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## Short Communication

## TLR5 signalling is hyper-responsive in porcine cystic fibrosis airways epithelium



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## ABSTRACT

Excessive lung inflammation and airway epithelium damage are hallmarks of cystic fibrosis (CF) disease. It is unclear whether lung inflammation is related to an intrinsic defect in the immune response or to chronic infection. We aimed to determine whether TLR5-mediated response is defective in the CF airway epithelium. We used a newborn CF pig model to study intrinsic alterations in CF airway epithelium innate immune response. Airway epithelial cells (AECs) were stimulated with flagellin or lipopolysaccharide to determine responses specific for TLR5 and TLR4, respectively. We observed a significant increase in cytokine secretion when CF AECs were stimulated with flagellin compared to wild type (WT) AECs. These results were recapitulated when AECs were treated with an inhibitor of CFTR channel activity. We show that TLR5-signalling is altered in CF lung epithelium at birth. Modulation of TLR5 signalling could contribute to better control the excessive inflammatory response observed in CF lungs.

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## 1. Introduction

Cystic fibrosis (CF), the most common lethal genetic disease in the Caucasian population, is a recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, rendering the protein non-functional [1]. It is characterized by chronic bacterial infection, mainly *Pseudomonas aeruginosa*, persistent inflammation with exacerbated recruitment of polymorphonuclear neutrophils (PMNs) into the lungs, excessive release of proteases and ultimately lung tissue destruction [1].

There is a long-standing debate regarding the links between *CFTR* mutations and the development of persistent infection and inflammation [1]. While some suggest that *CFTR* dysfunction leads to intrinsic inflammation [2], others hypothesize that excessive inflammation is rather the result of a lung environment that favours bacterial colonization [3]. The development of the mutated *CFTR*<sup>ΔF508/ΔF508</sup> pig [4] and the *CFTR*<sup>-/-</sup> knockout pig [5], which

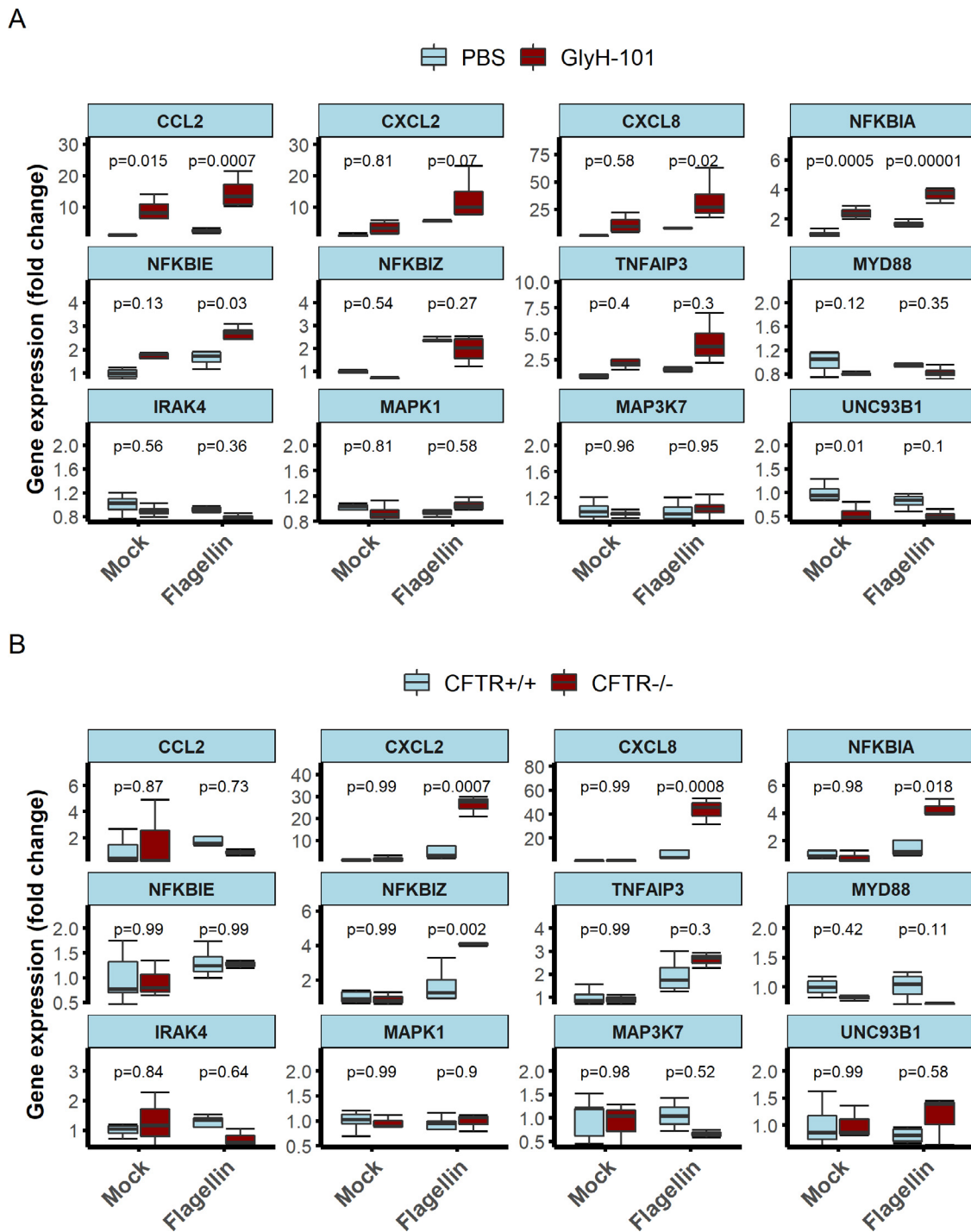
mimic the human CF symptoms show that there is no intrinsic airways inflammation in naive, non-infected lungs [4]. However, their immune response upon a bacterial challenge seems to be altered facilitating lung colonization by the pathogen and consequently a pro-inflammatory lung environment [6].

