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Original Article

Transcriptome analysis of the distal small intestine of *Cftr null* mice

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ABSTRACT

Cystic fibrosis (CF) is caused by mutations in the gene encoding the CFTR anion channel. Loss of CFTR function in pancreatic, biliary and intestinal epithelia, severely affects gastrointestinal function. Transcriptome analysis indicated the activation of an innate and adaptive immune response in the distal small intestine of *Cftr null* mice. Inflammation was associated with differential regulation of numerous genes involved in the transport and metabolism of nutrients and, particularly, lipids, that are targeted by ligand-dependent nuclear receptors and/or HNF4α. Among the most strongly down-regulated genes are the FXR targets *Fgf15* and *Nr0b2*, the PPARα target *Pdk4*, and the PXR target *Ces2a*, whereas expression of the CF modifier gene *Slc6a14* was strongly increased. Most changes in gene expression were reversed by bacterial containment. Our data suggest that the gut microbiota has a pervasive effect on gene expression in CF mice, affecting enterocyte maturation, lipid metabolism, and nutrient absorption in CF.

1. Introduction

Cystic fibrosis (CF) is a potentially lethal disease that is caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which encodes a phosphorylation-regulated chloride/bicarbonate channel that drives salt and water secretion across various epithelia, foremost those in the respiratory and the gastrointestinal tract. CFTR-mediated anion secretion contributes to bile formation, and is critical for exocrine pancreatic function and for salt and water secretion across the intestinal epithelium. Thus, the intestinal pathology of CF is characterized by luminal dehydration, luminal surface acidification and accretion of viscous mucus [1]. Mucus accumulation and dehydration are thought to play a key role in the development of the CF-typical obstruction of the distal small intestine. The abnormally viscous mucus layer that lines the epithelium may also provide a niche for bacterial colonization, leading to small intestinal bacterial overgrowth (SIBO) [2]. SIBO is relatively common among CF patients and may affect the luminal processing and uptake of nutrients (lipids in particular) and bile acids, potentially contributing to their malabsorption and to the growth retardation observed in CF. SIBO is likely to markedly impact host-microbe interactions, and trigger a local, innate immune response [2]. This is most evident from studies on CF mouse models, which report evidence for both SIBO and inflammation of the gut wall [3,4].

A limited number of studies have systematically assessed the effect of the CF condition on intestinal gene expression, using a DNA microarray approach [3,5–7]. Applied to a *Cftr null* mouse model with a severe intestinal phenotype (i.e. a strong predisposition to develop a lethal obstruction in early life), these microarray studies have shown that CF is associated with marked changes in intestinal gene expression. Some of these changes appear to be directly related to SIBO, e.g. the up-regulation of genes involved in host-microbe interactions [3]. For other differentially regulated genes this link with SIBO is less evident. For instance, it was shown that the expression of peroxisome proliferator activated receptor γ (PPARγ) targets is reduced in the CF intestine, conceivably because of a shortage of activating ligands [7]. Also, it was shown that antibiotic treatment did not restore expression of genes involved in the processing/uptake of macro- and micro-nutrients, although it did improve weight gain [4,6].

These studies assessing the CF intestinal transcriptome have yielded important insights into the pathophysiology of CF-related intestinal disease. However, one limitation is that they were performed on animals reared on a lipid-enriched, low-carbohydrate/protein elemental liquid diet (Peptamen), which may have intrinsic effects on the CF gastrointestinal phenotype and the gut microbiota [8,9]. Also, only one of these studies specifically addressed gene expression in the distal small intestine, i.e. the region which is most susceptible to obstruction and SIBO, and has a key role in bile acid uptake [5]. In the present study, to exclude confounding effects of the dietary regimen, we

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assessed the ileal transcriptome of CF mice reared on a solid diet with a standard nutrient formulation. The CF mouse model used by us differs from the one presented in previous CF-related microarray analyses in that, probably because of secondary genetic and environmental factors, it does rarely suffer lethal intestinal obstructions in the adult stage, even when maintained on a conventional regimen (i.e. a solid chow and drinking water without added osmolytes). We employed a next-generation sequencing technique, RNAseq, that allows the quantification of RNA transcripts with single base-pair resolution, while maintaining a high dynamic detection range. To minimize variation in gene expression resulting from secondary genetic and environmental factors, we used sex (and age) matched littermate couples that, from birth onwards, were housed together. Recent advancements in the analysis of high-throughput gene expression data, specifically the application of algorithms that assess causal networks that are assimilated based on prior published research, enabled us to identify differentially regulated signaling pathways and their upstream modulators [10,11].

