Flagellin from *Pseudomonas aeruginosa* increases the expression of the SARS-CoV2 entry protease TMPRSS2 in airway epithelial cells

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Abstract

The major challenge of the COVID-19 health crisis is to identify the factors of susceptibility to SARS-Cov2 in order to adapt the recommendations to the populations and to reduce the risk of getting COVID-19 to the most vulnerable people especially those having chronic respiratory diseases (CRD) including cystic fibrosis (CF) and chronic pulmonary respiratory diseases (COPD). Airway epithelial cells (AEC) are playing a critical role in the immune response and in COVID-19 severity. SARS-CoV-2 infects the airways through ACE2 receptor and the host protease TMPRSS2 was shown to play a major role in SARS-CoV-2 infectivity.

In this report we showed that *Pseudomonas aeruginosa* and its virulence factor flagellin (Fla-PA), a ligand of Toll-Like receptor 5 are able to increase TMPRSS2 expression in control and CF AEC. In contrast, no effect was observed with recombinant *Salmonella typhimurium* flagellin, used as an adjuvant in the clinical development of new vaccines against respiratory viruses. Considering the urgency of the health situation, this result is of major significance for patients with CRD (COPD, CF) which are frequently infected and colonized by *P. aeruginosa* during the course of the disease. In the general population, a *P. aeruginosa* ventilator-associated pneumonia in SARS-CoV-2 patients under intubation in intensive care units could be also deleterious and should be monitored with care.

Main text

As of August 24th, COVID-19 pandemic caused by the Severe Acute Respiratory Syndrome (SARS)-Coronavirus (CoV)-2 has infected globally over 21 million people with nearby 762,000 deaths (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports). One of the major challenges of this health crisis is to identify factors of susceptibility to this viral infection in order to adapt recommendations to the populations and to reduce the risk of getting COVID-19 to the most vulnerable people. This is particularly the case for patients having common chronic respiratory diseases (CRD) such as asthma and COPD, but also for patients with less common or rare CRD, such as cystic fibrosis (CF) or interstitial lung diseases. Even if it seems reasonable to think that, as they have a lung impairment, people with CRD would be at higher risk to develop a severe COVID-19, the magnitude of this risk is still uncertain [1]. Along with the clinical follow-up of these patients to have a better estimation of their risk, basic research regarding pathophysiology of SARS-CoV2 infection will be useful for a better understanding.

It is particularly true for patients with CF. This disease is caused by variants in the *CFTR* gene, the most frequent being F508del, which result in an abnormal function of the airway epithelial cells (AEC). During the course of CF, lungs of the patients are inflamed and chronically infected by various pathogens including *Pseudomonas aeruginosa,* which is the most prevalent (80% in CF adults) [2]. Up to now, a multinational report identified 40 cases of CF patients infected by SARS-CoV-2 with no death recorded [3]. Of these cases, 78% had symptoms for COVID-19 at presentation such as fever (77% of symptomatic patients), altered cough (48%) or dyspnea (32%). Importantly, 50% of CF patients tested positive for SARS-CoV-2 had chronic *P. aeruginosa* airways infection. AEC are known to play a critical role in the immune response and in COVID-19 severity [4]. SARS-CoV-2 infects the airways

through ACE2 receptor and two specific host proteases TMPRSS2 and FURIN were shown to play a major role in SARS-CoV-2 infectivity [5-9].

In this report we show that *P. aeruginosa* and its virulence factor flagellin (*PA*-F), a ligand of Toll-Like receptor 5 (TLR5) are able to increase TMPRSS2 expression in airway epithelial cells. Importantly, we demonstrated that this increase is higher in cells deficient for CFTR.

