Supplementary Online Information

Pseudomonas aeruginosa (*P.a*) LasB protease impairs innate immunity in mice and humans by targeting a lung epithelial CFTR-IL-6-antimicrobial-repair pathway

Saint-Criq V, Villeret B, Bastaert F, Kheir S, Hatton A, Cazes A, Xing Z, Sermet-Gaudelus I, Garcia-Verdugo I, Edelman A, Sallenave JM

METHODS

Metalloprotease enzymatic activity

LasB activity assay was performed in 384 wells black plates in a final volume of 30 µL and was determined using a fluorogenic substrate specific for metalloproteases, TACE substrate II ((5-FAM-Ser-Pro-Leu-Ala-Gln-Ala-ValArg-Ser-Ser-Ser-Arg-Lys(5-TAMRA)-NH2; Enzo Life Sciences: excitation and emission wavelength being 485 and 535 nm, respectively). Purified LasB and secretome samples (diluted in Tris 25 nM; $ZnCl_2$ 2,5 µM; Brij-35 0,005 %; pH 9,0 buffer) were incubated at room temperature in the presence of substrate (10 µM) and fluorescence was read over a 3 hrs period (one measurement taken every 10 min) with a *Varioskan™ Flash Multimode Reader* (Thermo scientific).

Protein extraction and Western blotting

Cells were washed twice with ice-cold PBS and lysed (lysis buffer composition: TrisHCl 50 mM, NaCl 150 mM, NP40 1 %, Glycerol 3 %, EDTA 2 mM, EGTA 2 mM). Lysates were centrifuged (14 000 rpm, 15 min, 4 °C) and pellets were discarded. Protein concentrations were measured using BioRad protein assay and the supernatants were stored at -80°C until used. They were then normalized for protein content, and 4X gel loading buffer (Biorad) was added to 20 µg of protein. After heating at 95°C for 10 min (STAT3 activation Western-Blots) or at 37°C for 30 min (CFTR Western-Blot), samples were separated by SDS-PAGE

and transferred to PVDF membranes. Membranes were blocked in 5 % blocking buffer [milk in Phosphate buffered saline with 0.1 % Tween (PBST)] for 60 min at room temperature, followed by incubation with primary antibody (anti-phospho-STAT3: 1/1000, Cell Signaling Technology, 9145, anti-STAT3: 1/1000, Cell Signaling Technology, 9139, anti-CFTR(596): 1/2000 CFF consortium) in blocking buffer overnight at 4 °C followed by a 2hr incubation at room temperature. After washing (4x) in PBST, membranes were incubated with HRP-conjugated secondary antibody to rabbit or mouse IgG in blocking buffer for 60 min at room temperature. After further washing (4x) in PBST, immunoreactive STAT-3 or CFTR were detected by enhanced chemiluminescence (Pierce ECL2 Western Blotting Substrate #80196, ThermoScientific). Membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific, 21059), blocked in 5 % nonfat dry milk in TBST for 60 min at room temperature, incubated with anti-GAPDH (mouse Covalab mab90009/00006357, 1/10000) or anti-β-tubulin primary antibody (Sigma-aldrich) overnight at 4 °C, washed 3 times in PBST, incubated for 1 hr at room temperature with HRP-conjugated secondary antibody, washed again 3 times in PBST and immunoreactive loading control was detected by enhanced chemiluminescence. Image acquisition was performed using a photosensible camera (PXi 4; Syngene), saved with the *GENESys* (version 1.4.0.0) software and analysis was done using ImageJ software.

Adenovirus constructs

The control 'Ad-null (Ad-dl70/3)' was used as described in (Ref E5). Ad-GFP-WT-CFTR, a kind gift of Pr Boulanger (Lyon) was described in (Ref S6). Ad-m-IL-6 (Ref S7) were also used *in vivo* in mice experiments.

