Toll-like receptor-4 and insulin-like growth factor pathways influence survival and intestinal homeostasis in cystic fibrosis mice

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To Issa and Pablo (JJ)
My support, My life, My everything

ABSTRACT

Cystic fibrosis (CF) is a common and lethal genetic disease affecting various organs such as lungs, pancreas and intestine. The intestinal disease severity widely varies among patients and plays a critical role as it regulates body weight gain, the characteristic growth deficit of these patients which is of great importance as it predicts for lung disease and survival. Modifier genes, apart from the disease causative gene, regulate the intestinal phenotype. Our objective was to use CF mice to identify genes or pathways influencing the severity of CF intestinal disease to better our understanding of the disease pathogenesis and to provide genes for clinical investigation.

We made use of the CF mouse model, as they also present the low body weight phenotype and share similar intestinal alterations characteristic of CF patients, to evaluate factors contributing to the growth deficit in CF.

Initially, in this thesis, we identified a negative correlation between body weight and the histological feature of crypt-villus axis (CVA) height in CF mice, thus uncovering a phenotypic response to non functional Cftr which is related to CF intestinal disease severity. We also identified specific genes of a proliferative response to be differentially expressed in the ilea of CF mice compared to non-CF controls, among these, were toll-like receptor 4 (*Tlr4*) and insulin-like growth factor-I (*Igf-I*). Based on these previous findings, we investigated the role of these candidates in the proliferative response of the CF intestine and their contribution to weight gain by i) creating and evaluating a Cftr/Tlr4 double mutant mouse and by ii) treating CF mice with an Igf-I modulatory recombinant protein. Interestingly, Tlr4 proved to be implicated in early survival due to mechanisms unrelated to the proliferative response but possibly due to immune and/or mucus secretory actions, whereas Igf-I may potentially play a critical role as an intestinal adaptive-inducing factor influencing mouse body weight.

In conclusion, in this thesis we have successfully identified two candidate modifier genes implicated in the CF intestinal response which modulate survival and intestinal adaptation, and provide strong evidence for their possible evaluation as targets for CF therapy.

RÉSUMÉ

La fibrose kystique (FK) est une maladie génétique commune et mortelle qui affecte plusieurs organes tels que le pancréas, les poumons et les intestins. Lorsque la maladie affecte les intestins, la sévérité des symptômes n'est pas la même chez tous les patients La sévérité des symptômes intestinaux joue un rôle très important car un déficit de croissance et de prise de poids sont fortement liés aux chances de survie et à la sévérité des symptômes pulmonaires. Les gènes modificateurs, en plus du gène qui cause la fibrose kystique, sont responsables de la sévérité des symptômes intestinaux. Notre objectif était d'étudier des souris qui ont la fibrose kystique afin d'identifier des gènes ou des processus qui affecteraient la sévérité des symptômes intestinaux afin d'améliorer nos connaissances des mécanismes pathogéniques et afin de suggérer des gènes qui pourraient être évalués cliniquement.

Nous avons utilisé des souris qui ont la fibrose kystique pour évaluer les facteurs qui contribuent au déficit de croissance de la FK, car elles représentent bien cette maladie puisqu'elles ont un faible poids ainsi que des malformations intestinales qui sont caractéristiques des patients qui ont la FK.

Premièrement, nous avons identifié une corrélation négative entre le poids de la souris et un marqueur histologique du rapport de la hauteur villosités / cryptes, ce qui est un phénotype qui indiquerait une influence du gène mutant Cftr sur la sévérité de symptômes intestinaux. Nous avons aussi identifié des gènes impliqués dans la prolifération cellulaire qui étaient exprimés de façon différente dans les iléons des souris FK que dans les iléons des souris non-FK tels que toll-like receptor 4 (*Tlr4*) et insulin-like growth factor-I (*Igf-I*).

Nous avons décidé de confirmer le rôle de ces candidats dans la réponse proliférative dans les intestins des souris FK et de leur contribution à la prise de poids des souris FK en i) créant et évaluant les souris double mutantes Cftr/Tlr4 et en ii) traitant des souris FK avec une protéine recombinante qui régule Igf-I. Nous avons découvert que le gène *Tlr4* était impliqué dans la survie des souris, possiblement de façon immunologique ou par la sécrétion des muqueuses, mais non de façon proliférative alors que Igf-I pourrait être un facteur d'adaptation des intestins qui influencerait le poids des souris.

En conclusion, dans cette thèse nous avons identifié deux gènes modificateurs qui seraient impliqués dans la réponse intestinale de la fibrose kystique et qui sont associés à la survie et à l'adaptation à la maladie, ainsi que démontré que ces gènes seraient de bons candidats thérapeutiques.

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Chapter 2: Intestinal phenotype of variable weight cystic fibrosis knockout mice

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JC Canale-Zambrano was responsible for data analysis and drafted the manuscript. MC Poffenberger collected tissue samples, prepared samples for histology and isolated RNA for microarray analysis. SM Cory and DG Humes were responsible for analysing the microarray raw data.

Chapter 3: Toll-like Receptor-4 genotype influences survival of cystic fibrosis mice

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Chapter 4: Insulin-like growth factor binding protein-3 treatment alters intestinal cell proliferation and body weight of Cftr deficient mice

JC Canale-Zambrano and CK Haston.

JC Canale-Zambrano was responsible for the study design, performed data analysis, and drafted the manuscript.

Dr. Christina K Haston conceived of and coordinated the studies and wrote the submitted manuscripts.

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ABBREVIATIONS

ATP	Adenosine triphosphate
B6	C57BL/6J
BALB	BALB/cJ
bp	Base pair
C3H	C3Hf/Kam or C3H/HeJ
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
Cl^-	Chloride
CVA	Crypt to villus axis
DIOS	Distal intestinal obstruction syndrome
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tags
GO	Gene ontology
H&E	Hematoxylin and eosin
IGF-I	Insulin-like growth factor 1
IHC	Immunohistochemistry
kb	kilobase
kDa	kilodaltons
KO	Knock-out
LPS	Lipopolysaccharide
MI	Meconium ileus
MSD	Membrane spanning domain
NBD	Nucleotide binding domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERT	Pancreatic enzyme replacement therapy

PI	Pancreatic insufficiency
PS	Pancreatic sufficiency
rhIGFBP-3	Recombinant human insulin-like growth factor binding protein 3
RNA	Ribonucleic acid
RT-PCR	Real-Time PCR
Sca10 (Atxn10)	Spinocerebellar ataxia 10 homolog (human)
SIBO	Small intestinal bacterial overgrowth
TLR4	Toll-like receptor 4
WT	Wild-type

CHAPTER 1: GENERAL INTRODUCTION

This dissertation explores the use of a mouse model of the Cystic Fibrosis (CF) disease to identify genes or pathways directly involved in the severity of CF intestinal disease. Even though pulmonary failure is the primary cause of morbidity and mortality in CF patients, the gastrointestinal tract is, as well, a major source of morbidity. CF patients suffer from malnutrition and failure to thrive. The identification of the mechanisms leading to the growth deficit in CF patients is of great importance as percent ideal body weight is prognostic of CF survival and lung disease, thus the identification of factors that influence this phenotype are important to understanding CF pathophysiology.

In the last decades the mean survival of CF patients has greatly improved and as it is currently normal to reach adulthood, these older patients are now faced with additional complications affecting their lifestyle. In this dissertation, the use of a CF mouse model was chosen as it presents the primary clinical intestinal disease, and as it is possible to manipulate candidate genes/pathways in order to elucidate their role in the disease in a controlled environment.

This approach enabled us to identify novel alterations in the expression of Toll-like receptor-4 (Tlr4) and Insulin-like growth factor (Igf) pathways in the small intestine of CF mice. We hypothesized that these two pathways could be particularly important in the severity of CF intestinal disease, since lately, Tlr4 has been the focus of intense research due to its critical role in the innate response against infection and more recently in intestinal homeostasis. In addition, the role of the Igf system is widely known to be implicated in somatic growth and intestinal adaptation. In this thesis, besides the identification of the altered intestinal state of these pathways, the novel important contribution of Tlr4 and Igf to the CF disease severity was further confirmed.

This was achieved by manipulation of the two candidate genes/pathways by 1) creating a double mutant mouse model of CF disease (Cftr/Tlr4 null mice) and by 2) administration of an Igf-I modulatory recombinant protein to CF mice.

Extensive previous studies of these two strong candidates have confirmed their involvement in other intestinal disorders while in this dissertation we reveal their novel critical role in the development of CF intestinal disease severity. These observations contribute to better our understanding of the disease pathogenesis and provide genes for clinical investigation.

The Cystic Fibrosis disorder

"*Woe to that child which when kissed on the forehead tastes salty. He is bewitched and soon must die".* This adage from the northern European folklore is the earliest reference to what we now know as Cystic Fibrosis (CF) disease. As it is implied, CF was once characterized by a salty sweat and early death.

Cystic fibrosis is currently established among the most common inherited disorders that result in the premature death of its sufferers. This autosomal recessive disease is present at a highest frequency, of 1 in 2,500 newborns, in the Caucasian population, with an extremely prevalent carrier frequency of 1 in 25 [1]. Of note, higher incidence has been reported in certain groups where an incidence of 1 in 895 live births is known to occur in French-Canadians from the Sauguenay-Lac St. Jean region [2]. The median predicted survival age, in North America, has been recently reported to be 37.4 years [3].

Cystic fibrosis is a complex disorder reflected in the heterogeneity of the disease presentation and in the several organ systems affected. The lungs, gastrointestinal tract, pancreas, liver and reproductive tract, among others, are all affected by CF [4].

In 1938, Dorothy Andersen was the first to comprehensively describe the post-mortem observations of the pancreas from CF children [5]. She noticed severe destruction and microscopic features of the tissue that led her to designate the syndrome as "cystic fibrosis of the pancreas". The pancreatic ducts are blocked with mucus where the released enzymes are trapped and autodigest the organ. Today, exocrine pancreatic insufficiency (PI) is evident in the majority (~85%) of CF patients. The remaining 15% display variable degree of mild pancreatic dysfunction and are referred to as pancreatic sufficient (PS). The consequence of PI is the deficiency in production and secretion of digestive enzymes which result in fat and protein maldigestion and malabsorption leading to poor weight gain, a hallmark of CF. These patients need to be under replacement therapy with pancreatic enzymes taken with each meal. As it will be later described, CF patients, even under appropriate pancreatic enzyme replacement therapy (PERT), are still growth retarded.

The main cause of mortality in CF patients, however, is due to complications of the respiratory system. Abnormalities in maintaining proper airway hydration lead to mucus accumulation creating an adequate niche for bacteria. Generally, the persistent inflammatory response, due to chronic infection by *Pseudomonas aeruginosa*, ultimately leads to lung failure [6].

Although respiratory failure and lung infections are the main cause of mortality in CF, complications due to gastrointestinal involvement are generally the first severe symptoms to be present in neonates [4], namely meconium ileus (MI), a form of intestinal obstruction in newborns. Intestinal complications, however, still prevail after childhood and are partly responsible for the growth retardation of CF patients [7], a critical factor in disease prognosis and survival.

Since factors influencing CF intestinal disease and growth failure are the main concern of this dissertation they will be described in more detail in subsequent sections.

Growth importance in CF

One of the many disease phenotypes of CF is failure to thrive, as CF patients present lower body weight relative to the non-CF population [8]. The combination of pancreatic insufficiency, intestinal disease and lung disease is thought to contribute to the low weight for height of CF patients [8, 9]. As early as the 1970s, evidence existed for good nutritional state to be associated with a better prognosis [10]. However, particularly high importance in improving nutritional status and growth in CF patients was attributable due to the discovery, in the late 1980s, of the influence of nutritional status in survival [11]. This study compared treatment and survival between two CF clinics in Boston and Toronto. The mean age of survival in Toronto was 30 years compared to 21 years in Boston. Treatment of CF patients in both clinics was fairly similar except for the difference in nutritional approach. Toronto had implemented a high-fat, high-calorie diet, whereas Boston supplied a low-fat, high-carbohydrate diet, this resulted in patients being shorter and lighter compared to those in Toronto.

After the awareness that improving weight gain by a high-fat diet resulted in enhanced survival in CF patients [11], implementation of aggressive nutritional therapies with optimal pancreatic enzyme replacement has since become a general practice in CF centres; however, despite these, the average height and weight of these patients still remain below the normal average of the healthy population [12, 13]. Achievement of normal growth in CF is a critical goal, nevertheless, evidence of malabsorption of proteins and fats despite appropriate pancreatic enzyme therapy [14-18], provides unquestionable evidence of the existence of non-pancreatic factors contributing to the maldigestion and malabsorption that prevent patients from reaching the desired growth. Deficiency of pancreatic enzymes and maldigestion are critical to malnutrition; however, persistent growth failure in CF is the consequence of several contributing factors. This impaired growth remains a major unresolved problem. CF patients with drastic decrease in body weight

must endure daily oral supplementation, and nasogastric or gastrostomy tube feeding, activities that greatly affect the quality of life of CF patients.

For these reasons, the identification of these poorly understood factors, which may involve intestinal factors, will be of great importance in helping achieve the universal goal of normal weight gain in CF patients. Consequently, as percent ideal body weight is prognostic of CF survival [19], and may predict for lung disease severity [20], the identification of these factors that influence this phenotype are important to understanding CF pathophysiology.

Additional evidence that supports the critical knowledge of modifiers affecting this phenotype is given by numerous reports that found strong association of improved nutritional status (enhanced growth) with better pulmonary outcome [20-26].

The *CFTR* gene

In 1989, after an intense search, collaborators from the Hospital of Sick Children in Toronto and the University of Michigan isolated the CF causative gene. In an elegant trilogy of papers they described their accomplishment using positional cloning including chromosome walking and jumping techniques, as well as linkage disequilibrium analysis and correlation of expression pattern between CF and non-CF tissues [27-29]. Finally, it was known that the "cystic fibrosis transmembrane conductance regulator" (*CFTR*) gene, as they named it, was located on chromosome 7q31.2, that it was comprised of 27 coding exons spanning over 250 kb, and that the transcriptional product was 6.5 kb (Figure 1). During this project it was also evident that a mutation resulting in a 3 bp deletion, causing a loss of phenylalanine at position 508 of the protein, was the most common mutation found in the CF population and named it ΔF508 [27], it has since been reported as being present in ~70% of the CF chromosomes worldwide [30]. This mutation is considered a severe mutation due to development of severe

phenotypic complications in patients with two CF alleles carrying this sequence variation. Over 1,000 different mutations resulting in abnormal CFTR have been identified so far [31].

The CFTR protein

Immediately after the newly discovered *CFTR* sequence, predictions were derived for the CFTR protein [32]. CFTR was predicted to be a chloride (Cl^-) channel expressed in the apical membrane of epithelial cells and to be part of the ATP-binding cassette (ABC) transporter proteins. As shown in figure 1, it comprises 1,480 amino acids with a molecular weight of ~170 kDa. CFTR is composed of two membrane-spanning domains (MSD1 and 2), each of these is composed of six transmembrane segments (TM1 and 2) which form the channel; two nucleotide binding domains (NBD1 and 2), which are capable of ATP hydrolysis; and finally a regulatory domain (R) with multiple phosphorylation sites [32, 33]. Activation of this Cl^- channel is regulated by cyclic adenosine monophosphate (cAMP) which regulates phosphorylation of the R domain and stimulates the hydrolysis of ATP by the NBDs [33]. Besides its main function as a Cl^- channel in epithelial cells, CFTR has also been implicated in the activity regulation of other ion channels [34].

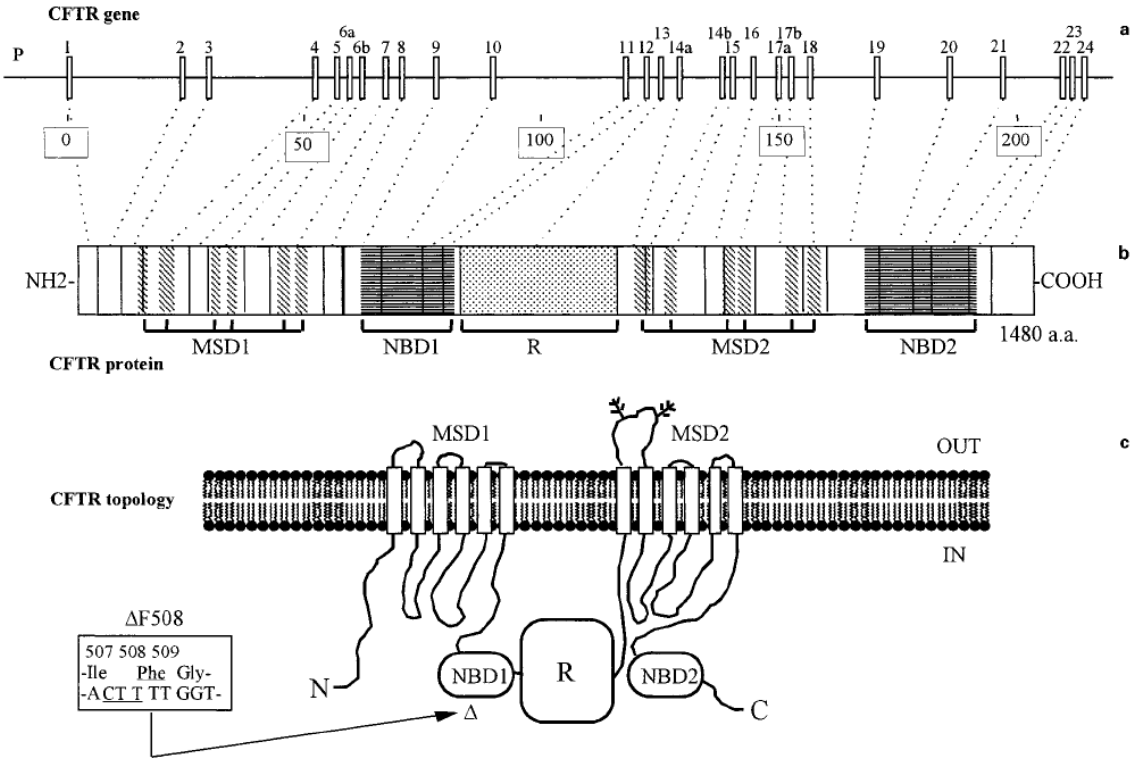

Figure 1. Schematic diagram of the CFTR gene and protein.
(a) Structure of the CFTR gene showing the distribution of the 27 exons. (b) CFTR protein with the 5 predicted domains. (c) Topology of the CFTR protein relative to the apical epithelial cell membrane and position of ΔF508 mutation. Copied from [35].

CF in the small intestine

CFTR is expressed in the normal mucosal epithelium throughout the small intestine [36], where it facilitates secretion of intestinal fluid to the lumen by efflux of Cl^- ions [37]. In CF, defective Cl^- efflux is a common feature [38] which results in abnormal fluid secretion, contributing to the intestinal symptoms of the disease. In addition to the secretory capacity, the major role of the small intestine is the absorption of nutrients, electrolytes and water; thus it is possible that lack of CFTR may affect weight gain by influencing intestinal absorption. This is supported by the fact that CF patients show fat malabsorption suggested to be due to reduced mucosal uptake of fatty acids [18].

The abnormal decreased water secretion facilitates the accumulation of mucus in the small intestine of CF patients which presumably explain the development of intestinal complications. Meconium ileus (MI) is a severe and complete intestinal obstruction by accumulation of abnormally viscid meconium, approximately 15-20% of CF newborns develop MI at birth [39, 40]. Distal intestinal obstruction syndrome (DIOS), which may be recurrent, is due to abnormal accumulation of inspissated and voluminous intestinal content in the terminal ileum and it is mostly specific to the CF disorder affecting 25% of CF adults [4].

CF genotype-phenotype correlations

CF has a highly variable spectrum of clinical symptoms, such that a fraction of patients carrying identical *CFTR* mutations develop widely divergent clinical outcomes. This shows that the association between the type of *CFTR* mutation (genotype) and the severity of clinical disease (phenotype) is in most cases not straightforward [1, 4, 41, 42]. This variability suggests the influence of other factors such as modifier genes and/or environmental factors having an effect on the disease manifestation. In this thesis, by using mice with a common *Cftr* disruption and in a controlled environment we proposed to identify modifier genes influencing intestinal disease severity. Modifier genes are genetic variants, apart from the disease causative gene (*CFTR*), that have an effect on the clinical manifestation of the disease**.**

Generally, two presentations of the disease are recognized: the classic CF presentation results from "severe" mutations that completely disrupt the production or function of the CFTR protein. Approximately 85-90% of CF patients suffer from these mutations, ΔF508 belongs to this class, and normally these patients suffer from pancreatic exocrine insufficiency (PI), higher risk of meconium ileus and poorer lung function. Individuals presenting the non-classic CF disease bear at least one *CFTR* allele with a "mild" mutation that allows some

residual CFTR functionality, thus, they normally present a milder form of CF disease with out the need of PERT.

Pancreatic function is the single phenotype that reasonably correlates with *CFTR* genotype; severe mutations are commonly associated with PI, whereas mild mutations are found mainly in patients with pancreatic sufficiency (PS) [43]. However, genotype/phenotype correlations for intestinal and pulmonary disease are less clear.

Several environmental factors such as socioeconomic status [44], exposure to tobacco smoke [45] and nutritional deficiencies have been associated with variability of CF outcomes. Importantly, infections with various bacterial agents such as *Pseudomonas aeruginosa* [46] and *Burkholderia cepacia* [47] lead to respiratory failure and deterioration of nutritional status. However, environmental factors alone cannot completely account for the entire clinical variability observed in CF patients with identical *CFTR* genotypes; therefore, in order to uncover modifier genes numerous studies have been completed.

Modifier gene studies in CF patients

Undeniable evidence for the role of additional non-*CFTR* modifier genes in determining CF phenotype severity is given by the observation that even siblings with identical *CFTR* mutations and who share the same environment show different disease severity [48, 49]. Studies on twins further confirm the presence and modulatory actions of modifier genes, where monozygous twins showed a notably higher concordance of nutritional status, lung disease severity [50] and incidence of MI [51] than dizygous twins and siblings. In support of this, the increased probability for a second CF child to develop MI in families where the first CF child had MI [39, 48] further demonstrates that intestinal modifiers genes independent of *CFTR* also influence intestinal phenotype.

Genetic analysis for the detection of modifier loci (in order to locate modifier genes) of intestinal disease in CF have been successfully completed, however, complete identification of the responsible gene(s) has proven to be complicated. The initial identification of the CF modifier 1 (*Cfm1*) locus in a CF mouse model (see below), led to linkage analysis using nine DNA markers on CF human chromosome 19q13 (homologous region of *Cfm1*) from CF siblings pairs that were concordant or discordant for MI. The results of this analysis showed the majority of concordant MI sib pairs to have two shared haplotypes at this region, whereas the majority of MI discordant sib pairs shared neither haplotypes. This confirmed that the *CFM1* locus is also linked to MI in CF patients [40]. However, *CFM1* does not predict for MI in all CF patients, indicating that other modifiers of this phenotype may exist and as subsequent reports have failed to replicate such region [51, 52] on different cohorts and the responsible gene has not yet been identified. Furthermore, additional suggestive linkage of regions with modifier genes that cause MI in CF patients have been reported on chromosomes 4q35, 8p23, and 11q25 [51] and 12p13 [52], and regions with protective MI effects on chromosomes 20p11, 21q22 [51] and 4q13 [52]. It can then be concluded, that development of intestinal disease in CF is regulated by the cumulative effects of modifier genes located in multiple loci.

Altogether, the wide heterogeneity in the severity of the affected organs in CF is explained by the type of *CFTR* mutation, modifier genes present elsewhere in the genome and by environmental factors (Figure 2).

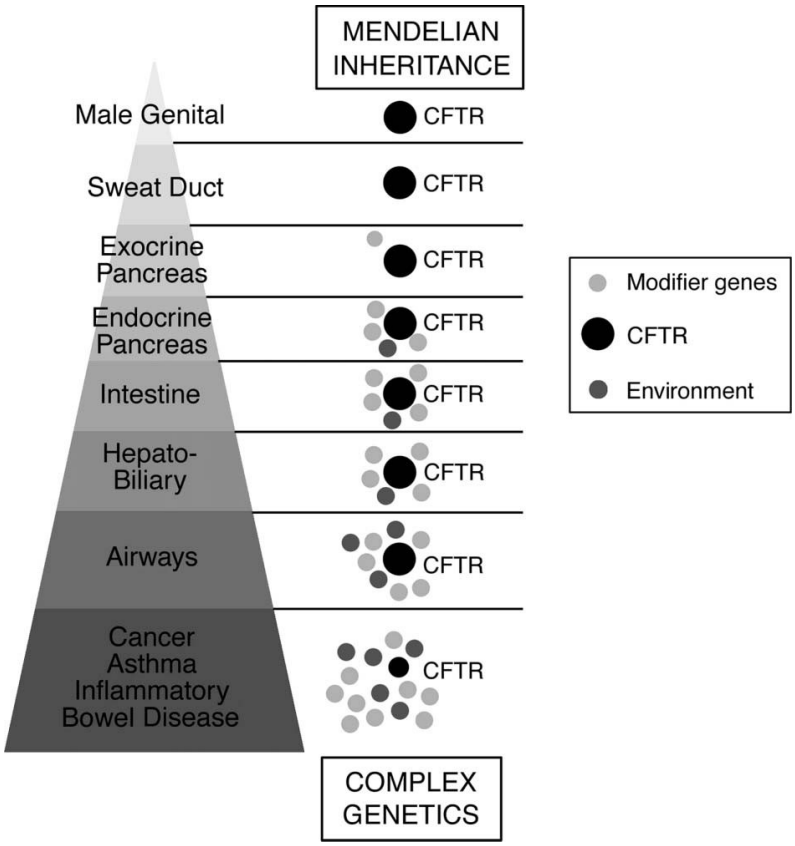

Figure 2. The relative contribution of secondary factors on the CF phenotype: mutations in CFTR, variations in modifiers genes and environmental factors. Copied from [53].

Mouse models of CF

Humans are the only reported species in nature shown to spontaneously develop CF disease. This and the discovery of the *CFTR* gene have led to the development of several genetically-manipulated mouse models of CF [54, 55]. The mouse model used in this thesis was the *Cftr*^{tm1Unc} knockout model. This CF model was created in 1992 [56] by introducing a premature stop codon in exon 10 to disrupt *Cftr*, this gene targeting strategy produces no detectable levels of *Cftr* mRNA. The absence of Cftr in mice produces, as well, a lack of Cl^- secretion mediated by cAMP [57], resembling the intestinal electrolyte transport abnormalities observed in CF patients.

CF mice, like patients, are also growth retarded which is mostly due to the severe pathological intestinal abnormalities [54, 57]. The most striking pathology of most CF mouse models is the frequently lethal intestinal obstructions. The majority of CF mice (50-90%) will succumb either early in life (resembling MI) or after weaning to solid chow (resembling DIOS). In order to ameliorate fatal obstructions and increase survival, these mice need to be fed a lipid-enriched liquid diet (Peptamen®) [58] or to add osmotic laxatives in their drinking water (polyethylene glycol-based) [59]. The intestinal disease in CF mice is more severe than that presented in humans; however, the pathophysiological processes are most likely the same and confirm the value of these mice as a model for human disease.

Upon histological examinations of the small intestine of CF patients, the most evident features are hyperplasia and hypertrophy of mucus-secreting glands associated with accumulation of eosinophilic material filling the lumen [60, 61]. Although the histological appearance of the intestinal mucosa in CF patients has been reported to be unaltered, reports on alterations in the crypts of Lieberkühn that appear dilated and containing dense material [61, 62] have also been observed. The CF mouse small intestine also displays pathological abnormalities such as goblet cell hyperplasia, mucus accumulation, crypt dilation and mucosal and muscularis thickening [54, 56-58]. The CF intestines shown in Figure 3, taken from a CF patient (A) and a CF child with MI (B), appear remarkably similar to that of a CF mouse intestine (chapter 2, 3).

As will be described in later chapters of this thesis, an interesting phenotype of the CF mouse intestine is the increased depth of the crypts, the augmented height of the villi, and the increased muscle thickness. In CF patients, using ultrasound technique, bowel wall thickness of the small intestine and colon has also been shown to be greater in CF patients compared to controls [63-65].

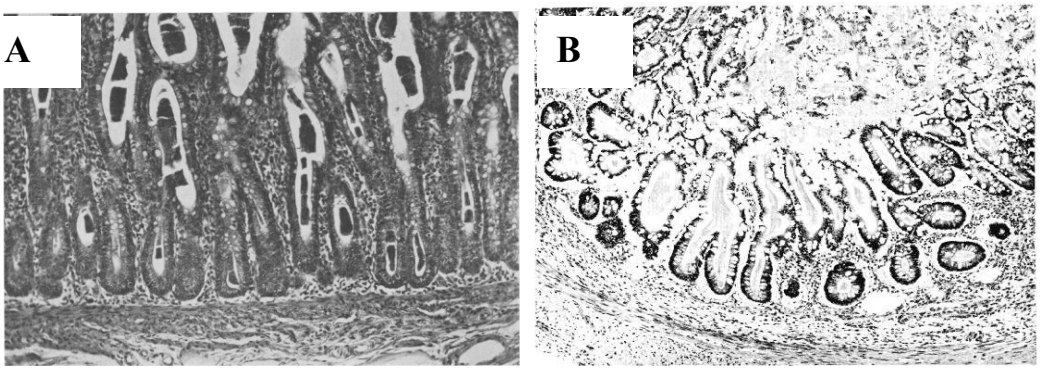

Figure 3. (A) Characteristic accumulation of dense secretion and dilated glands are evident in the small intestine of a CF patient. Copied from [61]. (B) Ileal tissue from a suspected CF infant that developed MI, note the mucus in lumen and distension of mucus glands. Copied from [66].

