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TLR5 as an Anti-Inflammatory Target and Modifier Gene in Cystic Fibrosis

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New treatments are needed to improve the health of people with cystic fibrosis (CF). Reducing lung-damaging inflammation is likely to be beneficial, but specific anti-inflammatory targets have not been identified. By combining cellular immunology with a population-based genetic modifier study, we examined TLR5 as an anti-inflammatory target and modifier gene in CF. Using two pairs of human CF and control airway epithelial cells, we demonstrated that the TLR5–flagellin interaction is a major mediator of inflammation following exposure to *Pseudomonas aeruginosa*. To validate TLR5 as an anti-inflammatory target, we analyzed the disease modifying effects of the *TLR5* c.1174C>T single nucleotide polymorphism (rs5744168) in a large cohort of CF patients ($n = 2219$). rs5744168 encodes a premature stop codon and the T allele is associated with a 45.5–76.3% reduction in flagellin responsiveness ($p < 0.0001$). To test the hypothesis that reduced TLR5 responsiveness would be associated with improved health in CF patients, we examined the relationship between rs5744168 and two clinical phenotypes: lung function and body weight. Adults with CF carrying the *TLR5* premature stop codon (CT or TT genotype) had a higher body mass index than did CF patients homozygous for the fully functional allele (CC genotype) ($p = 0.044$); however, similar improvements in lung function associated with the T allele were not statistically significant. Although follow-up studies are needed to confirm the impact of TLR5 on nutritional status, this translational research provides evidence that genetic variation in *TLR5* resulting in reduced flagellin responsiveness is associated with improved health indicators in adults with CF. *The Journal of Immunology*, 2010, 185: 7731–7738.

Cystic fibrosis (CF) is an autosomal recessive disorder resulting from mutations in the CF transmembrane conductance regulator (*CFTR*) gene encoding a chloride channel. Although both understanding and treatment of the disease have developed considerably during the past two decades, new treatment options for patients with CF are critically needed since the median age for survival is still only in the mid-30s (1).

Lung disease, the major cause of death in CF, is caused by chronic infection and inflammation. Current therapies for CF address airway infection with antibiotics and airway obstruction using

physiotherapy clearance techniques combined with mucolytics. Safe and clinically acceptable therapies to target airway inflammation are likely to augment current treatments and improve the clinical outcome in CF (2). Clinical trials have demonstrated that anti-inflammatory therapy is beneficial for patients with CF, improving important clinical outcomes such as lung function and body weight. Oral corticosteroids helped maintain pulmonary function, but unfortunately adverse side-effects render this approach unacceptable (3–5). The nonsteroidal anti-inflammatory ibuprofen, taken at high doses for a long duration, slowed the progression of lung dysfunction, and body weight was better maintained in the ibuprofen-treated group (6–8). In addition to corticosteroids and ibuprofen, increasing evidence shows that azithromycin, a macrolide antibiotic with both antimicrobial and anti-inflammatory activity, benefits people with CF (9–12).

Clinical experience with these relatively nonspecific anti-inflammatory medications provides “proof of concept” evidence that targeting inflammation can be beneficial in CF. Our challenge is now to identify new, specific anti-inflammatory targets. Modulating the function of the innate immune system is a particularly attractive treatment approach, since activation of the innate immune system is central to the inflammatory response occurring in the CF lung (13, 14). Innate immunity relies on a series of germline-encoded receptors, including TLRs, to sense infectious organisms and to trigger an acute inflammatory response. Work by our group and others suggests that modulation of TLR function, and particularly TLR5 inhibition, has therapeutic potential to improve the inflammatory manifestations of CF (15, 16). TLR5 is the receptor for flagellin, the protein component of bacterial flagella expressed by classic CF bacterial pathogens, particularly *Pseudomonas aeruginosa* (17). Emerging evidence indicates that the TLR5–flagellin interaction plays a central role in driving the inflammatory response

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Abbreviations used in this paper: BCC, *Burkholderia cepacia* complex; BMI, body mass index; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DoB, date of birth; FEV₁, forced expiratory volume in 1 s; LDH, lactate dehydrogenase; MOI, multiplicity of infection; PA, infected with *Pseudomonas aeruginosa*; PI, pancreatic insufficient; PS, pancreatic sufficient; PPIA, peptidylprolyl isomerase A; SNP, single nucleotide polymorphism; wt, wild-type.

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triggered by *P. aeruginosa* (16, 18–22), and inhibition of TLR5 normalizes the inflammatory response generated by CF airway epithelial cells following exposure to *P. aeruginosa* (16). In this study we use cellular model systems to further explore the central role of TLR5 in driving the inflammatory response of airway cells against *P. aeruginosa*. We have deliberately focused on the response of CF airway epithelial cells, because evidence suggests that radioresistant lung epithelial cells are essential for triggering the innate immune responses in the lung (23).