We first examined *ACE2*, *FURIN* and *TMPRSS2* expression from a previous transcriptomic study performed with primary airway epithelial cells (hAEC) isolated from control donors (non-CF) and CF patients homozygous for the F508del variant[10]. At baseline, similar *ACE2* (Fig. 1A) and *FURIN* (Fig. 1B) mRNA expression is observed between non-CF and CF pAEC, while *TMPRSS2* expression level is increased in CF hAEC (Fig. 1C). Importantly, *P. aeruginosa* induces *TMPRSS2* mRNA expression (Fig. 1C) in CF hAEC only, while *ACE2* and *FURIN* expression (Fig. 1A & 1B respectively) are not affected. In order to study the mechanism behind this increase, we used Calu-3 cell lines which express higher level of *ACE2* and *TMPRSS2* mRNA and proteins than Beas-2B and 16HBE cell lines (Fig. 1D, 1E, 1F). This result is consistent with the already observed higher capacity of SARS-CoV-2 to replicate in Calu-3 cells in comparison to Beas-2B [6]. Next, we observed that exposure of Calu-3 cells to *P. aeruginosa* flagellin (but not lipopolysaccharide, not illustrated), a bacterial component present in sputa of CF patients [11], has no effect on *ACE2* expression (Fig. 1G) while it increases TMPRSS2 transcript and protein more importantly in Calu3 deficient for *CFTR* (Calu3-*CFTR*-KD) than in in Calu3 sufficient (Calu3-*CFTR*-WT) (Fig. 1H, 1I). Flagellin induces the synthesis of inflammatory cytokine IL-8 (Fig. 1J) both in Calu3-*CFTR*-WT and Calu3-*CFTR*-KD. As already described [12], CF epithelial cells exhibit a more potent inflammatory response to flagellin. In contrast, exposure to VacciGrade™ recombinant flagellin from *Salmonella typhimurium* has no effect on *TMPRSS2* and *ACE2* mRNA expression, nor on IL-8 synthesis. This result is of major importance since recombinant *S.*

typhimurium flagellin, used as an adjuvant in the clinical development of new vaccines against respiratory viruses, including Influenza, was recently suggested to modulate the innate response in order to eliminate SARS-CoV-2 and resolve COVID-19 disease [13]. In order to ensure that our results are not restricted to Calu-3 cell line, we confirmed the induction of TMPRSS2 by *P. aeruginosa* flagellin in pAEC from one healthy donor (Fig 1K). Altogether, these results suggest that *P. aeruginosa* infection, through flagellin, may predispose people to SARS-CoV-2 infection by increasing TMPRSS2 expression, thus increasing SARS-CoV-2 entry into the airway epithelial cells (Fig. 1L). In fact, inhibition of TMPRSS2 by camostat mesylate is sufficient to prevent infection of Calu-3 cells by the virus *in vitro* [6]. This is particularly important for patients with CF as well as with COPD, whom airways are frequently infected and colonized by *P. aeruginosa* during the course of the disease [14]. Since we also observed an increased *TMPRSS2* expression in control AEC cells, a *P. aeruginosa* ventilator-associated pneumonia [15] in SARS-CoV-2 patients under intubation in intensive care units could be deleterious.

For CF patients already infected or colonized by *P. aeruginosa,* it will be necessary to further determine if this increased expression of TMPRSS2 supports SARS-CoV-2 replication and associated inflammation. The understanding of the molecular mechanisms that could promote SARS-CoV-2 interaction and replication with the host cells are critical for future therapeutic management.

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Author Contributions

LG, VB, MR and JB designed experiments, MR, JB, CC, VB and LG made the experiments. LG wrote the manuscript. MR, JB, VB, and HC revised the manuscript.

Conflict of Interest

Authors have no conflict of interest to declare.