Of note, clinical growth retardation is already present at birth, where weight and length of CF newborns is reduced compared to healthy infants [67]. Mice, like CF patients, also manifest the poorly understood phenotype of low body weight [56, 58, 68]. The CF mouse also presents the clinical feature of fat malabsorption; demonstrated by the study of Bijvelds *et al.* [69] who measured fecal fat excretion and lipid uptake, and observed fecal fat to be increased and free fatty acid uptake to be delayed in CF mice. This was accompanied by a reduction of absorption of triglycerides which was not attributable to impaired pancreatic secretions.

Initially, CF mice were characterised as not presenting pancreatic abnormalities. More recently, studies in long-living CF mice report the presence of anomalies in the pancreatic tissue, however, these are mild compared to that in humans [70-73]. Consequently, since CF mice are pancreatic sufficient and present milder lung disease compared to humans, these prove to be excellent tools for the identification of non-pancreatic growth related defects due to inherent intestinal abnormalities.

The importance of growth failure in CF is such that Rosenberg *et al.* [74] completed a study of growth and major growth regulating hormones in order to evaluate the CF mouse as a model for growth retardation. CF mice were shown to be smaller in weight and length and to have lower serum levels of insulin-like growth factor-I (Igf-I, a major growth promoting hormone) which significantly correlated with the body weight of mice. They concluded that the CF mouse could be a useful model of CF growth failure in humans. Importantly, the intestinal disease in CF mice is presumed to partly explain the growth failure, since CF mice with the corrected intestinal defect by *CFTR* transgenesis are of the same weight compared to non-CF littermate controls [75].

Modifier gene studies in CF mouse models

Linkage studies in humans require a large amount of subjects and a very precise phenotype evaluation in order for the study to reach significant power, not to mention the disadvantage of uncontrolled environmental factors. For these reasons, investigators have benefited from the use of CF mouse models and have used linkage analysis for the identification of CF intestinal modifier loci.

The first concrete evidence of CF modifier genes was first reported by Rozmahel *et al*. [76]. They created a CF mutant mouse line (*Cftrm*^{1HSC}, 126/Sv x CD1), by disrupting exon 1 of the *Cftr* gene, and reported the majority of their *Cftr* mutant mice to succumb at or before 3 weeks of age due to severe intestinal obstruction; however, a smaller fraction were able to live longer. The introduction of different genetic backgrounds, to the *Cftrm*^{1HSC} model, produced a milder phenotype and longer survival. This prolonged survival was conferred by introduction of alleles from the CD1, C57BL/6L and BALB/cJ mouse strains and not by those from 129/Sv and DBA/2J strains [76]. A genome scan perfomed on this CF mouse model (*Cftrm*^{1HSC}), led to the identification of a highly significant modifier locus on chromosome 7 (*Cfm1*) linked to the severity of intestinal disease, where allelic ratios in surviving CF mice differed from mice with lethal

intestinal complications. Subsequently, a modifier locus for MI was identified on the homologous region of human chromosome 19q13 [40]. This suggests that a gene in this region, although not yet identified, acts specifically in the small intestine to increase the probabilities to develop MI in CF patients.

Using an approach similar to that of Rozmahel *et al*. [76], additional genome-wide linkage studies on F2 (C57BL/6J x BALB/cJ) CF mice have uncovered other potential regions on chromosomes 10 and X that are associated with the severe phenotype of survival at 12 weeks, which mostly depends in the severity of intestinal disease [77]. Interestingly, these F2 CF mice presented a wide range of body weight which allowed the mapping of CF modifiers for this phenotype. Five loci influencing body weight in CF mice were identified and these are presumed to be specific to CF since control mice did not show linkage to these regions [68]. In another study comparing CF mice on a mixed genetic background (95% C57Bl/6 and 5% 129Sv) with CF mice on a C57Bl/6 background, a milder intestinal phenotype was observed in the mixed mice and a preliminary genome-wide scan identified potential modifier loci on chromosomes 1, 9 and 10 for this milder phenotype [78]. In conclusion, intestinal disease and body weight in CF are regulated by multiple genetic factors that need to be revealed in order to propose targets for therapy.

Nevertheless, since specific intestinal modifier genes have not yet been identified by linkage studies, either in humans or mice, an alternative is to directly investigate reasonable candidates suspected to have a role in CF pathophysiology. These candidates for intestinal disease can well be genes involved in intestinal homeostasis, immune response, mucus secretion and general intestinal alterations, which we hypothesize they will also help explain the growth deficit in CF and improve prognosis. This approach greatly benefits from the use of animal models of the disease where candidate genes can be manipulated and further studied.

By means of this hypothesis, and due to an identified adaptive response of the CF intestine which is associated with body weight (described in chapter 2), we completed analysis on two candidate genes (*Tlr4* and *Igf-I*), which we identified to be altered in expression and to have strong potential as modifiers of intestinal disease severity, and subsequently tested their involvement in CF.

Previous studies have also identified altered intestinal expression of genes in CF mice and this has served as basis to support the manipulation of such pathways and have proven to be successful in alleviating intestinal disease. A study by Young *et al.* [79] identified decreased intestinal expression of *Clca3*, a gene involved in mucus production/secretion. Next they created a CF transgenic model overexpressing *Clca3* specifically in the intestine, this avoided hypersecretion and accumulation of mucus in the lumen and also resulted in enhanced survival and notably increased body weight at 5 weeks of age. Consequently, in a study of a cohort of 682 CF patients (99 had MI) van der Doef *et al.* [80] investigated the possible modifier effect of the p.S357N variant of the *CLCA1* gene, the human orthologue of *Clca3*. They found a significant overrepresentation of this variant in patients with MI, suggesting the important role of this modifier gene in CF intestinal disease in mice and humans. Another example is the study by Bradford *et al*. [81] who hypothesized that by decreasing intestinal fluid absorption, and thus increasing hydration of the intestinal lumen, they could prevent intestinal obstructions and increase survival. They made use of mice lacking the NHE3 Na^+/H^+ exchanger which suffer from diarrhea due to increased luminal fluid. Indeed, *Cftr*/*Nhe3* null mice did not accumulate luminal mucus which prevented obstructions and significantly increased survival. Of note, *Cftr*/*Nhe3* null mice had improved body weight at weaning. Additional support for the role of intestinal fluidity is the observation that administration of the compound talniflumate to CF mice increases survival, possibly by inhibiting Cl^-/HCO_3^- exchanger(s) [82].

In this thesis, after the identification of altered expression of *Tlr4* and *Igf-I* in the small intestine of CF mice we also decided to manipulate such pathways to elucidate their role in CF disease, and as it will be described in chapters 3 and 4, these proved to be involved in disease severity by affecting survival, intestinal homeostasis and body weight in CF mice.

Bowel infection and inflammation in CF

The normal small intestinal mucosa is in constant complex relation with a wide variety of bacterial species which, in some cases, abnormal regulation of this relation causes small intestinal bacterial overgrowth (SIBO). The presence of SIBO has detrimental effects to the host such as inflammation, mucosal damage and weight loss, among others. Several studies have reported the presence of SIBO in 30-50% of CF patients [83-85], while SIBO is also present in the CF mouse with an increased 40-fold luminal bacteria colonising the mucus along the villi surfaces [86, 87]. Observations by Clarke *et al.* [88] suggested the innate immune response to be altered in the CF mouse intestine. Specifically, the overt mucus accumulation that blocks the intestinal crypt lumen prevented the proper distribution of Paneth cell granules into the lumen, this resulted in impaired clearance of an avirulent cryptidin-sensitive pathogen (*Salmonella typhimurium*) [88]. In addition, prevention of SIBO by treatment of CF mice with broad-spectrum antibiotics in the drinking water has been shown to decrease mucus accumulation [89] and to improve body weight gain in CF mice [86].

Intestinal microflora seems to be critical in the development of intestinal inflammation in CF. Indications of chronic inflammation of the small intestine in CF patients has been reported [90-92]. Similarly, the CF mouse shows evidence of small intestinal inflammation with increased expression of inflammatory markers and mucosal infiltration of mast cells and neutrophils [93, 94].

Toll-like receptors

Toll-like receptors (TLR) are a family of pattern-recognition receptors that play an important role in innate immunity.[95] These receptors recognize conserved molecules (ligands) present on different pathogens. Once pathogens invade the host and the ligands are bound to and recognized by the specific TLR, a downstream signaling pathway is activated leading to the production of cytokines, chemokines, and antimicrobial molecules that are crucial for the initial innate immune response against pathogens. Today, a total of 10 TLRs (TLR1-10) recognizing different ligands have been reported in humans [95] although a total of 13 are present in mice (Table 1).

Discovery of TLRs

The first identification of a Toll protein was in the fruit fly *Drosophila* where it was originally found to play a critical role in embryo dorso-ventral polarity [96]. This newly described protein was later found to be involved in *Drosophila*'s immune response to fungal [97] and bacterial infection [98]. Based on these observations in *Drosophila*, it was proposed that Toll could also activate an immune response in humans. In 1997, Medzhitov *et al.* [99] compared the sequence of Toll to a human expressed sequence tag (EST) database, and discovered and cloned a human Toll (hToll). They also reported that this human homologue of Toll induced the activation of NF-κB and expression of inflammatory cytokines in human cell lines. In a later study using a similar approach, Rock *et al.* [100] uncovered 5 genes with homologous sequences to Toll and were designated Toll-like receptors 1 to 5. In this screen hToll was found to be TLR4 and was later determined to be the receptor for lipopolysaccharide (LPS) signaling.

The mouse strains C3H/HeJ and C57BL/10ScCr are resistant to LPS challenge (a major constituent of the outer membrane of Gram-negative bacteria) yet highly susceptible to Gram-negative infection. These "*Lps* mutant" strains,

which present immune deficiencies upon infection, were used in several studies in order to identify, by means of physical mapping [101, 102] and mouse manipulation [103], the localization and function of the *Lps* gene. *Lps* turned out to be *Tlr4*. These studies reported the C3H/HeJ strain to contain a missense mutation while C57BL/10ScCr strain to contain a null mutation in *Tlr4*, resulting in a non-functional protein, thus attributing the immune deficiency response to a Tlr4 alteration.

Table 1. Mammalian Toll-like receptors and their ligands.

TLR family	**Ligands (origin)**
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria) Soluble factors (Neisseria meningitides)
TLR2	Lipoprotein/lipopeptides (a variety of pathogens) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) A phenol-soluble modulin (Staphylococcus epidermidis) Glycolipids (Treponema maltophilum) Porins (Neisseria) Zymosan (fungi) Atypical LPS (Leptospira interrogans)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria) Taxol (plant) Fusion protein (RSV) Envelope proteins (MMTV) HSP60 (Chlamydia pneumoniae, host) Type III repeat extra domain A of fibronectin (host) Oligosaccharides of hyaluronic acid (host) Fibrinogen (host)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopeptides (mycoplasma)
TLR7	Imidazoquinoline (synthetic compounds) Loxoribine (synthetic compounds) Bropirimine (synthetic compounds)
TLR8	Small synthetic compounds; single-stranded RNA
TLR9	CpG DNA (bacteria)
TLR10	Unknown
TLR11 mice	Profilin (Toxoplasma gondii)
TLR12 mice	Unknown
TLR13 mice	Unknown

TLR4 signaling

While the extracellular domain of TLRs provide specificity to the varied number of ligands, the cytoplasmic domains of all TLRs are characterized of having a common Toll/interleukin-1 receptor (TIR) motif that binds to adaptor proteins in the cytoplasm. In order to have full ligand sensitivity, TLRs may need the assistance of other co-receptors. Such is the case of TLR4 which requires the presence of MD-2, CD14 and LPS binding protein (LBP) in order to effectively initiate the activation of the receptor (we have also found *MD-2* and *CD14* to be upregulated in the CF mouse intestine). Activation of TLR4 in the intestine initiates intracellular recruitment of adapter molecules necessary for signaling events. A total of five adapter molecules have been identified: MyD88, Tirap, Trif, Tram and Sarm [104]. Upon activation, MyD88 associates with a serine-threonine kinase (IRAK), subsequently TRAF6 is activated which in turn activates MAPK kinases (MKKs) and the IKK complex. IκB is phosphorylated by the IKK complex rendering it accessible for ubiquitination and thus degraded. Ultimately, IκB degradation releases NF-κB which is now capable to translocate into the nucleus where it can induce the expression of inflammatory genes [105] (Figure 4). Several other molecules, with the capacity to down regulate TLR signaling pathway and confer cellular tolerance, have also been identified. Piao *et al*. [106], reported that LPS tolerant monocytes, as compared to controls, had an increased expression of suppressor of IκB kinase-ε (SIKE) and sterile α and Armadillo motif-containing molecule (SARM) in addition to having increased levels of negative regulators such as Toll-interacting protein (Tollip), suppressor of cytokine signaling (SOCS)-1, IL-1R-associated kinase-M, and SHIP-1.

Innate immune response in the intestine by TLRs

The recognition of pathogen-associated molecules by TLRs in the gut result in signals that release antimicrobial peptides, reinforce epithelial barrier, and stimulate epithelial cell proliferation. Thus intestinal injury requires the

presence of TLRs to conduct a proper healing. The intestinal tissue has been examined for the expression and function of several TLRs, including TLR4. The intestinal epithelial cells (IEC) along with other intestinal cell types (macrophages, dendritic cells, neutrophils) have been reported to express almost all of the TLRs 1-9 [107]. The intestinal lumen is constantly in direct contact with commensal microflora that normally inhabits the healthy gut, despite this; a chronic inflammatory response is not observed. A mechanism of the intestine to prevent a dysregulated inflammatory response in the healthy state appears to be a downregulation of TLR signaling by normal IEC [108, 109]. Studies by Abreu *et al.* have shown that expression of *TLR4* and *MD-2* in IEC is barely detectable and thus show hyporesponsiveness to LPS [110, 111]. In contrast, evaluation of inflammatory disorders of the intestine such as Crohn's disease and Ulcerative Colitis has revealed, by immunhistochemical assessment, a strong upregulation of TLR4 [112]. In addition, lamina propria macrophages from the inflamed intestine have shown increased expression of TLR4 [113]. Interestingly, initial results of this thesis project revealed increased expression of *Tlr4* and *MD-2* (*Ly96*) in the intestine of CF mice, which suggests, although not evaluated here, that the CF intestine is able to respond to luminal Gram-negative bacteria.

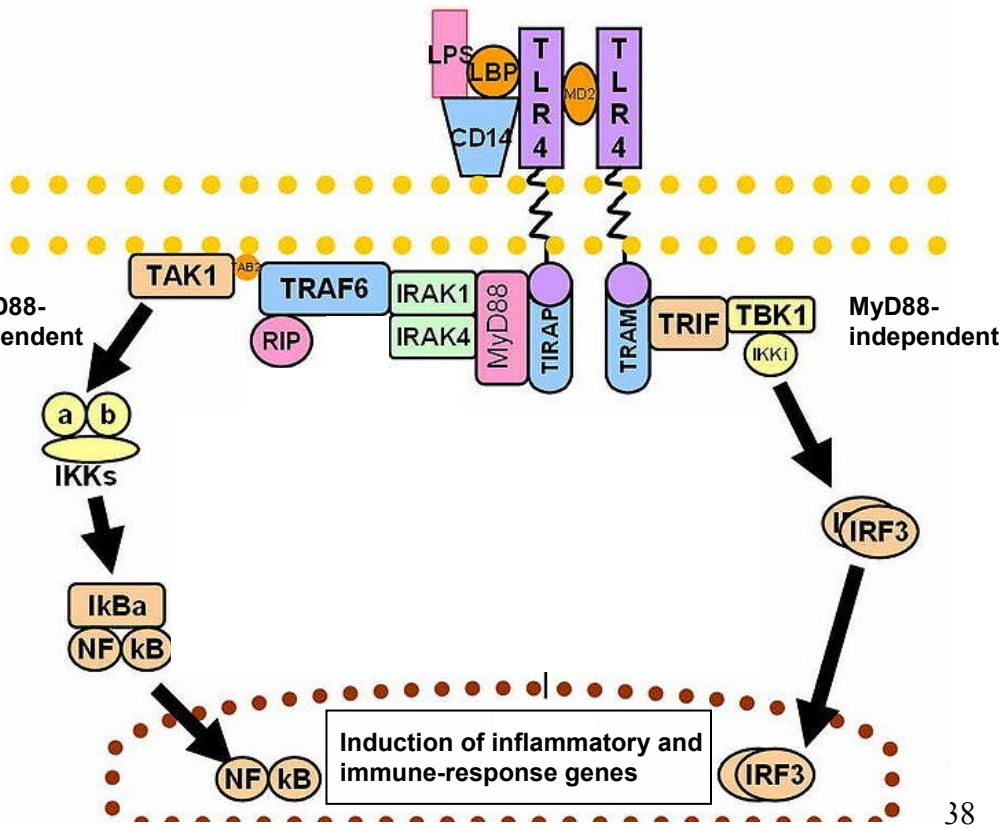

Figure 4. Diagram showing a simplified downstream signaling pathway of TLR4 once activated by LPS.

Homeostatic control by TLRs in the gut

A balanced proliferation and differentiation of cells along the crypt-villus axis in the intestine is one of the many factors that grant protection to intestinal injury [114]. The early observation that germ-free mice show a significant reduction of intestinal epithelial cell proliferation in the intestine compared to mice with conventional flora [115], suggests that TLR signaling, besides being important for the immune response, is also required for optimal intestinal homeostasis. A direct implication of TLRs in intestinal proliferation and repair to injury has been confirmed by several studies performed in challenged models of colitis with administration of dextran sodium sulphate (DSS) to cause injury.

Intestinal homeostasis was first described by Rakoff-Nahoum *et al.* [116] to require recognition of commensal microflora by TLRs. In this study, DSS induction of colitis in TLR2, TLR4, or MyD88 deficient mice (TLR2-/-, TLR4-/-, or MyD88-/-) resulted in higher mortality compared to wildtype mice. Furthermore, elimination of commensal bacteria in wildtype mice, by administration of oral broad-spectrum antibiotics prior to DSS treatment, was observed to resemble the increased mortality shown by MyD88-/- mice. In addition, they reported that MyD88-/- mice undergoing radiation-induced injury presented less proliferative response and shorter villus length compared to wildtype animals, confirming that TLRs are implicated in intestinal homeostasis. Fukata *et al.* [117] used TLR4-/- and MyD88-/- mice and administered DSS in their drinking water for 5 to 7 days. TLR4-/- mice showed a prominent reduction of acute inflammatory cell infiltrate in the intestine, increased gram-negative bacterial content in lymph nodes, and interestingly a decreased epithelial proliferation of crypt cells. Same observation was confirmed a year later where the decrease in proliferation was attributable to TLR4-mediated production of cyclooxygenase 2 (Cox-2) and prostaglandin E_2 (PGE_2) [118]. More recently,

blocking TLR4 with an antibody against the TLR4/MD-2 complex during DSS challenge also resulted in reduced macrophage infiltration to the lamina propria, decreased proinflammatory cytokine and chemokines expression. In contrast, blocking of TLR4 during the 7 days of recovery after discontinued administration of DSS impaired intestinal healing by decreasing epithelial proliferation [119]. They concluded that therapy against TLR4 could decrease intestinal inflammation but at a cost of impeding mucosal healing. The above observations suggest that for optimal intestinal epithelial cell proliferation, both TLRs and bacterial flora (both increased in the CF intestine) are required.

In this context, results described later in this thesis suggest a role for TLR4 as a factor influencing the characteristic hyperproliferative phenotype of augmented crypt-villus axis length in the small intestine of CF mice which could also be affecting body weight.

Possible implications of TLR4 in the CF intestine

Despite the well known evidence of the implication of TLR4 in intestinal disorders, there are no reports, up to date, evaluating the role of TLR4 in the CF intestinal disease. LPS from Gram-negative bacteria (via TLR4) has the ability to induce strong immune responses by activating the expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) in the intestine. This response is protective for the host by limiting propagation of the pathogen, however, high concentrations or chronic stimulation results detrimental to the host. This has been observed in a study by Bihl *et al.* [120], where transgenic mice overexpressing *Tlr4* showed higher sensitivity to LPS, and a higher percentage survived up to 12-14 days after *Salmonella* infection compared to the non-transgenic mice where the majority succumbed between 6-9 days postinfection. However, they observed that *Tlr4* overexpressing mice developed an excessive inflammatory response that was ultimately detrimental to the host.

In another study by Khan *et al.* [121], they reported that Tlr4 signaling in the intestine contributed to morbidity and mortality after *Citrobacter rodentium* infection. Specifically, Tlr4-deficient mice had a milder response to infection compared to wildtype mice, showing decreased expression of inflammatory markers and inflammatory cell infiltrate in the intestine which seemed to result in increased body weight and survival. Interestingly, the increased colonic crypt height after infection was attenuated in Tlr4-deficient mice. They concluded that Tlr4 signaling after *C. rodentium* infection is eventually maladaptive to the host [121]. These observations could be of great importance to CF intestinal disease due to the fact that CF patients and mice suffer from SIBO (mostly LPS-carrying Gram-negative bacteria) and that we have identified increased expression of *Tlr4* in the CF mouse intestine (Chapter 2 and 3). This suggests that the altered environment in the CF intestine may possibly dysregulate this pathway and could finally result in disease alteration. In this context, dampening *Tlr4* expression in CF mice could result in two possible outcomes: One, Tlr4 deficiency in CF may possibly leave the mouse unable to awake an adequate immune response and finally succumb to the inherent bacterial overgrowth in the CF intestine; or two, the absence of chronic Tlr4 signaling could result beneficial to the animal by preventing an overt inflammatory response and alleviating the hyperproliferative phenotype.

In conclusion from these previous studies, we believe for the reasons presented below, that *Tlr4* could be an intriguing candidate for the causal variation which influences proliferation of CF intestinal epithelial cells and host defence, and that a better understanding of these implications is of great importance. 1) We have found *Tlr4* and two of its co-receptors (*CD14* and *MD-2*) to be highly expressed in the intestine of CF compared to non-CF mice, as were *Tlr1*, *Tlr2* and genes of lipid metabolism, a pathway for which there is "crosstalk" with Tlr4 signaling.[122] 2) Tlr4 implication in mouse models of intestinal proliferation in response to challenge as been reported. 3) Tlr4 binds lipopolysaccharide (LPS), a membrane constituent of gram-negative bacteria

which are commonly found in high numbers in the small intestine of CF mice and which influence their weight gain [86]. 4) Other toll-like receptors have been investigated as potential modifiers of CF lung disease [123, 124].

The hyperproliferative CF intestine

As mentioned before and as it will be described in subsequent chapters, the CF intestinal disease is characterized by a hyperproliferative state resulting in increased crypt-villus axis length which was associated with body weight. Interestingly, these histological changes are consistent with the mitogenic effects of increased levels of insulin-like growth factor-I (Igf-I). For this reason and due to our observation that intestinal *Igf-I* and related genes were altered in expression in CF, we hypothesized the perturbation of this pathway to play an important role in the CF phenotype.

Intestinal adaptation

The mature intestine is characterized for being highly sensitive to different stressors posed by several physiological challenges; these can normally lead to atrophy of the organ. In response to these challenges, the intestine is armed with mechanisms to alter the rates of growth, renewal, and functional capacities of the mucosal epithelium, this is known as intestinal adaptation [125, 126]. Intestinal adaptation aims to repair digestive and absorptive capabilities lost during mucosal disease, bowel injury, or intestinal resection, and it does this partly by increasing crypt cell proliferation in order to enlarge the crypt-villus axis [125, 127], which is strikingly similar to the CF intestinal phenotype. Growth factors such as insulin-like growth factor-I (IGF-I) play an important role in regulating this adaptive process.

Insulin-like growth factors (IGF)

The IGF system is comprised of two ligands (IGF-I and IGF-II), a tyrosine kinase receptor (IGF-IR), and six IGF binding proteins (IGFBP-1 to -6). The intestinal growth can be regulated by actions of circulating IGF-I in the blood (endocrine), which is primarily produced in the liver, and/or by actions of locally expressed *IGF-I* in the intestine (paracrine/autocrine). All the members of the IGF axis are expressed throughout the intestine, once IGF-I binds to its receptor (IGF-IR) this autophosphorylates and leads to the activation of the downstream signaling pathway which eventually leads to changes in proliferation and apoptosis [128]. General body growth and growth of several organs, including the small intestine, are regulated by IGF-I. Previous studies have provided concrete evidence of the proliferative actions of IGF-I on intestinal fibroblasts and intestinal smooth muscle cells [129].

Insulin-like growth factor binding protein-3

The IGF binding proteins possess a dual role in modulating IGF-I actions: they can prevent IGF-I binding to its receptor and inhibit the proliferative actions or, in contrast, they can facilitate its binding and thus promote the mitogenic IGF-I effects [130]. IGFBP-3 is of great importance as it binds approximately to 80% of circulating IGF-I. In addition, IGFBP-3 has been shown to have potent inhibitory functions. Evidence for this are the studies by Kuemmerle *et al.* [131, 132] which reported decreased expression of *IGFBP-3,* by human intestinal muscle cells, during initial growing and proliferating stages, while *IGFBP-3* was greatly overexpressed once the cells reached confluence and stopped proliferating. Expression of *IGFBP-5* was found to behave oppositely, concluding that IGF-I-induced proliferation is inhibited by IGFBP-3 and augmented by IGFP-5. In addition, Alami *et al.* [133] used a colorectal tumor mouse model to evaluate the antiproliferative action of systemic administration of a recombinant human IGFBP-3 peptide for a period of 25 days. Interestingly, tumor growth in the

intestine was inhibited in the rhIGFBP-3 treated mice providing evidence for the successful *in vivo* use of this peptide as an antiproliferative agent in the intestine.

Systemic IGF-I action on intestinal adaptation (endocrine)

In addition, there is extensive evidence, from several animal models, confirming the systemic action of IGF-I in intestinal growth: Animals on total parenteral nutrition (TPN) normally suffer from mucosal hypoplasia associated with decreased crypt cell proliferation and increased apoptosis. Systemic administration of IGF-I to TPN-fed animals result in increase crypt cell proliferation and decrease apoptosis with consequent augmented villus height and crypt depth compared to untreated TPN-fed animals, this is also accompanied by improved body weight [134-136].

Furthermore, in animal models of bowel resection the systemic administration of IGF-I also promote intestinal growth by increasing crypt proliferation and decreasing apoptosis which improves body weight gain after surgery [137-139]. A three-day infusion of IGF-I to healthy intact rats also has the same proliferative effects [140].

Local insulin-like growth factor expression during intestinal growth (paracrine)

Our initial analysis of intestinal gene expression of CF compared to wildtype mice revealed alterations of the IGF system, including *Igf-I* overexpression and *Igfbp-3* downregulation. Interestingly, a number of situations where intestinal adaptive growth occurs (similar to the CF intestine) have also been reported to be accompanied with this pattern of local IGF expression. Specifically, mice undergoing surgical major bowel resection respond by increasing crypt cell proliferation in order to augment crypt-villus axis length. In

resection models, the intestinal expression of *Igf-I*, like our CF mice, has been found to be upregulated while *Igfbp-3* to be downregulated [139, 141, 142].

In addition, prolonged fast periods cause intestinal hypoplasia which is reversed after refeeding. In a study by Winesett *et al.* [143], they reported that mucosal growth in rats ad libitum-fed, fasted and then refed correlated with changes in IGF expression. After refeeding, the mucosal hyperplasia was associated with increased *Igf-I* and decreased *Igfbp-3* expression. The observations from these models suggest the hypothesis that increases in Igf-I is critical to stimulate the intestinal adaptive response to stressors and that a decrease in Igfbp-3 is necessary to enhance Igf-I bioavailability.

Intestinal phenotype of IGF-I transgenic mice

Most recently, and more relevant to the CF intestinal phenotype, the use of transgenic mice constructed to overexpress *IGF-I* either systemically [144, 145] or locally in the intestine [146] have confirmed similar results for the action of IGF-I as a mitogenic agent promoting proliferation of the intestine. Specifically, Ohneda *et al*. [144] compared the intestinal phenotype of transgenic mice that overexpress *IGF-I* systemically, this transgene is driven by the mouse metallothionein 1 promoter. The small intestine of these transgenic mice, which have increased body weight, is larger and bulkier than that of the wildtype mice. Importantly, the crypt-villus axis is greater in the transgenic intestine where an increase in crypt mitoses was also found. A few years later, the same group evaluated the response of the intestine to increased expression of *IGF-I* particularly in the intestine [146]. This transgenic mouse was created by fusing a fragment of the smooth muscle-α actin promoter to the rat IGF-I cDNA [147]. As a consequence, the transgene is expressed solely in smooth muscle cell-containing tissues (bladder, stomach, aorta, uterus, and intestine), and importantly these mice, in contrast to the systemically transgenic, do not have increased levels of IGF-I in the circulation. In the first evaluation of these mice [147], the wet weight of the intestine was selectively

increased along with the thickness of the muscularis externa layer. Subsequently, Williams *et al*. [146] performed a more in depth analysis of the intestine of these mice, and found mucosal growth (weight, sucrase activity, DNA and protein content), muscularis externa thickness and crypt cell mitoses to be increased in the ileum of transgenic mice. However, although crypt mitoses were increased in the ileum there was no evidence of crypt-villus enlargement. Overexpression of *IGF-I* in these mice also resulted in increased expression of *IGFBP-5* in the lamina propria of the ileum. These results suggest that overexpression of *IGF-I* in intestinal mesenchymal cells act in a paracrine fashion in the ileal mucosal epithelium and in an autocrine fashion in the muscle layer throughout the bowel.

Insulin-like growth factor-I and body weight

The circulating levels of IGF-I in the blood have been reported to be lower in CF patients [148-150] and CF mice [74] compared to healthy controls, and this to correlate with body weight. Most of the circulating IGF-I is produced in the liver, however, a study by Yakar *et al*. [151] where they specifically deleted *Igf-I* expression in the liver dramatically decreased by 70% the circulating IGF-I levels, and yet the mice had normal postnatal growth and weight. This indicates that locally expressed *IGF-I* is critical for promoting postnatal body and organ growth.

A key question is whether the altered intestinal phenotype observed in the CF disease could be influencing the failure to thrive. Several observations prompted us to hypothesize that Tlr4 and/or Igf-I actions play an important role in this hyperproliferative response to the lack of Cftr.