Although data from our group and others, in a variety of animal and cellular model systems, indicate that inhibition of TLR5 may decrease inflammation in CF, it would be much more compelling if the benefits of TLR5 modulation could be confirmed using an independent experimental design based on the outcome of people living with CF. The identification of CF modifier genes represents a powerful, clinically relevant strategy to discover and validate new therapeutic targets. Modifier genes are loci containing variants that affect the clinical manifestation of a disease. Modifier genes have received considerable attention in CF: patients with an identical *CFTR* genotype (e.g., $\Delta F508$ homozygous) show great variability in lung function (24–26), and twin studies have confirmed a significant non-*CFTR* genetic contribution to this variation in CF lung disease (27). To further validate TLR5 as an anti-inflammatory target, we analyzed the disease modifying effects of the *TLR5* c.1174C>T single nucleotide polymorphism (SNP) (rs5744168) in a large representative cohort of Canadian CF patients (28, 29). *TLR5* c.1174C>T encodes a premature stop codon (i.e., $TLR5^{392STOP}$) in the ligand-binding domain of *TLR5*. Allele T is found in 6.1% of Canadian CF patients and acts in a dominant fashion with respect to allele C to significantly reduce flagellin responsiveness (30–32). Consequently, through determining the disease-modifying effects of the *TLR5* c.1174C>T SNP, we were able to examine the impact of reduced TLR5 responsiveness on the health of a large cohort of Canadian CF patients.

The goal of this study was to empower the development of new therapies to treat the inflammatory component of CF lung disease by exploring the potential of TLR5 modulation to improve the outcome in CF. We hypothesized that reducing damaging inflammation, through the modulation of TLR5 responsiveness, would improve the clinical outcome in CF. By combining in vitro cellular immunology approaches with a large in vivo population-based assessment of *TLR5* c.1174C>T SNP as a genetic modifier in Canadian CF patients we provide further evidence that TLR5 may represent an effective therapeutic target for improving the outcome in CF.

Materials and Methods

Airway epithelial cell lines and PBMCs

Studies on the inflammatory response in the CF airway were performed on two well-characterized sets of CF and non-CF airway epithelial cells: 1) IB3-1 (compound heterozygote for the $\Delta F508$ and W1282X) and C38 (IB3-1 transfected with *CFTR* using an adeno-associated viral vector) (33); and 2) CuFi-1 ($\Delta F508$ homozygous) and NuLi (wild-type [wt] *CFTR* genotype) (34). It is essential to validate any findings in at least two pairs of CF and control airway epithelial cells, because subtle technical artifacts, possibly related to immortalization and long-term culture, may alter the proinflammatory phenotype of any cell line (reviewed in Ref. 35). Importantly, airway epithelial cells grown at air–liquid interface or as submerged cultures have been shown to have similar patterns of cytokine production (36, 37). IB3-1/C38 cells were grown in precoated flasks (100 μ g/ml BSA, 30 μ g/ml bovine collagen I, 10 μ g/ml human fibronectin) in LHC-8 basal medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% penicillin-streptomycin-amphotericin B solution at 37°C and 5% CO₂. NuLi and CuFi cells were grown in flasks (precoated with 60 μ g/ml human placental collagen type VI; Sigma-Aldrich, St. Louis, MO) in serum-free bronchial

epithelial growth medium (BEGM; Lonza, Walkersville, MD) supplemented with SingleQuot additives in a BEGM BulletKit (Lonza) and 50 μ g/ml G-418 (Invitrogen) at 37°C and 5% CO₂. Sixteen hours prior to use in stimulation assays, the NuLi/CuFi and C38/IB3-1 cell lines were seeded into precoated 96-well plates at a density of 3–5 $\times 10^4$ cells/well. To assess cell viability after stimulation with bacteria or TLR ligands, release of a cytoplasmic enzyme (lactate dehydrogenase [LDH]) by damaged cells was measured using a LDH cell cytotoxicity kit (Roche, Indianapolis, IN).

With approval of the University of British Columbia Clinical Research Ethics Board and subject consent, DNA samples from healthy volunteer donors were obtained and genotyped for the *TLR5* c.1174C>T SNP. Individuals with relevant *TLR5* genotypes subsequently donated blood, PBMCs were isolated, and the cells were seeded into 96-well plates at a density of 1 $\times 10^5$ cells/well, according to protocols described previously (38).

Stimulation assay with pure TLR ligands and whole bacteria

PBMCs were challenged with pure TLR ligands (ultrapure LPS from *Escherichia coli* 0111:B4 [1 ng/ml]; recombinant endotoxin-free flagellin from *Salmonella typhimurium* [40 ng/ml]; InvivoGen, San Diego, CA). Culture supernatants were harvested after 24 h and cytokine concentrations (IL-6 and IL-8) were quantified by ELISA. Airway epithelial cells were exposed to heat-killed whole bacteria (*P. aeruginosa* PAK wt or PAK Δ *flhC*, a nonmotile derivative of PAK wt in which *flhC* is interrupted by a gentamycin cassette) or live whole bacteria (*P. aeruginosa* PAO1 wt) at a variety of multiplicities of infection (MOIs). Stimulation time was 24 h for heat-killed bacteria but was reduced to 4 h for live bacteria to minimize pathogen-driven cytotoxicity. In some experiments TLR5 activation was inhibited using a neutralizing polyclonal Ab raised against the extracellular region of human TLR5 (PAb-hTLR5 [6–25 μ g/ml]; InvivoGen) with a purified rat IgG1 (eBioscience, San Diego, CA) serving as a control.