2. Materials and methods

2.1. Animal procedures and tissue collection

CF (*Cftr*−/−) mice (*Cftr*^{tm1Cam}; congenic FVB/n) and littermate controls (*Cftr* N/N) were obtained from a colony that was maintained by continual (> 20 generations) crossing of the heterozygous progeny. This colony was kept at an environmentally controlled facility at the Erasmus University Medical Center (microbial status: SPF; 12 h light/12 h dark cycle, 20–22 °C). CF/wildtype, sex-matched littermate couples were housed together in individually ventilated cages, and were reared on a low fiber diet (C1013; protein: 17.6%, fat: 5.1%, carbohydrates: 62.2%; Altromin) and a polyethylene glycol/electrolyte drinking solution to prevent intestinal obstruction in early life [12]. For transcriptome analysis, four such littermate couples were used (3F/1M), of which one couple (F) was administered drinking water supplemented with ciprofloxacin (0.3 g/L) and metronidazole (0.5 g/L) for 15 days [13]. Before tissue collection, animals were kept on normal drinking water (or, when applicable, drinking water supplemented with antibiotics) for ≥4 days. This procedure is well tolerated by the adult CF mice used presently for tissue collection (age: 12–17 weeks; body mass: 21–25 g), and none of the animals used showed signs of (imminent) intestinal obstruction. Behavior (e.g. gait, activity) preceding termination was normal, intake of food and water during this period was comparable between genotypes (not shown), and, when animals were sacrificed, the small intestine did not contain inspissated material. Experiments were approved of by the Independent Committee on Ethical Use of Experimental Animals, Rotterdam, according to national guidelines (141-1208; 120-0501/0902).

2.2. RNA isolation

Animals were anaesthetized (ketamine 120 mg/kg, xylazine 20 mg/kg; i.p.), and the intestinal tract was collected and flushed with ice-cold saline. Ileal sections (3–5 mm in length) were collected at 5–6 cm proximal to the ileocecal valve. Sampling from a CF animal (*Cftr*−/−) and a sex-matched littermate control (*Cftr* N/N) was performed within a time window of 20 min, and between 12:00–14:00 h, to control for diurnal variations in gene expression. Tissue was homogenized with a rotor-stator homogenizer in Trizol reagent (Qiagen), and total RNA was extracted using the Nucleospin RNA kit (Macherey-Nagel). The integrity of the extracted RNA was verified by gel electrophoresis.

2.3. Transcriptome sequencing

Transcriptome sequencing was performed at the Beijing Genomics Institute (BGI). In brief, after RNA integrity was verified (RIN > 9.0; Agilent 2100 Bioanalyzer), mRNA (200 ng) was isolated from total RNA (Truseq RNA sample preparation kit; Illumina), fragmented and subsequently used for cDNA synthesis (Superscript II cDNA synthesis kit; Invitrogen). End-repaired cDNA was purified (Agencourt Ampure XP beads; Beckman Coulter) and amplified by PCR. The resulting cDNA library was sequenced using an HiSeq 2000 sequencer and TruSeq SBS Kit v3-HS reagent kit (Illumina). Data were processed with CLC genomic workbench 7.5 (CLC Bio), and the sequence reads were mapped to the Genome Reference Consortium (GRC) genome data set GRCm38.76, using default parameters. Data depict reads per 1000 base pair transcript per million reads mapped (RPKM). Datasets are available through the NCBI-GEO repository (GSE92991).

2.4. Data analysis

Transcriptome data were analyzed using the Ingenuity® web-based software application (Version 1.10; Qiagen). Ingenuity pathway analysis (IPA) and upstream regulator analysis (URA) was applied to identify differentially regulated pathways and their upstream modulators, as described in detail elsewhere [10]. The enrichment of the dataset with up- or down-regulated genes comprised within a signal transduction network was assessed using Fisher's exact test (overlap P-value). To assess the activation state of a pathway and its upstream regulator, the consistency of the match between the observed and the predicted (based on established interactions within a network) expression pattern was calculated (activation Z-score). A positive Z-score signifies a regulator in the active state, a negative score indicates that its activity is repressed.