Understanding the processes leading to this altered inflammatory response would be crucial to improve CF therapies. Toll-like receptors (TLRs) are the main family of pattern recognition receptors (PRRs) of the innate immune system. In the CF context, TLR4 and TLR5, which interact with LPS and flagellin, respectively, are known to be major factors in the immune response to *P. aeruginosa* [7]. In particular, TLR5 response to flagellin has been suggested to play a role in the excessive inflammation observed in CF airway epithelial cells [8]. Here, we aimed to determine whether TLR5 signalling is defective in the CF airway epithelium using primary AECs from newborn *CFTR*<sup>-/-</sup> piglets.

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**Fig. 1. Newborn *CFTR*<sup>-/-</sup> piglets present an exacerbated TLR5 response.** (A) Gene transcription analysis in airway epithelial cells from wild-type pigs treated with 50  $\mu$ M of apical GlyH-101 or the vehicle for 24 h and stimulated for 5 h with 200  $\mu$ l of either 100 ng/ml of apical FliC $_{\Delta 174-400}$  or the vehicle. Gene expression levels are shown relative to the mock group (treated with the vehicle). (B) Real-time RT-qPCR analysis of airway epithelial cells from newborn *CFTR*<sup>+/+</sup> and *CFTR*<sup>-/-</sup> piglets stimulated for 5 h with either 200  $\mu$ l of 100 ng/ml of apical FliC $_{\Delta 174-400}$  or the vehicle. Data were analysed by two-way ANOVA, using pig genotype and flagellin stimulation as factors, followed by Tukey's HSD *post hoc* test. Data are representative of four experiments.

## 2. Materials and methods

### 2.1. Ethics statement

All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at INRAE. All experimental procedures were evaluated by the Ethics Committee of the Val de Loire (CEEA VdL, committee N°19) and approved by the Ministry of Higher Education and Research (APAFIS#1166-2015071615392426 Notification and APAFIS#10125-20170602162555 Notification).

### 2.2. Animals

Male and female *CFTR*<sup>+/-</sup> transgenic pigs were provided by the LMU Munich, Germany [5], transferred to INRAE, Centre Val de Loire at Nouzilly (UE-PAO, INRAE Centre de Recherche Val de Loire, Nouzilly, France) and mated to generate *CFTR*<sup>+/+</sup>, *CFTR*<sup>+/-</sup> and *CFTR*<sup>-/-</sup> piglets. Piglets were genotyped and sacrificed as described in Guillon et al. [9]. Samples were collected within 6 hours of birth.