Gene expression

Total RNA isolated from airway epithelial cells (RNeasy Plus Mini kit; Qiagen, Valencia, CA) was transcribed to cDNA with oligo(dT) primers (high-capacity cDNA reverse transcription kit; Applied Biosystems, Foster City, CA). Quantitative PCR primers were designed for SYBR Green chemistry to amplify peptidylprolyl isomerase A (i.e., cyclophilin A, *PPIA*), *IL6*, and *IL8* (Table I) under standard cycling protocols using a 7300 real-time PCR system (Applied Biosystems). Relative gene expression in the CF compared with non-CF airway cells was analyzed by the 2^{- $\Delta\Delta$ Ct} method using cyclophilin A as a reference gene.

Description of the Canadian CF Modifier Study patient cohort

The Canadian CF Modifier Study has been described in detail elsewhere (28, 29). Briefly, at the time of this study the cohort consisted of 2219 CF patients with samples available for genotyping who were recruited from 37 specialized CF clinics across Canada. The protocols for this study were approved by Ethical Review Boards at the University of British Columbia, Hospital for Sick Children, and all participating institutions. Informed consent was obtained from each individual or his/her guardian. Clinical parameters analyzed were: 1) the mean forced expiratory volume in 1 s (FEV₁) was calculated for each subject from all available FEV₁ measurements in the 3 y before recruitment (or FEV₁% predicted). At each visit, FEV₁% predicted was calculated using pediatric (39) and adult (40) prediction equations based on age, height, and sex. 2) Body mass index (BMI) expressed as SDs above or below median for age and sex based on the most recent height and weight measurement (or zBMI).

Genotyping

The *TLR5* c.1174C>T SNP ($TLR5^{392STOP}$, rs5744168) genotyping was performed in a 384-well format using a commercially available TaqMan assay (C_25608804_10; Applied Biosystems). Twelve positive and 12 negative controls were included in each plate. End-point analysis (allelic discrimination) was performed using an ABI Prism 7900 sequence detection system (Applied Biosystems).

Statistical analyses for the CF modifier study

To minimize the overlap of records in the transition from pediatric to adult CF clinics, patients were stratified into pediatric (<18.5 y of age) and adult groups by the current age, which was defined as the age at the last visit to the clinic. Genotypes for the *TLR5* c.1174C>T SNP in both pediatric and adult groups were in Hardy–Weinberg equilibrium, and after combining CT and TT genotypes a Fisher exact test showed no evidence of genotype difference by gender. Considering the small number of individuals with the TT genotype, it was most appropriate to fit dominant models instead of

Table I. Primer sequences used for quantitative PCR

Gene	Forward (5' → 3')	Reverse (5' → 3')
<i>IL6</i>	TGTGAAAGCAGCAAAGAGGCACTG	ACCAGGCAAGTCTCCTCATTGAAT
<i>IL8</i>	GTGCAGAGGGTTGTGGAGAAGTTT	TGCATCTGGCAACCCCTACAACAGA
<i>PPIA</i>	TAAAGCATACGGGTCTGGCATCT	ATCCAACCACTCAGTCTTGGCAGT

additive or genotypic models (Table II). Linear models were fitted to ascertain the modifying effect of *TLR5* c.1174C>T. Confounders were identified by univariate analyses, and sensitivity analyses were performed to ensure that univariate analyses results were not driven by extreme values. Confounders included mean age (i.e., mean of ages at which FEV₁% predicted was measured), number of clinic visits (i.e., the number of visits with FEV₁% predicted values), and zBMI. Considering the number of sibling pairs in the pediatric and adult group (46 and 15, respectively), both linear and mixed effects models were fitted to assess the effect of the within-family correlation in variance estimation. Because the outputs of the two models did not differ significantly, we only reported the results based on the linear regression. The data were analyzed using SAS version 9.1.2 (SAS Institute, Cary, NC) procedures GLM, MIXED, and GENMOD. The nonparametric Kruskal–Wallis test was considered to see whether the medians of the zBMI in the *TLR5* c.1174C>T genotype groups differed significantly, and, to deal with the within-family correlation, the test was repeated after excluding the younger siblings.