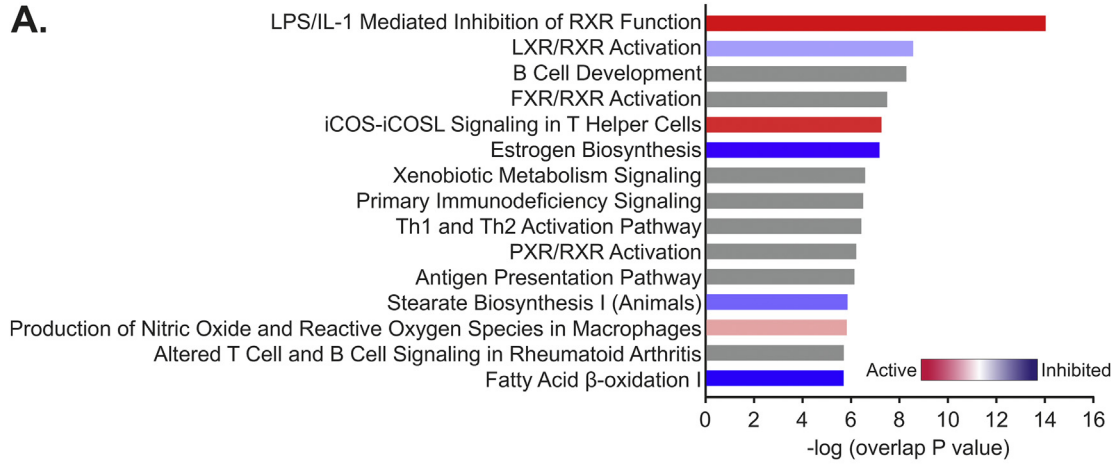
The gene set enrichment analysis (GSEA; Broad Institute) software platform (MSigDB version 6.1) was used to compare the observed expression profiles to a priori defined gene sets which represent well-defined biological processes (Hallmark and KEGG curated gene sets; Molecular Signatures database; Broad Institute) [14]. The degree to which genes in the predefined set are overrepresented at the extremes of the ranked list of transcripts in the sample is reflected by the enrichment score, as described in detail elsewhere [11]. After enrichment scores were normalized to allow for differences in gene set size, they were statistically evaluated using a correction for multiple hypotheses testing. The reported false discovery rate (FDR) is the estimated probability that the observed normalized enrichment score (NES) constitutes a false positive result.

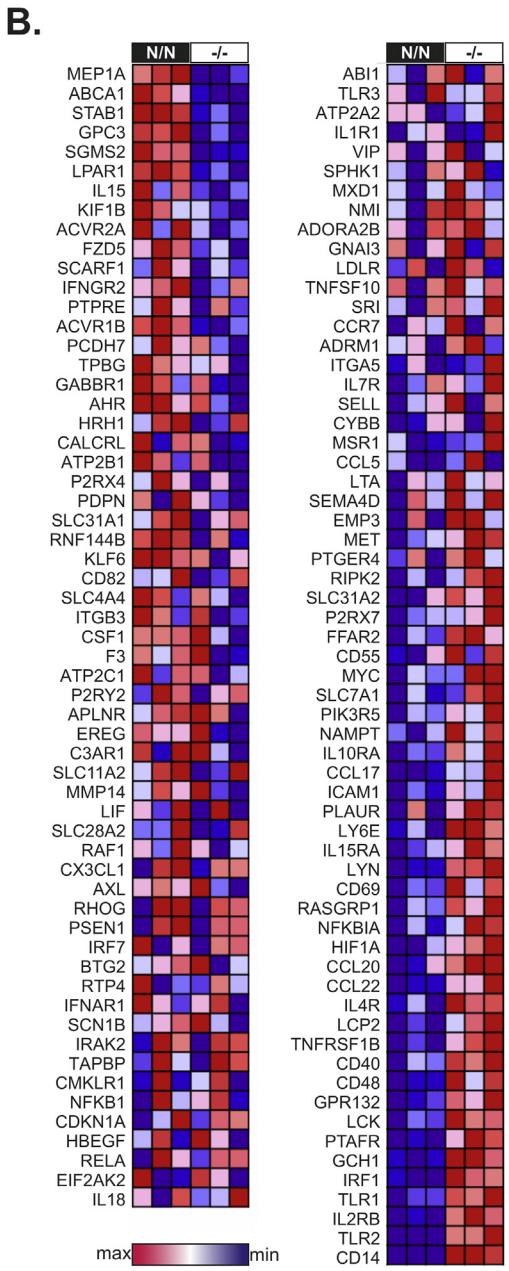
For identification of differentially regulated canonical signaling pathways in the ileum of CF mice (*Cftr*−/−), compared to controls (*Cftr* N/N), transcriptome data from 3 littermate couples (2F/1M) were used. For IPA, for each couple and transcript, expression in the CF animal was calculated relative to the respective control. Identification of differentially regulated signaling pathways was based on 2644 genes that were up- or down-regulated by a factor of > 1.25 in all couples. To assess the effect of the antibiotic treatment on ileal gene expression, for each genotype, the expression in one treated animal was expressed relative to the average expression level in the untreated animals. This analysis was performed on the 3937 and 2062 genes that were differentially regulated by a factor of > 1.5 in CF mice and controls, respectively. To minimize the contribution of genes with negligible expression, only genes expressed at > 1 RPKM in the conventionally reared group qualified for inclusion in the set of down-regulated genes, whereas only genes expressed at > 1 RPKM after antibiotic treatment were eligible for inclusion in the set of up-regulated genes. For GSEA, no prior selection was made and the analysis was based on the entire expression data set.

