Research paper

Lung immunoglobulin A immunity dysregulation in cystic fibrosis

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Abstract

Background: In cystic fibrosis (CF), recurrent infections suggest impaired mucosal immunity but whether production of secretory immunoglobulin A (S-IgA) is impaired remains elusive. S-IgA is generated following polymeric immunoglobulin receptor (pIgR)-mediated transepithelial transport of dimeric (d-IgA) and represents a major defence through neutralisation of inhaled pathogens like Pseudomonas aeruginosa (Pa).

Methods: Human lung tissue (n = 74), human sputum (n = 118), primary human bronchial epithelial cells (HBEC) (cultured in air-liquid interface) (n = 19) and mouse lung tissue and bronchoalveolar lavage were studied for pIgR expression, IgA secretion and regulation.

Findings: Increased epithelial pIgR immunostaining was observed in CF lung explants, associated with more IgA-producing plasma cells, sputum and serum IgA, especially Pa-specific IgA. In contrast, pIgR and IgA transport were downregulated in F508del mice, CFTR-inhibited HBEC, and CF HBEC. Moreover, the unfolded protein response (UPR) due to F508del mutation, inhibited IgA transport in Calu-3 cells. Conversely, pIgR expression and IgA secretion were strongly upregulated following Pa lung infection in control and F508del mice. Through an inflammatory host response involving interleukin-17.

Interpretation: A complex regulation of IgA secretion occurs in the CF lung, UPR induced by CFTR mutation/ dysfunction inhibiting d-IgA transcytosis, and Pa infection unexpectedly unleashing this secretory defence mechanism.

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Introduction

Cystic fibrosis (CF) is a lethal genetic autosomal recessive disease, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1], which encodes for an anion channel expressed in epithelial cells. Whereas all epithelia may be affected, lung disease is mainly responsible for the morbidity and mortality of patients with CF [2]. It is characterised by sticky mucus, chronic neutrophilic inflammation, recurrent opportunistic infections, and progressive formation of bronchiectasis. Pseudomonas aeruginosa (Pa) is the most common bacteria found in the CF adult lung and is associated with a worsening of lung function and vital prognosis [3,4]. The susceptibility to opportunistic Pa infection relates to impaired mucosal immunity underlying a permissive environment in CF airways. In
Cystic fibrosis (CF) is a lethal genetic autosomal recessive disease, which is caused by mutations on cystic fibrosis transmembrane conductance regulator (CFTR) gene and mainly affects the lungs. Lung disorder is characterised by airway inflammation and Pseudomonas aeruginosa (Pa) infection that affects almost 70% of patients with CF and is associated with poor clinical outcomes. Recurrent infections suggest impaired mucosal immunity in patients and the secretion of immunoglobulin A (IgA) is one of the defence mechanisms. IgA is transported across the epithelium by a transcellular routing mediated by the polymeric immunoglobulin receptor (pIgR). In the bronchial lumen, secretory IgA binds antigens and pathogens, and neutralises them through the so-called immune exclusion. pIgR has been studied in several chronic respiratory diseases, where its expression is downregulated. However, no data is available in CF. Previous studies reported increased IgA concentrations in serum or bronchoalveolar lavage of patients with CF. Conversely, decreased IgA secretion was described in CF saliva and in gastric luminal fluid.

Implications of all the available evidence

This study reconciled previous data by showing a complex interplay between epithelial cells, bacteria, and CFTR dysfunction, unfolded protein response, and a Th17 host innate immune response to IgA secretion in the lung. However, we observed in our study that a local specific humoral response to Pa leads to increased Pa-specific IgA in chronically infected patients in both sputum and serum. These measurements could provide a diagnostic tool for Pa chronic infection in the lungs of patients with CF.

Methods

Patients

Lung tissue were obtained from 74 patients, which included explants obtained from 36 patients with end-stage CF disease compared with lung tissue from 38 control patients undergoing lung surgery for a solitary lung tumour (n = 32) or unused lung donors (n = 6). Sputum and serum were obtained from 118 subjects, with 67 patients with CF at stable state compared with 51 age-matched control subjects with no clinical evidence of lung disease and normal lung function. 25 patients were enrolled for cell culture, which included seven control subjects (undergoing lung resection surgery for a solitary lung tumour) and 18 patients with severe CF (undergoing lung resection surgery for transplantation) (Table 1). Patients with CF were classified according to their state of infection by Pa as follows: 1) chronic infection, when having more than 50% of months, when samples had been taken, positive in a 12-months period, 2) intermittent infection, when 50% or less months, when samples had been taken, positive in a 12-months period, 3) free of infection, when samples had been taken, positive in a 12-months period, 4) never infected, when Pa was never isolated in culture. Among Pa-never infected patients, eight out of ten were infected by other bacteria (such as Staphylococcus aureus, Chromobacter, or Burkholderia species) and two patients were infected by Aspergillus fumigatus.