Results

TLR5–flagellin interaction is a major mediator of the inflammatory response generated by CF airway epithelial cells following exposure to P. aeruginosa

To assess the relative contribution of the TLR5–flagellin interaction to the inflammatory response generated by CF airway epithelial cells, our experimental strategy was to alternatively block both sides of the TLR5–flagellin host–pathogen interaction. To remove the pathogen contribution, we stimulated airway epithelial cells with PAKΔ*flhC*, a laboratory strain of *P. aeruginosa* that expresses a full complement of pathogen-associated molecular patterns but lacks flagella. Strikingly, the absence of flagellin significantly reduced proinflammatory IL-6 and IL-8 production at the mRNA ($p < 0.05$ and $p < 0.01$) and protein level ($p < 0.0001$) by the CF airway epithelial cells following exposure to *P. aeruginosa* (Fig. 1A–C). To remove the host contribution of the TLR5–flagellin interaction, we used a neutralizing polyclonal Ab raised against the extracellular region of human TLR5 that has been shown to efficiently and specifically inhibit TLR5 (16). The anti-TLR5 Ab significantly reduced proinflammatory IL-6 production by CF airway epithelial cells following exposure to *P. aeruginosa* (Fig. 1D, 1E). Importantly, the anti-TLR5 Ab was equally effective in reducing inflammation regardless of whether the bacteria were heat-killed (PAK wt strain, $p = 0.004$) or live (PAO1 wt strain, $p < 0.0001$). Experiments using live bacteria were performed using PAO1 rather than PAK, as PAK was profoundly cytotoxic to these cell lines. LDH release assays confirmed that there was no significant cytotoxicity to CF or control cells under our experimental conditions following stimulation with live PAO1 (data not shown).

TLR5 c.1174C>T SNP significantly reduces flagellin responsiveness

To facilitate our genetic modifier study, we first sought to identify a functional variant in *TLR5* that is common in the population.

Table II. *TLR5* c.1174C>T genotype data

Genotype	CC	CT	TT	Total
Pediatric	531	64	2	597
Adult	407	56	3	466

Hawn et al. (30) described the functional impact of the *TLR5* c.1174C>T SNP, which encodes a premature stop codon. To assess the functional impact of *TLR5* c.1174C>T we obtained PBMCs from individuals who were heterozygous for the premature stop codon (CT genotype) and control subjects homozygous for the common allele (CC genotype). Subjects homozygous for the premature stop codon (TT genotype) make up <1% of the population and were not included in this analysis. PBMCs were stimulated with the TLR5 ligand flagellin (endotoxin-free, 40 ng/ml) and the TLR4 ligand LPS (ultrapure, 1 ng/ml). Heterozygous carriers of the premature stop codon (CT genotype) demonstrated significantly decreased IL-6 and IL-8 production (76.3 and 45.5% reduction, respectively; $p < 0.0001$) after stimulation with flagellin compared with controls (CC genotype), whereas following LPS stimulation the inflammatory response was slightly lower in the controls (Fig. 2). These data confirm the functional impact of the *TLR5* c.1174C>T SNP on flagellin responsiveness.

Relationship between the TLR5 c.1174C>T SNP and clinical outcome variables in CF

We successfully genotyped the *TLR5* c.1174C>T SNP in 99.4% of the 2219 CF patient samples, and we stratified the subjects as outlined in the flowchart in Fig. 3. Specifically, to reduce genetic variability at the *CFTR* locus, we included only CF patients who had insufficiency of the exocrine pancreas (PI) and carried severe *CFTR* mutations on both alleles. For the purposes of this study, exocrine pancreatic function status was based on clinical status and *CFTR* mutations (29, 41, 42). PI status was assigned if both *CFTR* mutations were known to be associated with PI. Pancreatic sufficient (PS) patients carried at least one known PS mutation. Otherwise, those with *CFTR* mutations that were unidentified or not clearly associated with PS or PI were classified as PS or PI based on the reported clinical use of pancreatic enzymes. Furthermore, given that a modifying effect of *TLR5* would only be expected to occur in patients infected with flagellated bacteria, we excluded patients not infected with *P. aeruginosa*. Finally, patients infected with organisms of the *Burkholderia cepacia* complex (BCC) were excluded from analysis, because infections with BCC are associated with a worse prognosis at all levels of pulmonary function, and BCC may cause “cepacia syndrome,” which is characterized by rapid pulmonary deterioration, septicemia, and death in ~20% of infected CF patients (43, 44). Table III summarizes the basic demographic data of the subjects after stratification.

We examined the relationship between the *TLR5* c.1174C>T SNP and two clinical characteristics that have been associated with mortality among patients with cystic fibrosis: FEV₁ and body weight (24). These were relevant endpoints for examining the modifying impact of a *TLR5* variant known to reduce TLR5 function, as clinical trials evaluating the anti-inflammatory benefits of ibuprofen and azithromycin often demonstrated improvements in both FEV₁ and body weight (6–12).

In both the pediatric and adult CF patients, zBMI was not normally distributed. Nonparametric tests were considered to see whether the medians of the zBMI in the two genotype groups differed significantly (Table IV). Based on the nonparametric Kruskal–Wallis test, adults with CF carrying the *TLR5* premature