Hypothesis

This research project hypothesises that modifier genes alter the CF intestinal phenotype and the disease severity; and these, subsequently, influence the growth failure characteristic of the clinical disease.

Aims

The first aim of this research project was to identify intestinal alterations that could explain the poor body weight of CF mice, and to uncover evidence for possible causative candidate genes for further evaluation. To address this aim, we assessed the ileal pathology and gene expression from an F2 population of mice presenting a wide range in body weight. The histological and expression data were integrated to propose specific candidate genes/pathways leading to the intestinal phenotype in CF mice, and were evaluated in the subsequent aims.

The second aim was to determine whether toll-like receptor 4 genotype influenced the intestinal disease phenotype of CF mice. Based on findings from aim one, we hypothesized Tlr4 to be an intriguing candidate for the causal variation influencing intestinal epithelial cell proliferation. To accomplish this aim, we produced and phenotyped adult and neonate CF mice with non-functional Tlr4 and compared them to CF mice having intact Tlr4 and their corresponding littermate controls.

The third and final aim of this dissertation was to evaluate the role of the insulin-like growth factor axis in the CF intestinal phenotype. The hypothesis underlying this aim was that overexpression of *Igf-I* was responsible for the induced intestinal adaptation of the CF intestine. To investigate this hypothesis we treated CF and non-CF mice with recombinant human IGFBP-3, which possesses anti-proliferative and pro-apoptotic actions, and measured its effects both systemically and locally on the intestinal phenotype.

CHAPTER 2

Intestinal phenotype of variable weight cystic fibrosis knockout mice

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Abstract

Cystic fibrosis (CF) transmembrane conductance regulator (*Cftr*) knockout mice present the clinical features of low body weight and intestinal disease permitting an assessment of the interrelatedness of these phenotypes in a controlled environment. To identify intestinal alterations that are affected by body weight in CF mice, the histological phenotypes of crypt-villus axis height, goblet cell hyperplasia, mast cell infiltrate, crypt cell proliferation, and apoptosis were measured in a population of 12-wk-old (C57BL/6 x BALB/cJ) F2 *Cftr*^{tm1UNC} and non-CF mice presenting a range of body weight. In addition, cardiac blood samples were assessed, and gene expression profiling of the ileum was completed. Crypt-villus axis height decreased with increasing body weight in CF but not control mice. Intestinal crypts from CF mice had fewer apoptotic cells, per unit length, than did non-CF mice, and normalized cell proliferation was similar to control levels. Goblet cell hyperplasia and mast cell infiltration were increased in the CF intestine and identified to be independent of body weight. Blood triglyceride levels were found to be significantly lower in CF mice than in control mice but were not dependent on CF mouse weight. By expression profiling, genes of DNA replication and lipid metabolism were among those altered in CF mice relative to non-CF controls, and no differences in gene expression were measured between samples from CF mice in the 25th and 75th percentile for weight. In this CF mouse model, crypt elongation, due to an expanded proliferative zone and decreased apoptosis, was identified to be dependent on body weight.

Introduction

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (*CFTR*), a gene that encodes a chloride channel expressed in epithelial cells of various organs such as the intestine, pancreas, lungs, and reproductive organs [6]. One of the pathologies of CF is intestinal disease, which is manifest as meconium ileus (MI) in 15–20% of CF newborns [40] and as distal intestinal obstruction syndrome (DIOS) episodes in 25% of CF adults [4]. From clinical studies, it has been established that the course of CF is highly variable; this variance in CF intestinal disease is determined in part by the nature of mutation(s) in *CFTR* as well as by alleles in yet-undiscovered modifier genes located elsewhere in the genome [35].

Mouse models created to have nonfunctional Cftr protein develop a phenotype resembling MI [54] and DIOS [72], and studies in these mice have identified a genomic locus that has also been shown to predict for clinical MI [40]. Investigations of the intestinal phenotype in CF mice have revealed that histologically, most mice suffer from intestinal goblet cell hyperplasia, mucus accumulation, and crypt dilation and elongation [54]. In addition, gene expression analyses have demonstrated the C57BL/6 *Cftr*^{tm1UNC} (B6 CF) small intestine profile to include altered expression of genes of the innate immune system and involved in lipid metabolism [93].

An additional CF phenotype reflected in mice [58, 68] that is, in part, related to the intestinal defect is the low body weight of CF patients [152] relative to the non-CF population. A CF patient's failure to thrive is due to a composite of factors, including nutrition level and environment, as well as fat malabsorption, which persists in this population even with pancreatic enzyme replacement therapy [17]. Peretti *et al*. [17] proposed that fat malabsorption in CF may be the result of histological changes in the intestine or of altered regulation of transport or metabolic enzymes. The cause of the lower body weight of CF mice is unknown, but studies have revealed the mice to have the clinical phenotype of fat

malabsorption, possibly due to altered pH of the intestine [69], while severe pancreatic disease is not a feature of most CF mouse models [54]. A bacterial presence in the intestine has also been reported to affect the growth of CF mice [86], and this phenotype may be due to the abnormal dissolution of Paneth cell granules in CF intestinal crypts [88]. Knowledge of mechanisms governing the growth of CF patients is important as percent ideal body weight has been shown to be prognostic of both survival and lung disease severity [19, 20].

To investigate if the intestinal pathology of CF mice is dependent on their body weight we measured the ileal histology of variable weight C57BL/6 x BALB/cJ F2 UNC *Cftr*^{tm1UNC} mice and compared this to non-CF mice. F2 mice were studied as the population presents a greater range of the body weight phenotype than does a congenic CF population [68]. In addition, to identify a set of non-*Cftr* genes that may influence the development of intestinal complications (MI, DIOS, and related to growth), we assessed the ileal gene expression of CF and non-CF mice presenting high- or low-body weight phenotypes. These expression data were integrated with the histology and blood biochemistry results to propose specific genes and pathways leading to the intestinal phenotype in CF mice.

Materials and Methods

Animals

The mice of the C57BL/6 (B6) *Cftr*$^{+/tm1UNC}$ strain were provided by Dr. Danuta Radzioch of the Montreal General Hospital, and the BALB/cJ (BALB) strain was purchased from the Jackson Laboratory. F2 CF mice were created from these strains as the B6 *Cftr*$^{+/-}$ and BALB mice were crossed to create F1 mice, and the resultant F1 mice were intercrossed to create F2 CF$^{-/-}$ and *Cftr*$^{+/+}$ mice.

The *Cftr* genotype of the mice was determined using a previously described PCR assay [153], and genomic DNA was isolated from the tails of the mice, which were clipped at 16 days of age. All CF and control mice were

maintained on liquid diet (Peptamen) from the age of 18 days until euthanasia [69]. Peptamen is a complete liquid enteral formulation composed principally of medium-chain triglycerides, essential fatty acids (0.16% linoleic and 0.023% linolenic acids), carbohydrates, and hydrolyzed protein [69]. All mice were housed in microisolator cages in a specific pathogen-free room and handled according to standard husbandry of the animal facility at the McIntyre Building of McGill University and cared for under a protocol approved by the McGill University Animal Care Committee.

Mice were killed, by carbon dioxide exposure, at the age of 12 wk. At this time, their total body weight was measured, and blood was drawn by cardiac puncture. At dissection, a 2-cm portion of the terminal ileum was removed, its contents were eliminated, and 1 cm of the proximal ileum was immediately homogenized in 1 ml of TRIzol reagent (Sigma). The homogenate was kept on dry ice until it was stored at –85°C. A second 1 cm of the ileum was fixed in formalin and submitted for standard histological processing. Obvious obstructions observed upon inspection of the intestine at autopsy were recorded. The body weight of a total of 98 F2 CF (40 females and 58 males) mice was evaluated at 12 wk of age. Five mice were identified to have ileal obstructions and were not included in this study so that the reported body weight of a CF mouse was not influenced by the development of an intestinal obstruction.

Histology

Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin for the evaluation of general histological structure. For each section, the crypt-villus axis (CVA) height was measured, and the numbers of goblet cells were counted for an average of 20 CVAs/section. The CVA height measurements included the entire length of the crypt and villus, and only complete and intact CVAs were measured using image analysis of the histological sections (Olympus BX51, Image-Pro Plus 5.1, Media Cybernetics). In addition, the goblet cells per CVA count was normalized to a linear length of 100 μm of epithelium along the

CVA, and separate measurements of villus height and crypt depth were taken to calculate the villus-to-crypt ratio. From 3-μm sections stained with Toluidine blue, mast cell counts for each mouse were calculated as the average numbers of stained cells per millimeter squared (x400 magnification), for an average of 40 fields/section. For these phenotypes, one section from each of 13 control mice (7 males and 6 females) fed the liquid diet and 18 CF mice (9 males and 9 females) selected to represent the weight range of the F2 population was scored. All sections were scored by an observer blinded to mouse genotype.

Assessment of proliferation and apoptosis

To visualize cells undergoing proliferation or apoptosis, paraffin-embedded tissue sections (5 μm thick) were immunostained with antibodies directed against PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) and caspase-3 (Cell Signaling Technology), respectively. In brief, sections were initially deparaffinized and hydrated through graded ethanol. Antigen sites were unmasked by antigen retrieval treatment for 10 min at 98°C in a citrate buffer. Endogenous peroxidase activity was quenched using 3% (vol/vol) hydrogen peroxide in Tris-buffered saline (TBS) for 15 min. Nonspecific binding sites were blocked with 10% sheep serum (Cedarlane Laboratories, Hornby, ON, Canada) in TBS for 20 min at 37°C. Sections were then incubated overnight at 4°C with the primary antibody (PCNA, 1:50; and caspase-3, 1:100). After being washed, sections were incubated with 1:100 biotinylated sheep anti-rabbit secondary antibody (Serotec, Raleigh, NC) for 60 min at room temperature. Sections were then washed and incubated in an avidin-peroxidase solution (StreptABComplex/HRP, DakoCytomation, Mississauga, ON, Canada) for 45 min. Sections were developed using 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB Substrate Chromogen System, DakoCytomation), counterstained with hematoxylin, dehydrated, and then mounted. For negative control preparations, the primary antibody was replaced by TBS. Blinded scoring of 30–100 crypts/mouse was performed and presented as the average number of positively stained cells per crypt.

Blood analysis

Blood was collected by cardiac puncture on anesthetized animals, stored in plasma separator tubes containing EDTA, and centrifuged. The blood analysis was completed on a biochemistry analyzer Hitachi 911, and the levels of glucose, cholesterol, high-density lipoprotein, and triglycerides were determined.

Phenotypic data analysis

Tests for differences in mouse weight, blood biochemistry, and ileal morphology between groups were done by *t*-test. Association of body weight with histological features was assessed by regression analysis using Microsoft Excel software, where a value of $P < 0.05$ was considered significant. To use all F2 CF mice in one test, the weights of the F2 female mice were increased by the relative mean weight of male mice to female mice, as in Haston *et al*. [68].

Gene expression analysis

Gene expression profiles were determined for groups of mice defined by *Cftr* genotype, sex, and body weight. To ensure that potential differences in ileal gene expression by murine weight could be assessed, RNA from mice with body weights in the highest and lowest quartiles of the weight distribution was selected for hybridization as in Ref. [154]. The gene expression of high-weight CF mice was assayed with five chips, three of which represent the RNA of an individual male mouse (mouse weights = 25.6, 25.9, and 26.0 g) per chip and two of the pooled RNA from five mice (5 males on 1 chip and 5 females on the second chip), with weights from the 4th quartile of the CF population. For low-weight CF mice, the gene expression profile was assayed from four chips, two of individual male mice (weights = 19.4 and 19.5 g), one individual female mouse (17.1 g), and one of RNA pooled from female mice with weights within the 1st quartile of the weight distribution. The gene expression profile of liquid diet-fed control mice ($Cftr^{+/+}$) was assayed with two chips. Each represents pooled RNA of five mice (1 chip for males and 1 chip for females) that were not selected for extreme weight.

Total RNA was extracted, according to the manufacturer's (Sigma) instructions, from the homogenate stored after tissue harvest. The quality of the isolated RNA was assessed and confirmed by Agilent Bioanalyzer readings (Agilent Technologies). Hybridization to the Murine MOE430 2.0 GeneChip (Affymetrix) was performed by the Affymetrix Gene Chip Core Facility at the McGill University and Genome Quebec Innovation Centre, as previously described [155]. Lists of significantly differentially expressed genes were generated for comparisons between groups using routines from Bioconductor version 1.6 within the R version 2.1.0 statistical language as in Ref. [155]. The detection of significantly overrepresented Gene Ontology categories was performed using the GohyperG function in Bioconductor [156]. Raw and normalized expression data are available from the National Center for Biotechnology Information GEO website.

Results

Body weight phenotype

To study CF and non-CF control mice with variable weights, a population of B6 x BALB F2 mice was bred from F1 progenitors. The average weight (mean ± SE) of male CF mice at 12 wk of age was significantly lower than that of controls (23.2 ± 0.3 compared with 32.0 ± 1.8 g, $P = 0.001$), and this difference was also evident in females (20.3 ± 0.3 g for CF vs. 22.8 ± 0.6 g for control mice, $P = 0.003$).

Ileal histology

To document the pathological changes in the ileum of CF and non-CF mice, histological sections were made and subsequently scored for CVA height, number of goblet cells, and presence of mast cells. As shown in Figure 1, the CVA height in CF mice exceeded that of liquid diet-fed control mice [355 ± 10 μm (means ± SE) vs. 185 ± 7.7 μm, $P = 1.8 \times 10^{-13}$], and the ilea of CF mice contained more goblet cells than those of non-CF mice (goblet cells/CVA: 18.0 ± 0.6 vs. 5.7 ± 0.5, $P = 4.3 \times 10^{-15}$; goblet cells/100 μm of CVA: 2.5 ± 0.1 vs. 1.6 ±

0.13, $P = 8.3 \times 10^{-7}$), suggesting the presence of a hyperplastic state in CF mice. Intestinal sections from CF mice also featured more mast cells in the submucosa and muscularis externa layers than did the sections of non-CF mice (3.6 ± 0.4 vs. 0.02 ± 0.01 mast cells/mm^2, $P = 0.0003$). These histological observations are consistent with previous reports on CF mice, including B6 CF mice [72, 86, 88, 93].

Regression analyses were completed to determine if the histological changes in the CF intestine were dependent on mouse body weight. As shown in Figure 2, CVA height of both male and female CF mice decreased with increasing body weight ($r = –0.59$, $P = 0.01$) while a directly proportional correlation of CVA height to weight was evident in control mice ($r = 0.59$, $P = 0.03$). Goblet cell numbers per linear length of CVA were identified to be independent of weight ($r = 0.05$, $P > 0.05$; see Figure 2); correspondingly, numbers of goblet cells per CVA decreased with increasing body weight ($r = –0.50$, $P = 0.03$) in CF mice. These findings indicate that the ileum of a heavier CF mouse more closely resembles that of a control mouse than does the ileum of a lighter CF mouse, with the latter presenting more severe morphological alterations. Mast cell counts were independent of body weight in CF mice ($r = 0.2$, $P > 0.05$; data not shown).

To further investigate the relationship of CVA height to weight, the crypt depth and villus height of each mouse were measured. The ratio of villus height to crypt depth in CF mice was found to be significantly lower than that of control mice (1.6:1 ± 0.05 vs. 2.5:1 ± 0.13, $P = 1 \times 10^{-7}$) and to increase with increasing body weight in CF mice ($r = 0.5$, $P = 0.03$). The average crypt depth of CF mice was approximately twice that of control mice (1.82 times), whereas the villi of CF mice were 1.15 times the length of those in non-CF mice. Therefore, the increase in CVA height is primarily due to an elongation of the crypts in CF mice.

Ileal crypt proliferation and apoptosis

Crypt cell proliferation and upward migration have been shown to be increased in the CF mouse small intestine [157], and our observation of increased CVA height is consistent with such changes. To further characterize the observed difference in crypt length between high- and low-body weight CF and control mice, crypt cell proliferation and apoptosis were measured with immunohistochemical staining. As shown in Figure 3, there were significantly more PCNA-positive (proliferating) cells in the crypts of CF mice compared with the levels detected in non-CF controls (8.4 ± 0.8 PCNA-positive cells/crypt in CF mice vs. 4.5 ± 0.7 PCNA-positive cells/crypt in non-CF controls, $P = 0.003$). Due to the crypt elongation in CF mice, however, the numbers of proliferating cells per unit length of crypt did not differ between CF and control mice (Figure 4A). Furthermore, the number of proliferating cells per crypt was found to be independent of body weight in CF mice ($P = 0.6$), indicating a lower rate of proliferation to exist in smaller CF mice, which present a CVA of increased length.

In contrast, numbers of apoptotic cells in the crypts of CF mice were not different from control levels (0.13 ± 0.02 positive cells/crypt in CF mice vs. 0.15 ± 0.01 positive cells/crypt in non-CF controls, $P = 0.5$). In this case, the increased crypt length in CF mice resulted in a lower number of apoptotic cells per unit length of tissue in CF compared with control mice (Figure 4B). Based on these observations, the change in crypt length in the CF mice is due to a relative reduction in numbers of apoptotic cells and an expansion of the proliferative zone. Both of these features are more pronounced in the smaller CF mice and thus may be associated with variable weight in this F2 CF mouse model.

Blood biochemistry phenotype

To investigate if the blood biochemistry of CF mice differed from that of non-CF mice, and if this was influenced by body weight, cardiac blood samples were collected from a cohort of mice at death. The blood of CF mice of both sexes (n = 11 males and 9 females), representing the high and low ends of the weight distribution, and of liquid diet-fed control mice (n = 9 males and 8 females) was tested. Blood biochemistry was not dependent on the sex of the mice within either the CF or non-CF groups (P = 0.14–0.97; data not shown), with the exception of a high glucose measure in male control mice relative to that of females (20.1 ± 1.3 vs. 16.5 ± 0.9 mmol/l, P = 0.036), and, therefore, these data were combined for a comparison of CF mice to non-CF mice. As shown in Table 1, triglyceride levels were significantly lower in CF mice than in control mice, as was the body weight, and no other differences in blood biochemistry levels by *Cftr* genotype were evident. Blood glucose levels did not differ between female CF and non-CF mice (P = 0.77), but male CF mice had lower glucose levels than male non-CF controls (15.8 ± 1.6 vs. 20.1 ± 1.3 mmol/l, P = 0.055). Blood triglyceride levels were not significantly correlated with weight in either of the CF (P = 0.56) or control mouse groups (P = 0.23; data not shown).

CF intestinal gene expression profiles

Eleven Affymetrix Gene Chip microarrays (45,101 probes sets/chip) were used to determine which genes were differentially expressed in the ilea of (B6 x BALB) F2 CF mice compared with (B6 x BALB) F2 non-CF liquid diet-fed control mice and how this differential expression was influenced by the sex and weight of the mice.

To identify the set of genes involved in the intestinal disease of CF mice, data from nine arrays (samples from 6 male $Cftr^{-/-}$ and 3 female $Cftr^{-/-}$ mice) were compared with data from two control arrays (from $Cftr^{+/+}$ mice); 205

genes/expressed sequence tags (ESTs) were determined to be significantly differentially expressed in the ilea of CF mice compared with control mice (fold > 2, $P < 0.05$; Table 2 and Supplementary Table 1). The genes most significantly differentially expressed in the CF intestinal response included complement component factor i, CD177 antigen, and fucosyltransferase 2, which were of increased expression in CF mice relative to controls, whereas *Cftr* and genes of oxidoreductase processes (cytochrome *P*-450 family members and retinol dehydrogenase 7) were of lower expression in CF mice. The ileal CF response also included 51 solute carrier family members and 9 genes for ATP binding cassette transporters. By gene ontology analysis, among the biological processes most affected in the murine CF ileum were cellular physiological processes ($P = 1.3 \times 10^{-08}$); metabolism ($P = 1.5 \times 10^{-06}$), including the subcategories of cellular, macromolecule, protein, and organic acid metabolism; and DNA replication initiation (see Table 3 and Supplementary Table 2). In further analyses, completed by comparing data of CF male mice (6 chips) with those from CF female mice (3 chips), we determined the sex of the mouse to have a minimal influence on the intestinal gene expression as eight genes were found to be differentially expressed by sex ($P < 0.05$; Supplementary Table 3).

Finally, to ascertain if ileal gene expression differed with murine body weight, data from high-weight CF mice (weights > 75th percentile, assayed with 5 chips) were compared with data from low-weight CF mice (weights < 25th percentile, assayed with 4 chips). In this analysis, no probe sets were identified to be significantly differentially expressed ($P < 0.05$).

Discussion

In this study, we demonstrated that the CF intestinal phenotype of increased CVA height decreases with increasing body weight in B6 x BALB F2 CF mice, whereas mast cell infiltration and goblet cell hyperplasia did not change with mouse weight. We further showed the intestinal crypts from CF mice to have fewer cells undergoing apoptosis per unit length than did non-CF mice and to have an expanded proliferative zone. By gene expression analysis, DNA replication and metabolic biological processes were revealed to be affected in CF mice regardless of their weight. Finally, the CF defect of low blood triglyceride levels was shown to be strongly dependent on CF status and not on body weight.

Through histological evaluation, we showed the intestinal phenotype of B6xBALB F2 CF mice to be consistent with that previously reported for CF mice, which includes crypt elongation and dilatation as well as goblet cell hyperplasia [54, 56, 58, 86]. The evaluation of a group of F2 CF mice, which, on the genetically mixed B6 x BALB background present a range in body weight [68], revealed the known hyperplastic state of the CF intestine to decrease with increasing animal mass. The existence of the relatively increased crypt depth in smaller CF mice, which was due to both an expansion of the proliferative zone and a reduced rate of crypt cell apoptosis, suggests this phenotype to be associated with a more severe intestinal disease. These observations of crypt cell number changes are consistent with those of Gallagher *et al*. [157], who identified an increased rate of epithelial cell proliferation, but no difference in apoptosis, within the intestinal crypts in *Cftr*-null mice relative to non-CF mice.

In the present study, we also detected an increased number of PCNA-positive cells in the CF intestine and a similar number of apoptotic cells in CF and control mice. Differences between the studies occur based on normalization. In the work of Gallagher *et al*. [157], numbers of positively stained cells within the first 10 cells following the Paneth cells in the crypts were evaluated, and the CVA height of CF and control mice was found not to differ. In this study, apoptotic

cells per unit length of crypt decreased due to the difference in crypt depth between CF and non-CF mice. In addition, animals here were studied at 12 wk of age, whereas in Ref. [157], the age was not indicated; thus, events taking place earlier or later in mouse intestinal disease development might reflect in the reported differences. In our study, the increase in the proliferative zone and decrease in crypt cell apoptosis could have contributed to the disease in CF mice by altering the functional structure of the small intestine. Indeed, the increased numbers of goblet cells in smaller CF mice producing and secreting an increased or altered amount of mucus to the intestinal lumen could influence body weight through the creation of an extraphysical barrier to nutrient absorption. The histological changes in the lower-weight CF mice are also reminiscent of short bowel syndrome, which features CVA of increased height as an adaptation to a surgical or medical loss of intestinal length [158]. As the entire length of the small intestine was not measured in this study, it is not known if the observed histological change occurred through a similar mechanism, i.e., as an adaptation to reduced length of the small intestine. Further study is required.

The intestinal phenotype of F2 CF mice was also assessed through gene expression profiling, and this revealed genes of cell proliferation, including DNA replication, to be among those differentially expressed in CF mice, supporting the histological data. To determine if these gene expression and histological observations were consistent with reported intestinal crypt and villus patterns of gene expression, we compared our data with those of Mariadason *et al*. [159]. In their work, they analyzed the murine gene expression profile of intestinal cell maturation along the CVA and concluded that genes of cell proliferation and of RNA and protein synthesis decreased in relative expression from the crypt to the villus. Comparing our CF ileal gene expression profile with that of intestinal cell maturation, we found genes involved in RNA processing, translation, and protein folding, which were found by Mariadason *et al*. [159] to be decreased in villi and thus increased in crypts, were more highly expressed in CF mice; thus, the CF expression profile is consistent with the histological observations. Furthermore,

specific to the proliferative response, the gene for Eph receptor B_2, which is a key regulator of epithelial cell migration and proliferation [160], was more highly expressed in CF intestinal tissue than in controls.

The gene expression profile for the ileum of B6 x BALB F2 CF mice also agrees with, and extends, the previous report [93] made of the small intestine of B6 CF mice, in that 62% of the genes identified to be differentially expressed in the prior study were among those of altered expression in this report, and, as an additional, 609 genes were identified to be part of the F2 CF response, 92 of which had a fold change >2. By using the Affymetrix 430 2.0 chip, the expression of a greater number of genes was sampled, for an increased number of mice; thus, we were able to build on previous observations. For example, we confirm the observation of Norkina *et al*. [93] showing that components of the innate immune system are upregulated in the small intestine of CF mice, whereas genes of lipid metabolism are downregulated. In addition, we identified other innate response genes, such as S100 calcium-binding protein A11 (calizzarin) (*S100A11*), chemokine (C-C motif) ligand 9, and Toll-like receptor 4, to be part of the CF intestinal response, independent of weight. These data also indicate that the body weights of F2 CF mice could have been, in part, influenced by susceptibility or response to bacterial infection, as has been reported to affect the growth of a B6 CF mouse [86]. A further similarity to the findings from Norkina *et al*. [93] is the increased presence of mast cells in CF mice, which was evident histologically. We did not, however, uncover differential expression of mast cell genes, which may be due to the relative density of mast cells to the entire tissue sampled by gene expression or may reflect the fact that the cells were positive for toluidine blue staining but were not active and expressing secretory markers.

Our analysis of the gene expression data also revealed the profile of the ileal tissue of the heaviest CF mice not to differ from that of the lightest mice. This finding is likely due to histological change observed (increased crypt depth) being only a fraction of the tissue composition or may indicate, by our level of sampling (data of 5 microarrays compared with 4 microarrays), that the changes in

gene expression by mouse weight are more subtle than those induced by nonfunctional *Cftr*. It is also possible that gene expression profiles in the more proximal small bowel may differ in mice segregated by body weight due to the functional differences of segments of the small intestine. Such changes were not evaluated as in this investigation we focused on alterations in the ileum as this is the site of obstruction in the clinical CF intestinal phenotypes MI and DIOS. Importantly, the data of the gene expression profiles suggest that lipid metabolism is similarly altered in high-weight CF mice.

The intestinal phenotype of CF mice includes the altered expression of genes encoding solute and fatty acid transporters and genes of lipid metabolism. Among these were genetic factors implicated in the development of colitis or Crohn's disease (*multidrug resistance 1a*, *Slc22a4*, *and Slc22a5*) [161], which may suggest the existence of an overlap in the pathways leading to ileal inflammation and hyperplasia in these diseases. Also of reduced expression in CF mice was the gene for peroxisome proliferator-activated receptor-α, which is a transcription factor regulating levels of enzymes of fatty acid metabolism [162], as were fatty acid processing genes for phospholipases A2 (*Pla2g4a* and *Pla2g5*), the altered expression of which may influence both lipid metabolism and the development of the inflammatory component of the CF intestinal phenotype [163].

Finally, the lower body weight phenotype of CF patients is thought to be due in part to fat malabsorption [17]. Our analysis of absorption through blood biochemistry confirmed the CF mice to have lower triglyceride levels than Peptamen-fed non-CF mice, as reported in Ref. [69], and furthermore showed triglyceride levels to depend on CF status and not body weight. From the gene expression analysis, the reduced expression of the intracellular metabolic enzyme diacylglycerol *O*-acyltransferase 1 (*Dgat1*) could partially account for this phenotype, as *Dgat1* is one of two known enzymes that catalyze the final step in mammalian triglyceride synthesis [17]. Of significance, these data suggest that blood triglyceride levels remain lower in near-normal weight CF mice.

In summary, we demonstrated the CVA elongation phenotype of CF mice to feature an increase of the proliferative zone and decreased crypt cell apoptosis and to be related to body weight. The proliferative response to nonfunctional *Cftr* has also been shown in CF lung disease in mice [155] and thus may be mechanistically important to disease severity in CF. Furthermore, we showed the expression of genes for transporters and metabolic enzymes responsible for the uptake and processing of nutrients from the lumen to be reduced in the murine CF intestine, independent of weight, and blood triglyceride levels to be influenced by CF status, and not weight, in mice.

Grants

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Figure legends

Figure 1. Sections of ileal tissue from 12-wk-old F2 control and F2 cystic fibrosis (CF) mice (stained with hematoxylin and eosin).

A and *C*: F2 control mouse ileum. The crypt lumen was small, and goblet cells appeared compacted. *B* and *D*: F2 CF mouse ileum featuring greater crypt-villus axis (CVA) height, crypt elongation, and hyperplasia of goblet cells.

Figure 2. Intestinal features of CF and WT mice.

CVA height (*A*), numbers of goblet cells per CVA (*B*), and numbers of goblet cells per unit length of CVA (*C*) as a function of body weight in B6 x BALB F2 CF mice and non-CF mice. ▲, Individual male CF mice; △, individual male control mice; ○, individual female CF mice; ●, individual female control mice. In all graphs, the weights of F2 female mice were increased by the relative mean weight of male mice to female mice, as described in Materials and Methods.

Figure 3. Sections of ileal tissue from 12-wk-old F2 control and F2 CF mice stained against a proliferation marker (PCNA).

A: control mouse presenting PCNA-positive crypt cells with the characteristic nuclear staining. *B*: CF mouse section presenting a higher number of crypt cells positive for PCNA.

Figure 4. Quantitation of immunohistochemical measures of ileum crypt proliferation (*A*) and apoptosis (*B*) in CF and control mice.

Data are presented as means ± SE of positive cells/100 μm of crypt length and were analyzed for statistical significance between control and CF sections. *A*: PCNA (n = 9 control and 10 CF mice, $P > 0.05$). *B*: caspase-3 (n= 7 control and 10 CF mice, $P = 0.03$).