Immunohistochemistry for human and mouse pIgR, and human IgA

Lung samples were fixed in 4% formaldehyde and embedded in paraffin wax. Paraffin lung sections (5 μm-thick) were deparaffinised in toluene and rehydrated through a graded series from methanol to...
water. Except for human plgR/SC, sections underwent antigen retrieval treatment using a pressure cooker treatment during 5 min at a pressure of 15 psi. Endogenous peroxidase, biotin, and avidin solutions or Bloxall (Vector Laboratories Inc., USA), as appropriate. Non-specific protein binding sites were blocked with 5% bovine serum albumin or horse serum (Bio-Rad, USA) in tris-buffered saline (TBS) for 1 h, according to the secondary antibody. Rabbit polyclonal anti-human plgR (1 μg/mL; home-made Lp877), mouse anti-human IgA (4 μg/mL; Thermo Fisher Scientific, Germany), or goat anti-mouse plgR (R&D Systems, UK) were incubated overnight at 4°C. After washes with TBS 0.1% Tween 20, sections were incubated with goat anti-rabbit IgG (1:3000; Sigma-Aldrich, USA) – followed by streptavidin-horseradish peroxidase (HRP) amplification –, horse anti-mouse Ig (ImmPRESSSTM Reagent) or mouse anti-goat IgG (1:100; Sigma-Aldrich), respectively. Revelation was performed by using 3,3′-Diaminobenzidine (Sigma-Aldrich). Sections were counterstained with Hematoxylin (Sigma-Aldrich) and coverslipped. Slides were scanned using Leica SCN400 (Leica, UK) before selecting the ten preserved fields. Quantification was done with Leica software and expressed as percentage of positive area for plgR expression and for IgA immunostaining as the number of IgA positive cells per mm².

**Dual immunohistochemistry for IgA and cluster of differentiation (CD) 138, and for CD3 and RAR-related orphan receptor (ROR)γt**

As previously, unstained paraffin sections were deparaffinised, rehydrated, and subjected to antigen retrieval. Endogenous peroxidase activity was blocked by incubation in Bloxall (Vector lab, USA) for 15 min and 0.3% hydrogen peroxide in TBS 5% goat serum (Abcam) for 30 min respectively. Each section was subjected to two sequential stainings, each including a blocking with TBS 5% goat serum followed by primary antibody incubation and corresponding secondary HRP-conjugated polymer antibody (Invitrogen). Mouse anti-human IgA (Thermo Fisher Scientific) and rabbit anti-CD138 (Acris, Germany), or rabbit anti-CD3 (Cell Signaling, USA) and mouse anti-RORγt (Sigma) were successively used as primary antibody.

Each HRP-conjugated polymer mediated the covalent binding of a different fluorophore using tyramide signal amplification, as previously described [24]. Finally, sections were counterstained with Hoechst (Thermo Fisher Scientific) and mounted with fluorescence mounting medium (Dako, USA). As negative controls, we used corresponding control isotypes diluted at the same concentration as related primary antibody. Images were acquired with Panoramic P250 Flash III slide scanner (3DHistech, Hungary). Quantification of the CD3 and RORγt co-staining was performed with Author software (Visiopharm®) and was expressed as the number of positive cells per μm².

**Basal cell isolation**

The bronchial section was digested by pronase E from Streptomyces griseus 1 mg/mL (Sigma-Aldrich) in Roswell Park Memorial Institute (RPMI) medium (Lonza, Belgium) supplemented with 200 U/mL penicillin and 200 μg/mL streptomycin (Lonza) overnight at 4°C. Cells were labelled with anti-CD151 conjugated with phycoerythrin (Clone 14A2.H1, BD Biosciences) and anti-CD142 conjugated with fluorescein isothiocyanate (BD Biosciences). Basal cells (CD151+/CD142+) were selected and seeded in a 75 cm² cell culture flask in bronchial epithelial basal medium (BEBM) (Lonza), supplemented with 200 U/mL penicillin and 200 μg/mL streptomycin (Lonza) (as well as Primocin™100 μg/mL, Invitrogen, France) for CF cells), bovine serum albumin 1.5 mg/mL (Sigma-Aldrich) and retinoic acid 100 μM (Sigma-Aldrich), M (Sigma-Aldrich). Cells were cultured in a submerged condition for 15 days. Then air-liquid interface (ALI) condition was applied in a 75 cm² cell culture flask in bronchial epithelial basal medium (BEBM) (Lonza), supplemented with 200 U/mL penicillin and 200 μg/mL streptomycin (Lonza) overnight at 4°C. Cells were labelled with anti-CD151 conjugated with phycoerythrin (Clone 14A2.H1, BD Biosciences) and anti-CD142 conjugated with allopregocyanin (Clone HTF1, Miltenyi Biotec, Netherlands) antibodies after blocking with FcR Blocking Reagent (Miltenyi Biotec) and dead cells discrimination by Fixable Viability Stain 450 (BD Biosciences, USA). Cell sorting was performed with a FACSAria cell sorter (BD Biosciences). Basal cells (CD151+CD142+) were selected and seeded in a 75 cm² cell culture flask in bronchial epithelial basal medium (BEBM) (Lonza), supplemented with 200 U/mL penicillin and 200 μg/mL streptomycin (Lonza) (as well as Primocin™100 μg/mL, Invitrogen, France) for CF cells), bovine serum albumin 1.5 mg/mL (Sigma-Aldrich) and retinoic acid 100 μM (Sigma-Aldrich). Cells were cultured at 37°C and 5% CO₂ to reconstitute a primary bronchial epithelium. When reaching 90% confluence, cells were trypsinised and 100,000 cells were seeded in 0.33 cm² insert with 0.4 μm polyester membrane pore (Corning, USA) in submerged condition for 10–15 days. Then air-liquid interface (ALI) condition was applied for two weeks in BEBM:Dulbecco’s Modified Eagle Medium (DMEM) (Lonza) 1:1 supplemented as pure BEBM.