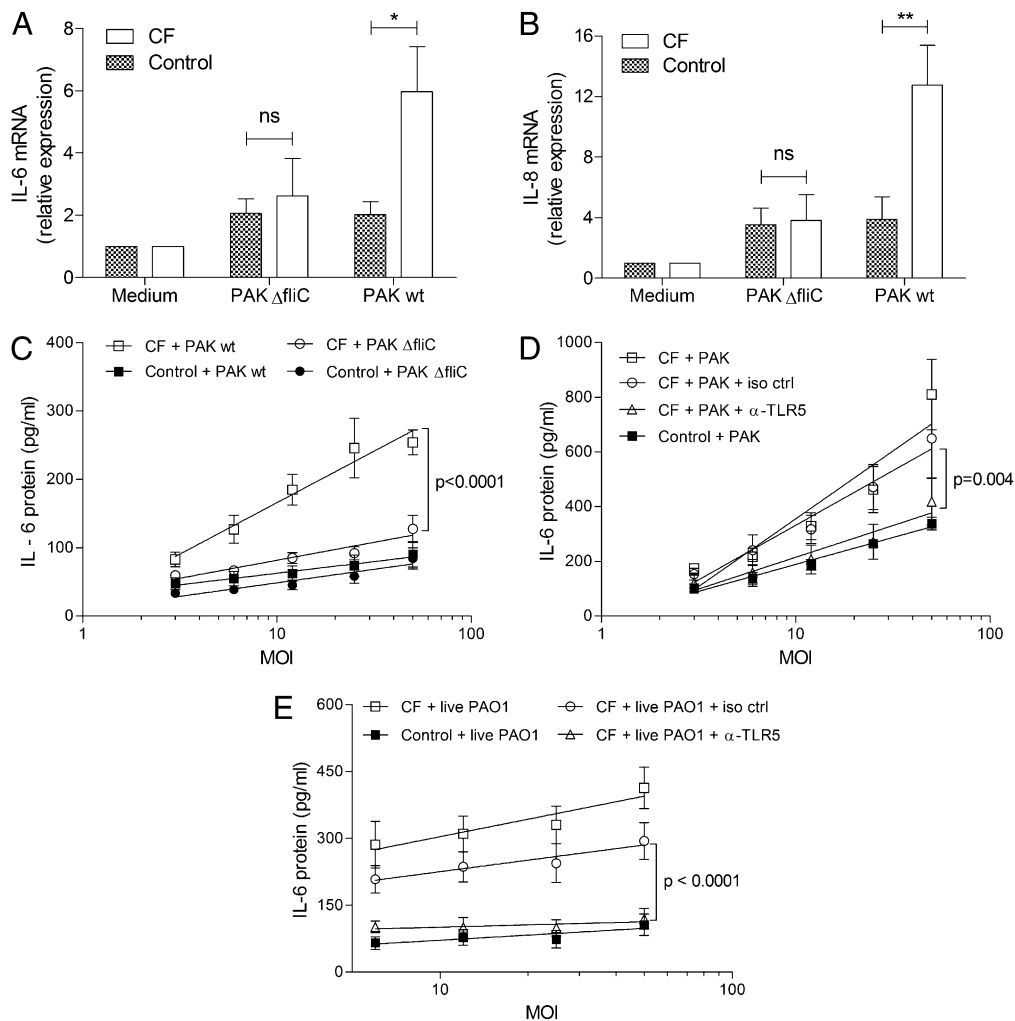


FIGURE 1. Inhibition of the TLR5–flagellin interaction significantly reduces the proinflammatory response of CF airway epithelial cells following exposure to *P. aeruginosa*. *A–D*, CF (CuFi-1) and control (NuLi) airway epithelial cells were stimulated with a heat-killed PAK or PAK Δ flhC at varying MOIs. mRNA expression was analyzed after 2 h, while supernatants were harvested after 24 h for cytokine quantification. mRNA expression is shown for the 50:1 MOI. In some experiments an anti-TLR5 Ab or isotype control was added. *E*, CF-corrected (C38) and CF (IB3-1) airway epithelial cells were stimulated for 4 h with live PAO1, and IL-6 secretion into the supernatant was quantified by ELISA. Values represent means \pm SEM of three to four separate experiments all performed in triplicate. Statistical analysis was performed by two-way ANOVA using the Bonferroni correction for multiple comparisons (*A, B*) and semilogarithmic nonlinear regression (*C–E*). * $p < 0.05$; ** $p < 0.01$.

stop codon (CT or TT genotype) had higher zBMI than did CF patients homozygous for the common fully functional allele (CC genotype) ($p = 0.044$). This difference between the CT/TT and CC genotypes was equivalent to CF patients carrying the premature stop codon (CT or TT genotype) having a BMI 1.18 kg/m² higher, which equates to a 3.3 kg weight increase for a person 167 cm (5 feet 6 inches) tall. To test the sensitivity of this association and assess the impact of within-family correlation, we repeated the analysis after excluding the younger sibling of each sibling pair ($n = 15$) and the association between the *TLR5* c.1174C>T genotype and zBMI was lost ($p = 0.119$). To account for the duration of infection with *P. aeruginosa*, we examined 1) the Spearman correlation between the duration of infection and BMI, stratified by *TLR5* genotype; and 2) the relationship between the duration of infection and *TLR5* genotype by the Kruskal–Wallis test. These analyses suggested that the direction and strength of association between zBMI and *TLR5* c.1174C>T genotype were not altered significantly when we accounted for the duration of infection with *P. aeruginosa*.

