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Short communication

Inactivation of the interleukin-22 pathway in the airways of cystic fibrosis patients

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ABSTRACT

Interleukin (IL)-22 plays a critical role in regulating the maintenance of the mucosal barrier. As airway epithelial regeneration is abnormal in cystic fibrosis (CF), we investigated IL-22 integrity in CF. We first demonstrated, using *Il-22-/-* mice, that IL-22 is important to prevent lung damage induced by the CF pathogen *Pseudomonas aeruginosa*. Next, IL-22 receptor was found normally expressed at the airway epithelial surfaces of CF patients. In wound-healing assays, IL-22-treated CF cultures had higher wound-closure rate than controls, suggesting that IL-22 signaling *per se* could be functional in a CF context. However, persistence of neutrophil-derived serine-proteases is a major feature of CF airways. Remarkably, IL-22 was found altered in this protease-rich inflammatory microenvironment; the serine protease-3 being the most prone to fully degrade IL-22. Consequently, we suspect an acquired deficiency of the IL-22 pathway in the lungs of CF patients due to IL-22 cleavage by the surrounding neutrophil serine-proteases.

1. Introduction

Cystic fibrosis (CF) is characterized by recurrent bronchial obstruction due to mucus accumulation, bacterial airways infections and persistent inflammation. *Pseudomonas aeruginosa* (PA) is the predominant pathogen responsible of chronic lung infection, resulting in lung tissue damage [1]. Also, human airway surface epithelial regeneration is abnormal in CF [2]. This CF feature is still not perfectly understood and could be a contributing factor to recurrent infections. IL-22, a member of the IL-10 family, is an important component of immune-epithelial cell cross-talk and plays a critical role in regulating the maintenance of the mucosal barrier [3]. IL-22 targets respiratory epithelial cells through its receptor IL-22R to control defense mechanisms and repair of respiratory epithelia damaged by infection [3]. The role of IL-22 in controlling PA infections is not completely clear. A protective role against PA lung infection is debated [1,4,5]. CF lung disease is characterized by non-resolving inflammation, driven by continuous recruitment of immune cells into airways [6]. In particular, neutrophil infiltration is a classic feature of CF and has been shown to be crucial to fight PA [7]. Neutrophils are a major source of serine proteases (elastase, cathepsin G, protease-3) [8]. Neutrophil serine proteases have beneficial roles in microbial killing but could also be harmful by destructing host tissue components [6]. We previously demonstrated a regulation of IL-22 signaling in chronic obstructive

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pulmonary disease by the proteolytic action of neutrophil enzymes [9]. In the context of CF lung disease, the protease burden overwhelms the anti-protease shields and causes a variety of damaging effects, for instance by cleaving immune proteins [8]. IL-22 production is evident in CF patients infected by PA [10], but the functionality of the IL-22-dependent pathway has not been examined in the CF context. Here, we aimed to specifically investigate whether IL-22 could be altered in the protease-rich microenvironment of CF airways.

2. Materials and methods

2.1. Ethical considerations

Animal experiments were conducted according to protocols approved by the *Comité d'Ethique en Expérimentation Animale Nord Pas-De-Calais (agreement number N°AF16/20090)*. The use of human tissues was authorized by the French bioethics authorities (*L'Espace de Réflexion Ethique Région Centre*) and was conducted in accordance with the ethical standards of the Helsinki Declaration. Informed written consent was obtained from each participant.

2.2. Mice and P. aeruginosa-induced lung infection

Il-22-/- C57BL/6 mice of both sexes, 6–8 weeks old and wild-type (WT) were challenged intranasally with 50 µL of PBS containing 5×10^7 CFU of PAKΔ*pscF* strain or PBS alone. Bronchoalveolar lavage (BAL) fluid and whole lungs were collected 72 h post-infection. The lungs were perfused and immersed in 10% formalin, dehydrated, paraffin-embedded. Sections (5 µm) were stained with H&E. A multiparameter cumulative scoring system was developed by the pathologist and validated in mice infected with various pathogens (grading details are provided in Online Table 1). Bacterial burden was evaluated by plating serially diluted samples of lung homogenate or BAL.