| Table 1 | Patient characteristics. |
|---|---|
| **Lung tissue series** | | |
| Subjects, n | 38 | 36 | 74 |
| Sex, F/M | 21/17 | 20/16 | 41/33 |
| Age, years | 58 ± 14 | 31 ± 9 | 45 ± 18 |
| Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 22/10/4 | NA |
| FEV1, % predicted (n = 68) | 94 ± 18 | 24 ± 8 | 60 ± 38 |
| **Sputum and serum series** | | | |
| Subjects, n | 51 | 67 | 118 |
| Sex, F/M | 35/16 | 36/31 | 71/47 |
| Age, years | 31 ± 10 | 34 ± 11 | 33 ± 11 |
| BMI, kg/m² | 23 ± 5 | 22 ± 3 | 22 ± 4 |
| Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 36/24/7 | NA |
| FEV1, % predicted | 100 ± 9 | 67 ± 23 | 81 ± 25 |
| VC, % predicted | 102 ± 10 | 87 ± 19 | 93 ± 17 |
| Stimulation | NA | 33/12/10/NA |
| **Human-derived epithelial cells series** | | | |
| Subjects, n | 7 | 18 | 25 |
| Sex, F/M | 1/6 | 9/9 | 10/15 |
| Age, years | 62 ± 8 | 35 ± 12 | 43 ± 16 |
| Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 97/14 | 48 ± 34 |
| Subjects, n (plgR expression) | 6 | 8 | 14 |
| Subjects, n (UPR activation) | 8 | 10 | 18 |
| Subjects, n (PuP supernatant stimulation) | 0 | 5 | 5 |
| Subjects, n (IL-17 stimulation) | 0 | 6 | 6 |

Definition of abbreviations: CF, cystic fibrosis; F, female; M, male; FEV1, forced expiratory volume in the first second; NA, not applicable; BMI, body mass index; VC, vital capacity; Pa, Pseudomonas aeruginosa; C, chronically infected by Pa; I, intermittently infected by Pa; F, free of Pa infection; N = never infected by Pa; plgR, polymorphic immunoglobulin receptor; UPR, unfolded protein response. N is specified when data are missing.
In vitro epithelial assays

Primary human bronchial epithelial cells (HBEC) were treated after culture for two weeks in ALI condition with 25 μL of CFTR inhibitors (GlyH-101 or PPQ-102) (CF Foundation) (1, 5, 10, 20 μM), or 25 μL of Pa supernatant, at the apical pole for 48 h. Basal media, apical washes, and cell lysates (for western blot or reverse transcription and real-time polymerase chain reaction (RT-qPCR)) were then harvested. HBEC cultures were also treated for 48 h with increasing doses of IL-17 (10, 20, 40 ng/mL). Media and cells were then collected and harvested.

For the transcytosis assay of d-IgA, after two weeks in ALI condition, 1 mg/mL d-IgA was added at basolateral pole for 72 h, and S-IgA was measured by sandwich Enzyme Linked Immunosorbent Assays in the apical wash (300 μL phosphate-buffered saline).

Pa supernatant preparation and stimulation

Pa mucoid clinical strain was processed according to Massion and colleagues to obtain Pa supernatant [25]. Briefly, Pa mucoid clinical strain was seeded in Tryptic soy Broth medium (BD, USA) and incubated during 72 h, at 37°C. Then, bacteria culture was centrifuged at 10,000 g during 50 min, at 4°C. Supernatant was filtered (0.2 μm) and stored at -80°C. After two weeks in ALI condition, ALI-HBEC were stimulated with 25 μL of Pa supernatant at apical pole for 48 h. Sterile phosphate-buffered saline was used as control condition. Basal media, apical washes, and cell lysates (for western blot or RT-qPCR) were then harvested.

Calu-3 cell culture

Calu-3 cells (passages 10–15) were cultured at 37°C and 5% CO2 in a RPMI medium (Lonza), supplemented with 10% foetal bovine serum, 200 U/mL penicillin and 200 μg/mL streptomycin (Lonza). Cells were maintained in a 75 cm2 cell culture flask and split when they reached 90% confluence. For unfolded protein response (UPR) activation, Calu-3 cells were stimulated with thapsigargin (Sigma-Aldrich) (0.2, 0.5, 1, 2 μM) or tunicamycin (Sigma-Aldrich) (2, 5, 10, 20 μg/mL), diluted in dimethyl sulfoxide (DMSO), during 6 h or 24 h. DMSO was used as control condition. Media and cell lysates (for western blot or RT-qPCR) were then harvested. For transcytosis assay of d-IgA, Calu-3 cells (50,000 per insert) were seeded in 0.33 cm2 insert with 0.4 μm pore nylon filter (Prosep, Belgium) and centrifuged at 450 g, during 5 min. Neutrophil elastase activity was measured in supernatant. Remaining supernatant was treated with PMSF Protease Inhibitor 2% v/v in order to inactivate elastase and was stored at -80°C. Cells were processed for cytospin (fixed in methanol and staining by Diff-Quick method (Kwik-DiffTM Stains, Thermo Fisher Scientific) and epithelial contamination was assessed. The blood was centrifuged at 650 g for 20 min, the serum was collected, and stored at -80°C.

F508del mice and model of Pa lung infection with Pa-coated beads

Mice (FVB/129) homozygous for the F508del mutation (F508del mice, kindly received from Dr T. Leal, UCLouvain Brussels, Belgium) and wild-type littermates (WT mice) were housed in a specific pathogen free animal facility, in ventilated cages. 15 F508del mice and 15 WT received no treatment (eight females and seven males in each group). In parallel, nine F508del (eight females and one male) and six WT (four females and two males) mice were instilled with agaroase beads, coated with a clinical strain of Pa (isolated from a patient with CF). Sterile beads were used to instil seven F508del (four females and three males) and eight WT (three females and five males), as control. Beads were prepared according to Martin and colleagues [27]. Dissolved agaroase (coloured with Indian ink) (Type XII, Sigma-Aldrich®) was mixed with mineral oil (Sigma-Aldrich®) at 50°C, and with around 100 colony-forming units of Pa in culture. This mix was cooled down with ice while mixing for 20 min. After rinsing by centrifugations at 4°C, beads were sieved twice to obtain a homogenous solution of 300 to 400 μm beads, a size considered optimal to penetrate the lower airways, but not the alveoli. In order to control the bacterial load, beads and Pa culture were spread on agar-agar and counted after incubation during 24 h at 37°C. A volume of 40 μL of beads solution at 5% in phosphate-buffered saline (sterile beads or Pa-coated) was instilled in mouse trachea after anaesthesia with Ketamine 15 mg/mL (Nimatek® 100 mg/mL Eurovet, Netherlands) and Xylazine 2 mg/mL (Rompun® 2%, Bayer, GmbH D24106, Germany) at 100 μL/10 g body weight. Eventual respiratory stress could appear after instillation and therefore mice were closely monitored and received oxygen supplementation if needed. After 15 days, mice were sacrificed, BAL was performed, and lungs were collected for immunohistochemistry, RT-qPCR and western blot, as well as blood to obtain serum.