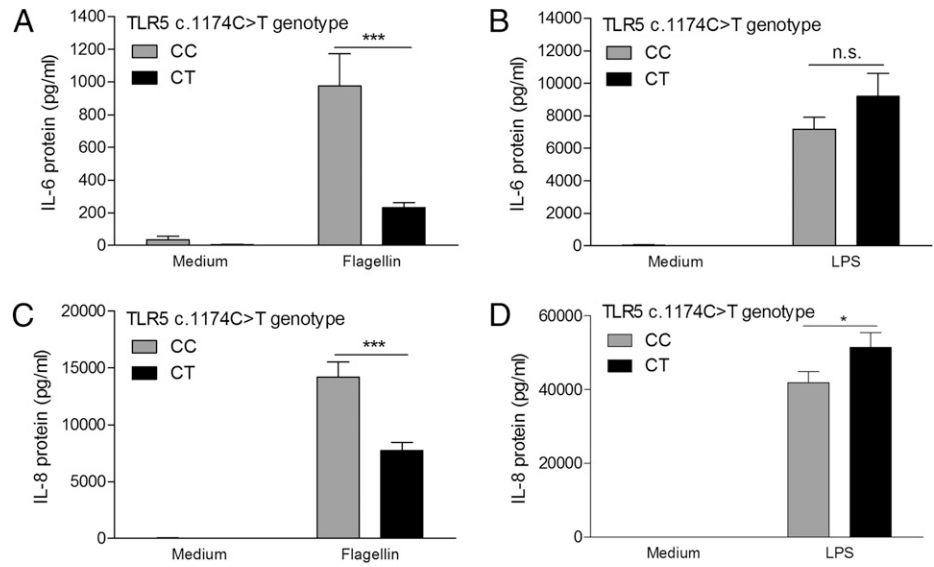
Whereas the *TLR5* c.1174C>T SNP was associated with differences in BMI in adults with CF, no significant modifying effect

of the *TLR5* c.1174C>T SNP on lung function was observed in either the pediatric or adult CF patients regardless of adjustment for the confounders (Table IV).

Discussion

The aim of this study was to explore the potential of TLR5 as an anti-inflammatory target to improve the clinical outcome in CF. Anti-inflammatory therapy has been shown in clinical trials to be beneficial for patients with CF, improving both lung function and body weight. To date, most attention has focused on the therapeutic potential of corticosteroids and ibuprofen. Unfortunately, safety concerns render these currently available anti-inflammatory medications unacceptable for routine use. Indeed, recently published guidelines concerning the use of chronic medications for maintenance of CF lung health recommend against the use of oral corticosteroids in children (45). Similarly, while guidelines now recommend the chronic use of oral ibuprofen to slow the loss of lung function, uptake of this therapy at CF centers has been very limited due to concerns about side-effects, particularly gastrointestinal ulceration and renal damage (46). To expand the repertoire of anti-inflammatory medications for CF, recent attention has turned to the

FIGURE 2. *TLR5* c.1174C>T SNP is functionally relevant, significantly reducing flagellin responsiveness. Proinflammatory cytokine production by PBMCs upon stimulation with pure recombinant flagellin (40 ng/ml) is markedly reduced in individuals carrying the *TLR5* premature stop codon (CT genotype, $n = 5$) compared with control individuals homozygous for the common allele (CC genotype, $n = 5$). Response to LPS (LPS, 1 ng/ml) was examined as a TLR5-independent control. Statistical comparisons made by two-way ANOVA using the Bonferroni correction for multiple comparisons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



immunomodulatory properties of macrolide antibiotics, especially azithromycin, with trials demonstrating improved lung function and body weight in those receiving azithromycin (9–12). However, there are also potential challenges in using azithromycin, including the risk of selection of resistant bacteria (12, 47) and our lack of mechanistic understanding of the beneficial anti-inflammatory actions of macrolides (48). Consequently, alternative anti-inflammatory agents with different modes of action and safety profiles are required to improve the outcome in CF.

Controlled inflammation is important for fighting infection, but in CF the inflammatory response is dysregulated and prolonged (reviewed in Ref. 49). Inflammation in the CF lung is characterized by an intense neutrophilic infiltrate, high levels of proinflammatory cytokines (IL-8, IL-6, TNF- α , IL-1 β), and evidence of activation of NF- κ B and other proinflammatory signaling cascades. Specifically targeting the molecular pathways driving these aspects of CF airway inflammation is a particularly attractive therapeutic approach. In previous work we had shown that activation of TLR5 by flagellin is required to trigger the exaggerated proinflammatory cytokine production seen in CF airway cells following exposure to *P. aeruginosa* (16). Two limitations of these previous experiments were our reliance on a single pair of immortalized CF and CF-corrected airway epithelial cells, and the fact that the bacteria used for the stimulation assays were heat-killed to control for differences in motility between wt and flagella-deficient strains of *P. aeruginosa*. To assess more thoroughly the potential of TLR5 as an anti-inflammatory target in CF, it was critical to address these weaknesses and expand our previous observations. In this study we used two pairs of CF and control airway epithelial cells to confirm that the interaction between TLR5 and flagellin plays a central role in triggering proinflammatory cytokine production by CF airway epithelial cells following exposure to the major CF pathogen, *P. aeruginosa* (Fig. 1). Our observation that blockade of TLR5 abrogated proinflammatory cytokine production by CF airway epithelia following exposure to live *P. aeruginosa* was of particular relevance to host–pathogen interactions occurring in the CF lung. While live bacteria have the capacity to activate a wide variety of innate immune detector systems, including NOD-like receptors, which can respond to both flagellin and elements of the bacterial type III secretion system (50–53), our data indicate that following exposure to *P. aeruginosa* the production of IL-6 and IL-8—key proinflammatory cytokines known to be present at high levels in the CF lung—is dependent on signaling through TLR5.