Figure 1

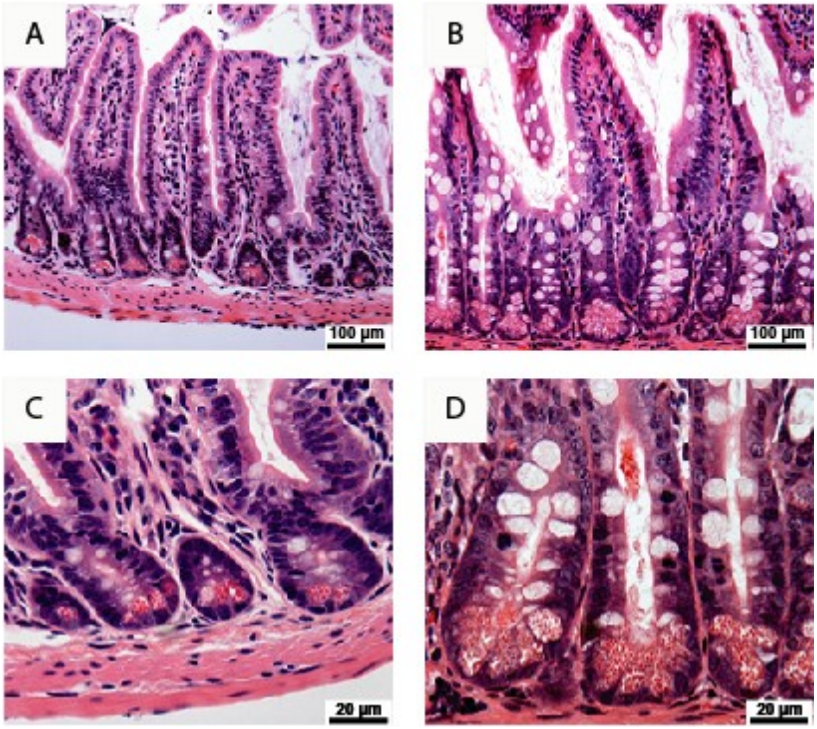

Figure 2

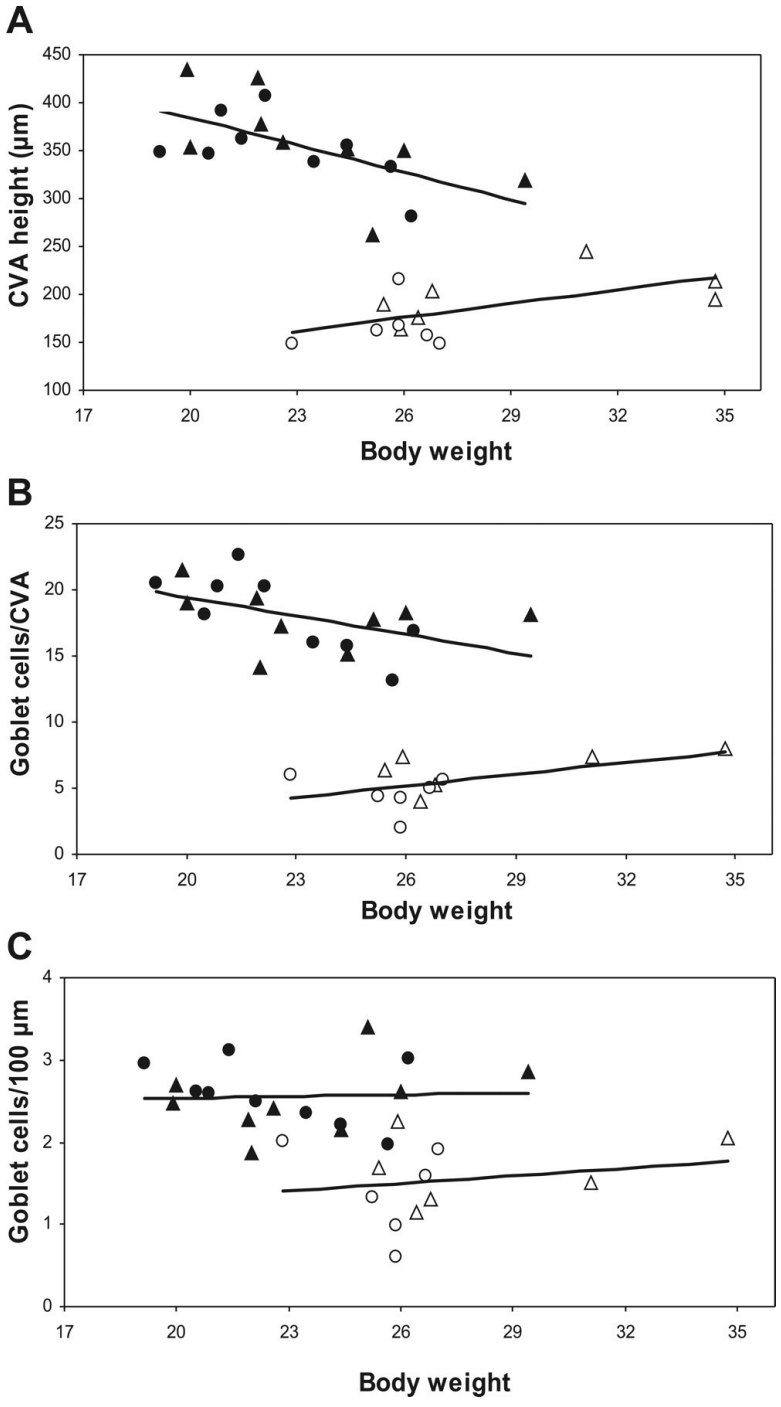
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Figure 3

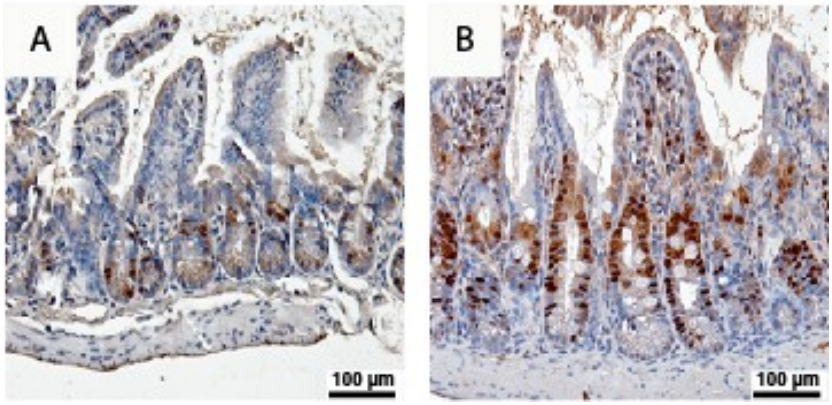

Figure 4

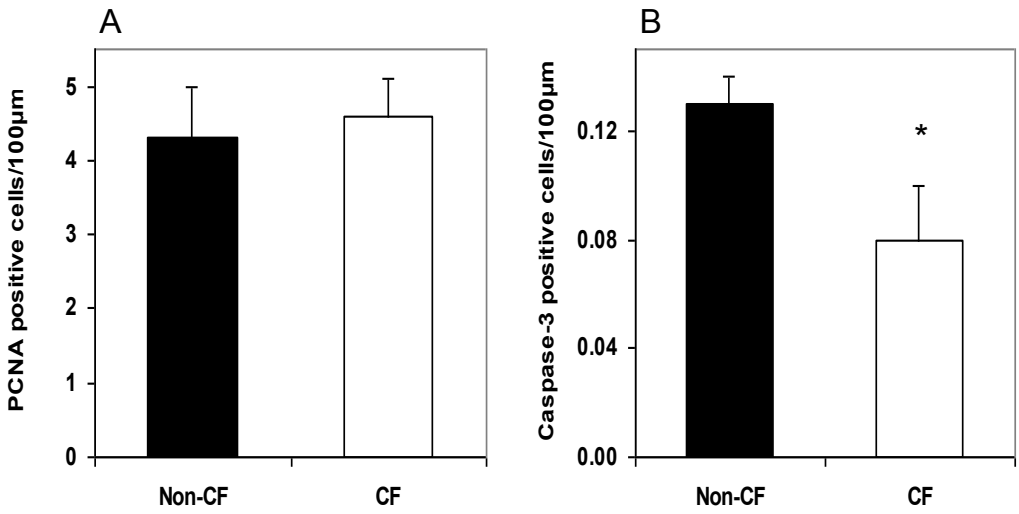

Table 1. *Blood Biochemistry in CF and liquid diet-fed non-CF mice at 12 weeks of age*

	CF (n=20)	**Non-CF (n=17)**	**CF vs non-CF P value**
Glucose (mmol/L)	15.8 ± 1.1*	18.4 ± 0.9	0.09
Triglycerides (mmol/L)	1.06 ± 0.12	1.69 ± 0.14	$1.5x10^{-3}$
HDL (mmol/L)	2.66 ± 0.13	2.59 ± 0.12	0.48
Cholesterol (mmol/L)	3.31 ± 0.19	3.06 ± 0.17	0.34
Body Weight† (g)	22.0 ± 0.5	32.0 ± 1.0	$4.6x10^{-9}$

*value ± standard error of the mean

†the weights of the F2 female mice were increased by the relative mean weight of male mice to female mice, as described in methods.

Table 2. *Genes most significantly differentially expressed in the Ilea of 12 week old B6xBALB F2 CF mice relative to non-CF mice*

Symbol	Gene Title	Increase (Fold)	UniGene ID	Symbol	Gene Title	Decrease (Fold)	UniGene ID
1600029D21 Rik	RIKEN cDNA 1600029D21 gene	9.2	Mm.29959	*Cyp3a25*	Cytochrome P450, family 3, subfamily a, polypeptide 25	21.5	Mm.384461
Tra2a	Transformer 2 alpha homolog (Drosophila)	6.8	Mm.196598	*Pdzk1*	PDZ domain containing 1	14.4	Mm.28015
Ceacam10	CEA-related cell adhesion molecule 10	5.4	Mm.30300	*Rdh7*	Retinol dehydrogenase 7	11.3	Mm.6696
Cd177	CD177 antigen	4.9	Mm.292848	*AW011956*	Expressed sequence AW011956	7.5	Mm.10034
Aqp4	Aquaporin 4	4.5	Mm.250786	*Cubn*	Cubilin (intrinsic factor-cobalamin receptor)	7.3	Mm.313915
Vip	Vasoactive intestinal polypeptide	3.9	Mm.98916	*Cyp4v3*	Cytochrome P450, family 4, subfamily v, polypeptide 3	7.1	Mm.245297
9030623N16 Rik	RIKEN cDNA 9030623N16 gene	3.9	Mm.171333	*Serpina1b*	Serine (or cysteine) preptidase inhibitor, clade A, member 1b	6.9	Mm.312593
Fut2	Fucosyltransferase 2	3.9	Mm.290046	*Slc7a15*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 15	6.2	Mm.46326
Cfi	Complement component factor i	3.7	Mm.117180	*Treh*	Trehalase (brush-border membrane glycoprotein)	6.2	Mm.45380
Mtac2d1	membrane targeting (tandem) C2 domain containing 1	3.6	Mm.275939	*Arg2*	Arginase type II	6.1	Mm.3506
Pla2g5	phospholipase A2, group V	3.2	Mm.23347	*Akr1b7*	Aldo-keto reductase family 1, member B7	5.9	Mm.90151
Tmprss2	transmembrane protease, serine 2	3.1	Mm.276145	*Susd2*	Sushi domain containing 2	5.8	Mm.247956
1810030J14	RIKEN cDNA 1810030J14 gene	3.1	Mm.7150	*1300013J15*	RIKEN cDNA 1300013J15 gene	5.7	Mm.100741
Mfsd2	major facilitator superfamily domain containing 2	3.0	Mm.331842	*Aadac*	Arylacetamide deacetylase (esterase)	5.4	Mm.24547
C1galt1	core 1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase	3.0	Mm.102752	*Cndp1*	Carnosine dipeptidase 1 (metallopeptidase M20 family)	5.1	Mm.23278
Evi1	ecotropic viral integration site 1	3.0	Mm.56965	*Cftr*	Cystic fibrosis transmembrane conductance regulator homolog	5.0	Mm.15621
Tlr4	toll-like receptor 4	2.9	Mm.38049	*Slc2a2*	Solute carrier family 2 (facilitated glucose transporter), member 2	4.4	Mm.18443

Table 3. *Most relevant categories of differentially expressed ileal genes of 12 week old CF vs. non-CF mice by Gene Ontology analysis*

Biological Process	*p* value	Molecular Function	*p* value
cellular physiological process	$1.3x10^{-08}$	catalytic activity	$4.6x10^{-10}$
metabolism	$1.5x10^{-06}$	RNA binding	$2.0x10^{-06}$
physiological process	$3.7x10^{-06}$	nucleotide binding	$3.2x10^{-06}$
intracellular protein transport	$3.3x10^{-05}$	protein transporter activity	$1.1x10^{-05}$
intracellular transport	$5.0x10^{-05}$	nucleoside-triphosphatase activity	0.00015
protein transport	$7.6x10^{-05}$	acyltransferase activity	0.00034
cellular metabolism	0.00011	exopeptidase activity	0.00037
interphase of mitotic cell cycle*	0.00012	transferase activity, transferring groups other than amino-acyl groups	0.00045
macromolecule metabolism	0.00014	pyrophosphatase activity	0.00046
DNA replication initiation*	0.00015	purine nucleotide binding	0.00049
primary metabolism	0.00020	ATPase activity	0.0011
cellular macromolecule metabolism	0.00023	symporter activity	0.0011
G1/S transition of mitotic cell cycle*	0.00035	protein carrier activity	0.0025
translation	0.00050	aldehyde dehydrogenase (NAD) activity	0.0025
RNA metabolism	0.00061	oxidoreductase activity	0.0067
biosynthesis	0.00076	metalloexopeptidase activity	0.0078
cell cycle*	0.00088		
DNA replication*	0.0012		
DNA-dependent DNA replication*	0.0021		
diacylglycerol metabolism	0.0028		
mitotic cell cycle*	0.0063		

*Categories involved in cell proliferation, RNA and protein synthesis.

CHAPTER TRANSITION

Presented in chapter 2, we have identified the hyperplasic state of the CF small intestine to be associated with body weight. Furthermore, intestinal gene expression analyses have also identified several intriguing candidates with altered expression that could be responsible for the observed phenotype.

In the next chapter 3, we proposed to study the role of the candidate gene Toll-like receptor 4 (Tlr4), which was highly expressed in data from chapter 2, and evaluated its possible implication on the proliferative response of the CF intestine and its subsequent effect in bacterial clearing, immune response and body weight.

CHAPTER 3

Toll-like Receptor-4 genotype influences survival of cystic fibrosis mice

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Abstract

Toll-like receptor (Tlr) 4 is a lipopolysaccharide (LPS) receptor which contributes to the regulation of intestinal cell homeostasis, a condition that is altered in the intestines of cystic fibrosis mice. Herein, we assessed whether *Tlr4* genotype influences cystic fibrosis intestinal disease by producing and phenotyping 12 week (adult) and 4 day (neonate) old mice derived from BALB cystic fibrosis transmembrane conductance regulator, *Cftr*+/^{tm1Unc} and C.C3-*Tlr4*$^{Lps\text{-}d}$/J (*Tlr4*-/-) progenitors. Intestinal disease was assayed through mouse survival, crypt-villus axis (CVA) length, cell proliferation, bacterial load, inflammatory cell infiltrate and mucus content measures. Of the 77 *Cftr*-/- (CF) mice produced only one *Cftr*/*Tlr4* double mutant mouse lived to the age of 12 weeks while the majority of the remainder succumbed at approximately 4 days of age. The survival of CF *Tlr4*+/- mice exceeded that of both CF *Tlr4*+/+ and *Cftr*/*Tlr4* double mutant mice. Adult CF mice presented increased *Tlr4* expression, CVA length, crypt cell proliferation and bacterial load, relative to non-CF mice, but no differences were detected in *Tlr4*+/-, compared to *Tlr4*+/+, CF mice. The double mutant neonates did not differ from *Tlr4*+/+ or +/- CF mice by intestinal CVA length or bacterial load but fewer *Tlr4*+/- CF neonates presented with luminal mucus obstruction in the distal ileum; and the intestinal mast cell increase of CF mice was not evident in double mutant neonates. We conclude that Tlr4 deficiency reduces the survival, but does not alter the intestinal phenotypes of extended CVA or increased bacterial load in BALB CF mice.

Introduction

Cystic fibrosis (CF) is the most common, fatal, autosomal recessive disease of Caucasians with an incidence of 1 in 2,500 newborns in this population [1]. Although it is well documented that the basic defect in CF lies in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, exactly how the encoded epithelial chloride-ion channel deficiency leads to the varied CF symptoms, including intestinal disease, is unclear. The intestinal disease is presumed to be due to reduced water secretion and increased mucus accumulation in this organ which results in the development of meconium ileus in 15-20 % of CF newborns [40] and distal intestinal obstruction syndrome episodes in 25% of CF adults [4].

Several mouse models have been made to investigate the complex CF phenotype [54], and as most Cftr deficient mice suffer from intestinal goblet cell hyperplasia, mucus accumulation, and crypt dilation, which ultimately lead to lethal obstruction [54, 57], these mice have been used as a model of clinical meconium ileus. One approach towards elucidating the mechanism leading to intestinal disease in CF mice has been to cross the mice with others deficient in specific physiological pathways such as NHE3 a sodium/hydrogen ion exchanger which alters intestinal content fluidity [81] or chloride channel Clca3 [79] to determine whether such deficiencies alter the CF disease course.

Toll-like receptors (Tlr), which are expressed on innate immune and epithelial cells and coordinate an immune response when bound by ligands, have been investigated as modifiers of cystic fibrosis lung disease [123, 124], but the potential influence of such receptors on cystic fibrosis intestinal disease has not been studied. Rakoff-Nahoum *et al.* [116] showed that recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis and given that both intestinal homeostasis [94] and gram-negative bacterial content [86], which contain the Tlr4 ligand, are altered in the intestine in CF mice we hypothesised

that mutations in *Tlr4* would alter the CF intestinal phenotype. Supporting this hypothesis, we have shown *Tlr4* expression to be increased in the intestines of BALB x C57BL/6 F2 Cftr deficient mice [94] as were components of its signaling pathway CD14 and Ly96 and genes of lipid metabolism, a pathway for which there is crosstalk with Tlr4 signaling [122]. In addition, the intestinal phenotype of the F2 CF mice featured both increased proliferation and less apoptosis in crypts compared to wildtype mice. These changes are consistent with the influence of increased Tlr4 signaling on the CF intestine as *Tlr4* deficient mice, when challenged with dextran sodium sulphate, have been shown to develop colitis with decreased proliferation and increased apoptosis [118]. A deficiency in this gene, in CF, could therefore spare the proliferation and apoptosis alterations in the intestine. Alternatively, a deficiency of Tlr4 in CF could leave the mouse unable to mount an adequate inflammatory response to the increased bacteria which would result in increased disease such as occurs in the development of sepsis [164].

Using the mutation in *Tlr4* originally identified in C3H/HeJ mice [101], which renders the gene product non functional, herein to investigate the hypothesis that *Tlr4* genotype alters the CF intestinal phenotype we bred and phenotyped a population of *Cftr/Tlr4* double mutant mice and their littermates.

Materials and Methods

Mice

To create *Cftr/Tlr4* double mutant mice in one genetic background we bred C.C3-*Tlr4*$^{Lps\text{-}d}$/J mice obtained from Jackson Laboratories (Bar Harbor, Maine), which have the C3H/HeJ point mutation in *Tlr4,* in the BALB background [165], with BALB *Cftr*^{tm1UNC} mice and genotyped the offspring for *Cftr* [94] and the C3H/HeJ-derived *Tlr4* donor region [165]. *Cftr*/*Tlr4* double mutants and littermate controls were then derived from *Cftr*/*Tlr4* double heterozygous and *Cftr* +/-/*Tlr4* -/- parents. *Cftr* and *Tlr4* are on separate chromosomes therefore these genes

segregated independently. Mice were maintained on Colyte® from the age of 21 days until sacrifice at 12 weeks of age [166]. Neonatal mice were similarly generated through timed matings of the parental mice and sacrificed at 4 days of age. All mice were housed in micro-isolator cages in a Specific Pathogen Free room and handled according to the standard husbandry of the animal facility at the Meakins-Christie Laboratories of McGill University. The mice were cared for under a protocol approved by the McGill University Animal Care Committee in accordance with the regulations set by the Canadian Council on Animal Care.

Histology

Mice were sacrificed, by carbon dioxide exposure, at the age of twelve weeks and their total body weight was recorded. At dissection, the entire small intestine was removed, flushed with PBS and a mucolytic agent (10 mM dithioreitol), the contents collected, and a 10-15 cm portion of the terminal small intestine was fixed in 10% buffered formalin and submitted for standard histological processing. Also at dissection, a 1 cm portion of the terminal ileum was removed and immediately homogenized in 1 mL of Trizol reagent (Sigma) and stored at -85°C. Neonatal mice were decapitated at 4 days of age and their entire small intestine was fixed and processed. Swiss roll paraffin embedded sections (5 μm) were stained with haematoxylin and eosin and the crypt-villus axis (CVA) length was measured. The entire depth of the crypt and the length of the villus were measured only from complete and intact CVAs using image analysis of the histological sections (Olympus BX51, Image-Pro Plus 5.1, Media Cybernetics). Goblet cells and mast cells were detected using Periodic acid-Schiff (PAS) and Toluidine blue staining, respectively. Goblet cells were quantified per intact CVAs and mast cells per high power field (HPF) from an average of 20 random sections viewed at 200X. The mucus obstruction was measured as the area filled by mucus plugs in PAS-stained sections using image analysis. All sections were scored by an observer blinded to mouse genotype.

Immunohistochemistry

To assess the numbers of proliferating cells, macrophages and T and B lymphocytes, and the expression of Tlr4, paraffin tissue sections (5-µm thick) were immunostained with antibodies directed against proliferating cell nuclear antigen (PCNA) as used previously [94], F4/80 (MCA497R), CD3 (MCA1477, AbD Serotec), CD20 (sc-7735, Santa Cruz Biotechnology), and Tlr4 (ab47093, Abcam), respectively, using antigen retrieval treatment as in [94]. Blinded scoring of an average of 25 CVAs per mouse was performed and presented as average number of positively stained cells per CVA or per HPF. The F4/80 expression in the mucosa was recorded as the percentage of stained tissue area in the entire measured area, as single cells were difficult to delineate.

Quantitative Real-Time PCR

RNA was extracted according to the manufacturer's (Sigma) instructions from 1 cm of the distal ileum collected at dissection. cDNA was generated and relative quantification of *Cftr* and *Tlr4* was completed with Assays-on-Demand™ TaqMan probes using the Applied Biosystems' 7500 Real Time PCR System™ as in Haston *et al.* [155].

Bacterial load measurement

DNA was extracted from 100 mg of the intestinal contents using a Stool DNA Kit (Qiagen) and the bacterial load contained was quantified by real-time PCR amplification of the 16S (small ribosomal subunit gene) as in [167, 168]. A standard curve relating the number of bacteria present to the amount of DNA was created by extracting and quantifying DNA from suspensions of *Escherichia coli* of known colony forming units (cfu)/mL. The bacterial load of 4 day old mice was determined by counting the number of colony forming units evident on agar plates (LB Agar, Lennox L Agar, Invitrogen) incubated with serial dilution of the entire

intestinal homogenate (tissue in 5 mL of cold sterile PBS) at 37°C for 24 hours.

Phenotypic Data Analysis

Tests for differences in mouse weight, gene expression and ileal morphology between groups were done by Student's *t*-test or by ANOVA in the case of multiple groups. Survival curves were generated by using the Kaplan-Meier method and were compared by the log-rank test.

Results

Survival of Cftr deficient mice is dependent on Tlr4 genotyp

To determine the effect of *Tlr4* genotype on cystic fibrosis intestinal phenotype a population of mice was bred from a cross of *Cftr* +/- and *Tlr4* +/- or *Tlr4* -/- progenitors. Of a total of 302 mice produced 77 were homozygous for the *Cftr* mutated allele. The survival of these CF mice was dependent on *Tlr4* genotype, as shown in Figure 1, where double mutant mice (*Cftr* -/- *Tlr4* -/-) showed lower survival compared to that of *Tlr4* +/+ or *Tlr4* +/- CF mice; and only a single double mutant mouse survived to 12 weeks of age. The majority of the *Cftr*/*Tlr4* double mutant mice succumbed at approximately 4 days of age (Figure 1). In addition, *Tlr4* +/- CF mice showed greater survival to 12 weeks of age than that of either *Tlr4* -/- or *Tlr4* +/+ CF mice. Of the non-CF littermate control mice, including 72 *Tlr4* -/- mice, a small percentage succumbed at an early age and the survival of these mice was independent of *Tlr4* genotype (p=0.10; data not shown).

Seventeen CF mice (16 females) lived to the age of 12 weeks and at this age the CF female mice were smaller than the non-CF mice (CF, 17.9 g ± 0.78 SE; non-CF, 22.1 g ± 0.28; p=0.0002), in agreement with previous data of this strain [68]

but no difference in body weight by *Tlr4* genotype in CF (p=0.4) or non-CF mice (p=0.1) was evident (data not shown).

Ileal expression of Tlr4 is increased in BALB Cftr deficient mice

To determine whether the ileal expression of *Tlr4* is altered in 12 week old BALB CF mice, as it is in (BALB x B6) F2 CF mice [94], real-time PCR analysis was performed. As depicted in Figure 2*A* CF mice had higher expression levels of *Tlr4* compared with levels in non-CF mice and the expression of *Tlr4* was not altered by the *Tlr4* mutation in CF (p=0.44) or non-CF (p=0.11) mice, as has been shown in C3H/HeJ mice [101]. The expression level of *Tlr4* did not differ between *Cftr* +/+ and *Cftr* +/- mice (p=0.15, data not shown) thus the data of these groups were combined in Figure 2. Secondly, the expression level of *Cftr* was greater in non-CF mice than in CF mice and this difference was independent of *Tlr4* genotype (Figure 2*B*).

We used immunohistochemistry to confirm the increased expression of *Tlr4* in the CF intestine and to identify the cells expressing this protein. As shown in Figure 2*C*, Tlr4 staining was evident in mononuclear cells in lamina propria and submucosa with faint staining in enterocytes and epithelial crypt cells. Quantification of this staining revealed the number of Tlr4 positive mononuclear cells to be increased in the CF mice, compared to non-CF littermates as shown in Figure 2*D*. This difference in number of Tlr4 positive cells was evident for both *Tlr4* +/+ and *Tlr4* +/- mice.

Intestinal phenotypes unaltered by Tlr4 haploinsufficiency in 12 week old CF mice

We [94] and others [56, 58, 157, 169] have shown the intestinal phenotype of *Cftr* deficient mice to include an extended crypt-villus axis and increased proliferation compared to non-CF mice. To determine whether this phenotype was evident in

BALB CF mice and was influenced by *Tlr4* haploinsufficiency histological measures were completed. Comparison of the intestinal phenotypes showed CVA length and proliferative cell number to differ between BALB CF and non-CF mice, but not to be affected by *Tlr4* haploinsufficiency either in CF or non-CF mice, as shown in Figure 3 *A-D*. CVA length and cell proliferation counts did not differ between *Cftr* +/+ and *Cftr* +/- mice (p=0.17 and p=0.34 respectively; data not shown) thus, the data of these groups were combined in Figure 3.

Next, bacterial loads in the small intestines were recorded to determine whether *Tlr4* genotype influenced this phenotype. As shown in Figure 3*E*, and in agreement with Norkina's report of C57BL/6J CF mice [86], more bacteria colonized the small intestine of 12 week old CF mice compared to that of non-CF littermates, but no difference in bacterial counts was found in mice grouped by *Tlr4* genotype.

Finally, Shang *et al* [170] reported transgenic mice created with the *Tlr4* gene constitutively active in the intestinal epithelium to have increased B cell numbers in the proximal region of this tissue compared to control mice. As *Tlr4* was of higher expression in CF mice we investigated the presence of intestinal B cells in our 12 week old CF and non-CF mice. Numbers of CD20 positive cells did not differ in mice grouped by CF status and this result did not depend on *Tlr4* genotype (data not shown).

Phenotypic assessment of Cftr/Tlr4 neonates

As *Cftr*/*Tlr4* double mutant mice had low survival to the age of 12 weeks we assessed the influence of *Tlr4* genotype on the intestinal phenotype of CF mice at the age of 4 days. A population of mice was bred from a cross of *Cftr* +/- and *Tlr4* +/- or *Tlr4* -/- progenitors and of the 166 mice produced 42 were homozygous for the *Cftr* mutated allele which is consistent with expected Mendelian ratios. CF

mice, independent of *Tlr4* genotype, had lower body weight compared to non-CF mice (CF, 2.1 ± 0.08 SEM; non-CF, 3.5 ± 0.05, $p < 0.0001$).

To determine whether phenotypic alterations are present in the intestines of *Cftr*/*Tlr4* double mutant neonates which might account for their reduced survival to 12 weeks of age we measured the CVA length, bacterial content, goblet cell count, intestinal mucus obstruction and inflammatory infiltration of this tissue. Histological assessment of the intestine revealed the distended phenotype of CF mice to be evident at this age, as shown in Figure 4*A*, but that the greater crypt depth in CF mice was not dependent on *Tlr4* genotype (p=0.81) (Figure 4*B*). Similarly, the intestinal bacterial load in the neonates revealed higher counts for the CF mice, compared to non-CF littermates, and this was independent of *Tlr4* genotype in CF (p=0.45) and non-CF (p=0.58) mice as shown in Figure 4*C*.