Western blot for plgR/SC and (phospho-)euukaryotic initiation factor 2α ((P-)eIF2α)

Cells were lysed with Laemmli’s Blue. After boiling for 5 min, lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel and proteins were transferred to a nitrocellulose membrane. After blocking with TBS 5% bovine serum albumin, the membrane was incubated with rabbit anti-plgR (1:6000; homemade Lp877), rabbit anti-GAPDH (1:3000; Sigma-Aldrich), mouse anti-eIF2α (1:2000; Cell Signaling), rabbit anti-P-eIF2α (1:1000; Cell Signaling), and mouse anti-β-actin (1:1000; Sigma-Aldrich) overnight at 4°C, then with goat anti-rabbit IgG (1:2000; Cell Signaling) or anti-mouse IgG (1:5000; Sigma-Aldrich) for 1 h at room temperature. Detection of immunoreactivity was performed with the ECL™ Prime Western Blotting Detection Chemiluminescence reagent (Amer sham™ ECL, GE Healthcare, UK) blot and quantification was performed by using Quantity One software.

Enzyme Linked Immunosorbent Assays for SC, S-IgA, S-IgM, specific IgA to Pa and IL-17

SC, S-Ig, and total Ig concentrations were measured in HBEC apical washes, sputum supernatants or sera, in plates coated with anti-SC (1:2000; Ch606), anti-IgA (1:1000; ACP17) or anti-IgM (Sigma-
Aldrich) as capture antibody. After washes, samples and standard incubated 2 h, at 37°C, and detection was performed with biotinylated anti-SC (1:4000; Ch606) – followed by Streptavidin-HRP amplification – or HRP-linked anti-IgA (1:5000; Sigma-Aldrich) or anti-IgM (1:5000; Sigma-Aldrich).

In sputum supernatants, specific IgA to Pa were determined by coating 2.25 μg of antigens from serotypes 001-015 (Statens Serum Institute, Denmark) and detection was performed with the antibody mentioned above, and IL-17 was measured by Duoset (R&D Systems).

In mouse BAL and serum, concentrations of mouse total IgA, as well as (only in BAL) mouse S-IgA and mouse SC, were measured. Plates were coated with anti-mouse IgA (1:1000; Sigma-Aldrich) or anti-mouse SC (1:200; R&D Systems). After blocking, samples and standard (IgÃ­k myeloma (Sigma-Aldrich) or pure BAL) were loaded and incubated 2 h, at 37°C. Immunodetection was carried out by using biotinylated anti-mouse IgA (SYnAbs, Belgium) and Streptavidin-HRP amplification. For mouse SC, samples and standard (R&D Systems) were coated in plates and incubated overnight at 4°C. Mouse SC was captured with goat anti-mouse SC (R&D Systems) and detected by biotinylated anti-goat IgG (Sigma-Aldrich) and Streptavidin-HRP amplification.

Reverse transcription and real-time polymerase chain reaction

After extraction, ribonucleic acid (RNA) was reverse-transcribed with RevertAid H minus Reverse transcriptase kit (Thermo Fisher Scientific, UK) with 0.3 μg of random hexamer, 20 U of RNase inhibitor, and 1 mM of each dNTP following the manufacturer’s protocol in a thermocycler (Applied Biosystems). For the expression measurement, the reaction mix contained 2.5 μL of complementary deoxyribo- nucleic acid diluted 10-fold, 200 nM of each primer (Table S1), and 2x iTaq UniverSybr Green® Supermix (Bio-Rad) in a final volume of 20 μL. The cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. To control the specificity of the amplification products, a melting curve analysis was performed. Copy number was calculated from the standard curve. Expression levels of target genes were normalised to the geometric mean of the values for the housekeeping genes (ribosomal protein S13, S18 and L27 in human lung tissue and primary HBEC, S18 and hypoxanthine-guanine phosphoribosyltransferase 1 in Calu-3, and TATA-box binding protein, hypoxanthine-guanine phosphoribosyltransferase 1 and tyrosine 3-monoxygenase/tryptophan 5-monooxygenase activation protein zeta in mouse tissue).

Transmission electron microscopy

ALI-HBEC were fixed overnight at 4°C in 2.5% glutaraldehyde BEBM: DMEM 1:1 (Lonza), supplemented as mentioned before, washed in cacodylate buffer (pH 7.4) and postfixed twice in 1% osmium tetroxide (and 1.5% ferrocyanide) for 1 h. Then, cells were stained with 1% uranyl acetate for 1 h at room temperature, dehydrated in ethanol and embedded in epoxy resin (Agar 100 resin; Agar Scientific, UK). Quantification...
of the ER area was performed with ImageJ software. Results were expressed as the percentage of ER area.