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2.3. Human airway tissues

Non-infected human airway tissues were collected after nasal polypectomy from 11 CF subjects (median age: 22; range: 12–26; male gender: 9/11; genotypes: F508del/F508del: 7; F508del/G85E: 1; F508del/I507del: 1; F508del/N1303K: 1; F508del/Y1092X: 1) and 7 non-CF subjects who did not suffer from any other disease at the time of experiments were carried out (median age: 58; range: 40–71; male gender: 6/7).

2.4. Human airway epithelial cell dissociation and culture at the air-liquid interface (ALI)

Non-CF and CF human airway epithelial (HAE) cells were obtained after dissociation by 0.05% type XIV collagenase (Pronase E; Sigma Aldrich, St Louis, MO) in RPMI 1640 medium supplemented with 20 mM HEPES (Gibco, Life technologies, Paisley, UK) and antibiotics (200 UI/ml penicillin, 200 µg/ml streptomycin; Gibco) overnight at 4 °C. HAE cells from 7 non-CF and 7 CF donors (genotypes: F508del/F508del: 5; F508del/G85E: 1; F508del/N1303K: 1) were suspended in CnT17 medium (CELLnTEc, Bern, Switzerland) and seeded (5×10^4 cells per membrane) on type IV collagen (Sigma Aldrich)-coated porous 12 mm diameter Transwell polyester membranes (Transwell-Clear, 0.4 µm pores; Corning, Acton, MA). Cells were grown in liquid-liquid conditions in CnT17 medium until confluence. The culture medium was then definitively removed from the upper compartment and the epithelium was allowed to differentiate at the ALI for 35 days in medium consisting in 1:1 DMEM/F12 (Gibco), and BEGM (Lonza, Walkersville, MD) with the Lonza supplements for hEGF, epinephrine, BPE, hydrocortisone, insulin, triiodothyronine and transferrin, and supplemented with 200 UI/ml penicillin, 200 µg/ml streptomycin (Gibco), 0.1 nM retinoic acid (Sigma Aldrich) and 1.5 µg/ml bovin serum albumin (BSA; Sigma Aldrich). All the cultures were incubated at 37 °C in a 100% humidified incubator in the presence of 5% CO_2.

2.5. RNA isolation, cDNA preparation, and RT-PCR

For details, see [11].

2.6. Immunohistochemical analysis of airway tissues and ALI cultures

Immunohistochemistry was performed on formalin-fixed paraffin-embedded non-CF and CF human nasal tissues and well-differentiated ALI cultures. Five µm sections were blocked with 10% BSA in PBS for 30 min at room temperature. The slides were then incubated overnight at 4 °C with rabbit polyclonal anti-IL-22R1 (Abcam, Cambridge, United Kingdom; 10 µg/ml in 10% BSA in PBS). Slides were washed with PBS and incubated with donkey anti-rabbit Alexa Fluor 488 secondary antibodies (Molecular Probe, Eugene, OR) and cell nuclei stained using DAPI (Sigma Aldrich). Negative controls were performed by omitting the primary antibodies. Images were taken by Confocal Zeiss LSM710 microscope (20X DIC/1.40 oil).

2.7. Human airway epithelial cell culture for wound-healing assay

HAE cells from 4 CF donors (genotypes: F508del/F508del: 2; F508del/I507del: 1; F508del/Y1092X: 1) and from 3 non-CF donors were suspended in BEGM medium and seeded (13×10^3 cells per cm^2) on type IV collagen-coated 24-well plates. When confluence was reached, epithelial monolayers were injured mechanically with a P20 pipette tip. After injury, the monolayers were washed to remove detached injured cells, and fresh BEGM medium containing only 10% of each supplement was added. This medium was supplemented or not with human recombinant IL-22 (20 ng/ml; ImmunoTools GmbH; Friesoythe, Germany). A mark on the Petri dishes allowed us to photograph the wounds exactly at the same place at various times and to measure with ImageJ software, the wound area initially and after repair. Our results are reported as percentages of the initial wound area. Each experiment was realized in triplicate.