Figure Legend

Figure. 1. (**A**) *ACE2*, (**B**) *FURIN* and (**C**) *TMPRSS2* mRNA expression in pAEC infected by *P. aeruginosa* (RNAseq data extracted from[12]) (non-CF vs. CF at T0, *padjBH=1.76x10^{-3}; T0 vs T6h in CF, *padjBH=0.048). (**D**) *ACE2* and *TMPRSS2* mRNA expression in submerged cultures of Calu-3, Beas-2B (reference group) and 16HBE14o- cell lines (n=3, ANOVA Dunnett's multiple comparison test, ***p<0.001). (**E**) Western blot of ACE2 protein expression in submerged cultures of Calu-3, Beas-2B and 16HBE14o- cell lines. (**F**) Immunofluorescence analysis of ACE2 and TMPRSS2 protein expression in submerged cultures of Calu-3 cells. Nucleus are stained with DAPI and (-) denotes negative control with secondary antibody alone. (**G**) *ACE2* and (**H**) *TMPRSS2* mRNA expression in Calu-3-*CFTR*-WT (reference group) and *CFTR*-KD grown at the air-liquid interface (ALI) and either unstimulated (-) or stimulated 3 h and 6 h by *P. aeruginosa* flagellin (*Pa*-F, 50 ng/ml) or recombinant *Salmonella typhimurium* flagellin (*St*-F, 50 ng/ml) (n=5), ANOVA Bonferroni's multiple comparison test, *p<0.05, ***p<0.001, ****p<0.001). (**I**) Immunofluorescence analysis of TMPRSS2 protein expression in Calu-3 (ATCC) cells grown at the ALI and stimulated by *Pa*-F for 18 h. (**J**) IL-8 production of Calu-3-CFTR-WT and CFTR-KD grown at the ALI and unstimulated or stimulated 3 h and 6 h by *Pa*-F (50 ng/ml) or *St*-F (50 ng/ml)

(n=5, ANOVA Bonferroni's multiple comparison test, *p<0.05, ***p<0.001, ***p<0.0001). (**K**) *ACE2* and *TMPRSS2* mRNA expression analysis in submerged pAEC (n=1) incubated with control media (reference group) or 6 h by *Pa*-F (50 ng/ml). (**L**) Proposed model of the impact of *P. aeruginosa* infection in SARS-CoV2 susceptibility.

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Supplementary information

Ethics

This project is approved (Opinion number 20-688) by the Inserm Institutional Review Board (IRB00003888, IORG0003254, FWA00005831).

Materials and Methods

Reagent	**Source**	**Reference number**	**Working Concentration/dilution**
Calu-3 cells	ATCC	HTB-55™/Lot: 62657853	-
BEAS-2B	ATCC	CRL-9609™/Lot 59227035	
Human airway epithelial cells of bronchial origin hAEC	Epithelix	EP51AB/Batch: 02AB077201F2	-
Flagellin from *P. aeruginosa*	Invivogen	TLRL-PAFLA	50 ng/ml
Flagellin FliC VacciGrade™	Invivogen	VAC-Fla	50 ng/ml
LPS *P. aeruginosa*	Sigma-aldrich	L8643	1 µg/ml
Anti-TMPRSS2	ThermoFisher scientific	14437-1-AP	IF: 1/100
Anti-ACE2	RnD sytsems	AF933	Western-blot:1/200 IF: 1/60
Anti-β-actin	Sigma-aldrich	A2228	Western-blot:1/5000
Anti-rabbit Alexa 488	Cell signaling technology	4412	IF: 1/2000
Anti-goat Alexa 488	ThermoFisher scientific	A11078	IF: 1/2000
Anti-rabbit-HRP	Cell signaling technology	7074	Western-blot:1/10000
Anti-goat-HRP	ThermoFisher scientific	A27014	Western-blot:1/2000

Taqman Gene Expression Assay ACE2	ThermoFisher scientific	Hs01085333_m1	-
Taqman Gene Expression Assay TMPRSS2	ThermoFisher scientific	Hs00237175_m1	-
Taqman Gene Expression Assay GAPDH	ThermoFisher scientific	Hs00237175_m1	-
Human IL-6 DuoSet ELISA	RnD sytsems	DY206	-
Human IL-8 DuoSet ELISA	RnD sytsems	DY208	-