Statistical analysis

GraphPad Prism 8.0.2 software (GraphPad Inc., USA) and SPSS (IBM) were used for statistical analysis. Normality of the data distribution was first assessed. Comparisons between two groups were performed using an unpaired Student’s t test, except when data distribution were not normal. In such case, comparisons between two groups were performed using the Mann-Whitney U-test, whereas comparisons between more than two groups were performed using the Kruskal-Wallis test followed by a Dunn’s post-hoc test or Friedman test in case of paired values. Comparisons were considered as significant if p-value was under 0.05.

Ethics statement

All patients received information and signed a written consent to the study protocol, which was approved by our local clinical ethical committee (Ref. protocol “CLARA” 2005/22SEP/149 – update 29/11/2016, S51577 S52174 S55877) and CPP lle de France II #1072 for lung tissue samples, Ref. 2015/09Jan/014 for sputum samples). Murine experiments were approved by our local Faculty Ethical committee (Ref. 2018/UCL/MD/04).

Results

Upregulation of epithelial plgR and IgA expression in the human CF lung

plgR expression was increased in the bronchial epithelium from patients with CF, compared with controls, in both large and small airways (Fig. 1a–c) while no significant difference was observed at the mRNA level in lung homogenates (Fig. 1d).

As an increased number of lymphoid aggregates was confirmed in our CF lungs, compared with controls (Fig. S1), IgA immunostaining was carried out on lung tissue, and confirmed a large increase in the number of IgA+ cells in CF lungs in subepithelial areas (Fig. 2a) and in the surrounding submucosal glands (Fig. 2b). A dual immunostaining for IgA and CD138 indicated that >70% of IgA+ cells consisted of IgA+ plasma cells (Fig. 2c).

Upregulation of total and Pa-specific IgA in human CF sputum

Despite raised neutrophil elastase activity in sputum from patients with CF (Fig. S2a), which is able to cleave IgA and SC [28], the concentration of IgA was increased in sputum and serum from patients with CF, compared with controls, especially in chronically Pa infected patients (Fig. 3a–b). Neber Pa infected patients were nevertheless infected with other bacteria (such as Staphylococcus aureus, Acromobacter, or Burkholderia species) or Aspergillus fumigatus (in eight and two out of ten patients, respectively). A similar increase was observed for IgM (Fig. S2b), also secreted following plgR-mediated transport. In addition, a trend for increased S-IgA and no change for SC (Fig. 3a) were noticed. Of note, higher concentrations of IgA and SC were measured in sputum from F508del homozygous patients when compared with patients without this mutation (Fig. S2c–f).

In addition, a Pa-specific IgA response was observed in sputum and serum from patients with CF, chronically or intermittently infected with Pa (Fig. 3c–d), and in patients carrying at least one F508del mutation (Fig. S2ag–h).

Downregulation of plgR expression following CFTR dysfunction in the human bronchial epithelium

In order to avoid the major risk of infection of primary CF HBEC, airway basal cells were sorted (by using CD142 and CD151 staining), and cultured in ALI for two weeks to reconstitute in vitro a differentiated CF bronchial epithelium [29]. In contrast to tissue data, a reduced plgR expression was observed in CF HBEC, compared with the epithelium derived from control lungs both at protein (Fig. 4a) and mRNA level (Fig. 4b). In addition, the CF-derived epithelium displayed strong decreases in SC and S-IgA release in the apical washes (Fig. 4c–d). To confirm this negative link between CFTR dysfunction and plgR expression, control HBEC were treated at 2 weeks in ALI culture with increasing concentrations of two CFTR inhibitors (GlyH-101 and PPQ-102). After 48 h of treatment, a dose-dependent decrease in plgR/SC production and SC release in apical washes was observed (Fig. 4e–f).

Downregulation of plgR expression and IgA secretion following CFTR dysfunction in F508del mice

In order to confirm plgR downregulation in “sterile” environment upon CFTR dysfunction in vivo, we studied plgR expression in F508del mice. A decreased plgR expression was observed, as compared with WT mice (Fig. 5a), as well as a lower concentration of total IgA in BAL (Fig. 5b). As in human lung, S-IgA in BAL (Fig. 5c) and Plgr mRNA expression in lung homogenates were not significantly changed (Fig. 5d).

UPR is activated in CF-derived HBEC and F508del mice, and affects SC secretion

To explore the mechanism of plgR downregulation observed upon CFTR dysfunction, in both human CF HBEC and F508del mice, we hypothesised that the ER stress and UPR could be involved. Indeed, F508del mutation has been associated with an accumulation of misfolded proteins in the ER, resulting in ER stress and UPR activation [30]. UPR activation assessed by the mRNA ratio of active, spliced/unspliced, XBP-1 was confirmed in F508del mouse lungs (Fig. 6a), which received no treatment. A trend was also observed in the reconstituted epithelium from patients with CF (most of them harbouring at least one copy of the F508del mutation) as compared with controls (Fig. 6b). In addition, ER stress was also evidenced by transmission electron microscopy showing dilated ER cavities in CF, while this feature was absent in control HBEC (Fig. 6c). Therefore, we stimulated UPR in Calu-3 cells (by thapsigargin or tunicamycin), an epithelial cell line which stably expresses plgR at high level, to evaluate whether this regulates plgR expression. UPR activation leading to phosphorylation of eIF2α (Fig. 6d–e), increased spliced/unspliced XBP1 mRNA ratio (Fig. 6f) and dose-dependently decreased plgR protein expression (Fig. 6g) and SC release (Fig. 6h). In addition, d-IgA transcytosis was impaired in Calu-3 cells treated with tunicamycin (Fig. 6i). These data indicate that UPR activation per se (or that follows ER stress) could recapitulate the downregulation of plgR expression and IgA transport observed in CF cell cultures and in CF mouse lungs.