Cells, cell cultures and protocols

NCI-H292 cell line was grown in RPMI-1640 + Glutamax, Hepes (Gibco) supplemented with 10 % Fetal Bovine Serum (FBS, Gibco), 1 % Antibiotic-Antimycotic (Gibco). For CFTR overexpression, the cells were serum-starved and infected with Ad-vector coding for GFP-WT CFTR at Multiplicity of Infection (MOI) 150 pfu for 1 hr in serum-free medium. Complete medium was then added and left for 24 hrs prior to a 16 hrs treatment with the secretomes. NCI-H292 cells were pre-treated with either IL-1β (1 µg/mL) or IL-6 (1-50 ng/mL) before addition of LB medium, WT-SEC or ΔLasB-SEC (5 % in serum-free medium)

for 4 hrs (IL-6, IL-8, trappin-2 secretion and mRNA levels, STAT3 activation) or 16 hrs (wound healing assay).

Calu-3 cell line was expanded in T75 flasks in DMEM:F12 supplemented with 10 % FBS, 1 % Antibiotic-Antimycotic and 1 % non-essential amino acids (Gibco). All experiments were performed on Calu-3 cells seeded on semi-permeable supports (Millipore) to induce polarization and development of high TransEpithelial Electric Resistance (TEER). All treatments with secretomes were made in FBS-free conditions either for 4 hrs (trappin-2, IL-6, IL-8 secretion and STAT3 activation) or 24 hrs (CFTR expression).

WT-CFTR and F508del-CFTR CFBE cell lines (derived from the parental CFBE41o- cells, D.C. Gruenert, Cal. Pacific Med. Cent. Res. Inst.) were used. The CFBE41o- cell line was originally derived from a bronchial tissue isolate of a CF patient homozygous for the F508del CFTR mutation and immortalized with the pSVori- plasmid that contained a replication-deficient simian virus 40 (SV40) genome (Ref S8). Stably transfected CFBE41o- (WT and F508del-CFTR (Ref S9)) cells were grown in the presence of 600 μg/mL hygromycin B to select for clones of cells that contained the transfected plasmid. For Ussing chamber studies, WT-CFTR CFBE were cultured in Eagle's Minimal Essential Medium (MEM) containing 10 % Fetal Bovine Serum (FBS); 100 U/mL penicillin and 100 U/mL streptomycin. Cells were expanded in flasks coated with an extracellular matrix cocktail comprised of fibronectin (Corning), collagen (Sigma-Aldrich), and bovine serum albumin (Sigma-Aldrich) and further grown on semi-permeable Snapwells (Corning® Costar®, Sigma-Aldrich).

Cell scratching and cell culture RNA preparation

Wound healing assay on NCI-H292 and WT-CFTR overexpressing CFBE cells was performed by producing a wound using a 10 μl pipet tip. Cells were then washed with PBS to remove detached cells and debris and treated with different concentrations of IL-6, LB, WT-SEC or ΔLasB-SEC. Monolayers were photographed at time 0 and 16 hrs after wounding and images were analyzed by measuring the area wounded using ImageJ software. Cells were thereafter washed and mRNA extracted in order to perform qPCR analysis.

RNA isolation from cells was performed using PureLink® RNA Mini Kit (12183018A, Ambion, Life technologies), following the manufacturer's instructions. Briefly, lysates were mixed with 70 % ethanol and loaded onto a silica-membrane column. After different washings, total RNA was eluted in DNAse-RNAse-free water and stored at -80°C until use.

DNase treatment was performed prior to Reverse transcription polymerase chain reaction (RT-PCR) using RNAse-free DNAse I (Roche) at 37°C for 10 min. DNAse was then inactivated by increasing the temperature to 70 °C for 10 min. Complementary DNA (cDNA) was synthesized from total RNA (500 ng) using M-MLV Reverse Transcriptase (Promega) as per the supplier's protocol (1 hr at 37°C followed by 10 min at 70 °C).

Real-time quantitative PCR was performed in a total volume of 15 µL using 2x Fast SYBR® Green Master Mix (Life Technologies), 2 µL of diluted cDNA, 2 µmol forward primer, 2 µmol reverse primer in a 96-well plate. PCR was run with the standard program: 95°C 10 min, 40 times of cycling 95°C 15 sec and 60°C 1 min in a 96-well plate. Results are shown as relative quantity of mRNA copies to the determined control condition, with HPRT expression used as internal control and as dCT values.