2.8. Analysis of proteolytic degradation of IL-22

Sputum from four CF patients were collected (3/4 colonized by *P. aeruginosa*, 2/4 colonized by *S. aureus*). Sputum were homogenized in three volumes (w/v) of PBS, incubated with 1 mM dithiothreitol for one hour, and then centrifuged. Purification of human blood neutrophils and assessment of serine proteinase activity were performed as described [12]. Total serine proteinase activity in activated cells was inhibited with 1 µM of α1Pi (Preparatis, Krakow, Poland) and Pr3 was specifically inhibited with 10 µM of Ac-PYDAP(O-C6-H4-4-Cl)2 [13]. Human recombinant IL22 (50 ng) were incubated at 37 °C with 20 µL of homogenized sputum or supernatant of purified neutrophils or 50 µL of different concentrations of purified elastase (Preparatis), cathepsin G (Preparatis) and proteinase 3 (Athens Research, Athens, USA). Reaction was stopped by adding of 6X Laemmli then freezing (−20 °C) at different time points. IL-22 concentration was determined using DuoSet ELISA assay kits (R&D Systems). SDS-PAGE was performed using anti-IL22 antibody diluted 1/3000e (Abcam, Paris, France).

2.9. 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 test (two groups) or the Kruskall-Wallis test with Dunn's multiple comparison test (more than two groups). Statistical analyses was performed using GraphPad