Upregulation of plgR expression and (S-)IgA production upon Pa infection

In order to reconcile the apparent discrepancy between upregulated plgR expression in human CF lungs and its downregulation upon CFTR dysfunction in HBEC and in F508del mice, we reasoned that the negative CFTR-plgR link could be overcome during lung infection with Pa. We observed that WT mice instilled with Pa-coated beads displayed increased plgR expression in the airway epithelium compared with sterile beads (Fig. 7a). In addition, a strong upregulation of IgA and S-IgA concentrations was observed in BAL upon Pa
infection both in WT and F508del mice (Fig. 7b), associated with an increase in PIGR mRNA expression (Fig. 7d).

Pa infection upregulates plgR expression through an indirect mechanism involving IL-17

To address the mechanisms by which Pa could upregulate plgR expression and IgA secretion, CF HBEC were incubated with the supernatant of a Pa clinical strain culture. Only marginal and not significant changes in PIGR mRNA expression, plgR protein, SC release and d-IgA transcytosis were observed in CF-derived cells treated for 48 h with Pa supernatant (Fig. S3). An indirect mechanism was then explored by looking at the inflammatory response induced by Pa infection. In particular, we evaluated IL-17 as it is reportedly increased upon Pa infection, and it is able to upregulate PIGR mRNA expression in intestinal cells [31,32]. An induction of Il17 mRNA expression was noticed in the lungs from mice instilled with Pa-coated beads, compared with mice instilled with sterile beads (Fig. 8a). While IL-17 upregulation was not recapitulated in sputum or lung tissue from patients with CF (Fig. 8b–c), immune cells producing IL-17 (RORγt+ cells, including CD3+ RORγt+ Th17 cells) [33,34] were increased in subepithelial areas of patients with CF, compared with controls (Fig. 8d), as well as in total lung tissue (Fig. S4a). When CF-derived cells were stimulated by increasing concentrations of IL-17A, we observed a dose-dependent increase in plgR expression (Fig. 8e), associated with a trend to increased PIGR mRNA expression, SC release and d-IgA transcytosis (Fig. 8f–h), which did not influence UPR activation through XBP-1 splicing (Fig. 8i). Upregulated PIGR mRNA was also measured in Calu-3 cells stimulated by IL-17A (Fig. S4b–d). As shown in CF-derived HBEC, no influence on UPR activation through XBP-1 splicing was observed upon IL-17A treatment (Fig. S4e). These data suggest that Pa infection could stimulate plgR expression and S-IgA production in the CF lung by activating an inflammatory host response that involves IL-17.

Discussion

This study shows that epithelial plgR expression, IgA production, and IgA+ B cells are all upregulated in the CF lung, sputum and serum. This IgA response includes specific antibodies to Pa which represents a major opportunistic bacterium in this disease. Secondly, experiments in airway epithelial basal/stem cell-derived cultures from CF lungs and in F508del mice revealed a constitutive downregulation of plgR/SC production and d-IgA transcytosis, as recapitulated by adding CFTR inhibitors in control cells. This negative CFTR-plgR pathway involved UPR activation following the ER stress observed in CF. Finally, an in vivo model of chronic lung infection by Pa-coated microbeads enabled to identify that infection could restore plgR expression and IgA production in the lungs from F508del mice, through an IL-17 inflammatory host response (Fig. 9).

In contrast to previous data in COPD or chronic rhino-sinusitis, this study shows that bronchial plgR expression is
Fig. 3. IgA and Pa-specific IgA concentrations are increased in sputum and serum from patients with CF. (a) IgA concentration in sputum from 51 controls and 65 patients with CF (**p = 0.0092, unpaired Student’s t-test), distributed following Leeds criteria (**p < 0.001, *p < 0.01, Kruskal-Wallis test followed by Dunn’s test); S-IgA concentration in sputum from 51 controls and 65 patients with CF (p = 0.1146, unpaired Student’s t-test); SC concentration in sputum from 51 controls and 65 patients with CF (p=0.2516, unpaired Student’s t-test). (b) IgA concentration in serum from 51 controls and 66 patients with CF (***p = 0.0006, unpaired Student’s t-test), distributed following Leeds criteria (***p < 0.001, Kruskal-Wallis test followed by Dunn’s test). (c) Pa-specific IgA concentration in sputum from 51 controls and 65 patients with CF (**p = 0.0107, unpaired Student’s t-test), distributed following Leeds criteria (**p < 0.001, *p < 0.05, Kruskal-Wallis test followed by Dunn’s test). (d) Pa-specific IgA concentration in serum from 51 controls and 66 patients with CF (***p = 0.0003, unpaired Student’s t-test), distributed following Leeds criteria (***p < 0.001, **p < 0.01, *p < 0.05, Kruskal-Wallis test followed by Dunn’s test). Bars indicate mean and standard deviation or median and interquartile ranges (Leeds distribution). IgA, immunoglobulin A; Pa, Pseudomonas aeruginosa; CF, cystic fibrosis; S-IgA, secretory immunoglobulin A; SC, secretory component; C, chronically infected by Pa; I, intermittently infected by Pa; F, free of Pa infection; N, never infected with Pa.
upregulated in CF [16–18,35]. Numbers of IgA+ B cells are also increased in this disease, as previously observed in COPD [36]. Whereas decreased SC concentration in saliva from patients with CF was previously reported [37], Marshall et al. observed an increased concentration and altered glycosylation of SC in CF sputum [21]. Our study could reconcile the apparent discrepancy between previous studies regarding IgA [20–23], by suggesting that IgA upregulation may selectively occur in the lungs (but not in the digestive tract) as those are the target of acquired bacterial infection (e.g. Pa) in CF. This increased IgA response is particularly noticed in patients with severe disease, and includes the production of serum/systemic and respiratory/secretory Pa-specific IgA [9,38–42]. Our study robustly confirms increased IgA levels in CF airways, as well as increased serum IgA, and that this response includes a specific reactivity to Pa at both levels.