When considering TLR5 as a novel anti-inflammatory target in CF, several potential limitations must be addressed. The first is the observation that most environmental *P. aeruginosa* strains and those isolated early in CF infections are highly motile, whereas some strains isolated from older, chronically infected CF patients are immotile (54, 55). However, the potential therapeutic value of a TLR5 inhibitor is supported by our demonstration that 75% of clinical isolates of *P. aeruginosa* obtained from the lungs of CF patients aged from 1 to 36 y retained the capacity to activate TLR5



FIGURE 3. Flowchart outlining CF patients included in the analysis of TLR5 as a modifier gene in CF. Flowchart provides an overview of subjects included in the analysis. Subjects were considered to be infected with *P. aeruginosa* if they had one or more positive cultures, and subjects who subsequently eradicated *P. aeruginosa* were not excluded from analysis. PA, infected with *P. aeruginosa*.

Table III. Demographic and clinical characteristics of the CF population

	Pediatric (n = 597)	Adult (n = 466)
Gender, M/F	280/317	216/250
Age at diagnosis, y	0.36 (−0.57 to 12.72)	0.75 (−0.003 to 59.4)
Age, y	11.54 (6.02 to 18.50)	25.04 (16.33 to 61.31)
BMI, kg/m ²	17.83 (11.89 to 29.33)	21.41 (14.54 to 35.98)
zBMI	−0.24 (−5.16 to 2.13)	−0.31 (−4.99 to 2.26)
Mean FEV ₁ % predicted	88.82 (19.18 to 132.40)	61.11 (15.34 to 127.50)

Values represent the median (and range) for the cohort.

(16). Despite the significant loss of motility of *P. aeruginosa* during the evolution of chronic CF lung infection, the TLR5 activating capacity of *P. aeruginosa* is relatively retained in the CF lung across the patient's lifespan. A second factor to consider is that in the CF lung, *P. aeruginosa* appears to exist as biofilms (56). These structured communities of *P. aeruginosa* encased in a self-produced polymeric matrix may fail to interact with the TLR5-expressing airway epithelium; nevertheless, biofilms are dynamic and flagellated planktonic organisms that break free from the bacterial community are likely to shed flagella within the airway lumen to activate TLR5. Finally, LPS (the ligand for TLR4) is often considered to be the most potent immunostimulatory molecule expressed by *P. aeruginosa*. While unique lipid A modifications occur in *P. aeruginosa* during chronic infection of the CF lung (57), accumulating evidence indicates that the TLR4-mediated response to LPS is minimal in airway cells because of a lack of the necessary coreceptors, including MD2 and CD14 (58–60).

With multiple lines of evidence in a variety of model systems all implicating TLR5 as a major driver of inflammation following pulmonary infection with *P. aeruginosa* (16, 18–22, 61), the second focus of this study was to explore the contribution of TLR5 to the health outcomes of patients living with CF. A powerful translational research approach for identifying and validating new therapeutic strategies is through the identification of genetic variants that modify CF clinical phenotypes. Our data establish *TLR5* as a biologically plausible candidate gene that may modify clinical outcomes in CF. The *TLR5* gene harbors a polymorphism, *TLR5* c.1174C>T (rs5744168), that encodes a premature stop codon at amino acid position 392 in the ligand-binding domain of TLR5. Because allele T has been shown by our group (Fig. 2) and others (30–32) to be associated with significantly impaired flagellin responsiveness, we predicted that CF patients carrying the T allele would have improved clinical outcomes due to the endogenous anti-inflammatory effects of this *TLR5* variant.

We elected to investigate the disease-modifying impact of *TLR5* c.1174C>T on two important prognostic indicators in CF: pulmonary function and nutritional status (24, 43, 62, 63). In our large representative CF cohort, adults with CF carrying the *TLR5* premature stop codon (CT or TT genotype) had improved nutritional status, as measured by zBMI, compared with CF patients homozygous for the common fully functional allele (CC genotype) (Table

IV). In contrast to adults, the *TLR5* c.1174C>T SNP was not associated with differences in the age- and sex-adjusted zBMI of children with CF. When examining pulmonary function, we found no evidence that the *TLR5* premature stop codon modified lung function in our CF cohort. Specifically, although the CT/TT genotype was associated with a higher mean FEV₁% predicted (2.0 and 2.5% in pediatric and adult CF patients, respectively), these differences were not statistically significant (Table IV). Nevertheless, our results must be interpreted with some caution given that the association between the *TLR5* c.1174C>T SNP and zBMI in adult CF patients was modest ($p = 0.044$) and this association was lost during sensitivity analysis that excluded the younger sibling of each sibling pair, perhaps because of the loss of power caused by excluding 15 subjects. Although our in vitro data establish *TLR5* as a plausible candidate modifier gene in CF, the modifying impact of *TLR5* will only be firmly established through replication studies in other CF cohorts. Moreover, formal establishment of a link between any observed clinical benefits and modulation of TLR5 function will require the use of animal models, such as mice and pigs with engineered defects in both *CFTR* and *TLR5*.