Primary human bronchial epithelial cells

After the consent of F508del-CFTR CF patients (IRB Ile de France II), bronchial epithelial cells (HBE) were cultured from explants obtained after lung transplantation. HBE cell cultures had the cobblestone appearance typical of epithelial cell cultures after 2-3 weeks in air-liquid interface (ALI). The protocol used allows for the differentiation of cells into ciliated and secretory goblet cell types, labeled with alpha-tubulin and Muc5AC proteins respectively, with polarization of reconstituted epithelium, observed by specific apical staining of ZO-1 (Zona occludens-1) and alpha-tubulin. This was confirmed by the presence of cytokeratin 8 (K8), a protein that is specific for simple epithelia (Ref S10) and the presence of tight junctions, demonstrated by specific pattern of ZO-1 protein. Reconstituted epithelium also formed ion transport and barrier function reflected by high transepithelial electrical resistance (TEER). After one week of growth in ALI, the TEER was measured regularly with a chopstick voltmeter to define the time of culture necessary to obtain an adequate resistance > 600Ω/cm^2. Analysis of TEER and immunofluorescent staining of markers have revealed that cells remained well polarized after 16 – 30 days of ALI culture. For all types of experiments, 16 – 30 days of ALI culture HNE and HBE cell cultures were used. This age of culture corresponds to full development of ciliated cells and balanced ratio of ciliated to goblet-secretory cells (Ref S11). With microscopic analysis in brightfield or immunofluorescent staining of cilia, we estimated that 50-70 % of ciliated cells developed with our protocol. Additionally, analysis of CFTR mRNA level in primary cells in culture, reported by Prulière-

Escabasse et al (Ref S12), show that the epithelial differentiation at 16 – 30 days is accompanied by CFTR expression.

In order to overexpress WT-CFTR in primary bronchial and nasal epithelial monolayers, cells were serum-starved and infected with either Ad-null or Ad-GFP-WT CFTR (MOI 100 pfu) for 1 hr. Complete medium was then added and left for 24 hrs prior to treatment with the SECs.

Short-Circuit Current Measurements in Ussing Chambers

Cells were plated on (F-C-B)-coated semi-permeable Snapwell cell culture inserts (Corning® Costar®, Sigma-Aldrich) at a density of 1 $x10^6$ cells/cm^2 that were used 15 to 25 days after growing in ALI. Under these conditions, CFBE cultures display phenotypic properties most characteristic of the respiratory epithelium.

Transepithelial short-circuit current (Isc) and TEER measurements were carried out in the short-circuit mode and in the presence of an asymmetrical physiological solution to create a chloride (Cl^-) gradient. The inserts were mounted in Ussing chambers (0.33 cm^2 aperture). Hemichambers were connected to a DVC-1000 voltage clamp (World Precision Instruments, Inc., Sarasota, FL) via Ag/AgCl electrodes and 3 M KCl agar bridges for recording of short-circuit current. Currents were stored on computer using analog-to-digital converter (PowerLab) and LabChart software 5.0. (AD Instruments).

Prior to the experiment, prostaglandin generation was blocked by adding 10 µM indomethacin to the apical and basolateral compartments. Transepithelial Isc was calculated as $\mu Amp.cm^{-2}$ insert surface area. The apical solution contained (in mM): 145 Na Gluconate, 3.3 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 10 Hepes and the basolateral solution contained (in mM): 145 NaCl, 3.3 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 10 Hepes). Both solutions were gassed with 95 % O_2 - 5 % CO_2 (pH 7.4). After current stabilization, amiloride (100 µM) was added to the apical bath in order to inhibit ENaC current; forskolin (10 µM) and IBMX (100 µM) were then added apically and basolaterally to induce chloride current, which was then inhibited by adding CFTRInh172 (5 µM) to the apical bath (Ref S13).

RESULTS

LasB is the main protein in the *Pseudomonas aeruginosa* secretome

We first investigated, in an unbiased fashion, the protein content of the PAO1 secretome (SEC) by SDS-PAGE analysis, followed by Coomassie blue staining. One protein was present in huge excess compared to others, with an apparent MW of 33 kDa (Fig S1A, lanes 2-7). This suggested that this protein may correspond to LasB, an important T2SS secreted product, with metalloprotease activity. We confirmed that this protein was LasB (Refs S1-S2), since the band was absent in secretomes from ΔLasB PAO1 (Fig S1A, lanes 8-13).