Inflammatory cell phenotyping revealed the number of CD3+ cells to be greatest in the small intestine of *Cftr*/*Tlr4* double mutants, but no statistically significant differences in lymphocyte count by *Cftr* genotype (p=0.34 by *t* test) or *Tlr4* genotype within CF mice (p=0.22 by ANOVA) were evident (Figure 5*A*). As shown in Figure 5*B*, and in agreement with previous reports on adult CF mice [93, 171], the number of mast cells was increased in the small intestine of CF compared to non-CF mice (p=0.038 by *t* test); and within the CF mice the double knockouts had the fewest mast cells (p=0.068 by ANOVA), p=0.033 compared to *Tlr4*+/+ CF. An increase of mucosal F4/80+ cells was evident in the non-CF when compared to the CF mice (p=0.018 by *t* test) but no difference in this phenotype by *Tlr4* genotype was observed within the CF mice (p=0.64 by ANOVA) (Figure 5*C*). The findings in submucosal macrophages, however, did not reveal a significant difference in cell numbers by either *Cftr* or *Tlr4* genotype (p=0.38 by ANOVA) (Figure 5*D*).

Goblet cell hyperplasia is a feature of CF intestinal disease in mice [56] and our assessment of the neonates showed this trait to be evident in the proximal intestine of CF mice (p=0.005), independent of *Tlr4* genotype (ANOVA p=0.50); whereas in the distal half, which includes the terminal ileum, the number of goblet cells did not differ by CF status (p=0.36; Figure 6*A*). Most CF neonates also had histological evidence of dilated mucus filled crypts and the accumulation of mucus material in intervillous spaces and in the intestinal lumen, while a few presented with a non-obstructed ileum (Figure 6*B*). By *Tlr4* genotype the incidence of intestinal obstruction was 8 of 9 double mutants; 5/5 *Tlr4* +/+ CF mice and 3/6 *Tlr4* +/- CF mice. To assess whether the obstructed area was dependent on *Tlr4* genotype, we measured the total obstructed area in the ileum of PAS-stained sections of these mice. As expected, CF mice had a greater obstructed area compared to the level in non-CF, and we observed a non significant decrease in the affected area of CF *Tlr4* +/- compared to CF *Tlr4* homozygous mice (Figure 6*C*).

Discussion

In this work we show the CF intestinal disease traits of extended crypt villus axis, goblet cell hyperplasia and greater bacterial load to be present in the BALB *Cftr*^{tm1UNC} model of cystic fibrosis and that these changes are evident in 4 day old CF neonates. By creating and analysing a population of mice carrying *Cftr* and/or *Tlr4* mutations we demonstrate the survival of CF mice to be altered by mutations in *Tlr4* where, specifically, the incidence of lethality was increased in *Tlr4* homozygous mutant CF mice while survival was enhanced in *Tlr4* heterozygous CF mice. Finally, our studies revealed the level of *Tlr4* expression to be increased in the intestinal tissue of adult BALB CF mice but that haploinsufficiency of *Tlr4* did not alter the CVA length, crypt proliferation or bacterial content in these adult CF mice.

In this population the survival of CF mice with the *Tlr4* +/+ genotype agrees with that reported for the original *Cftr*^{tm1UNC} mouse model [56] by both the early wave of perinatal lethality and in that approximately 46% of mice survived to weaning. In contrast, the CF mice with homozygosity for the *Tlr4* mutation succumbed to early lethality in this study, while the greater survival to 12 weeks of age of *Tlr4* +/- CF mice was associated with perinatal deaths in only 25% of the population. The mechanisms contributing to this enhanced (for *Tlr4* +/- CF mice) or reduced (for *Tlr4* -/- CF mice) survival were most likely Tlr4 dependent development of mucus obstructions, and a reduced inflammatory response in double mutant mice, respectively, as extensive analysis of the intestinal disease of 4 day old CF and *Cftr/Tlr4* mutant mice revealed no differences in CVA length or in intestinal bacterial content between double mutant and CF mice. In particular, for the histologically evaluated neonates only 4 CF mice, 3 of which were of the *Tlr4* +/- genotype, did not have evidence of complete mucus plugging of the intestinal lumen. Given that manipulations which reduce the mucus in the CF intestine increase the survival of these mice [79, 81] and that the administration of LPS, a Tlr4 ligand, can reduce mucus levels in the lung [172], we speculate that the

improved survival of *Tlr4* +/- CF mice is due to a reduction in intestinal mucus. Further analysis of the intestinal disease of 4 day old CF mice revealed a decrease in mast cell infiltration in *Tlr4* -/-, compared to the level in *Tlr4* +/+, CF mice. The reduced mast cell infiltration in the double mutant mice, which may be due to the deficiency in Tlr4 signaling [173, 174] coupled with the reduced inflammatory response of Tlr4-deficient mast cells [175] could result in an inadequate immune response to the gram-negative bacterial burden in the intestines of CF mice [86], such as occurs in sepsis [175] and thus the reduced survival of the *Cftr/Tlr4* double mutant mice.

Established functions for Tlr4 include initiating an innate immune response to bacterial challenge, which ultimately decreases tissue bacterial load [176], and in the intestine, signaling to maintain epithelial cell homeostasis [116] but in this CF model neither the epithelial cell proliferation nor amount of luminal bacteria were altered by mutant *Tlr4*. That intestinal bacterial load was not dependent on functional Tlr4 has been reported for related animal models of ileitis and colitis [121, 177] and distinguishes Tlr4 function in this tissue from that in a systemic response where bacterial levels are related to *Tlr4* genotype [176, 178, 179]. Secondly, the influence of Tlr4 on intestinal cell homeostasis and disease phenotype in challenge models is not obvious as the deficiency has been shown to increase [116-118] or to lessen [121, 177, 180] disease. Regarding the latter, Leaphart *et al.* [180] reported C3H/HeJ mice to be spared necrotizing enterocolitis due to reduced apoptosis and increased proliferation compared to the levels in wildtype mice. Despite carrying the same C3H/HeJ mutation in *Tlr4*, the crypts of our *Cftr/Tlr4* mutant mice did not display altered levels of apoptosis or proliferation, relative to CF *Tlr4* +/+ animals. The absence of a Tlr4 effect on proliferation/apoptosis in the CF intestine may have occurred as the level of injury in CF is not of the order of that in the induced colitis models or may indicate this *Tlr4*-regulated tissue response does not contribute to CF intestinal disease.

In this work we confirmed our previous finding of higher levels of *Tlr4* expression in the ileal tissue of CF mice [94] and extended this work to show this increase to be due in part to greater numbers of Tlr4 positive cells in the lamina propria in CF mice relative to control. The localization of Tlr4 positive cells to the intestinal lamina propria in mice has been previously reported [181, 182] as has increased Tlr4 expression levels in the intestinal diseases of necrotizing enterocolitis [180] and inflammatory bowel disease [112] indicating a similarity in tissue response across syndromes. Despite the increased expression of intestinal *Tlr4* in CF mice, we did not detect an increase in intestinal B lymphocytes as was reported by Shang *et al* [170] for mice with the *Tlr4* gene constitutively active in the intestine, although in their work this increase was evident in the proximal small intestine and here we evaluated the more distal intestine. Nevertheless we report B cell numbers in the intestine to be unaltered in CF mice.

In summary, the results of this study show the increased expression and presence of Tlr4 in the CF mouse small intestine, and, significantly, that functional interruption of this protein (through mutation) severely reduced the survival of CF mice. Despite evidence of *Tlr4* mutations altering tissue bacterial levels and intestinal cell homeostasis, neither of these traits was identified as *Tlr4*-dependent in the CF intestine, rather the reduced survival of Cftr/Tlr4 deficient mice was associated with decreased mast cell numbers. Finally, we also showed the CF newborn mouse to acquire an almost immediate small intestinal bacterial overgrowth, crypt elongation and mucus accumulation compared to non-CF neonates, resembling the phenotypes found in CF adult mice.

Grants

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Figure legends

Figure 1. **Survival of cystic fibrosis knockout mice is dependent on *Tlr4* genotype.**

A population of Cftr deficient mice was bred from BALB/c *Cftr* +/^{tm1Unc} and C.C3-*Tlr4*$^{Lps\text{-}d}$/J progenitors and their survival to the age of 12 weeks recorded. * Indicates a significant difference in survival between groups, $p < 0.05$.

Figure 2. Expression of *Tlr4* and *Cftr* in ileal tissue of 12 week old mice derived from BALB/c *Cftr* +/^{tm1Unc} and C.C3-*Tlr4*$^{Lps\text{-}d}$/J progenitors.

A 1 cm section of the distal ileum was collected at autopsy and the expression levels of *Tlr4* (*A*) and of *Cftr* (*B*), relative to the ataxia 10 control gene, were measured by real time PCR analysis (n = 3-10 mice/group). (*C*) Representative immunostaining for Tlr4. Note the localization of positive mononuclear cells in the lamina propria of CF mice (arrows). Original magnification of 200X; inset image is a higher magnification of area in box. (*D*) Average ± SEM number of Tlr4 positive cells in the lamina propria and submucosa of 12 week old mice (n = 4-5 mice/group). * Indicates a significant difference between groups, $p < 0.05$.

Figure 3. Intestinal histopathology and bacterial load were not affected by *Tlr4* haploinsufficiency in 12 week old CF mice.

(*A*) Representative sections of ileal tissue, stained with hematoxylin and eosin. In the control mice the crypt lumen is small and goblet cells appear compacted. In CF mice the ileum features greater CVA length, crypt elongation and hyperplasia of goblet cells. (*B*) Crypt-villus axis length measurements, made by image analysis of histological sections, in CF and non-CF mice by *Tlr4* genotype. (*C*) Representative immunostaining for the proliferation marker PCNA. (*D*) Proliferating cell counts for the crypts of CF and non-CF by *Tlr4* genotype. Data are presented as average of group ± SEM (n = 3-14 mice/group). (*E*) Bacterial counts of the small intestine from 12 week old BALB mice. Bacterial load was measured by absolute quantification of 16S DNA in luminal contents of the small

intestine. Each dot represents one mouse and the dotted line represents the average of the group. * Indicates a significant difference between CF and non-CF mice, $p < 0.05$. Original magnification of 200X.

Figure 4. Increased crypt-villus axis length and bacterial load of the small intestine of 4 day old CF compared to non-CF mice, independent of *Tlr4* genotype.

Mice were euthanized at 4 days of age and crypt-villus axis length measurements made by image analysis of histological sections. (*A*) Representative sections of the terminal ileum of a CF and non-CF mice, H&E staining. (*B*)The increased CVA length in CF mice is largely due to the presence of elongated and fully developed crypts in these mice (n = 3-6 mice/group). (*C*) Bacterial load was measured by plating the tissue homogenate of the entire intestine and counting the number of colony forming units at 24 hrs post plating. Each dot represents one mouse and the dotted line represents the average of the group. * indicates significant difference between CF and non-CF mice, $p < 0.05$.

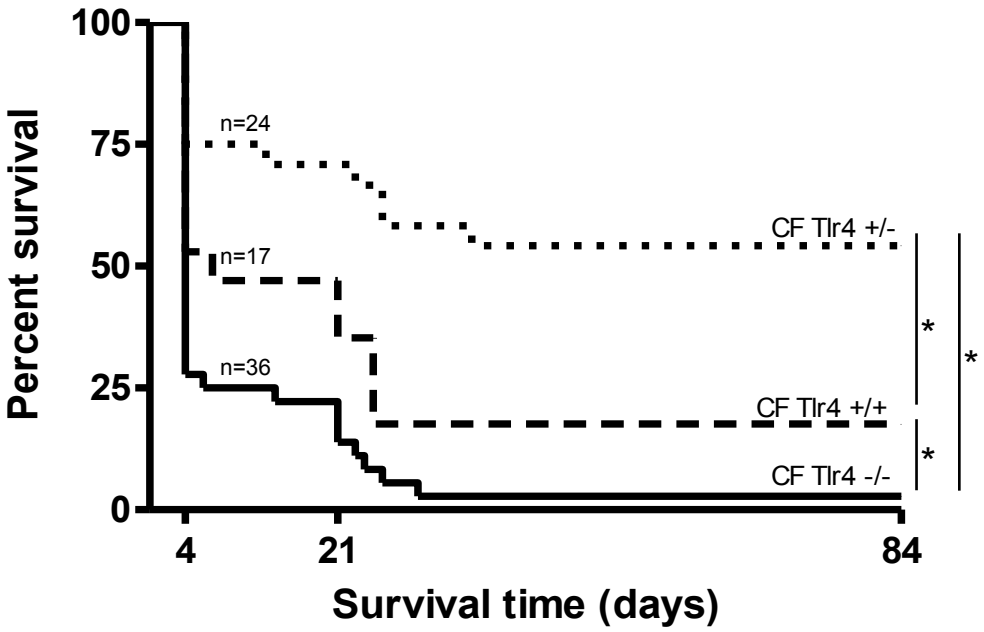
Figure 5. Inflammatory cell numbers in the intestine of 4 day old CF, and non-CF neonates, by *Tlr4* genotype.

Mice were euthanized at 4 days of age, intestinal sections immunostained or stained with the indicated markers and positive cells per high power field counted. (*A*) CD3+ (*B*) mast cells by toluidine blue staining (*C*) Percentage of F4/80 positive stained area in the mucosa and (*D*) F4/80+ cells in submucosa. Data are presented as average of group ± SEM; n = 3-5 mice/group. * Indicates a significant difference between groups, $p < 0.05$.

Figure 6. Increased mucus accumulation and obstruction in distal ileum of 4 day old CF compared to non-CF neonates.

(*A*) Number of goblet cells per CVA in the proximal and distal sections of the small intestine of CF and non-CF mice. (*B*) Representative sections of the terminal ileum of: an unobstructed non-CF mouse; a *Cftr/Tlr4* +/+ obstructed mouse; an unobstructed CF *Tlr4* +/- neonate and a *Cftr/Tlr4* double mutant mouse presenting increased mucus accumulation and obstruction of the lumen PAS staining; Original magnification of 200X. (C) Obstructed area measured by image analysis of histological samples. Data are presented as average of group ± SEM. N = 4-5 mice/group. * Indicates a significant difference between CF and non-CF mice, $p < 0.05$.

Figure 1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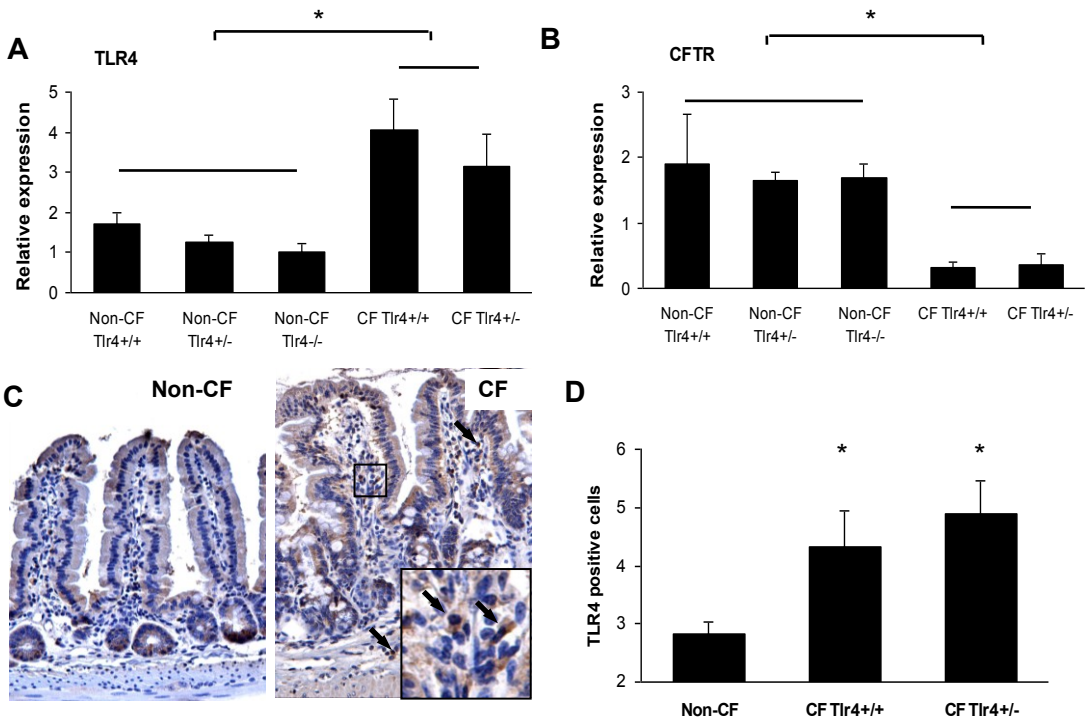
Survival time (days)

Figure 2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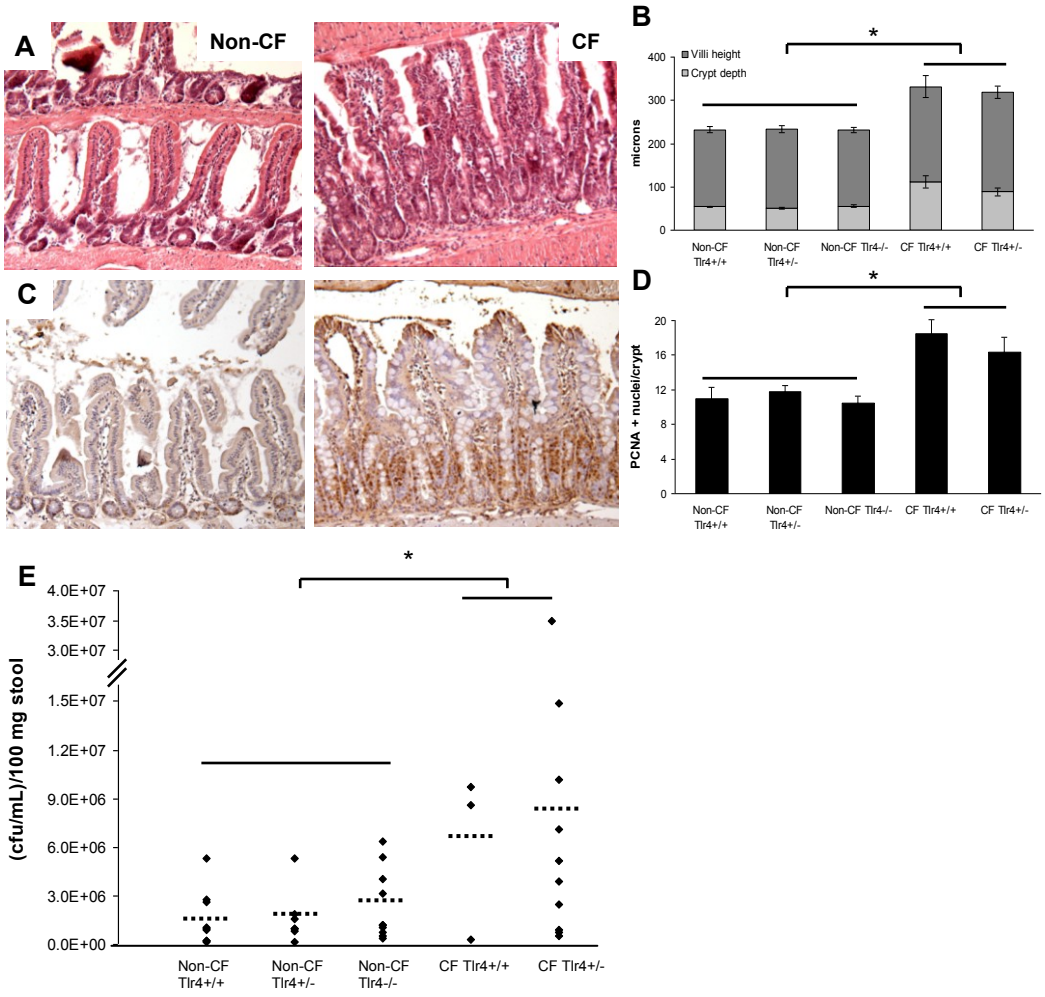
CF Tlr4+/-

Figure 3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Non-CF
CF
B
Villi height
Crypt depth
microns
400
300
200
100
0
*
Non-CF Tlr4+/+
Non-CF Tlr4+/-
Non-CF Tlr4-/-
CF Tlr4+/+
CF Tlr4+/-
C
D
PCNA + nuclei/crypt
20
16
12
8
4
0
*
E
(cfu/mL)/100 mg stool
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3.5E+07
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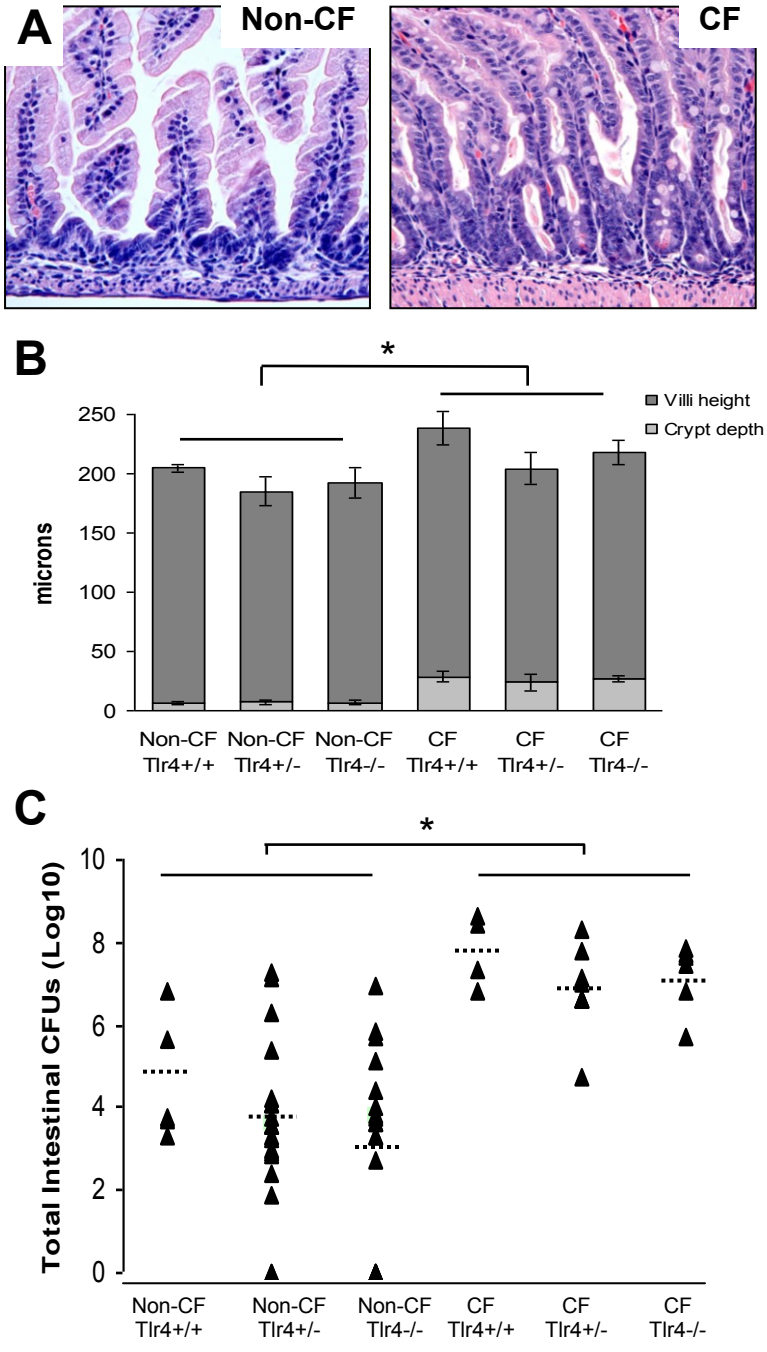
Figure 4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B
*
Villi height
Crypt depth
microns
250
200
150
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Non-CF Tlr4-/-
CF Tlr4+/+
CF Tlr4+/-
CF Tlr4-/-
C
*
Total Intestinal CFUs (Log10)
10
8
6
4
2
0
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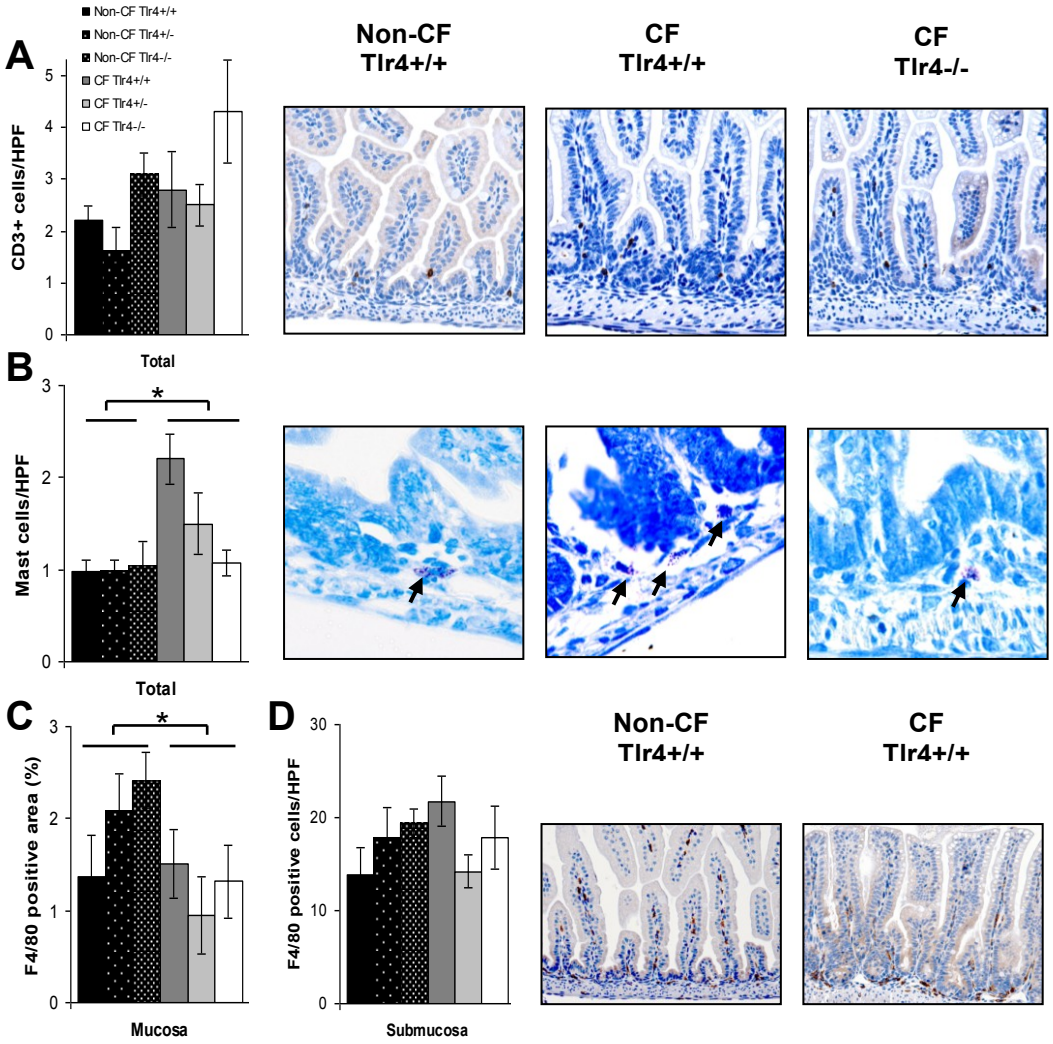
Non-CF Tlr4+/-
Non-CF Tlr4-/-
CF Tlr4+/+
CF Tlr4+/-
CF Tlr4-/-

Figure 5


A
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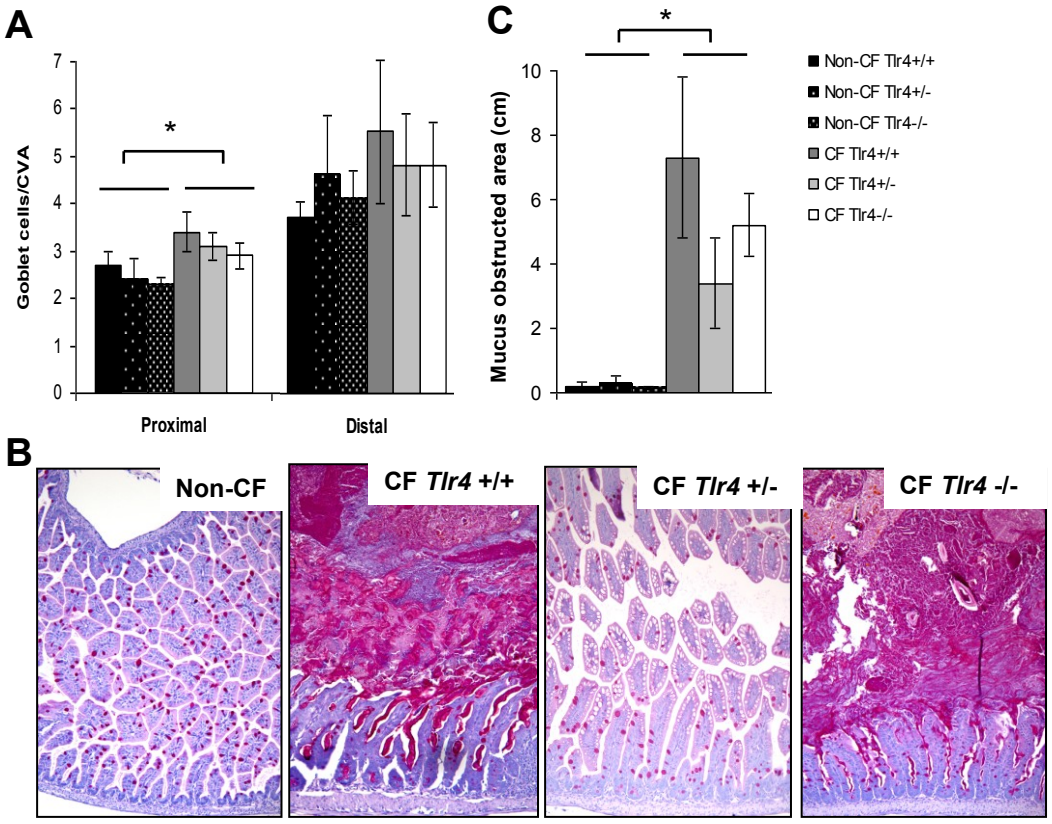
Non-CF Tlr4+/-
Non-CF Tlr4-/-
CF Tlr4+/+
CF Tlr4+/-
CF Tlr4-/-
CD3+ cells/HPF
Total
Non-CF Tlr4+/+
CF Tlr4+/+
CF Tlr4-/-
B
Mast cells/HPF
Total
C
F4/80 positive area (%)
Mucosa
D
F4/80 positive cells/HPF
Submucosa
Non-CF Tlr4+/+
CF Tlr4+/+

Figure 6


A
Goblet cells/CVA
0
1
2
3
4
5
6
7
*
Proximal
Distal
C
Mucus obstructed area (cm)
0
2
4
6
8
10
*
Non-CF Tlr4+/+
Non-CF Tlr4+/-
Non-CF Tlr4-/-
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CF Tlr4+/-
CF Tlr4-/-
B
Non-CF
CF Tlr4 +/+
CF Tlr4 +/-
CF Tlr4 -/-

CHAPTER TRANSITION

In chapter 3 we described Tlr4 as a plausible candidate affecting survival of CF mice. Due to the low survival of this model, evaluation of an effect of Tlr4 on body weight of adult mice was not possible. Insulin-like growth factor (IGF) system was another intriguing candidate pathway with altered expression in the CF intestine observed from data in chapter 2.