### 2.3. Production of recombinant flagellin

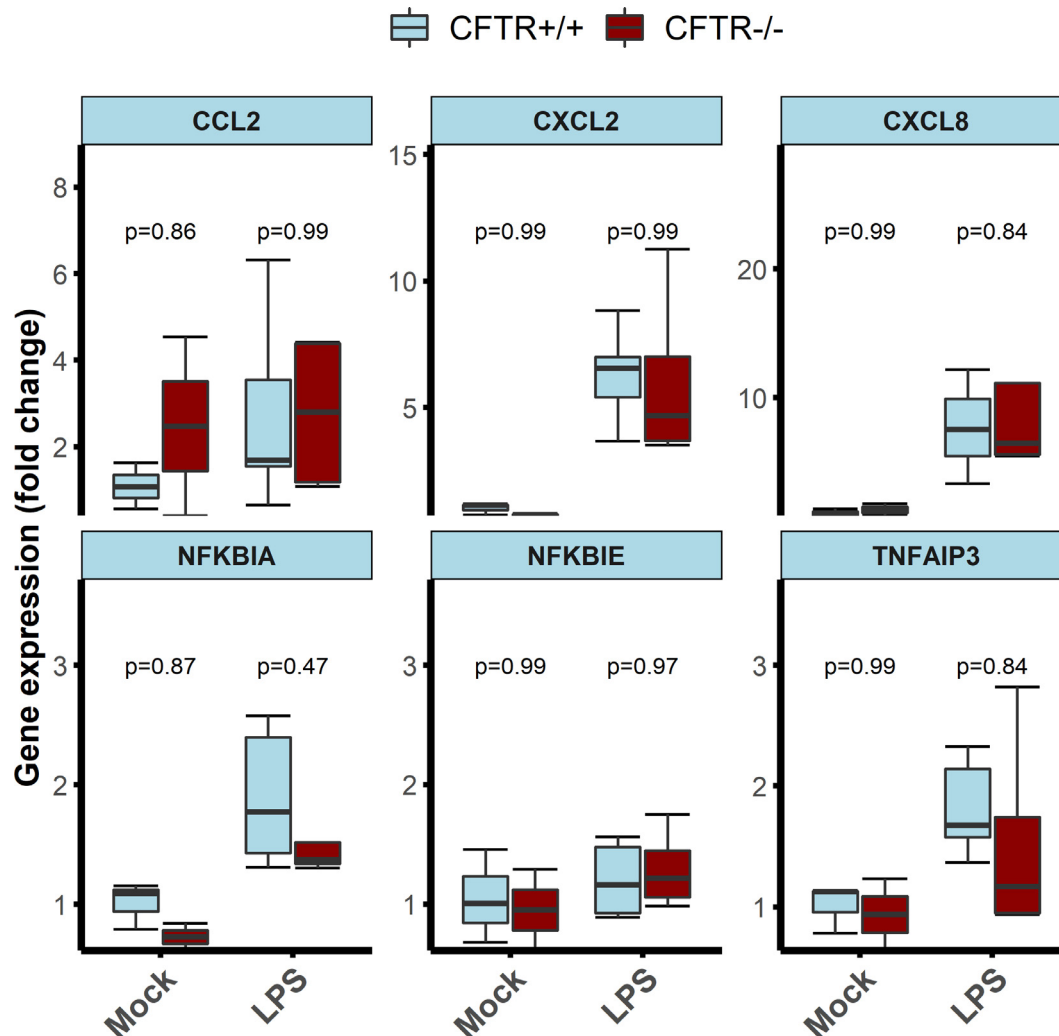
The recombinant flagellin FliC<sub>Δ174-400</sub> came from *S. enterica* serovar Typhimurium FliC and was produced with an histidine tag, as described previously [10].

### 2.4. Development of a differentiated primary culture of airway epithelia (AECs) from newborn cystic fibrosis piglets

Bronchial epithelial cells from newborn *CFTR*<sup>+/+</sup> and *CFTR*<sup>-/-</sup> piglets were differentiated under air-liquid-interface conditions as described in [11].