A major finding of this study is that epithelial pIgR expression and IgA transport are disrupted in primary cultures of HBEC from patients with CF, as well as in the lung from F508del mice. In human lung homogenates, Pigr gene expression was not affected, maybe due to the large amount of non-pIgR expressing cell types into the lung. On the contrary, the use of CF-derived HBEC showed impaired pIgR expression when cultivating in the absence of inflammatory stimulus and allows to distinguish slight difference (notably for PIGR mRNA levels). *In vitro* experiments using selective CFTR inhibitors could confirm that CFTR dysfunction induces pIgR protein downregulation and defective pIgR
protein expression is also demonstrated in non-infected F508del mice. F508del mutation, which was also carried by most of our patients with CF from whom cells were derived, is associated with improper folding and accumulation of the CFTR protein in the ER. Therefore, we hypothesised and confirmed that ER stress and subsequent UPR activation could represent a mechanism involved in plgR downregulation, in line with previous reports in short-term cultures of CF-derived cells and in a CF cell line [30,43]. We could also demonstrate UPR activation through XBP-1 splicing in the lungs from F508del mice even in the absence of infection. Furthermore, our data show that UPR activation in epithelial cells induces a downregulation of plgR/SC production as well as of d-IgA transcytosis. In addition, it has been shown that UPR activation in CF not only fails to resolve the ER stress but also sensitises the inflammatory response to Toll-like receptor stimulation [30]. Interestingly, plgR downregulation (among several other proteins) was also linked to UPR activation in the context of ulcerative colitis [44], whereas another recent study showed that mice with a restricted deletion of XBP-1 in the intestinal epithelium (thus enable to develop UPR to counteract ER stress in these cells) display T cell-independent accumulation of intestinal epithelial cells [32]. Interestingly, Th17-mediated responses (through IL-17A production) were shown to upregulate plgR expression in the airway epithelium and IgA/M concentrations in BAL from mice [54]. Other inflammatory cytokines such as IL-1β and TNF-α, which are also upregulated upon Pa infection and able to stimulate plgR transcription in airway cell lines [55,56], could also be implicated. Similarly, the observation of increased levels of IgA in sputum from never infected patients (Fig. 3a) suggests that other opportunistic bacteria (e.g., *Staphylococcus aureus, Burkholderia cepacia*) could drive similar mechanisms as those evidenced with *Pa*. However, increased concentration of IL-1β and TNF-α as well as IL-8, and neutrophil infiltration has been demonstrated in BAL of young infants, without the presence of detectable pathogens, suggesting “sterile inflammation” [57,58]. But these statements remain controversial [59,60]. We could not assess this in our patients with CF cohort (for lung tissues and sputum) since these adult patients (although few were never infected by *Pa*) were carriers...
Fig. 6. UPR is activated in CF-derived human HBEC and in lungs from F508del mice at baseline and it downregulates SC secretion in Calu-3 cells. (a) Xbp1s/Xbp1u mRNA ratio (corrected for housekeeping genes) in lung from ten F508del mice receiving no treatment, compared with ten WT mice (*p = 0.0133, Mann-Whitney test). (b) XBP1s/XBP1u mRNA ratio (corrected for housekeeping genes) in HBEC (cultured in sterile condition) from nine patients with CF, compared with seven controls (p = 0.1738, Mann-Whitney test). (c) Transmission electron microscopy in HBEC (cultured in sterile condition) from one control and from one patient with CF and quantification of ER (arrows) surface in one control and from one patient with CF (**p = 0.0094, unpaired Student’s t-test). (d) Representative western blot for eIF2α, P-eIF2α and pIgR in Calu-3 cells treated with increasing concentrations of thapsigargin, tunicamycin or DMSO as control condition. (e) Quantification of P-eIF2α/eIF2α protein expression in Calu-3 cells after treatment with increasing concentrations of thapsigargin and tunicamycin, compared with DMSO as control condition (n = 3). (f) XBP1s/XBP1u mRNA ratio (corrected for housekeeping genes) in Calu-3 cells treated with increasing concentrations of thapsigargin and tunicamycin, compared with DMSO as control condition (n = 3). (g) SC secretion upon treatment with increasing concentrations of thapsigargin and tunicamycin, compared with DMSO as control condition (n = 3). (h) SC secretion upon treatment with increasing concentrations of thapsigargin and tunicamycin, compared with DMSO as control condition (n = 3). (i) After transcytosis assay, S-IgA release upon thapsigargin treatment (*p = 0.0286, Mann-Whitney test) (n = 4). Bars indicate median and interquartile ranges, except for (c) (mean and standard deviation). UPR, unfolded protein response; XBP1s, spliced X-box binding protein 1; XBP1u, unspliced X-box binding protein 1; eIF2α, eukaryotic initiation factor 2 α; P-eIF2α, phospho-eukaryotic initiation factor 2 α; pIgR, polymeric immunoglobulin receptor; SC, secretory component; S-IgA, secretory immunoglobulin A; Tha, thapsigargin; Tun, tunicamycin. Scale bar, 500 nm.
of other pathogens. Recent studies related to CF sterile inflammation (in CF animal models and CF samples) showed the potential responsibility of hypoxic injury, resulting in necrosis, IL-1β release and sterile neutrophilic inflammation [61]. The possible induction of IL-1β and activation of NF-κB by IL-1α might be responsible of plgR expression early in patients with CF, even before infection detection. As mentioned before, despite showing no CF-like lung phenotype, F508del mice develop a minor but spontaneous inflammation in the lungs. However, we could notice that plgR expression was still affected in this model although the decrease was less marked than in cellular model. This could be due to isolation of cells from inflammatory microenvironment.