As an extensive area of study has revealed that the adaptive intestinal response, in various altered states, is strongly dependent on the local expression of the IGF system we decided to evaluate, in chapter 4, its implication in the CF intestinal response and body weight gain.

CHAPTER 4

Insulin-like growth factor binding protein-3 treatment alters intestinal cell proliferation and body weight of Cftr deficient mice

JC Canale-Zambrano and CK Haston

Modified version of manuscript under review in *Pediatric Research*

Abstract

The intestinal phenotype of cystic fibrosis (CF) transmembrane conductance regulator deficient mice includes altered cell homeostasis and a distended crypt-villus axis, which, in previous work, was associated to body weight. To investigate whether a causal relationship exists between these features we treated CF mice with insulin-like growth factor binding protein-3 (Igfbp-3), a protein which, as it has potent effects on cell proliferation and apoptosis, we hypothesized would alter the intestinal cell homeostasis, and as a consequence, body weight. Six week old C57BL/6J x BALB F2 CF mice and wild type littermates received systemic treatment of recombinant human IGFBP-3 (20 mg/kg), or vehicle control, daily for 28 days and weight gain, serum protein levels, and intestinal histology were assessed. Intestinal expression of insulin-like growth factor (IGF) pathway genes was altered in untreated CF mice compared to wildtype with increased *Igf-I* and decreased *Igfbp-3* levels detected. Administration of rhIGFBP-3 to CF mice significantly increased the number of Igfbp-3 positive cells in the intestine and partially reversed the hyperproliferative phenotype of intestinal crypts and muscularis externa. Apoptosis levels did not differ between rhIGFBP-3 treated CF mice and mice receiving vehicle control. Serum Igfbp-3 levels were increased and Igf-I, albumin and triglycerides measures were decreased in CF compared to wild type mice. After rhIGFBP-3 treatment, despite a significant increase of serum albumin and triglycerides, body weight of CF female mice was reduced throughout the duration of the treatment, but only significant at days 14 and 21. In this study, we have identified rhIGFBP-3 treatment to decrease intestinal crypt cell proliferation, crypt depth and muscularis externa thickness in the CF mice, resulting in reduced body weight in female mice.

Introduction

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, and is a common, lethal autosomal recessive disease principally affecting Caucasians.[4] One of the pathologies of CF is intestinal disease, which is presumed to be due to reduced water secretion in this organ and which results in the development of meconium ileus in 15-20% of CF newborns[40] and distal intestinal obstruction syndrome episodes in 25% of CF adults.[4] Cystic fibrosis patients also have a lower body mass index than those without CF and this is influenced by lung, intestinal and pancreatic disease and nutritional status.[7, 21]

Mouse models wherein *Cftr* has been knocked out have been evaluated to investigate the mechanisms through which mutations in *Cftr* produce intestinal disease, and may contribute to the growth defect. Specifically, Snouwaert *et al*. showed *Cftr*^{tm1Unc} mice to be smaller than their littermates and to develop an intestinal phenotype resembling meconium ileus in that the mice had mucous build up associated with lethal intestinal plugs.[56] Kent *et al.*, using a liquid diet which reduced the incidence of the lethal intestinal complication, reported the intestinal crypts of older CF mice to be distended and the mucosal thickness of the intestine to be increased relative to non-CF littermates.[58] In addition, we previously assessed the relationship of the CF growth defect to intestinal disease in a population of genetically mixed CF mice and showed crypt elongation, due to an expanded proliferative zone and decreased apoptosis, to be dependent on body weight.[94]

The histological changes evident in the intestines of CF mice are consistent with the mitogenic effects of increased levels of insulin-like growth factor-I (Igf-I) have on this tissue, suggesting the IGF pathway may be perturbed in CF mice. In detail, increases in Igf-I achieved through recombinant protein

administration[183] or transgenesis[144-146] have been shown to enhance crypt cell mitosis, crypt depth, villus height and intestinal mucosal and muscularis mass and to decrease the apoptotic index in animal models while strong over expression of the Igf-I receptor, *Igf-Ir*, has been demonstrated to occur in colorectal carcinoma.[184] Igf-I is regulated by a family of proteins, known as the Igf-binding proteins (Igfbp), which modulate Igf-I action either by presenting Igf-I to its receptor or by sequestering the peptide and reducing receptor interaction.[130] Igfbp-3 accounts for 80% of IGF binding in the circulation,[185] and may modulate Igf-I action in the intestine.[143] Independent of the Igf-I interaction, increased levels of Igfbp-3 have been shown to inhibit the proliferation of intestinal smooth muscle cells[131, 132] and to induce apoptosis in colonic adenoma-derived cells.[186]

We hypothesized that the anti-proliferative and pro-apoptotic actions of Igfbp-3 would attenuate the hyperproliferative phenotype which characterises the intestines of CF mice, and as a consequence, alter their body weight. To investigate this hypothesis we treated CF and non-CF mice with recombinant human IGFBP-3 and measured its effects both systemically, and on the intestinal phenotype.

Materials and Methods

Mice

C57BL/6J (B6) x BALBc/J F2 *Cftr*^{tm1unc} (F2 CF) & wildtype (*Cftr*$^{+/+}$) mice were derived from a cross of B6 *Cftr*$^{+/-}$ to BALB *Cftr*$^{+/-}$ mice through two generations of breeding. Their *Cftr* genotype was identified by PCR as in our previous study.[94] To prevent fatalities due to intestinal obstructions common in CF mice,[56, 187] all mice were maintained on liquid diet (Peptamen) from the age of 21 days until sacrifice at the age of 10 weeks. At the same time on each experimental endpoint day the body weight of each mouse was measured, the

mice were anesthetized by intraperitoneal delivery of sodium pentobarbital and euthanized by exsanguination. Mice were handled according to the standard husbandry of the animal facility at the Meakins-Christie Laboratories of McGill University and cared for under a protocol approved by the McGill University Animal Care Committee.

IGFBP3

Recombinant hIGFBP-3 was a generous gift from Insmed Incorporated (Richmond, VA). Six week old mice received rhIGFBP-3 (20 mg/kg, diluted in acetate buffer) through intraperitoneal injections once a day for 28 consecutive days. Similar doses of this protein have been shown to influence colorectal tumour growth in vivo.[133] The control group was injected with acetate buffer once daily for 28 consecutive days.

Quantitative Real-Time PCR

Total RNA was extracted from 1 cm of the terminal ileum according to the manufacturer's (Sigma) instructions. To generate the cDNA for real-time PCR, 3 μg of total RNA from each tissue sample was reverse transcribed with oligo(dT) Primer using Superscript™ II RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), in a 20 μL total volume.

We performed Taqman relative quantification on the following IGF axis genes: *Igf-I* (Insulin-like growth factor-I), *Igf-1r* (Insulin-like growth factor-I receptor), *Igfals* (Insulin-like growth factor binding protein, acid labile subunit), *Igfbp-1, 2, 3, 4* and *5* (Insulin-like growth factor binding proteins 1-5). Taqman probes were obtained from Applied Biosystems (Foster City, CA) as Assays-on-Demand™. Relative quantification was performed with the Applied Biosystems' 7500 Real Time PCR System™. Each 25 μL reaction on a 96 well plate contained 0.1 μg of cDNA template, 12.5 μL of TaqMan Universal PCR Master Mix (at a

2X concentration) and 1.25 μL of Assays-on-Demand™ Gene Expression Assay Mix, which contained forward and reverse primers and labelled probe. The manufacturer's default thermal cycling conditions for PCR were used. Relative gene expression data analysis was carried out with the standard curve method[188] using the expression of the reference gene *Atxn10* (Ataxin 10). Relative quantification values were obtained by using the Applied Biosystems' software and a calibrator sample was employed in each run to correct for run to run variation.

Serum phenotype

Blood from the cardiac puncture was stored in serum separator tubes and centrifuged. Serum Igf-I and Igfbp-3 levels were determined by enzyme-linked immunosorbent assays using commercially available kits from R&D Systems (Minneapolis, MN, USA). Serum samples were assessed for glucose, albumin and triglycerides levels using a biochemistry analyzer Hitachi 911.

Histology

At dissection, the entire small intestine was removed, flushed with PBS, and a 10-15 cm portion of the terminal small intestine was fixed in 10% buffered formalin and submitted for standard histological processing. Swiss roll paraffin embedded sections (5 μm) were stained with haematoxylin and eosin and the crypt-villus axis (CVA) length was measured. The entire depth of the crypt and the length of the villus were measured from an average of 40 complete and intact CVAs using image analysis of the histological sections (Olympus BX51, Image-Pro Plus 5.1, Media Cybernetics). For muscle thickness, the muscularis externa layer was measured at 50 regular intervals throughout the small intestine. All sections were scored by an observer blinded to mouse *Cftr* genotype and treatment.

Immunohistochemistry

For antibody staining the paraffin tissue sections were initially deparaffinized and hydrated through graded ethanol. Antigen sites were unmasked by an antigen retrieval treatment. Antibodies against proliferating cell nuclear antigen (PCNA, sc-9857, dilution 1:100), Igf-Irβ (sc-713, dilution 1:600), Igfbp-3 (sc-9028, dilution 1:500) and p-Igf-Ir (sc-101703, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, California) and Caspase-3 (1:75, Cell Signalling Technology) were used as in [94].

Endogenous peroxidase activity was quenched using 3% (v/v) hydrogen peroxide in TBS for 15 minutes. Non-specific binding sites were blocked with 10% normal serum (Sigma) in TBS for 45 minutes. The sections were then incubated overnight at 4°C with each of the antibodies. After washing, sections were incubated with 1:100 biotinylated secondary antibody for 45 minutes at room temperature. Sections were then washed and incubated in a Streptavidin-Horseradish Peroxidase solution (BD Biosciences) for 45 minutes. Sections were developed with 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB substrate chromogen system, DakoCytomation Inc.) counterstained and mounted with Cytoseal. For negative control preparations, the primary antibody was replaced by TBS. Blinded scoring of an average of 20 crypt-villus axes (CVA) per mouse was performed and presented as the average number of positively stained cells per CVA or percentage of positive stained cells of total crypt cells for PCNA staining.

Phenotypic Data Analysis

Tests for differences in serum protein levels, gene expression and histological measures between groups of mice defined by genotype, treatment or sex were completed with Student's *t* test, where a *p* value of ≤ 0.05 was considered significant. For body weight change evaluation, mice were followed for 28 days post start of rhIGFBP-3 treatment. Mice were weighed every week for

the determination of percent weight change which was calculated as: % weight change = (weight at day X – day 0/weight at day 0) X 100.

Results

Expression of IGF pathway genes is altered in intestines of CF mice

To investigate whether genes of the IGF family are altered in expression in the CF intestine we assayed the level of *Igf-I* and related genes in this tissue. As shown in Figure 1, the intestinal expression of *Igf-I* and its binding proteins 2 and 5 was increased while the levels of binding proteins 1 and 3 were decreased in the intestines of CF compared to WT mice. The expression of *Igf-Ir*, Igf binding protein 4 and Igf binding protein acid labile subunit did not differ between CF and WT mice.

rhIGFBP-3 treatment increases numbers of Igfbp-3 positive cells in intestinal tissue

The decreased intestinal expression of *Igfbp-3* with increased *Igf-I* suggests the proliferative response of the CF intestine could be due in part to increased bioavailability of Igf-I. To investigate this we treated CF and non-CF mice with rhIGFBP-3, over a period of 28 days, and measured the CF intestinal disease phenotype.

As shown in Figure 2, the recombinant protein treatment effectively increased the number of Igfbp-3 positive cells in the intestines of CF mice compared to those receiving the vehicle control ($p = 0.022$). Numbers of cells positive for the Igf-I receptor were not affected by the protein treatment ($p = 0.66$) but, the number of cells positive for the phosphorylated (i.e., activated) form of the Igf-I receptor was found to be decreased in rhIGFBP-3 treated CF mice ($p = 0.003$).

rhIGFBP-3 treatment decreases crypt depth and muscle thickness in CF mice

To investigate whether altered levels of Igfbp-3 influenced the CF intestinal distension phenotype histological measures were made. As shown in Figure 3, and in agreement with previous reports,[56, 58, 94] the CF intestinal mucosa was distended and muscularis externa layer increased compared to WT mice. This distension was partially reversed by the administration of rhIGFBP-3 which decreased the CVA length significantly ($p = 0.011$), primarily due to the near 25% reduction of the crypt depth ($p = 0.002$), in CF mice (Figure 3A,B). The thickness of the intestinal muscularis externa layer was also reduced by rhIGFBP-3 treatment ($p = 0.004$) (Figure 3A,C).

rhIGFBP-3 treatment decreases crypt cell proliferation but not apoptosis in CF mice

Increased levels of Igfbp-3 have been shown to inhibit proliferation[131] and to promote apoptosis[189] in vitro therefore to investigate the mechanism through which the decrease of CVA length occurred in rhIGFBP-3 treated CF mice, immunohistochemistry with proliferative and apoptotic markers was performed. The decreased CVA length in rhIGFBP-3 treated mice was mainly due to reduced proliferation of crypt cells in rhIGFBP-3 treated versus vehicle treated CF mice ($p = 0.035$) (Figure 4A) while numbers of apoptotic cells were not significantly altered ($p = 0.2$) (Figure 4B).

rhIGFBP-3 treatment-related changes in blood biochemistry

To determine whether rhIGFBP3 treatment affected systemic levels of Igf-I or Igfbp-3 the serum levels of these proteins were measured. As shown in Figure 5A, Igf-I was lower in serum from CF versus non-CF mice ($p = 0.003$) as has been reported for CF patients[148, 150, 190] and CF mice,[74] while Igfbp-3 levels were

significantly increased ($p = 0.002$). Serum levels of Igfbp-3 have not been reported for CF mice while in CF patients these measures have been shown to be lower[150, 191, 192] or unaltered;[193, 194] neither measure was significantly altered by rhIGFBP-3 treatment.

As an assessment of the nourishment level in these mice, which can influence the IGF system,[195] serum albumin was assayed[196] and, as prior studies report lower serum triglycerides in CF mice,[69, 94] we determined whether the treatment affected this phenotype. The serum levels of albumin and triglycerides were found to be lower in CF compared to WT mice ($p = 0.0007$ and $p = 0.02$, respectively) and to increase by administration of rhIGFBP-3 in CF mice ($p = 0.03$ and $p = 0.015$, respectively) (Figure 5B,C). The serum levels of glucose did not differ among mice grouped by *Cftr* genotype or rhIGFBP-3 treatment (Figure 5D). No differences in serum protein levels by sex were identified (data not shown).

rhIGFBP-3 treatment and body weight

As we had previously determined body weight to be inversely correlated with CVA length in B6xBALB F2 CF mice[94] we next assessed whether rhIGFBP-3 treatment altered the weight gain. The CVA length of this population of CF mice presented a direct association with body weight (CF rhIGFBP-3 treated mice $r = 0.68$, $p = 0.01$; CF buffer treated mice $r = 0.50$, $p = 0.08$) (Figure 6). As shown in Figure 7, after 14 and 21 days of rhIGFBP-3 treatment the female CF mice showed less weight gain compared to CF vehicle treated mice, while weight gain in male CF mice was unaltered by treatement. In the wildtypes, however, rhIGFBP-3 treated male mice significantly gained more weight over the 4 weeks than vehicle control treated mice ($p > 0.035$), while in females weight gain was significantly increased in vehicle control treated mice at days 14 and 21.

Discussion

In this work we demonstrate the altered proliferative response which results in crypt-villus axis distension and increased muscularis thickness in the intestines of CF mice to be partially reversed by rhIGFBP-3 treatment, which inhibited Igf-Ir activation in the CF intestinal crypt cells, and for this change to produce decreased weight gain on female CF mice.

The changes in IGF pathway gene expression measured in the CF intestine are consistent with those of models of adaptive growth of the intestine, such as in response to small bowel resection,[141, 197] to refeeding after a fast,[143] or to *Igf-I* overexpression.[146] The intestinal phenotype of CF mice resembles that of rats after major small bowel resection where the terminal ileum undergoes dilatation, muscle wall hypertrophy, and mucosal and goblet cell hyperplasia with increased cell turnover and resultant villus enlargement.[198, 199] *Igfbp-3* mRNA levels in the intestine are decreased after major small bowel resection[141] or following post-fast refeeding where the decrease coincides with a similar growth response in the intestinal mucosa.[143] In addition, Gillingham *et al*.[197] reported increased expression of *Igf-1* and *Igfbp-5*, which were also more highly expressed in the CF intestine, in sections of intestinal growth of a jejunoileal resection model. Increased intestinal expression of *Igfbp-5* has also been reported in response to induced Igf-I levels achieved through Igf-I treatment[135, 200, 201] or transgenesis;[146] the latter effect also producing increased intestinal mucosa and muscularis, as was detected in the CF mice of the current study. These models suggest the hypothesis that a decrease in *Igfbp-3* is necessary to enhance Igf-I bioavailability in order to stimulate the adaptive response. The increased expression level of *Igf-I* and *Igfbp-5* in the CF intestine, reported here, with a decrease in the tissue level of *Igfbp-3* is therefore consistent with the enhanced proliferation reported for the CF intestine[94, 157] and for intestinal challenge models.

Administration of rhIGFBP-3 reduced the crypt-villus axis distension and the thickness of the muscularis layer in the CF intestine, but whether this occurred through Igf-I-dependent or independent actions is not clear. The finding that rhIGFBP-3 treatment reduces the number of proliferative crypt cells in the CF intestine agrees well with data of Edmondson *et al*.[202] who showed keratinocyte proliferation to be decreased in the skin of *Igfbp-3* transgenic mice and Alami *et al.*[133] who reported systemic administration of rhIGFBP-3 to significantly inhibit tumor growth in a mouse model of colon cancer. Related to mechanism, evidence for an Igf-I-dependent effect of Igfbp-3 on crypt cell proliferation and the muscularis layer is the observation that rhIGFBP-3 treatment also decreased the number of intestinal epithelial cells with the phosphorylated Igf-I receptor. As this form of the receptor indicates Igf-I binding,[203] the reduction in the number of cells positive for the phosphorylated Igf-I receptor would similarly indicate reduced Igf-I action in the treated mice which exhibited a lessened proliferative response. It is also possible that Igf-I-independent actions of Igfbp-3, which also inhibit proliferation[204] could have occurred.

We found the increased number of Igfbp-3 positive cells to be localized in the lamina propria of treated CF intestine while the decreased phosphorylated Igf-Ir positive cells were found predominately in neighboring epithelial crypt cells, suggesting a paracrine Igfbp-3 anti-proliferative effect in the CF intestine. Williams *et al.*[146] reported *Igfbp-3* to be expressed in the lamina propria of the small intestine and previous evidence for paracrine effects of Igfbp-3 in human intestinal smooth muscle cells as an anti-proliferative factor have also been reported. [131, 132]

In contrast to effects on the proliferative response of the intestine, rhIGFBP-3 treatment did not alter circulating Igf-I or Igfbp-3 levels, although systemically administered, this observation potentially excludes an endocrine effect of the treatment in the observed phenotypes. That circulating levels of Igf-I were lower while Igfbp-3 was higher in CF mice, which is the opposite of the

endogenous expression of these proteins in the intestine, suggests tissue specific rather than systemic alterations of the IGF system are responsible for the hyperproliferative state in CF intestine. We previously reported alterations in intestinal crypt distention to be indirectly proportional to the body weight of CF mice,[94] however, in CF mice from the present study, this association was found to be directly proportional to body weight. Therefore, the anti-proliferative actions of rhIGFBP-3 treatment on crypt cells (decreased CVA) and muscle thickness, resulting in diminished weight gain in female CF mice, suggest the CF intestine to undergo an adaptive response as a mechanism to maintain body weight.[197, 199]

The low levels of serum albumin and triglycerides in the CF mice of this study indicates a compromised nutrition level and reduced lipid absorption,[69] both of which were ameliorated by rhIGFBP-3 treatment. A positive association between increased IGFBP-3 levels and each of serum triglycerides[205, 206] and albumin levels[207-209] has been reported but a causal mechanism linking these measures, if such exists, is unknown. Igfbp-3 is known to interact with transcription factors retinoid-X-receptor-alpha or peroxisome proliferator activated receptor gamma[185, 204] whose actions lead to increased plasma triglycerides[210, 211] therefore this pathway, although not evaluated in the current work may have augmented the serum triglyceride levels in rhIGFBP-3 treated CF mice.

Finally, this study was completed in mice of a mixed genetic background, B6 x BALB F2, to build on our prior observation of intestinal distention as correlated to body weight,[94] which was made in CF mice of this background. One limitation created by the use of F2 mice is the possibility that the phenotypic changes (decreased CVA, muscle mass and proliferation) are due to differences in genetic background between rhIGFBP-3 and vehicle treated mice. The probability that our two populations (rhIGFBp-3 and vehicle treated mice) are comprised of mice which each have the disparate genotypes at the locus responsible for the observed phenotypes (or any one locus) is, however, extremely low ($\sim 10^{-8}$),

making the phenotypic differences observed due to treatment and not genetic background.

In summary, we report the hyperproliferative intestinal phenotype of cystic fibrosis mice to include the altered expression of insulin-like growth factor pathway genes, and for the recombinant human IGFBP-3 treatment to partially reverse this response in both the crypts and muscularis layer of this tissue, with a resulting diminished weight gain in female CF mice. This suggests a mechanistic response in which the CF intestine alters proliferation, through IGF, in an attempt to adapt and increase its absorptive surface area as it is evident in resection[141] and refeeding[143] models.

Figure legends

Figure 1. **Expression of IGF pathway genes in B6xBALB F2 CF and wildtype (WT) mice.**

RNA was isolated from ileal tissue harvested from 10 week old mice at necropsy. Expression relative to the *Atx10* reference gene is presented as the mean ± SE (n = 11-12 mice/group). * Indicates a significant difference between groups, $p < 0.05$.

Figure 2. **Immunohistochemical evaluation of IGF pathway proteins in ileal tissue of rhIGFBP-3 or vehicle treated B6xBALB F2 CF mice.**

Ileal tissue harvested from 10 week old mice was stained for Igfbp-3, Igf-Ir and phosphorylated Igf-I receptor, p-Igf-Ir. (A) Representative immunostaining of CF ileal tissue. Original magnification of 400X. (B) Number of positively stained cells per CVA, for CF BP3 = CF mice treated with rhIGFBP-3 and CF Buffer = CF mice injected with diluent buffer. Data are presented as the mean ± SE (n = 4-6 mice/group); * indicates a significant difference between groups, $p < 0.05$.

Figure 3. **Intestinal histology of rhIGFBP-3 or vehicle treated CF and WT mice**.

Mice received rhIGFBP-3 or vehicle daily for 4 weeks and intestinal tissue was harvested from 10 week old B6xBALB F2 CF mice following euthanasia. (A) Representative sections of ileal tissue, stained with hematoxylin and eosin. Original magnification of 100X. (B) Crypt-villus axis height and (C) muscle thickness were measured by image analysis of histological sections. CF, WT Buffer = CF and wildtype mice injected with diluent buffer. CF, WT BP3 = CF and wildtype mice treated with rhIGFBP-3. Data are presented as the mean ± SE (n =8-13 mice/group); * indicates a significant difference between groups, $p < 0.05$.

Figure 4. **rhIGFBP-3 treatment decreases crypt cell proliferation but not apoptosis in B6xBALB F2 CF mice**.

Mice received rhIGFBP-3 or vehicle daily for 4 weeks and the intestinal tissue harvested from 10 week old B6xBALB F2 CF mice at euthanasia was stained with (A) PCNA or (C) active caspase 3. (B, D) The number of positive cells per unit crypt villus axis was counted and is presented as the mean ± SE (n = 7-10 mice/group). Groups as defined in Figure 3. * Indicates a significant difference between groups, $p < 0.05$.

Figure 5. **IGF pathway proteins and serum biochemistry of rhIGFBP-3 or vehicle treated B6xBALB F2 CF and WT mice**.

Mice received rhIGFBP-3 or vehicle daily for 4 weeks and blood was drawn from 10 week old mice by cardiac puncture at euthanasia. Serum Igf-I and Igfbp-3 were measured by ELISA, and serum biochemistry on a Hitachi 911 analyzer. Groups as defined in Figure 3. Data are presented as the mean ± SE (n =11-13 mice/group); * indicates a significant difference between groups, $p < 0.05$.

Figure 6. Intestinal feature of CVA height as a function of body weight in CF mice.

Regression analyisis showed positive correlation of CVA height and body weight in (A) buffer treated and (B) rhIGFBP-3 treated CF mice.

Figure 7. **Effect of rhIGFBP-3 or vehicle treatment on weight gain in B6xBALB F2 CF and WT mice.**

Mice were weighed every week for the duration of the treatment and the percent weight change determined as described in Material and Methods. Data are presented as the mean ± SE (n = 6-7 mice/group). * Indicates a significant difference compared to vehicle treated mice, $p < 0.05$.