Our study design does not allow a direct investigation of the mechanism by which the *TLR5* premature stop codon (CT or TT genotype) may mediate an improvement in the nutritional status of adults with CF. Nevertheless, inflammation has been linked to energy expenditure in CF. Patients with CF have been demonstrated to have elevated energy expenditure leading to energy imbalance and the development of undernutrition (reviewed in Ref. 64). Of the many factors contributing to increased energy expenditure in CF patients, inflammation appears to be responsible for increases in resting metabolic rate. Indeed, Bell et al. (65) demonstrated that i.v. antibiotic therapy for CF pulmonary exacerbations resulted in parallel reductions of host systemic inflammatory markers and resting energy expenditure together with an improvement in lung function and an increase in body weight. A variety of other studies have revealed that acute respiratory exacerbations caused increased resting energy expenditure in many patients, which reverts to lower values after antibiotic therapy or administration of aerosolized dornase alfa (reviewed in Ref. 66). Taken together, these data suggest that inflammation and metabolic rate are coregulated, and consequently the downregulation of the inflammatory response mediated by the *TLR5* premature stop codon (CT or TT genotype)

Table IV. Relationship between *TLR5* C.1174C>T genotype and clinical outcomes

	Pediatric (n = 597)			Adult (n = 466)		
	CC	CT/TT	p Value	CC	CT/TT	p Value
zBMI	−0.239	−0.241	0.690 ^a	−0.335	−0.053	0.044 ^a
Mean FEV ₁ % predicted	88.720	90.765	0.51 ^b , 0.75 ^c	60.263	62.736	0.27 ^b , 0.77 ^c

^aBased on the Kruskal–Wallis test.

^bObtained from fitting a linear model unadjusted for confounders.

^cObtained from fitting a linear model adjusted for confounders: mean age, number of visits, and zBMI.

would be predicted to be accompanied by a reduction in the metabolic state and higher zBMI in CF patients. Clearly, due to the complex relationship between immunity, nutrition, and metabolic events, additional studies are needed to explore the relationship with *TLR5* variants and nutritional status in CF patients.

A strength of this study is the translational research design. Starting with in vitro models of the host–pathogen interaction occurring in the CF lung, we identified *TLR5* as a major mediator of the inflammation occurring after exposure to *P. aeruginosa* (Fig. 1). We then explored the potential in vivo consequences of *TLR5* inhibition by determining whether the functional *TLR5* c.1174C>T SNP influences clinical outcomes in people living with CF. Our demonstration that partial loss of *TLR5* function appears to improve nutritional status in adults with CF is likely to be applicable generally, because our patient cohort was recruited from a population-based sample representing nearly 75% of the current Canadian CF population. When compared with the Canadian CF Patient Registry, the demographic and clinical data in the recruited patient cohort were found to be nationally representative (28, 29). A slightly surprising outcome of this study was our finding that, while the *TLR5* c.1174C>T SNP appeared to modify BMI in adults with CF, this *TLR5* variant was not associated with differences in lung function. In our cohort there was a significant correlation between zBMI and mean FEV₁% predicted ($p = 0.0001$), which has been previously reported (e.g., see Ref. 67). However, the Pearson product-moment correlation coefficient between the two variables was 0.35 and 0.38 for the pediatric and adult CF patients, respectively, indicating that the correlation between zBMI and mean FEV₁% predicted was not perfect. It has been reported recently that mice genetically deficient in *TLR5* develop a metabolic syndrome associated with increased weight that appears to be related to changes in the composition of the gut microbiota. These mouse data indicate that *TLR5* dysfunction may modulate metabolic activity independently of lung function (68). Intriguingly, the disease-modifying impact of the *TLR5* c.1174C>T SNP is reminiscent of the results of a recent trial testing azithromycin in CF patients who were not infected with *P. aeruginosa* in which this anti-inflammatory agent improved BMI but not lung function (12).

In summary, using complementary in vitro and population-based in vivo approaches, we have demonstrated that: 1) inhibition of *TLR5* responsiveness is associated with a reduced inflammatory response of CF airway epithelial cells to *P. aeruginosa*; and 2) the functional *TLR5* c.1174C>T SNP, which significantly decreases *TLR5* responsiveness, is associated with improved nutritional status in adults living with CF. Although follow-up studies are needed to specifically examine the impact of *TLR5* on nutritional status, our study provides further preclinical evidence that strategies to inhibit *TLR5* may improve the health outcome for patients with CF.

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Disclosures

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