The preserved IgA response to pathogens was already suggested by increased Pa-specific IgA antibodies in saliva and nasal secretions from patients with CF [9], as well as by increased serum specific IgG correlated to the presence of Pa in the sinuses [62]. Such Pa sinussitis may precede intermittent lung infection, suggesting that parasinus sinuses may represent a bacterial reservoir able to re-contaminate the patient after lung eradication [63]. Pa-specific IgA in saliva was also assessed as a predictor of Pa chronic infection [64,65]. Similarly, we observed in our study that a local specific IgA response to Pa is consistently observed in chronically infected patients, both in serum and sputum secretions. Whether those IgA assays could provide the added value to IgG antibodies and bacteriology for the diagnosis of Pa (chronic) infection should be further studied in larger and multicentric clinical cohorts.

One limitation of our study first relates to the end-stage status of patients with CF recruited for tissue samples (lung explants). It is however remarkable that sputum data from a large spectrum of disease severity retrieved consistent findings, indicating that S-lgA upregulation encompasses the full spectrum of adult patients with CF. Second, we encountered difficulties to observe differences in IL-17 levels (and did not observe changes in PIGR expression despite SC alterations) in lung homogenates. While increased number of IL-17-producing cells were shown in CF, we could not confirm increased IL-17 expression or secretion possibly as a result of the dilution of the signal by the nature of the sample (tissue homogenate). As mentioned above, while plgR mRNA changes were observed in isolated broncho-epithelial cells, no difference was measured in lung homogenates, possibly due to a similar dilution of the signal. In addition, this study did not directly assess whether IgA plays a protective role in the lung from patients. It was reported that overglycosylation of SC in CF reduces its capacity to bind and neutralise IL-8/CXCL8, hence favouring neutrophilic inflammation [21]. It is further possible that accumulated S-lgA in the bronchial lumen (Fig. 2b), following increased production and impaired mucociliary clearance, could form immune complexes that – instead of being eliminated to achieve immune exclusion – activate FcγRI-bearing phagocytes [66]. Thus, the exact role of upregulated lung IgA responses during CF should be further investigated. Finally, plgR dysregulation in the absence of lung infection was assessed in F508del patients but the state of IgA secretion (through plgR) remains unclear in non-F508del patients and could be further addressed in a dedicated study of such patients even though these mutations are rarer and most patients suffer from early infection. It would be interesting to also address this point in CFTR-KO mice, compared to F508del mice.

In conclusion, this study in CF reveals a complex interplay between epithelial cells, B cells, and bacteria (Pa), which connects CFTR dysfunction, UPR activation, and a (Th17) host innate immune response to IgA secretion in the lung.
Fig. 8. IL-17 is increased in lungs from mice infected with Pa and upregulates pIgR expression in ALI-HBEC derived from patients with CF. (a) IL17 mRNA expression (corrected for housekeeping genes) in lungs from six WT mice instilled with Pa-coated beads, compared with eight WT mice instilled with sterile beads (*p = 0.0373, Mann-Whitney test). (b) IL-17 concentration in sputum from 65 patients with CF, compared with 51 controls (p = 0.3088, Mann-Whitney test). (c) IL17 mRNA expression (corrected for housekeeping genes) in lung homogenates from 15 patients with CF, compared with 16 controls (p = 0.0000, Mann-Whitney test). (d) RORγt staining (and CD3) in the subepithelial area from one representative control and one representative patient with CF, and number of RORγt+ cells (filled and no-filled arrows), including CD3+ RORγt+ cells (filled arrows), in the subepithelial area from 9 patients with CF, as compared with 10 controls (*p = 0.0279, p = 0.0551, Mann-Whitney test). (e) Representative western blot for pIgR in CF ALI-HBEC, treated with increasing concentrations of IL-17 and its pIgR-SC/GAPDH expression quantification in six CF ALI-HBEC, treated with increasing concentrations of IL-17 (*p = 0.0437, Friedman test). (f) pIgR mRNA expression (corrected for housekeeping genes) in five CF ALI-HBEC, treated with increasing concentrations of IL-17. (g) SC release by six CF ALI-HBEC, treated with increasing concentrations of IL-17. (h) XBP1s/XBP1u mRNA ratio (corrected for housekeeping genes) in five CF ALI-HBEC, treated with increasing concentrations of IL-17. Bars indicate median and interquartile ranges. IL-17, interleukin 17; Pa, Pseudomonas aeruginosa; pIgR, polymeric immunoglobulin receptor; CF, cystic fibrosis; ROR, RAR-related orphan receptor; CD, cluster of differentiation; SC, secretory component; S-IgA, secretory immunoglobulin A; XBP1s/u, spliced/unsliced X-box binding protein 1. Scale bar, 50 μm.
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Declaration of interests

The authors have declared that no conflict of interest exists.

Author contributions

A.M.C. conducted the experiments and analysis and wrote the paper; M.L. performed mice sacrifice, RT-qPCR for human lung tissues and helped with cell cultures and tissue processing for immunochemistry; S.N. and T.L. provided technical knowledge, F508del and WT mice and CFTR inhibitors; B.D. helped with sputum processing and ELISA; F.C. helped with FACS sorting protocol setting and supervised patients database; F.A.N. helped with tyramide multiplex immunohistochemistry; C.B. helped with quantification of immunohistochemistry; M.V. performed transmission electron microscopy; V.D. provided some sputum samples; L.R., C.M. and P.R.B. provided some lung tissues for immunochemistry, designed and performed Pseudomonas aeruginosa infection in mice; D.H. and S.V. participated in the collection of lung tissue samples for culture, immunochemistry and RNA analysis; A.F. reviewed the paper; C.P. and S.G. designed the study, supervised patient recruitment, data analysis and the writing of the paper.

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Supplementary materials

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