Figure 1

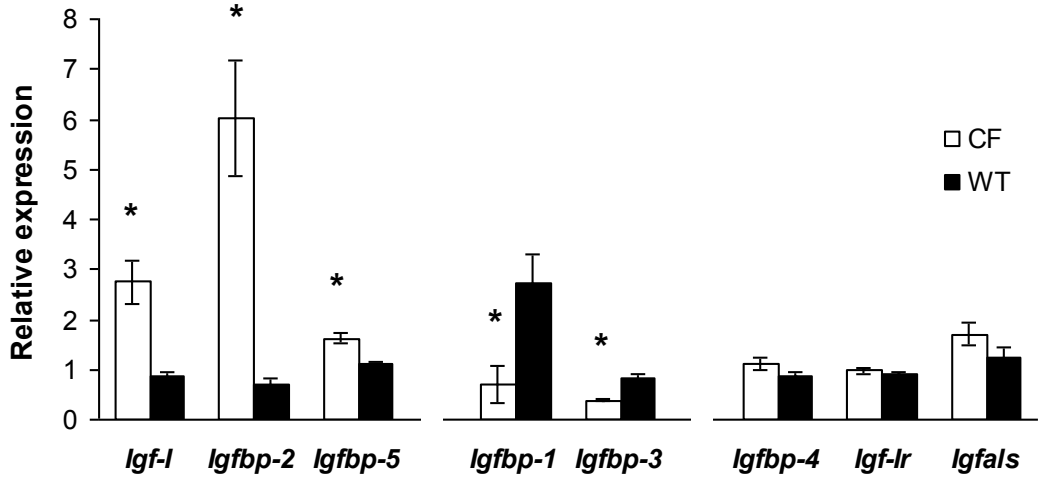
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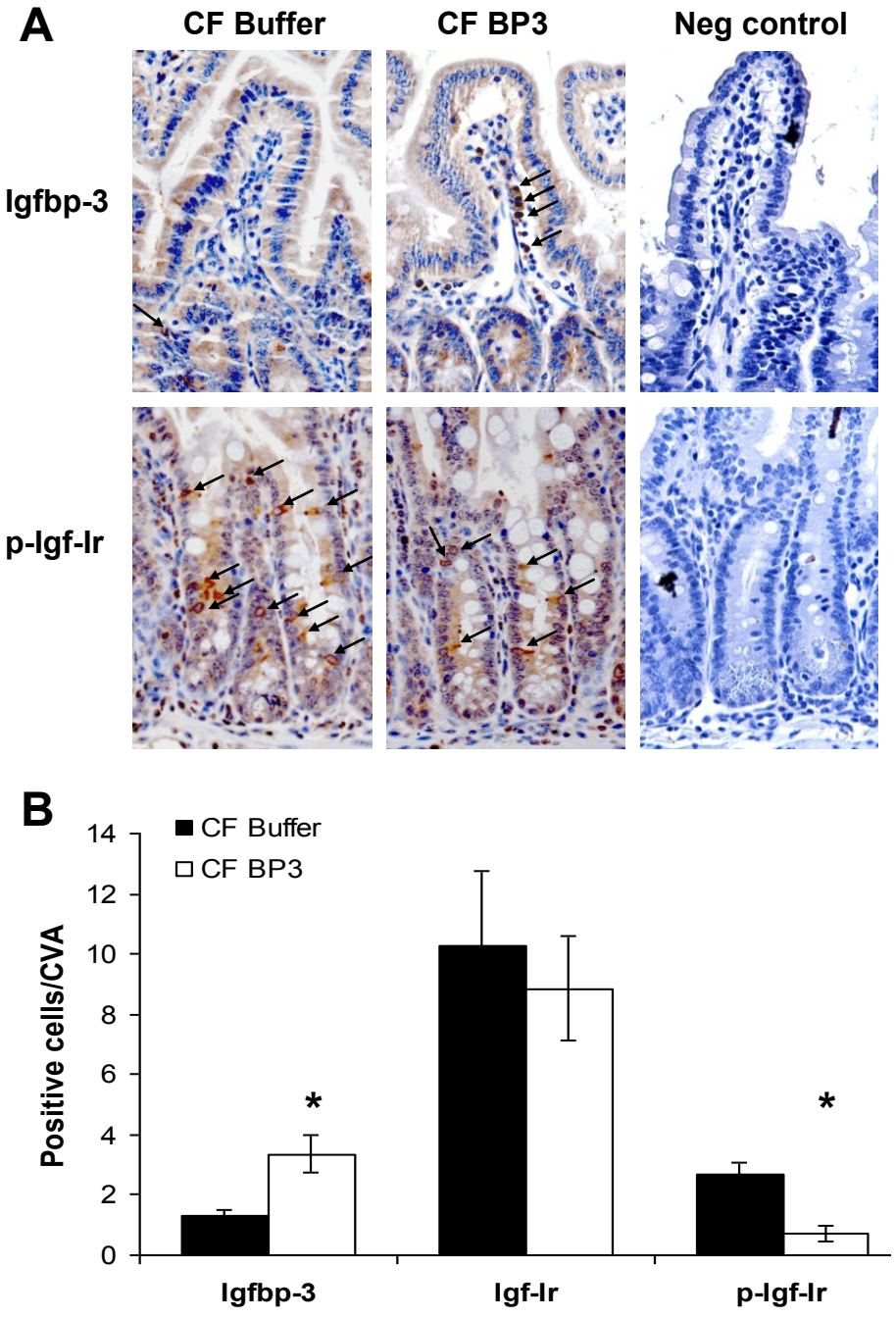
Figure 2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p-Igf-Ir

Figure 3

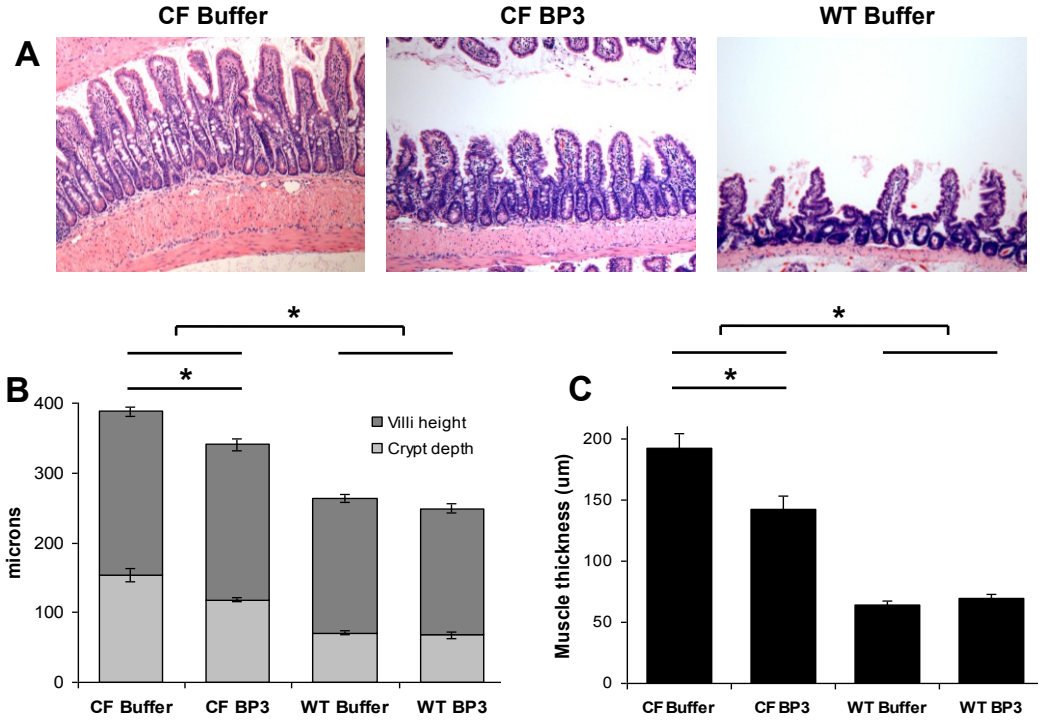

CF Buffer
CF BP3
WT Buffer
A
B
C
*
*
*
*
Villi height
Crypt depth
microns
400
300
200
100
0
CF Buffer
CF BP3
WT Buffer
WT BP3
Muscle thickness (um)
200
150
100
50
0
CF Buffer
CF BP3
WT Buffer
WT BP3

Figure 4

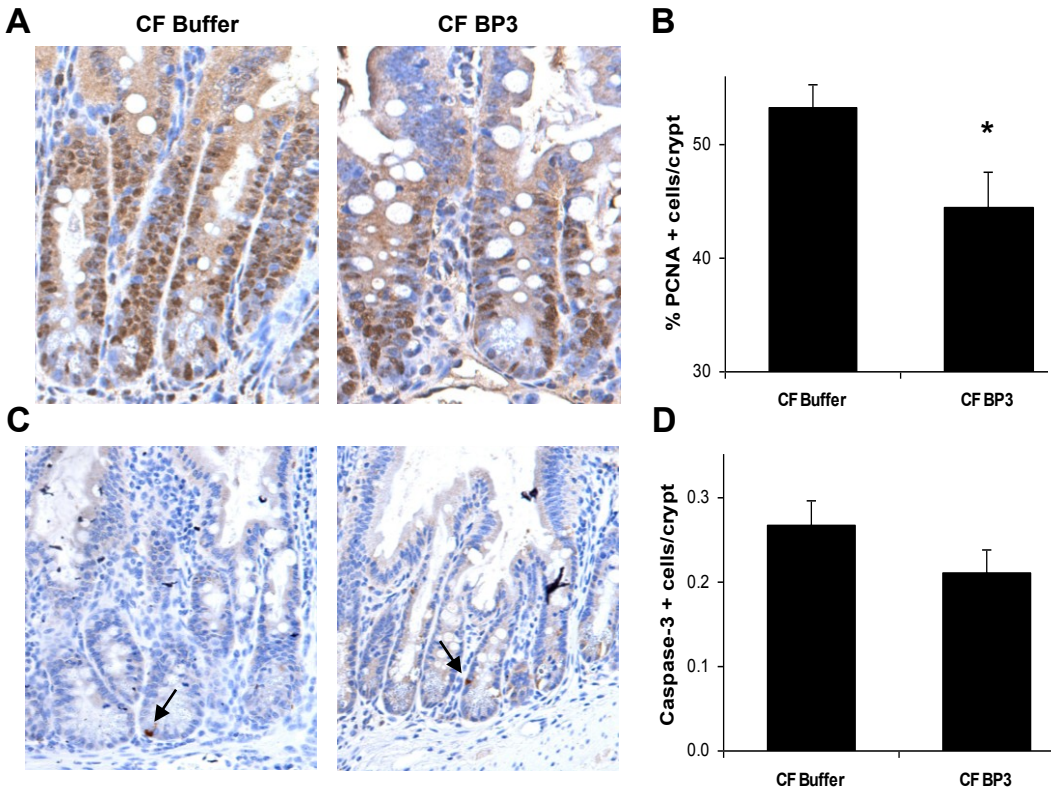

A
CF Buffer
CF BP3
B
% PCNA + cells/crypt
50
40
30
*
CF Buffer
CF BP3
C
D
Caspase-3 + cells/crypt
0.3
0.2
0.1
0.0
CF Buffer
CF BP3

Figure 5

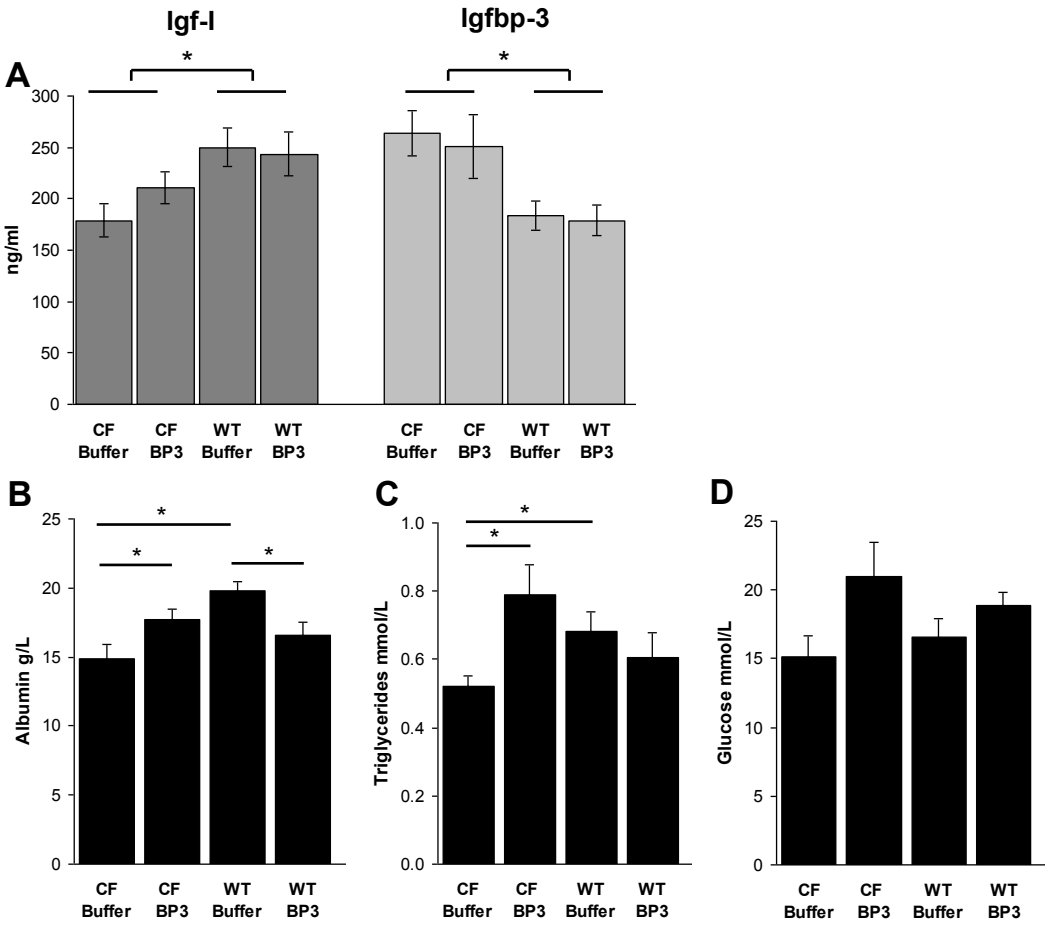

A
Igf-I
Igfbp-3
ng/ml
300
250
200
150
100
50
0
*
*
CF Buffer
CF BP3
WT Buffer
WT BP3
B
Albumin g/L
25
20
15
10
5
0
C
Triglycerides mmol/L
1.0
0.8
0.6
0.4
0.2
0.0
D
Glucose mmol/L

Figure 6

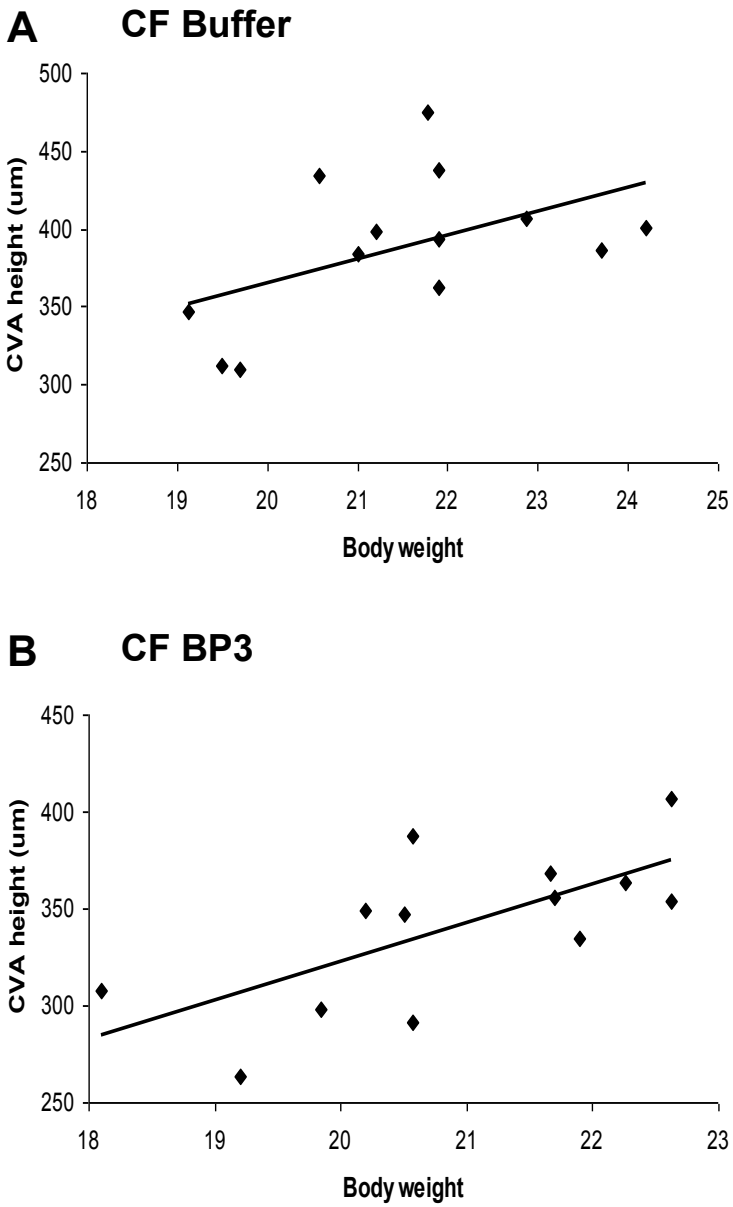
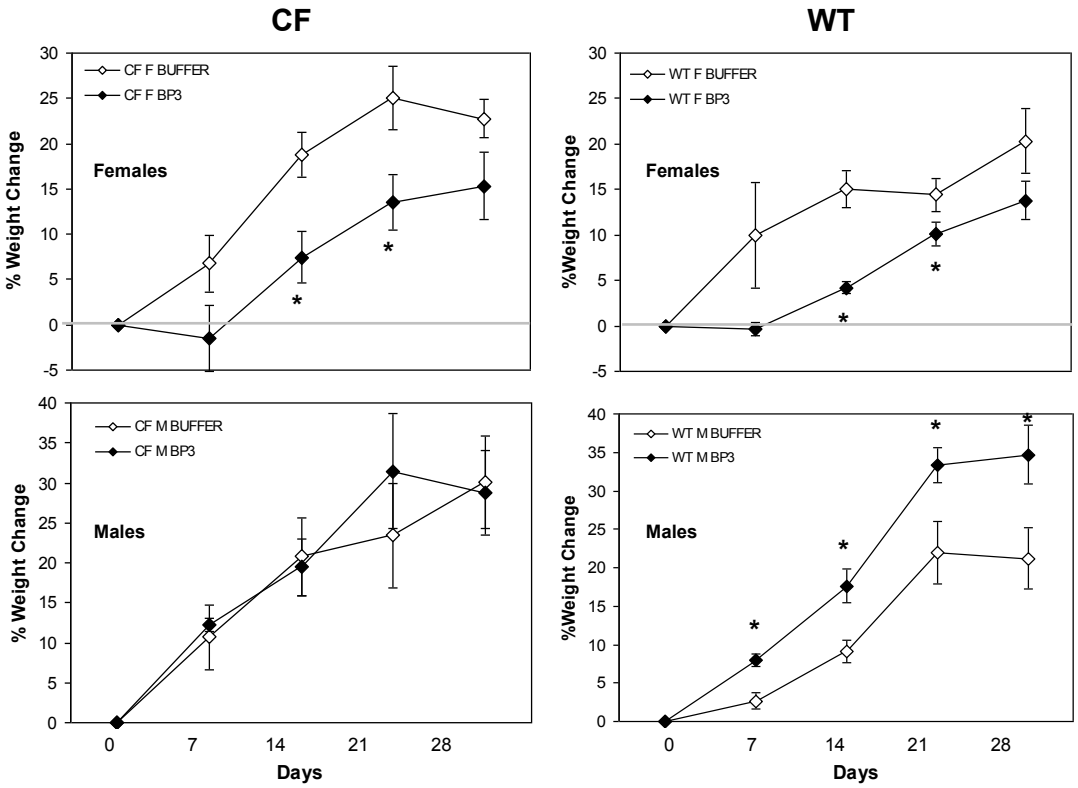

A
CF Buffer
CVA height (um)
500
450
400
350
300
250
18
19
20
21
22
23
24
25
Body weight
B
CF BP3
CVA height (um)
450
400
350
300
250
18
19
20
21
22
23
Body weight

Figure 7


CF
WT
CF F BUFFER
CF F BP3
Females
% Weight Change
WT F BUFFER
WT F BP3
%Weight Change
CF M BUFFER
CF M BP3
Males
WT M BUFFER
WT M BP3
Days
0
7
14
21
28

CHAPTER 5: GENERAL DISCUSSION

This discussion chapter is not intended to reiterate what in previous chapters has been discussed; instead, it will address the most relevant findings and place them in the context of CF intestinal disease severity. The possible interpretations of these novel results along with the study limitations will be discussed. Finally, future experiments to advance this field of research will be proposed.

The phenotypic variability among CF patients with identical *CFTR* mutations highlights the importance of additional factors influencing the severity of the disease. The main goal of this research project was to identify factors contributing to the weight gain deficit characteristic of the Cystic Fibrosis disease. To accomplish this, we initially evaluated the intestinal phenotype of variable weight CF mice to attempt to identify candidate modifier genes responsible for the morphological alterations. These intestinal alterations, originally identified to be associated with mouse body weight, might explain additional non-pancreatic factors affecting the failure to thrive characteristic of the disease.

The initial observation of the proliferative intestinal phenotype of CF mice, described in chapter II, strikingly resembles that of an intestinal adaptive process. In this study we found this adaptive process, of enlarged CVA, to be negatively associated with body weight. The influence of this association with body weight, if any, could be interpreted in two ways: first, the smaller CF mice apparently suffer from a more severe intestinal histopathology (greater CVA), which could be affecting their ability to gain weight, since shorter CVA height is evident in heavier CF mice. Secondly, the unknown factors altering body weight could be more severe in a fraction of the population (smaller mice) and milder in the other (heavier mice), thus the former mice, in order to compensate, respond with a greater intestinal adaptation, however it seems not to suffice and the need to correct other responsible mechanisms is required. We investigated the relation

of CVA and body weight from mice evaluated in chapters III and IV and found these parameters either not or to positively correlate. In addition, the grouping of data from mice of chapters II and IV, which were both B6xBALB F2 mice, showed a lack of association between CVA length and body weight. Despite of these results, however, the observation that CF mice, in general, differ from healthy controls in the presentation of intestinal hyperplasia (enlarged CVA), in the great number of altered expressed genes and in the growth retardation provides sufficient evidence to warrant further studies of candidate genes possessing intestinal proliferative effects and to evaluate their possible contribution to body weight. Overall, this first study provided a list of interesting genes with altered expression in the CF intestine; specifically, *Tlr4* and *Igf-I* were further evaluated due to their potential role as causative agents of intestinal proliferation.

In addition, in chapter II, we found the expression of several genes to be altered in the CF intestine that have also been implicated in the development of inflammatory bowel disease (IBD),[161] this may suggest an overlap in the pathways leading to ileal inflammation and hyperplasia in these diseases. Consequently, breakthroughs in the extensive research area of IBD might also prove useful for CF intestinal disease and vice versa.

In this thesis, the ileum was studied as it is the site of intestinal obstruction in CF patients and mice. However, it can be discussed that due to its anatomical site obstructions are more prone to occur here. The phenotypic alteration of CVA distension was also evident in the more proximal intestine of mice evaluated in chapter IV, this time tissues were prepared according to the Swiss roll technique in order to evaluate a larger section in a single slide. The increased muscle thickness was evident throughout the small intestine. Importantly, bacterial content in the small intestine normally increases gradually from proximal to distal, where the ileum harbours greater concentrations compared to the more proximal sections,[107] and since bacteria-receptor interaction is required for

intestinal homeostasis the ileum is a crucial tissue to evaluate CF severity. In addition, goblet cell numbers also increase from proximal to distal site and as shown in figure 6 of chapter III, the hypersecretion/accumulation of mucus is only evident in the distal ileum, suggesting this site to be more severely affected than the most proximal.

As a consequence of the previous data, in chapter III, we evaluated the role of Tlr4 in the severity of CF intestinal disease. For this purpose, the creation of a *Cftr*/*Tlr4* double mutant mouse was decided upon. These mice were created by mating BALB *Cftr* heterozygous mice with C.C3-*Tlr4*$^{Lps\text{-}d}$/J mice; this strain carries the *Tlr4* point mutation originally identified in C3H/HeJ mice, which renders the gene product non-functional, in a complete BALB background.

After the production of 13 CF adult mice, only a single double mutant mouse survived to adulthood. The single double mutant adult mouse did not differ from the rest in any of the evaluated phenotypes. At assessing the survival curve of the total CF population we discovered that a great number of mice succumbed at or around 4 days after birth, the majority of these comprised mice of the double mutant genotype. As a consequence, we decided to evaluate 4 day old double mutant neonate mice as an attempt to elucidate the cause behind the low survival.

To our surprise, CF *Tlr4* heterozygous mice revealed increased survival compared to CF *Tlr4* +/+ mice. This suggests the amount of functional Tlr4 in CF mice to affect the disease severity, as apparently, a balanced amount of functional Tlr4 (+/-) seems to result favourable by increasing survival, although the evaluated phenotypes did not differ between these mice. A complete lack of Tlr4 function might leave the mouse unresponsive to fight bacterial infection while overexpression and a complete Tlr4 function might result deleterious to CF mice. Our data suggest, but further studies are needed, that a complete lack of Tlr4 results in improper mast cell (innate) response while the increased survival of CF *Tlr4* heterozygous mice might be accounted for the decreased mucus obstructions.

These observations were not further evaluated herein; however they deserve special attention and the design of further experimental approaches to elucidate their possible implications.

The observation that double mutant mice had decreased mast cell infiltration, which suggest these cells to play a critical role in CF survival, could be addressed by creating mast cell-deficient CF mice or treat CF mice with drugs that stabilize mast cells and evaluate their survival. Genetically mast cell-deficient mice are commercially available. A similar mating approach as to that employed with the *Tlr4* mutant strain would also be of use. If mast cells do have a role in CF severity these mice would be expected to also suffer from low survival just as *Cftr*/*Tlr4* double mutants do. If this proves informative, reconstitution using adoptive transfer of bone marrow-derived mast cells into CF mice, as an attempt to rescue the phenotype, would further confirm their implication in disease severity.

A possible reason for the lack of proliferative phenotypic differences between Tlr4 sufficient and deficient CF mice could be explained by the presence of other TLRs that upon activation also regulate proliferative effects, such as Tlr2.[212, 213] The microarray gene expression data from chapter II (and qRT-PCR data not shown here) also revealed increased expression of *Tlr1* and *Tlr2* in the CF intestine. One of the ligands for Tlr2 is lipoteichoic acid (LTA), a major constituent of the cell wall of Gram-positive bacteria, which upon binding to Tlr2, also activates kinases that regulate cell growth, proliferation, survival and differentiation.[212, 214] Of note, Tlr2 has been shown to protect against DSS-induced colitis.[215] Protection against DSS-induced colitis has also been attributed to Tlr3, 5 and 9.[216-218] Therefore, activation of other TLRs could compensate for the lack of Tlr4 in maintaining the hyperplasic state observed in the CF intestine.

Furthermore, ex vivo studies could be attempted by isolating intestinal epithelial cells from CF mice to inhibit Tlr2, Tlr4 and MyD88 and stimulate with their respective ligands to evaluate their proliferative response. This should be carefully planned as normal intestinal epithelial cells are difficult to culture for prolonged periods, thus making it difficult to study the role of TLRs in the normal epithelium in isolation. Additionally, since the double mutants evaluated here lack Tlr4 systemically an attempt could be made to create CF mice deficient in Tlr2, Tlr4 and MyD88 specifically in the intestine.

Given that germ-free animals have shorter crypts and less crypt cell proliferation than conventional animals[115, 219-221] and that CF mice have increased bacterial content and expression of bacterial receptors, a plausible idea to directly study if this inherent increased bacterial concentration is responsible for the hyperproliferative CF phenotype (and as a consequence body weight) would be to evaluate germ-free neonatal CF mice or to treat adult mice with broad spectrum antibiotics and assess intestinal changes, this has been previously performed in order to evaluate the relation of SIBO and mucus secretion and inflammatory response.[86, 89] Furthermore, if bacteria does affect the adaptive response the administration of LTA and LPS to these mice would reveal which toll-like receptor is responsible.

The fact that *Drosophila*'s Toll protein (which shares structural similarities with Tlr4) was originally identified to play a critical role in dorso-ventral polarity during fly development might suggest a possible developmental alteration, triggered by the lack of Cftr, resulting in early postnatal lethality of double mutant mice. This is merely an assumption, since mice with deficiency in Tlr4 signaling develop normally, however, in the absence of Cftr unidentified complications could severely affect mouse survival. Therefore, mechanisms by which Tlr4 alters development through Cftr, if any, would be an interesting area to study.

Due to the extensive amount of evidence supporting the contribution of the IGF system during intestinal alterations such as inflammatory bowel disease, colon cancer, and injuries (resection, malnutrition etc) which present similar alterations to that of the CF intestine, we decided to evaluate its role in our CF model, the results of which are described in chapter IV.

Initially we considered the CF intestinal hyperplasia to be a severe detrimental phenotype while later results pointed us to believe it was more of an adaptive effect. The observation that this striking hyperplasic state evident in the small intestine of the runted CF mice was decreased (CVA and muscle thickness) after rhIGFBP-3 administration along with body weight gain of CF female mice, demonstrates this phenotype to be a compensatory mechanism of adaptive response to the lack of Cftr probably as an attempt to maintain or gain body weight. This discovery is of great importance as we now know that therapy to increase the intestinal adaptive process in CF could potentially help in preserve body weight.

Regarding the sex specific response to rhIGFBP-3 treatment in that only CF female mice had diminished body weight, several studies have reported the fact that CF female patients suffer from more severe disease than males (greater decline in both pulmonary function and survival)[222, 223] thus the way females and males respond to the disease may differ. In addition, CF girls undergo menarche (first menstrual cycle) approximately 2 years later than healthy girls,[224, 225] and since the IGF axis is modulated by hormonal changes this could also affect such response. In contrast, CF male body weight was not changed by rhIGFBP-3 administration, thus intestinal adaptation is a response to lack of Cftr, however, in male mice, does not rescue from the growth deficit phenotype, suggesting that body weight is a complex phenotype that depends on a variety of factors.

Besides the alteration of CF intestinal expression of *Igf-I* and *Igfbp-3*, additional members of the IGF system were also altered. *Igfbp-2* was highly expressed in the CF intestine compared to WT levels. This binding protein, in general, is considered to inhibit IGF actions,[130] however, studies showing a proliferative capacity have also been reported, arguing for a dual functionality of this protein depending on the system evaluated. Specifically, Igfbp-2 has been shown to have potent stimulatory effect on growth and proliferation on prostate cancer cells[226, 227] and to increase the mitotic activity and activation of the Akt pathway in two types of glioma.[228] Interestingly, Igfbp-2 has also been suggested to contribute to mononuclear cell proliferation and activation.[229] The other upregulated expressed binding protein in the CF intestine was *Igfbp-5* which has been reported to facilitate Igf-I/Igf-Ir binding and to augment Igf-I simulated growth of muscle cells,[230] and its increase has been associated with jejunal growth after Igf-I treatment.[197] Furthermore, *Igfbp-1* which was found to be decreased in the CF intestine has been shown to have Igf-I inhibitory actions,[130] further producing an effect similar to than observed in the CF intestine. This pattern of local IGF expression may explain the lack of complete rescue of the hyperplasic phenotype in the CF intestine, since other IGF members, some which potentiate and other which inhibit Igf-I mitogenic actions, are also altered in CF. This data shows that the IGF system is greatly altered in the CF intestine accompanied with a drastic adaptive response that is partly attenuated with the administration of rhIGFBP-3.

A very active area of IGF research is to determine whether the local or the systemic action of IGFBP-3 has more relevance and its dependence or independence on IGF to produce its effects. Regarding potential evidence for an Igf-I-dependant action of rhIGFBP-3 in decreasing the activation of the IGF pathway and consequently resulting in the observed phenotype, is the reported role of the Igf-Ir activation in human colon cancer cells, where treatment with EGCG green tea extract increased the levels of Igfbp-3 with a decrease in Igf-Ir phosphorylation and a significant reduction of cell proliferation.[231] Interestingly,

decreased receptor phosphorylation and cell proliferation were also evident in the rhIGFBP-3 treated CF mice of this thesis. In addition, Igfbp-3/Igf-I affinity is greater than Igf-Ir/Igf-I affinity,[204] therefore, it is likely that, in the treated CF intestine, the inhibition of Igf-Ir phosphorylation of crypt cells is mainly due to Igfbp-3 sequestration of Igf-I from its receptor. However, we cannot rule out the Igf-I-independent effects of Igfbp-3 reported to inhibit proliferation of intestinal smooth muscle cells[132] and the growth of other different cell types;[232, 233] most importantly, the fact that Ricort *et al.* reported IGFBP-3 to inhibit the autophosphorylation of IGF-IR independent of IGF-I and this inhibitory effect not to involve IGFBP-3/IGF-IR interaction.[234] Treatment with mutant IGFBP-3 that is incapable of binding to IGF-I would clarify these actions.

A significant problem when studying effects of the IGF system is the complexity in attributing changes due to the local (paracrine/autocrine) tissue expression as compared to the actions of the circulating (endocrine) protein levels. One disadvantage of the models used in this research project is that both candidates (Tlr4 and IGF) were altered systemically as opposed to tissue-specific. Future experiments to circumvent this issue could be to make use of the fusion of the intestinal fatty acid-binding protein promoter and the IGFBP-3 gene (I-FABP-IGFBP-3). The I-FABP gene promoter is commonly employed to create transgenic mice overexpressing, specifically in the small intestine, a gene of interest. This way, the effect of the systemically administered rhIGFBP-3 seen in chapter IV could be confirmed. Nevertheless, these models were extremely useful in providing clear evidence of the important implication of Tlr4 and IGF in the CF intestinal disease, validating their further and more thorough study.