This was further confirmed through zymography analysis by studying the gelatinolytic activity of purified (p)-LasB, Fig S1B, lane 2), WT SEC (Fig S1B, lanes 3-5), and ΔLasB-SEC (Fig S1B, lanes 6-8). Interestingly, and confirming previous reports (Refs S1-S2), the observed lytic zone, with a molecular weight around 180 kDa, did not correspond to the native 33 kDa LasB protein. This could mean, as suggested by others, that either LasB is active as a polymer, or that it aggregates in the presence of gelatin, or that a direct interaction between LasB and gelatin occurs within the copolymerized gel. Also, when pLasB was added to ΔLasB-SEC, the 180 kDa lytic zone was restored (Fig S1B, lane 9). Notably, a minor band was also present with a molecular weight of 50 kDa in both WT- and ΔLasB-SEC, which probably represents alkaline protease (AprA), another *P.a* metalloprotease, in PAO1 SEC (refs S1-S3). Furthermore, the enzymatic activity of LasB in the WT (but not ΔLasB) secretomes was demonstrated using a metalloprotease specific substrate (Fig S1C).

LasB down-regulates IL-6 and the antimicrobial molecule trappin-2 in primary CF lung epithelial cells

Bronchial epithelial cells derived from 4 independent F508del-CFTR individuals with CF were either 'corrected' with Ad-WT-CFTR or treated with Ad-null control, and stimulated with WT- or ΔLasB-SEC. Although there were no major differences in terms of trappin-2, IL-6 or IL-8 secretion between Ad-treatments, we observed, as above, that WT-SEC (but not ΔLasB-SEC) had, on each patient sample, a similar deleterious effect on the recovery of trappin-2 (Fig S2 A, D) and IL-6 (Fig S2 B, E), but not on that of IL-8 (Fig S2 C, F).

Up-regulation of IL-6 and trappin-2 induces lung repair factors in mice

Because the IL-6-IL-17-IL-22 pathway has been shown to induce some mucins (Muc1, Muc3, Muc10, Muc13) in the gastrointestinal tract (for a review, see (S14)), we evaluated the effect of overexpression of IL-6 or trappin-2 and treatment with the secretomes on MUC5B and

MUC5AC (two important respiratory mucins with bacteria-trapping and repair activities (S15)) expression. We showed that IL-6 significantly induced their expression and that, even at the basal state (PBS/PBS), eTg mice had higher levels of these mucins. Moreover, there was a trend for increased mucins expression in eTg mice over-expressing IL-6 and infected with PAO1, when compared to similarly treated WT mice (Fig 9I-J).

In addition, we showed that MRC-1 (murine CD206, a 'M2 marker'), but not iNOS (a 'M1 marker') was up-regulated by IL-6, notably in WT mice (Fig 10A-B). As M2 macrophages are often described as anti-inflammatory and pro-repair macrophages, this suggests that IL-6 over-expression induced reparative processes. There were also strong positive correlations between the expression of IL-6 and MRC-1 and those of trappin-2 and MRC-1 (Fig S6 panels A-B), when all mice were assessed together.

Furthermore, HGF (FGF-7), Fn1, and ColA1, 3 markers of proliferation/repair were also clearly up-regulated by IL-6 alone or by IL-6 + PAO1, when compared to the respective controls (Fig 10 C-E). Again, within the eTg group alone, there were strong correlations between IL-6 and Col1A1 and between trappin-2 expression and these repair markers (Fig S6 panels C-F). Interestingly, although IL-6 over-expression did not affect EGF expression *per se*, EGF expression was higher in eTg mice expressing Ad-IL-6 and infected with PAO1, compared to C57BL/6 mice treated similarly (Fig 10F).

FIG LEGENDS

Figure S1: LasB is the main secreted protein in PAO1 WT secretome.