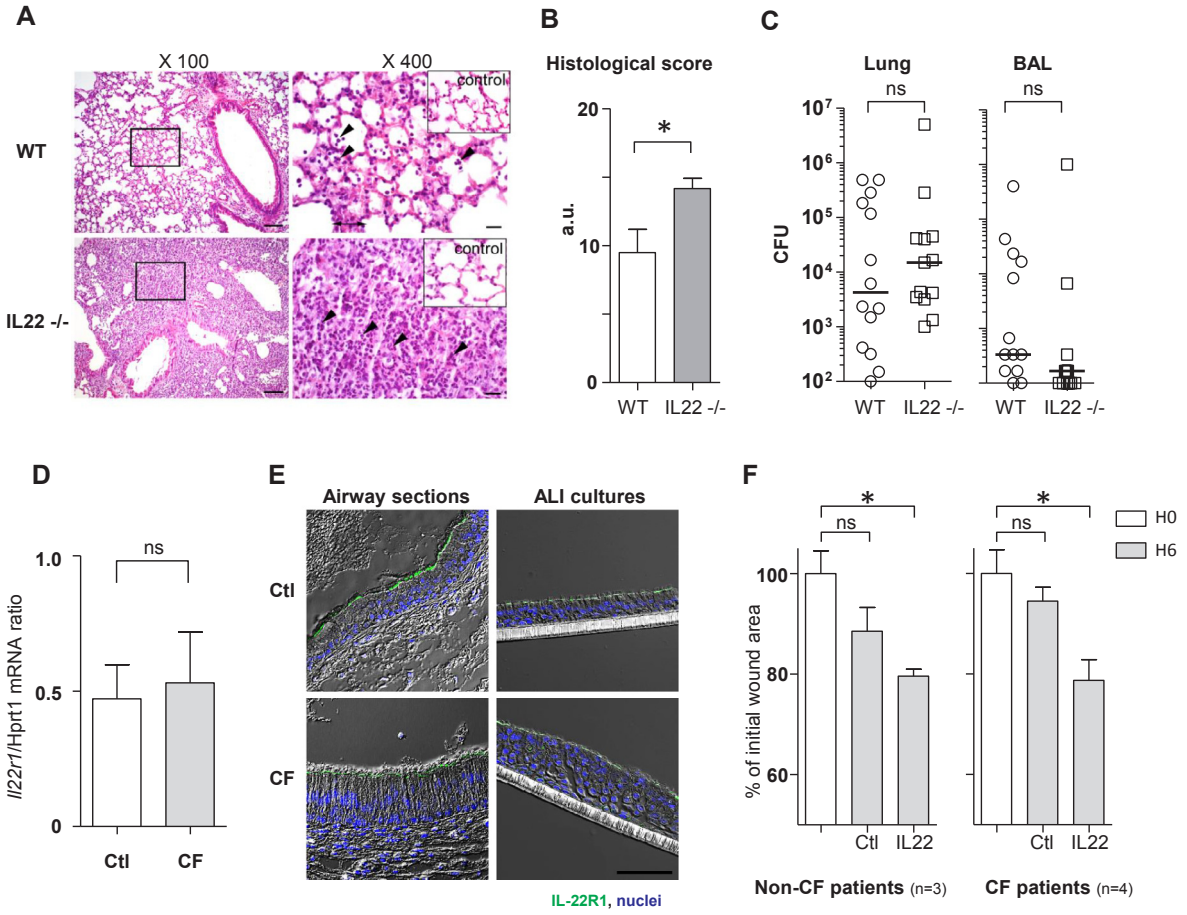

Fig. 1. IL-22 pathway is determinant to limit *P. aeruginosa*–induced lung damage and to heal wound in airway epithelia of CF patients. C57BL/6 wild type (WT) and *Il-22-/-* mice were instilled intranasally with 5×10^7 CFU PA mutant (deleted for T3SS) and sacrificed 3 days post-infection (n = 15 wt mice, n = 14 *Il-22-/-* mice, from two independent experiments). (A) Representative histologic staining shows extensive lung lesions with severe neutrophilic bronchopneumonia causing consolidation (arrow heads) in *Il-22-/-* mice, while the lungs of WT mice had moderate neutrophilic exsudation (arrow heads) and slight alveolar wall thickening (double arrow). Inserts in A show lung of non-infected mice. Scale bars × 100 = 100 µm; scale bars × 400 = 20 µm. (B) The global histological assessment of lung injury is represented by composite scores that summarize alveolar, bronchiolar and vascular lesions. (C) Bacterial loads in the bronchoalveolar lavage (BAL) or lung homogenates 72 h after instillation of PA. (D) IL-22R1 RNA expression was detected in differentiated ALI cultures of airway epithelial cells from nasal polypes by quantitative reverse transcriptase PCR and normalized to reference genes (Hprt1) for 7 CF and 7 non-CF patients. (E) IL-22R1 protein expression and localization were assessed by immunofluorescence (Alexa fluor 488, green) on sections of non-CF and CF airways and differentiated ALI cultures. Nuclei are counterstained using DAPI. Bar: 64,41 µm. (F) Airway epithelial cells from three non-CF donors and four CF donors were suspended in BEGM medium and seeded on type IV collagen-coated plates. When confluence was reached, epithelial monolayers were injured mechanically, and fresh medium containing only 10% of each supplement was added. This medium was supplemented or not with human recombinant IL-22 (20 ng/ml). The monolayers were photographed at time 0 and after 6 h. The wound areas were measured initially and after repair using ImageJ software. Data are shown as median (C) or mean ± SEM (B, D) and reported as percentage of the initial wound area (F). Ctl: control; ns: non-significant.

Prism5®software. A p value of < 0.05 was considered to be significant.

3. Results and discussion

We first sought to clarify the role of IL-22 in PA-associated pulmonary disease using *Il-22-/-* and wild-type (WT) mice. Lack of representative models of human lung disease due to PA infection is a significant hurdle to translational research. Administration of PA to murine lungs results in either rapid bacterial clearance or immediate animal death, both situations uninformative in regard to host-pathogen interaction studies. The PA bead model has been widely used to study pulmonary PA infection but has limitations in accurately mimicking human disease. These limitations led us to develop an inflammatory model of non-lethal PA infection in *Il22-/-* mice with PAK *ΔpscF*, a PA mutant lacking the type III secretion system (T3SS). Lungs from WT and IL-22-/- mice were examined three days post-infection with PAK *ΔpscF*. Infected *Il-22-/-* mice exhibited greater lung injury and more inflammatory cell recruitment (mainly neutrophils) than infected WT controls (Fig. 1A, B, Online Fig. 1). The infected *Il-22-/-* mice tended to have a higher bacterial load than infected WT mice, but the difference was not statistically significant (Fig. 1C). These results led us to conclude that the IL-22 pathway is likely important to prevent PA–induced lung damage.