### 2.5. cDNA pre-amplification and RT-qPCR on the BioMark HD real-time PCR platform

Extracted total RNA was converted to cDNA by reverse transcription of 20 ng of RNA using the Nucleospin® RNA XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Pre-amplification was performed with cDNA diluted 1:10 in TE (10 mM Tris, 0.1 mM EDTA, pH 8.0, Fluka Bio-



**Fig. 2. Real-time qPCR analysis of differentiated airway epithelial cells from newborn *CFTR*<sup>+/+</sup> and *CFTR*<sup>-/-</sup> piglets stimulated with LPS.** Real-time RT-qPCR analysis of airway epithelial cells from newborn *CFTR*<sup>+/+</sup> and *CFTR*<sup>-/-</sup> piglets stimulated for 5 h with 200  $\mu$ l of 1  $\mu$ g/ml of apical LPS. Gene expression was normalized against the unstimulated (Mock) *CFTR*<sup>+/+</sup> group. Data were analysed by two-way ANOVA, using pig genotype and LPS stimulation as factors, followed by Tukey's HSD *post hoc* test. Data are representative of three experiments.

chemika #93283) using a Preamp Master Mix (Fluidigm, # 100-5581). The pre-amplification thermal cycle conditions were: 95°C for 2 min followed by 14 cycles of 95°C for 15 seconds and 60°C for 4 min. Real-time quantitative PCR was performed in a 48 × 48 Dynamic Array Integrated Fluidic Circuits (Fluidigm). The quantitative RT-PCR data were analyzed using the  $2^{-\Delta\Delta C_t}$  method. The results are expressed as relative fold change (Fc) in comparison with the wild type unstimulated control.

## 2.6. Statistical analysis

Statistical analysis were performed using the statistical package R version 4.0.1 and the packages ggplot2 and ggpubr. Inter-group differences were analysed using two-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* test. A p-value <0.05 was considered statistically significant.

## 3. Results

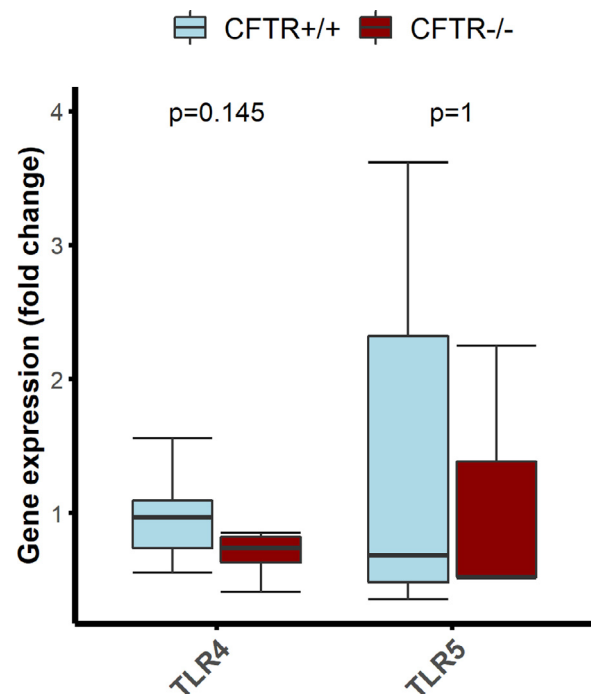
### 3.1. Flagellin induces an inflammatory response in airway epithelial cells that is exacerbated in cystic fibrosis

Airway epithelial cells from WT piglets (cultured under ALI conditions) were treated for 24 h with GlyH-101 (a CFTR inhibitor) and then stimulated apically with the recombinant flagellin FliC $_{\Delta 174-400}$ . We observed that inhibition of CFTR activity with GlyH-101 upregulated the expression of genes coding for inflammatory cytokines (*CCL2*, *CXCL2*) and regulation of the immune response (*NFKBIA*, *NFKBIE*) after flagellin administration (Fig. 1A). We also observed that a 24h supplementation of the AECs with GlyH-101 was enough to increase the gene expression of the evaluated genes in the absence of an inflammatory stimulus (Fig. 1A).

Our data suggest that TLR5 signalling could be altered after pharmacological inhibition of the CFTR channel. However, GlyH-101 could also affect other types of Cl<sup>-</sup> conductance [12]. To confirm the data, we used AECs from newborn *CFTR*<sup>+/+</sup> or *CFTR*<sup>-/-</sup> pigs. Stimulation of *CFTR*<sup>-/-</sup> AECs with FliC $_{\Delta 174-400}$  resulted in a significantly increased expression of *CXCL2* *CXCL8*, which are known to play an important role in the recruitment of neutrophils into the lungs, *NFKBIA* and *NFKBIZ* genes (Fig. 1B). This excessive response to an inflammatory stimulus seemed to be specific for TLR5 since LPS stimulation did not induce a significant overexpression of inflammatory genes in *CFTR*<sup>-/-</sup> cells (Fig. 2). In addition, no differences were found in either *TLR4* or *TLR5* gene expression between *CFTR*<sup>+/+</sup> or *CFTR*<sup>-/-</sup> AECs (Fig. 3).