A) Coomassie blue staining of SDS-PAGE analysis of purified LasB (pLasB), PAO1 WT and PAO1 *ΔlasB* mutant strains secretomes (WT-SEC and ΔLasB-SEC respectively) at different dilutions. (B) Zymography analysis of purified LasB (pLasB, 1µg) and WT-SEC and ΔLasB-SEC at different dilutions. (C) Metalloprotease activity reported in WT-SEC and ΔLasB-SEC using the TACE substrate II assay over a 3 hrs kinetic by spectrofluorometry (excitation wavelength 485 nm; emission wavelength 535 nm). Metalloprotease concentrations were calculated using the slope of fluorescence and a standard curve established using known concentrations of purified LasB.

Figure S2: LasB reduces trappin-2 and IL-6 recovery, but not that of IL-8, in the supernatants of primary epithelial cells from F508del homozygous patients.

Primary bronchial epithelial cells from 4 independent homozygous F508del-CFTR CF patients (dark circles, dark squares, upper triangles, lower triangles) were obtained from explants during lung transplants and were cultured at an air-liquid interface. Cells were either transfected with Ad-null or Ad-WT-CFTR (MOI =100), and 24 hrs later, cells were stimulated with either LB medium or with PAO1 WT- or ΔLasB-SEC (2.5 %) for a further 24 hrs. Supernatants were then obtained and analyzed for trappin-2, IL-6 and IL-8 content by ELISA, as above. Upper panels represent trappin-2, IL-6, and IL-8 production in pg/mL (absolute amounts) for each individual patient sample (NB: this form of representation was chosen to emphasize that despite variability between samples, the treatments had similar effects on each individual sample). Lower panels depict the relative expression of these mediators, plotted relatively to the Ad-null + LB treatment (fold increase = 1). * represents statistical significance ($p < 0.01$).

Fig S3 Broncho-alveolar lavage differential cell count

Mice treated as explained in Figs 9-10 were culled and bronchoalveolar lavage (BAL) was performed with 2 x 1 mL of PBS. Typically, a volume of 1.7 mL of BALF on average was retrieved and centrifuged at 2,000 rpm for 10 min. Cell pellets were resuspended in 400 µL of

PBS for total cell counts assessment. Cytospin centrifugation and Diff-Quik staining (Medion Diagnostics) was used for cell differential analysis (Diff-Quick, Dade Diagnostika GmbH, Unterschleissheim, Germany).

Fig S4 PCR array analysis (clustergram) of the expression of a gene panel from experimental lungs (see Figs 8-9).

An aliquot of RNA from individual mice (same absolute amount of RNA) within each of the groups represented in Fig 9 was pooled together, to constitute 10 new samples: 'B6 PBS/PBS'; 'eTg PBS/PBS'; 'B6-Ad-null/PBS'; 'eTg Ad-null/PBS'; 'B6 Ad-mIL-6/PBS'; 'eTg Ad-mIL-6/PBS'; 'B6 Ad-null/PAO1WT'; 'eTg Ad-null/PAO1 WT'; 'B6 Ad-mIL-6/PAO1 WT'; 'eTg Ad-m-IL-6/PAO1 WT'. These RNAs were retro-transcribed into cDNA and amplicons were amplified using primers for 84 genes, using the RT2 Profile 'Mouse Wound Healing PCR Array', according to the manufacturer's instructions. The clustergram presented was generated with the software also provided by the manufacturer (Quiagen) and differential colouring indicates levels of transcriptional activities.

Fig S5 Correlations between the expression of mediators from the 'IL-6/IL-17/IL-22/trappin-2 pathway'

Correlations between some of the key mediators analysed in Figs 9-10 are presented here. When the mRNA expression between most mediators was correlated with each other, all mice (C57BL/6 and eTg) were analysed together. However, when trappin-2 was correlated with cytokines/mediators, only eTg mice were considered since trappin-2 is not expressed in C57BL/6 mice. In all cases, correlations represented are those where the r value is ≥ 0.5, and which are statistically significant (non parametric Spearman correlation, with a 0.05 p value cut-off. The confidence interval is given in brackets. In almost all cases, the p value was < 0.0001).

Fig S6 Correlations between the expression of IL-6 or trappin-2 and that of repair molecules See legend of Fig S5

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