Cell culture

Calu-3 (ATCC), Calu-3-*CFTR*-WT and Calu-3-*CFTR*-KD (generously given by Pr. M. Chanson, University of Geneva, Switzerland) cells were cultured in MEM-Glutamax (ThermoFisher scientific) supplemented by 10% FCS (Eurobio, Les Ulis, France), 1% non-essential amino acids (ThermoFisher scientific), 10 mM HEPES (ThermoFisher scientific), 1% sodium pyruvate (ThermoFisher scientific) and 1% antibiotics (ThermoFisher scientific) as previously described[16]. Primary hAEC (from a male Caucasian donor aged 63 with no pathology nor smoker activity) were cultured as recommended by the manufacturer using hAEC complete culture media (Epithelix, Geneva, Switzerland). Beas-2B cells were cultured in F12 media supplemented by 10% FCS, 10 mM HEPES, and 1% antibiotics. 16HBE14o- were generously given by Dieter Gruenert (originator) and Dr. Beate Illek (provider) from Unversity of California San Francisco (UCSF). They were cultured in MEM-Glutamax supplemented by 10% FCS, and 1% antibiotics as recommended by the provider.

RT qPCR

RNA was isolated using a NucleoSpin RNA/miRNA (Macherey Nagel, Duren, Germany). RT was performed using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA, USA).

Real-time qPCR was performed with an ABI QS3, using Sensifast Probe Lo-Rox Kit (Bio-technofix, Guibeville, France), TaqMan probes and cDNA as a template. For relative quantification, the amount of target genes was normalized to the expression of GAPDH relative to reference group (specified in the figure legends) used as a calibrator and was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Total proteins were extracted using RIPA buffer (Euromedex, Souffelweyersheim, France). An equal amount of proteins (20 µg) was reduced, size-separated on 12% stain-free precast SDS-polyacrylamide gels (Biorad, Hercules, CA), and transferred to nitrocellulose membranes using iBlot2 apparatus (ThermoFisher scientific). The membranes were blocked in 5% milk in TBS-Tween 0.1% and incubated with specific primary antibodies O/N at 4°C. Bound antibodies were detected using clarity chemiluminescent substrate (Biorad). Images were recorded with a Fujifilm LAS-3000 bioimaging system (Fujifilm, Stamford, CT, USA).

Immunofluorescence

Cells were plated in 24-well plate on 12 mm diameter #1.5 coverslips (Marienfeld, Lauda-Königshofen, Germany) or on filters at the air-liquid interface and growth as previously described. After treatments, cells were rinsed with PBS and fixed with ice cold PFA 4% 20 min. The cells were permeabilized 10 min with 0.1% Triton X-100 in PBS; and then, washed with PBS and incubated in saturation solution (PBS+BSA 5%) for 1h. Cells were then incubated overnight at 4 °C with primary antibodies in PBS supplemented with 1% BSA. The following day, the cells were washed 3 × 5 min with PBS and incubated for 1h at room temperature with secondary antibodies, followed by DAPI staining. Coverslips were sealed

with ProLong diamond mounting media (ThermoFisher scientific). Fluorescence microscopy was achieved using a Olympus fluorescent microscope.