## 4. Discussion

Regulation of innate immunity in CF has been a topic of controversy throughout the years. We have investigated this question in a relevant translational model of cystic fibrosis, the CF pig model [5]. Porcine lungs share many physiological features with those of humans. Moreover, the pig model also displays a similar lung inflammation upon infection by *P. aeruginosa*, the main CF pathogen, as observed in humans [13]. Also, recent findings show that newborn CF piglets do not present intrinsic inflammation in the lungs, which spontaneously develops later in life [4]. This feature makes the newborn CF pig model especially well-suited to evaluate early defects in innate immune signalling, avoiding confounding effects that could modify the airway epithelium response to an inflammatory stimulus. Our data, however, points to an exacerbated inflammatory response in the airway epithelium driven by TLR5 signalling dysregulation that does not seem to be related with a receptor overexpression. This is in line with earlier studies that describe an overproduction of pro-inflammatory cytokines airway epithelia from CF patients [14]. This fits also with the known major



**Fig. 3.** Real-time qPCR analysis of TLR4 and TLR5 expression in differentiated airway epithelial cells from newborn *CFTR*<sup>+/+</sup> and *CFTR*<sup>-/-</sup> piglets. Gene expression was normalized against the *CFTR*<sup>+/+</sup> group. Data were using the Mann-Whitney–Wilcoxon test. Data are representative of four independent experiments.

role played by TLR5 signalling in the exaggerated cytokine production [8]. In addition, a recent study showed a decreased secretion of airway surface liquid in response to flagellin-expressing *P. aeruginosa* in the CF pig airways *in vivo*, suggesting a possible defect in TLR5 signaling [15]. No differences were observed when *CFTR*<sup>-/-</sup> AECs were stimulated with LPS, which could be related to a decreased sensitivity of AECs to LPS [16]. A possible mechanism explaining the *CFTR*<sup>-/-</sup> AECs increased response to flagellin could be the existence of endoplasmic reticulum (ER) stress in the CF airways. ER-stress have been linked to chronic inflammatory diseases, such as CF [17]. In this regard, *in vitro* induction of ER-stress in the AECs increases their inflammatory response to flagellin [18]. Moreover, we observed that thapsigargin-induced ER-stress in the AECs leads to increased response to flagellin but not LPS (unpublished results).

Our results differ somehow from a study by Bartlett et al [6] who analysed the impact on airway epithelium of heat-killed *Staphylococcus aureus* [6], which does not express flagellin [15], and which is therefore unable to activate TLR5 signalling. Very consistently, a recent study showed opposite effect in the induction of IL-8 by *S. aureus* and *P. aeruginosa* in BEAS-2B airway epithelial cells, with *P. aeruginosa* strongly inducing IL-8 secretion, and *S. aureus* having a minimal effect [19].

In conclusion, our results suggest that TLR5 response is dysregulated in the CF airway epithelium. We point to TLR5 signalling as a key player in this exacerbated inflammatory response. Our data open new leads toward the design of novel immune-modulatory therapies targeting TLR5 that could improve the management of lung inflammation in CF.

## Declaration of Competing Interest

JC Sirard is inventor or co-inventor of Patent Applications WO2009156405, WO2011161491, WO2015011254 and

WO2016102536 directed to recombinant flagellins for the prophylactic and therapeutic use in infectious diseases.

### Credit author statement

**Isabelle Fleurot:** Methodology, Validation, Formal analysis, Writing – Review & Editing

**Raquel Lopez-Galvez:** Methodology, Validation, Formal analysis, Writing – Review & Editing

**Pascal Barbry:** Investigation, Writing – Review & Editing, Resources, Supervision, Project administration, Funding acquisition

**Antoine Guillon:** Investigation, Writing – Review & Editing

**Mustapha Si-Tahar:** Validation, Writing – Original draft, Writing – Review & Editing, Supervision, Funding acquisition

**Andrea Bähr:** Resources

**Nikolai Klymiuk:** Resources, Funding acquisition

**Jean-Claude Sirard:** Methodology, Validation, Formal analysis, Investigation, Writing – Original draft, Writing – Review & Editing, Resources, Project administration, Funding acquisition

**Ignacio Caballero:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original draft, Writing – Review & Editing, Visualization, Resources, Supervision, Project administration, Funding acquisition

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