 ng) was incubated at 37°C with \textit{P. aeruginosa} supernatants or purified proteases. Reactions were stopped by adding an excess of protease inhibitor cocktail. Proteolytic processing of IL-22 was analyzed by western blotting. The biological activity of IL-22 was determined by measuring phospho-STAT3 in bronchial epithelial BEAS-2B cells treated with an optimal concentration of IL-22 (20 ng/mL).

**Analysis of virulence of \textit{P. aeruginosa} lacking protease IV on human cell culture**

We exposed BEAS-2B cells with 2 strains of \textit{P. aeruginosa}: one which expressed protease IV (PAK\textit{ΔpscF}) and the other one did not (PAK\textit{ΔpscF-Δpiv}). Of note is that both strains lacked a functional type III secretion system (T3SS) as the T3SS-dependent virulence factors would drastically trigger a mortality on BEAS-2B epithelial cells, that would not allow to accurately discriminate the impact of protease IV \textit{per se}. BEAS-2B cells were seeded into 12-well culture plates and incubated 48 hours at 37°C and 5% CO\textsubscript{2}. Cells were incubated with or without PAK\textit{ΔpscF} and PAK\textit{ΔpscF-Δpiv} at multiplicity of infection (MOI) of 0.1 for 20 hours in F-12K medium. The medium with bacteria was then removed and epithelial cells washed with PBS. Medium with gentamycin (25 µg/mL) was further added. We assessed the viability of the cells after incubation with the bacteria by a colorimetric method for determining...
the number of viable cells in proliferation (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, USA).

**Analysis of substrate susceptibility to proteases**

The substrates Abz-HPVPYAFSPQ-EDDnp and N-p-Tosyl-Gly-Pro-Lys-pNa were used to determine the proteolytic capacities of neutrophil and *P. aeruginosa* proteases, as previously described.44,45

**Analysis of the efficacy of various protease inhibitors on protease IV**

Protease IV (5 nM) was incubated 30 min in PBS with or without the different inhibitors: 10⁻⁷ M α1AT, α1ACT, or elafin; 10⁻⁶ M SLPI or Pepstatin; 10⁻⁴ M Aprotinin or 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF); 10⁻⁵ M Tosyl-L-lysyl-chloromethane hydrochloride (TLCK); 20% plasma. Protease IV activity was then measured using the substrate N-p-Tosyl-Gly-Pro-Lys-pNa as described.

**Human respiratory fluid samples**

Tracheal aspirates of ventilated patients were collected prospectively and considered to be purulent when the neutrophil count was greater than 10⁶ cells/g.46 Patients with purulent tracheal secretions were assigned to one of 2 groups: (i) patients whose tracheal secretions were recently positive for *P. aeruginosa* (≥10⁴ cfu/mL) and (ii) patients with tracheal secretions free of *P. aeruginosa* (with or without other pathogens). Patients with a new, persistent (>48-h) infiltrate, visible by chest X-ray, were not included. This study was approved by the French bioethics authorities (L’Espace de Réflexion Ethique Région Center) and was conducted in accordance with the ethical standards of the Helsinki Declaration. Informed consent was obtained from each participant.

**Degradation of IL-22 by tracheal secretions**

Human respiratory fluids were homogenized in 3 volumes (w/v) of PBS, incubated with 1 mM dithiothreitol for one hour, and then centrifuged. This concentration of dithiothreitol has no impact on the measurement of protease activity or IL-22 detection (data not shown). The human respiratory fluids were incubated for 15 min with human recombinant 10 ng IL-22 cytokine and the IL-22 quantified by ELISA.

**Statistical analysis**

Results are expressed as means ± SEM for the indicated number of experiments performed independently. Statistical significance between the different values was analyzed by the Man-Whitney or Kruskal-Wallis test (and Dunn’s test for post-hoc comparisons) depending on the number of groups to analyze. Relationships between variables were characterized with the Pearson’s correlation test. Statistical analyses was performed using GraphPad Prism® software. A *p* value of <0.05 was considered to be significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**ORCID**

Antoine Guillon http://orcid.org/0000-0002-4884-8620  
Brice Korkmaz http://orcid.org/0000-0002-5159-8706  
Francois Trottein http://orcid.org/0000-0003-3373-1814  
Richard J. O’Callaghan http://orcid.org/0000-0001-9931-6061  
Mustapha Si-Tahar http://orcid.org/0000-0002-5792-7742

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