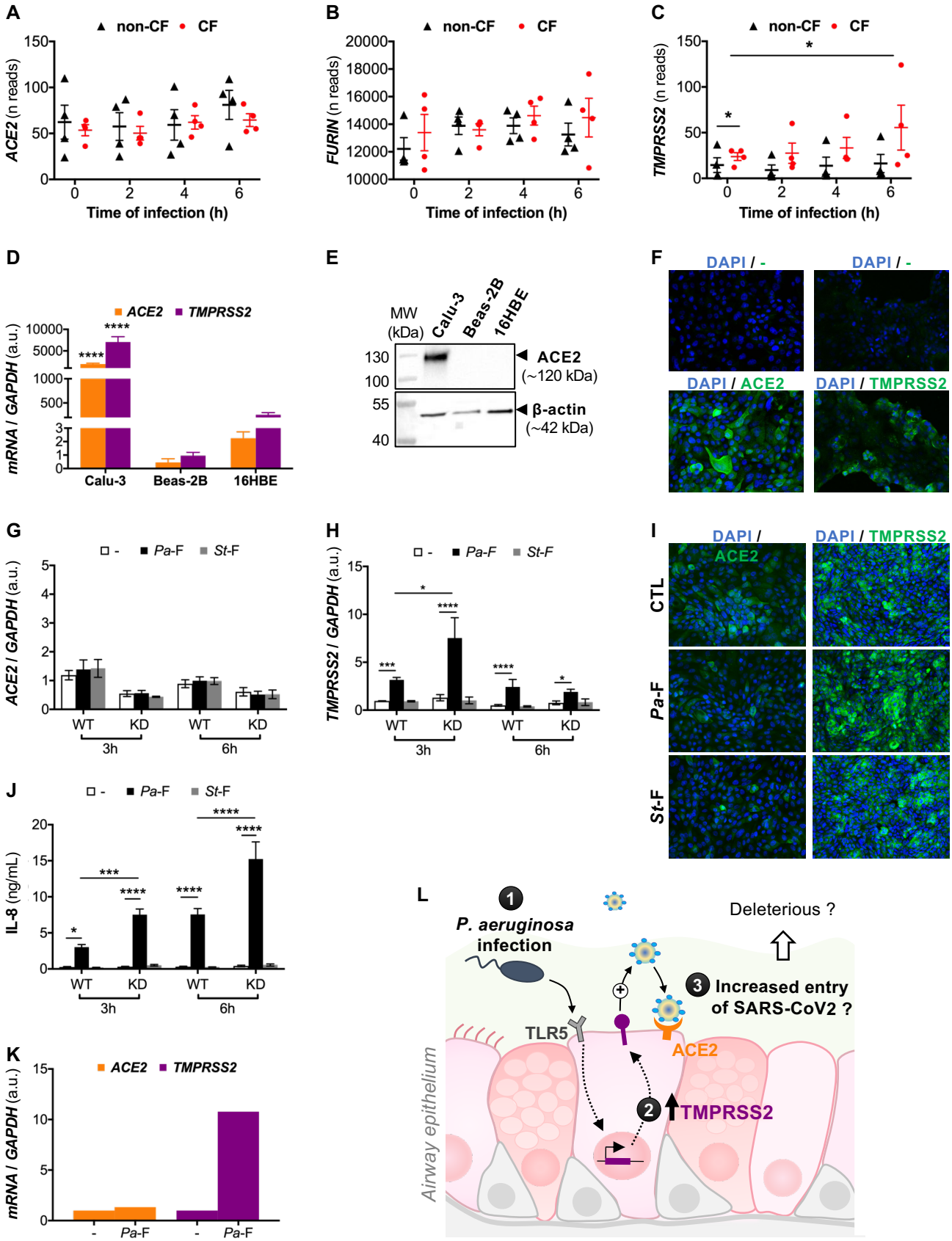
ELISA

Concentrations of human IL-8 (R&D, Minneapolis, MN, USA) were measured in cell supernatants using ELISA kit according to the manufacturer's instructions. The 3,3′,5,5′-tetramethylbenzidine substrate was from Cell signaling technology.

Statistical analysis

Differences among groups were assessed for statistical significance using Prism 7.00 software (GraphPad Software, La Jolla, CA, USA) as indicated in the Figure Legends. Differences with $p < 0.05$ were considered statistically significant.

Figure. 1


A
non-CF
CF
ACE2 (n reads)
Time of infection (h)
B
FURIN (n reads)
C
TMPRSS2 (n reads)
D
ACE2
TMPRSS2
mRNA / GAPDH (a.u.)
Calu-3
Beas-2B
16HBE
E
MW (kDa)
ACE2 (~120 kDa)
β-actin (~42 kDa)
F
DAPI / -
DAPI / ACE2
DAPI / TMPRSS2
G
ACE2 / GAPDH (a.u.)
WT
KD
3h
6h
H
TMPRSS2 / GAPDH (a.u.)
I
CTL
Pa-F
St-F
J
IL-8 (ng/mL)
K
L
P. aeruginosa infection
Deleterious ?
Increased entry of SARS-CoV2 ?
TLR5
ACE2
TMPRSS2
Airway epithelium