Data from this thesis project indicated an increase in nutritional markers but a decrease in body weight after rhIGFBP-3 treatment. Besides the anti-proliferative effects, Igfbp-3 has also been suggested to play a role in enterocyte differentiation,[235, 236] and as its been previously suggested that expression of *CFTR* plays a role in the differentiated functions of intestinal epithelial cell

types,[237] it is possible that rhIGFBP-3 treatment alters the differentiation of enterocytes, increasing fatty acid absorption and thus increasing circulating triglyceride levels. The observation that rhIGFBP-3 treatment increased circulating levels of the nutritional markers albumin and triglycerides but decreased weight gain, of female CF mice, indicates that stimulation of an adaptive intestinal response in CF mice is more important for maintaining weight than the circulating levels of these proteins.

In this last chapter, we have not uncovered the reason why CF mice fail to gain proper weight; instead, we have potentially identified a mechanism by which CF mice attempt to maintain body weight. The lack of complete weight gain despite this adaptive process could be due to multiple unidentified but often proposed factors such as transport deficiency, alteration in cell differentiation, increased energy expenditure, maldigestion and malabsorption, among others.

As most CF mice succumb to intestinal plugs, the relevance of this investigation towards elucidating this phenomenon is attributed to our findings implicating the IGF system as a contributor to the hyperproliferative state. By increasing the CVA, with the purpose to adaptively increase the absorptive surface area, there is also an increase in the number of mucus-producing cells (goblet cells). In addition, the CF intestinal muscle activity and contractility is known to be irregular resulting in slower intestinal transit and dysmotility. Our finding that rhIGFBP-3 administration decreased the intestinal muscle hypertrophy should be further examined in order to determine if this partial recovery results in improved intestinal transit. This is crucial since SIBO, which stimulates mucus secretion and accumulation, is probably due to slowed small intestinal transit, thus an optimal intestinal muscle layer is essential to prevent SIBO and mucus accumulation.

The major original findings of this dissertation are:

1) An altered local expression of Toll-like receptor 4 (and downstream signaling molecules) which, by means of a novel *Cftr*/*Tlr4* double mutant model, was determined to contribute to CF survival probably due to mucus accumulation.

2) An altered expression of the local IGF system reflecting the hyperproliferative adaptive state which was identified, by administration of rhIGFBP-3, to contribute to weight gain in the CF female mice.

In conclusion, resultant data from this thesis reveal new insight into the compensatory mechanisms responsible for the intestinal response to the lack of Cftr. As a consequence, although additional studies are needed, alterations to promote or control these mechanisms could potentially be targeted in order to relieve symptoms of the disease severity. Therefore, this research project successfully identified potential factors altering CF disease severity and opened a new area of research in the field of CF intestinal disease.

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APPENDIX

Supplementary Table 1. Complete data set of differentially expressed genes of the ileal tissue of F2 CF mice relative to controls.

Complete list of ileal genes differentially expressed (fold>2; p<0.05) in CF mice relative to control mice				
Gene Symbol	**Gene Title**	**Increase fold change CF/Control**	**P.Value**	**UniGene ID**
1600029D21Rik	RIKEN cDNA 1600029D21 gene	9.21	0.0254	Mm.29959
1500010G04Rik	RIKEN cDNA 1500010G04 gene	6.84	0.0033	Mm.88790
Ceacam10	CEA-related cell adhesion molecule 10	5.42	0.0149	Mm.30300
Cd177	CD177 antigen	4.88	0.0061	Mm.292848
Aqp4	aquaporin 4	4.45	0.0247	Mm.250786
Vip	vasoactive intestinal polypeptide	3.90	0.0076	Mm.98916
9030623N16Rik	RIKEN cDNA 9030623N16 gene	3.89	0.0313	Mm.171333
Fut2	fucosyltransferase 2	3.85	0.0406	Mm.290046
Cfi	complement component factor i	3.71	0.0032	Mm.117180
Mtac2d1	membrane targeting (tandem) C2 domain containing 1	3.63	0.0070	Mm.275939
LOC434220	NA	3.49	0.0037	Mm.323218
Pla2g5	phospholipase A2, group V	3.19	0.0061	Mm.23347
Tmprss2	transmembrane protease, serine 2	3.14	0.0028	Mm.276145
1810030J14Rik	RIKEN cDNA 1810030J14 gene	3.07	0.0438	Mm.7150
Mfsd2	major facilitator superfamily domain containing 2	3.01	0.0056	Mm.331842
C1galt1	core 1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase	3.00	0.0006	Mm.102752
Evi1	ecotropic viral integration site 1	2.96	0.0447	Mm.56965
Tlr4	toll-like receptor 4	2.94	0.0062	Mm.38049
Tat	tyrosine aminotransferase	2.91	0.0349	Mm.28110
Serpinb5	serine (or cysteine) peptidase inhibitor, clade B, member 5	2.89	0.0446	Mm.268618
Car8	carbonic anhydrase 8	2.79	0.0098	Mm.119320
Oact1	O-acyltransferase (membrane bound) domain containing 1	2.68	0.0488	Mm.89682
Socs3	suppressor of cytokine signaling 3	2.62	0.0378	Mm.3468
Cd44	CD44 antigen	2.59	0.0026	Mm.330428
Slc39a8	solute carrier family 39 (metal ion transporter), member 8	2.59	0.0018	Mm.30239
Mmp7	matrix metallopeptidase 7	2.57	0.0140	Mm.4825
Prom1	prominin 1	2.56	0.0068	Mm.6250
B3gnt5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	2.54	0.0087	Mm.33935
Ang4	angiogenin, ribonuclease A family, member 4	2.52	0.0433	Mm.343899
Aspn	asporin	2.49	0.0049	Mm.383216
Wtip	WT1-interacting protein	2.46	0.0176	Mm.27482
Gmds	GDP-mannose 4, 6-dehydratase	2.46	0.0134	Mm.247143
Tfrc	transferrin receptor	2.45	0.0497	Mm.28683
Slc6a14	solute carrier family 6 (neurotransmitter transporter), member 14	2.45	0.0400	Mm.253984
Habp2	hyaluronic acid binding protein 2	2.43	0.0116	Mm.25791
Ap4s1	adaptor-related protein complex AP-4, sigma 1	2.40	0.0019	Mm.116858
Gna14	guanine nucleotide binding protein, alpha 14	2.32	0.0364	Mm.313181
Blnk	B-cell linker	2.30	0.0062	Mm.9749
Gch1	GTP cyclohydrolase 1	2.29	0.0137	Mm.10651
Steap1	six transmembrane epithelial antigen of the prostate 1	2.29	0.0080	Mm.85429
Rab27b	RAB27b, member RAS oncogene family	2.27	0.0370	Mm.246753
Tspan1	tetraspan 1	2.26	0.0066	Mm.45994
Slk	STE20-like kinase (yeast)	2.23	0.0047	Mm.281011
MGI:2182965	Traf2 binding protein	2.22	0.0144	Mm.31852
Cd24a	CD24a antigen	2.21	0.0270	Mm.29742
Mbnl2	muscleblind-like 2	2.20	0.0034	Mm.238266
Lum	lumican	2.19	0.0063	Mm.18888
6720463M24Rik	RIKEN cDNA 6720463M24 gene	2.18	0.0482	Mm.23503
Tm4sf20	transmembrane 4 L six family member 20	2.17	0.0412	Mm.46325
Zwilch	Zwilch, kinetochore associated, homolog (Drosophila)	2.17	0.0150	Mm.44082
Capg	capping protein (actin filament), gelsolin-like	2.14	0.0154	Mm.18626

P4ha1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	2.13	0.0392	Mm.2212
Rgmb	RGM domain family, member B	2.11	0.0241	Mm.293466
Ociad2	OCIA domain containing 2	2.10	0.0047	Mm.274892
Slc39a10	solute carrier family 39 (zinc transporter), member 10	2.10	0.0106	Mm.233889
Nudcd1	NudC domain containing 1	2.10	0.0007	Mm.292021
Tnfaip8	tumor necrosis factor, alpha-induced protein 8	2.09	0.0044	Mm.27740
Rrm1	ribonucleotide reductase M1	2.09	0.0073	Mm.197486
Ly96	lymphocyte antigen 96	2.08	0.0449	Mm.116844
Gusb	glucuronidase, beta	2.08	0.0054	Mm.3317
Abce1	ATP-binding cassette, sub-family E (OABP), member 1	2.07	0.0106	Mm.5831
Ccl9	chemokine (C-C motif) ligand 9	2.05	0.0131	Mm.2271
Gstk1	glutathione S-transferase kappa 1	2.04	0.0229	Mm.267014
Gas5	growth arrest specific 5	2.04	0.0183	Mm.270065
Creb3l4	cAMP responsive element binding protein 3-like 4	2.03	0.0037	Mm.299952
2810028N01Rik	RIKEN cDNA 2810028N01 gene	2.02	0.0044	Mm.163339
Galnt7	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7	2.02	0.0116	Mm.62886
Mlstd2	male sterility domain containing 2	2.01	0.0063	Mm.206919
Pla2g10	phospholipase A2, group X	2.00	0.0403	Mm.4214
Ncl	nucleolin	2.00	0.0062	Mm.154378
Gene Symbol	**Gene Title**	**Decrease fold change CF/Control**	**P.Value**	**UniGene ID**
Cyp3a25	cytochrome P450, family 3, subfamily a, polypeptide 25	21.54	0.0028	Mm.301900
Pdzk1	PDZ domain containing 1	14.38	0.0053	Mm.28015
Rdh7	retinol dehydrogenase 7	11.53	0.0005	Mm.6696
AW011956	expressed sequence AW011956	7.52	0.0110	Mm.10034
Cubn	cubilin (intrinsic factor-cobalamin receptor)	7.32	0.0019	Mm.313915
Cyp4v3	cytochrome P450, family 4, subfamily v, polypeptide 3	7.09	0.0037	Mm.245297
Serpina1b	serine (or cysteine) preptidase inhibitor, clade A, member 1b	6.93	0.0062	Mm.347501
2010001P20Rik	RIKEN cDNA 2010001P20 gene	6.24	0.0017	Mm.383302
Treh	trehalase (brush-border membrane glycoprotein)	6.22	0.0067	Mm.45380
Arg2	arginase type II	6.14	0.0074	Mm.3506
Akr1b7	aldo-keto reductase family 1, member B7	5.93	0.0093	Mm.90151
Susd2	sushi domain containing 2	5.81	0.0041	Mm.247956
1300013J15Rik	RIKEN cDNA 1300013J15 gene	5.68	0.0028	Mm.100741
Aadac	arylacetamide deacetylase (esterase)	5.39	0.0093	Mm.24547
Cndp1	carnosine dipeptidase 1 (metallopeptidase M20 family)	5.05	0.0032	Mm.23278
Cftr	cystic fibrosis transmembrane conductance regulator homolog	4.98	0.0007	Mm.15621
Slc2a2	solute carrier family 2 (facilitated glucose transporter), member 2	4.37	0.0087	Mm.18443
Angptl4	angiopoietin-like 4	4.31	0.0014	Mm.196189
Ugt2b5	UDP glucuronosyltransferase 2 family, polypeptide B5	4.23	0.0257	Mm.29157
Bst1	bone marrow stromal cell antigen 1	4.08	0.0134	Mm.246332
Dio1	deiodinase, iodothyronine, type I	3.98	0.0185	Mm.148342
Maob	monoamine oxidase B	3.82	0.0010	Mm.241656
Mme	membrane metallo endopeptidase	3.72	0.0229	Mm.296022
Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	3.70	0.0062	Mm.207354
Tyki	thymidylate kinase family LPS-inducible member	3.62	0.0044	Mm.271839
Abcc2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	3.59	0.0147	Mm.39054
Xpnpep2	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	3.51	0.0020	Mm.129279
Fbp1	fructose bisphosphatase 1	3.51	0.0053	Mm.246512
Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7	3.38	0.0082	Mm.14609
Slc16a10	solute carrier family 16 (monocarboxylic acid transporters), member 10	3.24	0.0024	Mm.186778
BC021608	cDNA sequence BC021608	3.19	0.0176	Mm.325487
Slc5a4a	solute carrier family 5, member 4a	3.17	0.0080	Mm.154797
Slc6a19	solute carrier family 6 (neurotransmitter transporter), member 19	3.15	0.0119	Mm.271635
Ces6	carboxylesterase 6	3.12	0.0016	Mm.212983
Dnmt2	DNA methyltransferase 2	3.05	0.0024	Mm.6979
Slc22a4	solute carrier family 22 (organic cation transporter), member 4	3.00	0.0034	Mm.274590
Mpst	mercaptopyruvate sulfurtransferase	2.97	0.0044	Mm.294215
2010001E11Rik	RIKEN cDNA 2010001E11 gene	2.97	0.0052	Mm.235981
Gpr172b	G protein-coupled receptor 172B	2.96	0.0010	Mm.28597
Cyp3a11	cytochrome P450, family 3, subfamily a, polypeptide 11	2.91	0.0001	Mm.332844

Amn	amnionless	2.90	0.0038	Mm.197639
1200009I06Rik	RIKEN cDNA 1200009I06 gene	2.89	0.0075	Mm.248640
Mapk4	mitogen-activated protein kinase 4	2.88	0.0044	Mm.254517
Dusp12	dual specificity phosphatase 12	2.83	0.0019	Mm.34365
Slc5a11	solute carrier family 5 (sodium/glucose cotransporter), member 11	2.83	0.0024	Mm.211838
Guca2b	guanylate cyclase activator 2b (retina)	2.83	0.0031	Mm.278
Khk	ketohexokinase	2.82	0.0016	Mm.22451
1200006F02Rik	RIKEN cDNA 1200006F02 gene	2.82	0.0096	Mm.153218
Dfna5h	deafness, autosomal dominant 5 homolog (human)	2.80	0.0239	Mm.248361
Slc7a8	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	2.78	0.0010	Mm.276831
Trpm6	transient receptor potential cation channel, subfamily M, member 6	2.77	0.0154	Mm.215171
H2-Tw3	NA	2.73	0.0487	Mm.14109
Slc5a6	solute carrier family 5 (sodium-dependent vitamin transporter), member 6	2.67	0.0103	Mm.205463
MGI:2143217	X transporter protein 3 similar 1 gene	2.67	0.0084	Mm.27208
2810439F02Rik	RIKEN cDNA 2810439F02 gene	2.65	0.0023	Mm.52526
3110049J23Rik	RIKEN cDNA 3110049J23 gene	2.65	0.0056	Mm.368563
Hsd17b13	hydroxysteroid (17-beta) dehydrogenase 13	2.63	0.0016	Mm.284944
Dhrs8	dehydrogenase/reductase (SDR family) member 8	2.58	0.0062	Mm.46019
BQ952480	expressed sequence BQ952480	2.58	0.0038	Mm.374909
Cyp2d22	cytochrome P450, family 2, subfamily d, polypeptide 22	2.58	0.0027	Mm.157435
2010003K15Rik	RIKEN cDNA 2010003K15 gene	2.56	0.0333	Mm.380612
3110048E14Rik	RIKEN cDNA 3110048E14 gene	2.56	0.0119	Mm.247535
Lipe	lipase, hormone sensitive	2.56	0.0050	Mm.333679
Crym	crystallin, mu	2.56	0.0012	Mm.9114
Slc5a12	solute carrier family 5 (sodium/glucose cotransporter), member 12	2.53	0.0249	Mm.277148
Ephx2	epoxide hydrolase 2, cytoplasmic	2.52	0.0204	Mm.15295
Tspan5	tetraspanin 5	2.51	0.0062	Mm.31927
Pdk2	pyruvate dehydrogenase kinase, isoenzyme 2	2.48	0.0076	Mm.29768
Cml4	camello-like 4	2.48	0.0054	Mm.154782
Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1	2.47	0.0201	Mm.250866
A530016O06Rik	RIKEN cDNA A530016O06 gene	2.46	0.0264	Mm.211255
Aspa	aspartoacylase (aminoacylase) 2	2.44	0.0067	Mm.293574
MGI:2672795	liver-expressed antimicrobial peptide 2	2.44	0.0038	Mm.24283
C530046L02Rik	RIKEN cDNA C530046L02 gene	2.43	0.0174	Mm.301585
Fmo4	flavin containing monooxygenase 4	2.43	0.0282	Mm.155164
Neu1	neuraminidase 1	2.43	0.0027	Mm.8856
Anpep	alanyl (membrane) aminopeptidase	2.40	0.0038	Mm.4487
Pep4	peptidase 4	2.40	0.0131	Mm.69751
E030004N02Rik	RIKEN cDNA E030004N02 gene	2.39	0.0078	Mm.270304
Ihh	Indian hedgehog	2.37	0.0111	Mm.2543
AW125753	expressed sequence AW125753	2.36	0.0082	Mm.227253
Dp1l1	deleted in polyposis 1-like 1	2.34	0.0014	Mm.28147
1810054O13Rik	RIKEN cDNA 1810054O13 gene	2.34	0.0024	Mm.27338
Aplp1	amyloid beta (A4) precursor-like protein 1	2.33	0.0018	Mm.2381
Cgref1	cell growth regulator with EF hand domain 1	2.32	0.0047	Mm.45127
Rasd2	RASD family, member 2	2.32	0.0037	Mm.179267
Slc34a2	solute carrier family 34 (sodium phosphate), member 2	2.31	0.0380	Mm.284891
Slc27a4	solute carrier family 27 (fatty acid transporter), member 4	2.30	0.0085	Mm.330113
Slc16a5	solute carrier family 16 (monocarboxylic acid transporters), member 5	2.29	0.0323	Mm.25773
Plcd1	phospholipase C, delta 1	2.28	0.0025	Mm.23963
Clic5	chloride intracellular channel 5	2.28	0.0375	Mm.37666
Slc25a15	solute carrier family 25 (mitochondrial carrier ornithine transporter), member 15	2.26	0.0197	Mm.200907
Abp1	amiloride binding protein 1 (amine oxidase, copper-containing)	2.26	0.0466	Mm.213898
Mmd	monocyte to macrophage differentiation-associated	2.25	0.0053	Mm.277518
Slc30a2	solute carrier family 30 (zinc transporter), member 2	2.25	0.0373	Mm.358876
E230025K15	NA	2.25	0.0008	Mm.17670
Cln8	ceroid-lipofuscinosis, neuronal 8	2.25	0.0042	Mm.254027
Ntn4	netrin 4	2.24	0.0044	Mm.291158
Sct	secretin	2.23	0.0228	Mm.4723
Pipox	pipecolic acid oxidase	2.23	0.0051	Mm.8543
Vwa1	von Willebrand factor A domain containing 1	2.22	0.0052	Mm.26515
Cndp2	CNDP dipeptidase 2 (metallopeptidase M20 family)	2.21	0.0046	Mm.29646

Slc43a2	solute carrier family 43, member 2	2.20	0.0093	Mm.11186
Npc1l1	NPC1-like 1	2.19	0.0396	Mm.212492
Thsd6	thrombospondin, type I domain containing 6	2.19	0.0037	Mm.268527
Dpep1	dipeptidase 1 (renal)	2.17	0.0038	Mm.20388
Rsad2	radical S-adenosyl methionine domain containing 2	2.16	0.0215	Mm.24045
AI118064	expressed sequence AI118064	2.16	0.0131	Mm.212789
Atg9l1	autophagy-related 9-like 1 (yeast)	2.15	0.0164	Mm.358931
Tcn2	transcobalamin 2	2.14	0.0009	Mm.20948
Gpt1	glutamic pyruvic transaminase 1, soluble	2.14	0.0022	Mm.30130
Slc2a5	solute carrier family 2 (facilitated glucose transporter), member 5	2.14	0.0155	Mm.260220
G6pc	glucose-6-phosphatase, catalytic	2.14	0.0202	Mm.18064
Mcoln1	mucolipin 1	2.14	0.0401	Mm.8356
Ggt1	gamma-glutamyltransferase 1	2.13	0.0034	Mm.4559
A930008A22Rik	RIKEN cDNA A930008A22 gene	2.13	0.0060	Mm.138434
Ddhd2	DDHD domain containing 2	2.13	0.0018	Mm.246875
Gnpda1	glucosamine-6-phosphate deaminase 1	2.12	0.0191	Mm.22374
Hexb	hexosaminidase B	2.11	0.0074	Mm.27816
Nnat	neuronatin	2.11	0.0384	Mm.233903
Ppara	peroxisome proliferator activated receptor alpha	2.11	0.0397	Mm.212789
Oas3	2'-5' oligoadenylate synthetase 3	2.09	0.0231	Mm.204887
Ptpn21	protein tyrosine phosphatase, non-receptor type 21	2.09	0.0063	Mm.4420
Itpka	inositol 1,4,5-trisphosphate 3-kinase A	2.08	0.0162	Mm.65337
Faah	fatty acid amide hydrolase	2.08	0.0285	Mm.256025
Ccl25	chemokine (C-C motif) ligand 25	2.08	0.0111	Mm.7275
Irf7	interferon regulatory factor 7	2.07	0.0447	Mm.3233
Enpp3	ectonucleotide pyrophosphatase/phosphodiesterase 3	2.06	0.0049	Mm.338425
Sox13	SRY-box containing gene 13	2.06	0.0081	Mm.8575
Dgkq	diacylglycerol kinase, theta	2.05	0.0074	Mm.260921
Capn10	calpain 10	2.04	0.0044	Mm.294315
Dhcr24	24-dehydrocholesterol reductase	2.04	0.0120	Mm.133370
Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	2.03	0.0073	Mm.330745
AW491445	expressed sequence AW491445	2.01	0.0060	Mm.5323
Gpr133	G protein-coupled receptor 133	2.01	0.0156	Mm.379431

Supplementary Table 2. Complete data set of gene ontology categories of the gene expression response of the ileal tissue of F2 CF mice relative to controls.

Gene Ontology categories significantly represented (p<0.01) in the CF mice compared to control mice					
GO number	**GO Name**	**GO Type***	**No. changed**	**No. on chip**	**P Value**
GO:0003824	catalytic activity	MF	515	4240	4.68E-10
GO:0003723	RNA binding	MF	69	398	2.07E-06
GO:0000166	nucleotide binding	MF	194	1455	3.22E-06
GO:0008565	protein transporter activity	MF	29	126	1.08E-05
GO:0017111	nucleoside-triphosphatase activity	MF	61	388	0.0002
GO:0008415	acyltransferase activity	MF	24	116	0.0003
GO:0008238	exopeptidase activity	MF	19	83	0.0004
GO:0016747	transferase activity, transferring groups other than amino-acyl groups	MF	24	118	0.0005
GO:0016462	pyrophosphatase activity	MF	62	412	0.0005
GO:0017076	purine nucleotide binding	MF	165	1316	0.0005
GO:0016817	hydrolase activity, acting on acid anhydrides	MF	62	414	0.0005
GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	MF	62	414	0.0005
GO:0016787	hydrolase activity	MF	208	1721	0.0007
GO:0004177	aminopeptidase activity	MF	11	38	0.0008
GO:0016805	dipeptidase activity	MF	5	9	0.0008
GO:0016746	transferase activity, transferring acyl groups	MF	24	124	0.0010
GO:0016887	ATPase activity	MF	39	239	0.0011
GO:0015293	symporter activity	MF	17	77	0.0011
GO:0042623	ATPase activity, coupled	MF	37	224	0.0012
GO:0016407	acetyltransferase activity	MF	12	46	0.0013
GO:0008320	protein carrier activity	MF	5	11	0.0026
GO:0004029	aldehyde dehydrogenase (NAD) activity	MF	4	7	0.0026
GO:0048037	cofactor binding	MF	11	44	0.0030
GO:0008131	amine oxidase activity	MF	3	4	0.0036
GO:0015290	electrochemical potential-driven transporter activity	MF	26	154	0.0045
GO:0004028	aldehyde dehydrogenase activity	MF	5	13	0.0061
GO:0016491	oxidoreductase activity	MF	77	592	0.0068
GO:0030554	adenyl nucleotide binding	MF	128	1057	0.0072
GO:0008235	metalloexopeptidase activity	MF	10	43	0.0079
GO:0015291	porter activity	MF	25	153	0.0080
GO:0005524	ATP binding	MF	125	1038	0.0094
GO:0008451	X-Pro aminopeptidase activity	MF	2	2	0.0097
GO:0019807	aspartoacylase activity	MF	2	2	0.0097
GO:0004815	aspartate-tRNA ligase activity	MF	2	2	0.0097
GO:0005737	cytoplasm	CC	372	2863	5.91E-09
GO:0005622	intracellular	CC	695	6075	2.74E-07
GO:0005623	cell	CC	970	9040	2.62E-06
GO:0005903	brush border	CC	7	12	5.45E-05
GO:0043226	organelle	CC	596	5275	6.82E-05
GO:0043229	intracellular organelle	CC	596	5275	6.82E-05
GO:0043227	membrane-bound organelle	CC	533	4659	6.97E-05
GO:0043231	intracellular membrane-bound organelle	CC	533	4659	6.97E-05
GO:0005795	Golgi stack	CC	50	299	0.0003
GO:0045177	apical part of cell	CC	12	41	0.0005
GO:0016324	apical plasma membrane	CC	9	27	0.0010
GO:0005794	Golgi apparatus	CC	60	400	0.0012
GO:0012505	endomembrane system	CC	29	171	0.0039

GO:0005783	endoplasmic reticulum	CC	66	485	0.0078
GO:0019717	synaptosome	CC	9	36	0.0083
GO:0005768	endosome	CC	13	62	0.0084
GO:0016282	eukaryotic 43S preinitiation complex	CC	6	19	0.0092
GO:0050875	cellular physiological process	BP	836	7520	1.34E-08
GO:0008152	metabolism	BP	627	5485	1.54E-06
GO:0007582	physiological process	BP	902	8388	3.77E-06
GO:0006886	intracellular protein transport	BP	60	352	3.31E-05
GO:0046907	intracellular transport	BP	76	482	5.05E-05
GO:0015031	protein transport	BP	80	520	7.61E-05
GO:0044237	cellular metabolism	BP	581	5177	0.0001
GO:0051325	interphase	BP	13	41	0.0001
GO:0051329	interphase of mitotic cell cycle	BP	13	41	0.0001
GO:0043170	macromolecule metabolism	BP	308	2556	0.0001
GO:0006270	DNA replication initiation	BP	6	10	0.0002
GO:0044238	primary metabolism	BP	557	4961	0.0002
GO:0044260	cellular macromolecule metabolism	BP	294	2442	0.0002
GO:0045184	establishment of protein localization	BP	80	539	0.0003
GO:0043283	biopolymer metabolism	BP	279	2305	0.0003
GO:0000082	G1/S transition of mitotic cell cycle	BP	10	29	0.0004
GO:0043037	translation	BP	25	123	0.0005
GO:0008104	protein localization	BP	81	561	0.0006
GO:0016070	RNA metabolism	BP	51	319	0.0006
GO:0009058	biosynthesis	BP	118	883	0.0008
GO:0019752	carboxylic acid metabolism	BP	54	347	0.0008
GO:0006082	organic acid metabolism	BP	54	347	0.0008
GO:0007049	cell cycle	BP	74	511	0.0009
GO:0044267	cellular protein metabolism	BP	269	2268	0.0013
GO:0006260	DNA replication	BP	20	96	0.0013
GO:0009059	macromolecule biosynthesis	BP	72	503	0.0014
GO:0019538	protein metabolism	BP	270	2284	0.0015
GO:0006396	RNA processing	BP	41	253	0.0016
GO:0006412	protein biosynthesis	BP	65	451	0.0020
GO:0006261	DNA-dependent DNA replication	BP	9	30	0.0022
GO:0046339	diacylglycerol metabolism	BP	4	7	0.0028
GO:0006807	nitrogen compound metabolism	BP	41	262	0.0030
GO:0005975	carbohydrate metabolism	BP	46	304	0.0034
GO:0043161	proteasomal ubiquitin-dependent protein catabolism	BP	4	8	0.0052
GO:0051234	establishment of localization	BP	248	2131	0.0057
GO:0051179	localization	BP	250	2152	0.0060
GO:0046467	membrane lipid biosynthesis	BP	11	47	0.0062
GO:0000278	mitotic cell cycle	BP	27	162	0.0063
GO:0009308	amine metabolism	BP	38	249	0.0065
GO:0006810	transport	BP	245	2108	0.0065
GO:0044249	cellular biosynthesis	BP	100	782	0.0073
GO:0006400	tRNA modification	BP	11	48	0.0073
GO:0006519	amino acid and derivative metabolism	BP	33	212	0.0080
GO:0008654	phospholipid biosynthesis	BP	9	36	0.0082
GO:0006081	aldehyde metabolism	BP	3	5	0.0088
GO:0006882	zinc ion homeostasis	BP	3	5	0.0088
GO:0008380	RNA splicing	BP	22	128	0.0091
GO:0000398	nuclear mRNA splicing, via spliceosome	BP	21	121	0.0096
GO:0000375	RNA splicing, via transesterification reactions	BP	21	121	0.0096
GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	BP	21	121	0.0096
* MF- Molecular					

Function					
CC - Cellular Compartment					
BP - Biological Processes					

Supplementary Table 3. The data set of intestinal genes of the F2 CF mice which were differentially expressed by sex.

Complete list of ileal genes of the F2 CF mice which were differentially expressed by sex (p<0.05).

Gene Symbol	Gene Title	Fold change	p value	UniGene ID
Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	4.72	5.84E-09	Mm.250909
D1Pas1-rs1	DNA segment, Chr 1, Pasteur Institute 1, related sequence 1	4.48	8.23E-11	Mm.302938
Jarid1d	jumonji, AT rich interactive domain 1D (Rbp2 like)	3.63	4.20E-08	Mm.262676
Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	3.42	6.30E-07	Mm.302938
Uty	ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	2.52	4.71E-06	Mm.20477
C030026M15Rik	RIKEN cDNA C030026M15 gene	1.58	0.007	Mm.20477
Eif2s3x	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	-0.59	0.0002	Mm.218851
Xist	inactive X specific transcripts	-4.61	2.20E-08	Mm.350914