We next took into consideration that pathological IL-22R expression at epithelial surfaces has been previously demonstrated and could contribute to the pathogenesis of certain diseases [11,14]. The IL-22R complex consists of the receptor chains IL-22R1 and IL-10R2. As the IL-10R2 subunit is ubiquitously expressed, the expression of IL-22R1 by epithelial cells determines cellular sensitivity towards IL-22. We sought to investigate whether IL-22R1 was expressed in CF airway epithelium. Human airway tissues were collected after nasal polypectomy from seven CF and seven non-CF subjects. To determine whether IL-22R1 subunit expression was regulated at the transcriptional level, epithelial cells of all samples were cultured at the air-liquid interface (ALI) until complete differentiation, then processed for qRT-PCR. As shown in Fig. 1D, IL-22R1 mRNA expression was similar in CF and control

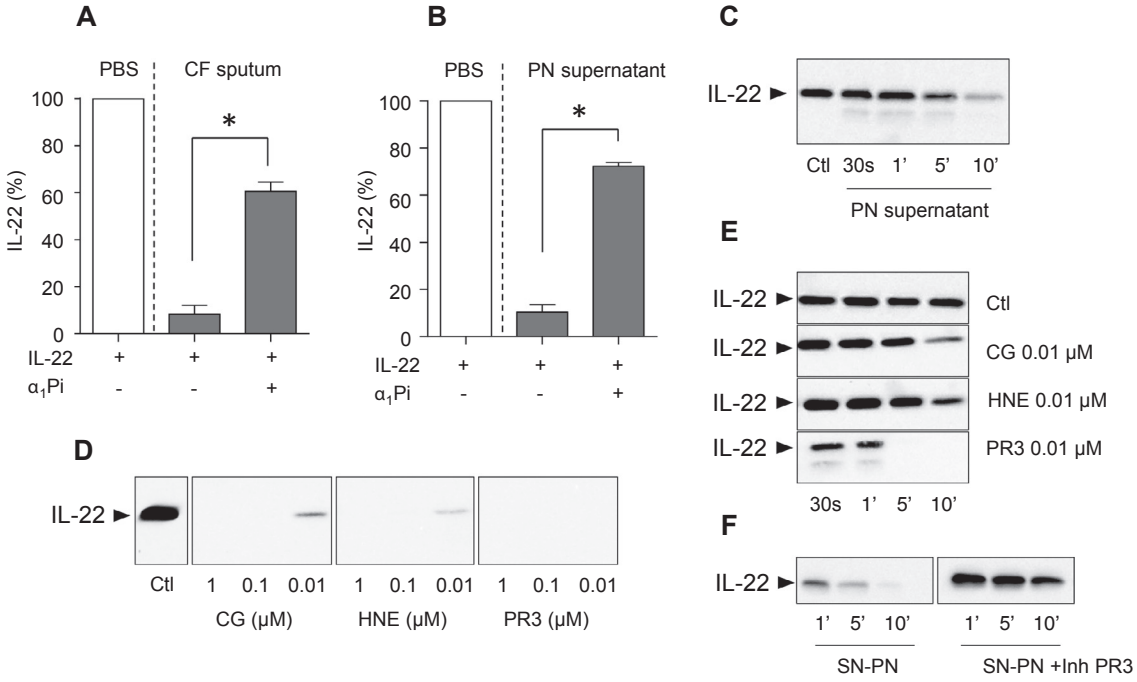

Fig. 2. IL-22 is degraded by neutrophil serine proteases. (A, B) IL-22 (50 ng) was incubated for 5 min in sputum from four CF patients (A) or in supernatants from human purified neutrophils (SN-PN) (B). α1-protease inhibitor (α1-Pi) was used to inhibit neutrophil serine proteases (including elastase, cathepsin G and proteinase 3). The final concentration of IL-22 was quantified by ELISA. (C, D, E, F) IL-22 (50 ng) was incubated with supernatant from neutrophils, or elastase, cathepsin G and proteinase 3 with the mentioned concentrations and times, and analyzed by western blotting using a specific anti-IL-22 antibody. (F) Proteinase 3 (Pr3) was specifically inhibited with 10 μM of Ac-PYDA^{P}(O-C_6-H_4-4-Cl)$_2$ [13]. Results are representative of two to three independent experiments. ns: non-significant; α1-Pi: α1-protease inhibitor; PN: neutrophils; SN-PN: supernatants from human purified neutrophils; Inh PR3: proteinase 3 specific inhibitor; Pr3: proteinase 3; HNE: human neutrophil elastase; CG: cathepsin G.

patients. We next assessed the IL-22R1 protein expression at epithelial surface by immunofluorescence analysis of CF and non-CF airways samples, along with non-CF and CF differentiated ALI cultures (Fig. 1E). Immunostaining revealed an expression of the IL-22R1 protein at the apical surface of ciliated cells that seemed to be comparable in CF versus control patients. We concluded that the CFTR mutation did not alter the IL-22R1 epithelial expression. In addition, we conducted wound-healing assays on nasal epithelial cells obtained from four CF patients and three non-CF patients. We assessed the effect of IL-22 on epithelial repair and observed that the initial wound area was significantly reduced over a 6-h period in IL-22-treated cultures but not in non-treated ones (Fig. 1F). This result suggested that the IL-22-dependent signaling was functional in *ex-vivo* airway epithelia, including CF epithelium.

However, since the IL-22 pathway seems to be particularly sensitive to proteases [9], we hypothesized that the serine protease-rich environment of CF airway may be hardly compatible with a functional IL-22 signaling. To verify this issue, we assessed the stability of IL-22 in sputum of CF patients (Fig. 2A) or in supernatant of neutrophils (Fig. 2B). Sputum and human neutrophils were collected and conditioned as described previously [12,15]. Recombinant IL-22 was incubated in CF sputum or supernatant of neutrophils for 5 min and its concentration was measured by ELISA. We observed a clear decrease of IL-22 concentrations in these two protease-rich fluids (Fig. 2A, B). Consistently, the degradation of IL-22 by the supernatant of neutrophils was confirmed by western-blot (Fig. 2C). The addition of a specific serine protease inhibitor (α_1Pi) prevented IL-22 degradation in both fluids (Fig. 2A, B). We further focused on the three major neutrophil serine proteases and observed that elastase, cathepsin G and protease-3 have the ability to cleave IL-22 (Fig. 2D). However, protease-3 was more prone to destroy IL-22 in a time- (30 s) and concentration (0.01 μM)-dependent manner (Fig. 2D, E). Of note, it has been previously demonstrated that the activities of these proteases in CF sputum were in the 10–100 nM range, with protease-3 and cathepsin G being the most active (> 0.1 μM) [12]. Finally, we exposed IL-22 to a neutrophil supernatant in the presence of a specific protease-3 inhibitor and observed that the proteolytic destruction of IL-22 was prevented (Fig. 2F).

In conclusion, our findings suggest that IL-22 signaling is likely not directly affected by CFTR deficiency *per se*, notably because IL-22R1 is normally expressed at the epithelial surfaces of CF airways. However, the inflammatory proteases released by activated neutrophils, in particular protease-3, have the ability to rapidly and completely degrade IL-22. Consequently, we suspect an acquired deficiency of the IL-22 pathway due to the persistent presence of neutrophils in the lungs of CF patients. These observations may have broader implications for other neutrophilic airway diseases.

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Declaration of interest

None.

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