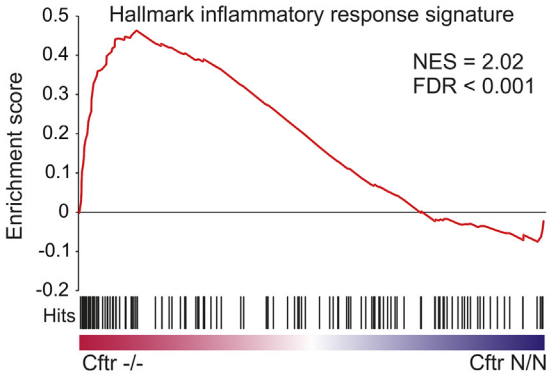
3. Results

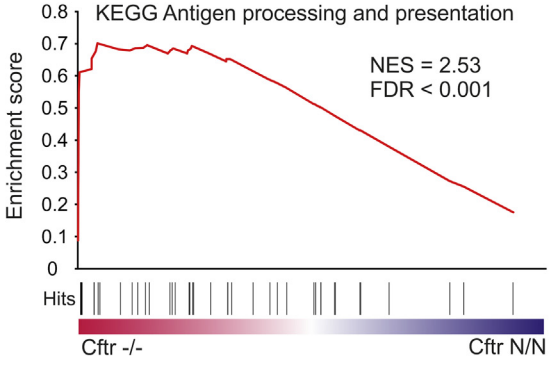
3.1. Activation of the innate and adaptive immune response in the CF ileum

IPA employs prior knowledge of cause-effect relationships in signaling networks to interpret gene-expression datasets, providing









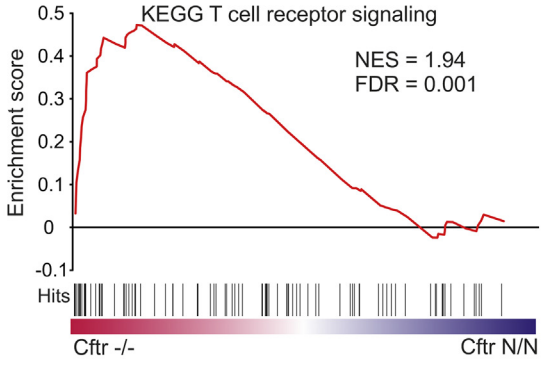


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Fig. 1. Differential regulation of canonical signal transduction pathways in the murine CF ileum. A. IPA was used to assess the enrichment of the dataset with up- or down-regulated genes that make up predefined signal transduction networks. Depicted are the 15 canonical pathways with the lowest overlap P-value. Bars are colored to indicate the activation Z-score of the pathway. Gray bars indicate pathways that are ineligible for a prediction. B. Heat map depicting relative transcript levels of genes comprised in the GSEA hallmark inflammatory response set, in CF (−/−) and wildtype (N/N) mice. Gene symbols refer to the human orthologues. C. Corresponding enrichment plot of the hallmark inflammatory response gene set. D. Enrichment plots of the KEGG antigen processing and presentation, and T cell receptor signaling gene sets.

information on the direction of regulation of such signaling networks. We compared the ileal transcriptome of CF mice and wildtype littermates by IPA. This identified numerous differentially regulated canonical signaling pathways. The most affected canonical pathways (based on the overlap P-value) represent immunological responses and pathways involved in lipid metabolism that are regulated by ligand-dependent nuclear receptors (Fig. 1A). Many of the up-regulated genes comprised in these partially overlapping pathways encode proteins involved in antigen processing or (co-)receptors and ligands that form the immunological synapse between antigen-presenting and CD4+ cells (Table S1). This includes molecules involved in antigen association with MHC molecules and their presentation to the T cell receptor (TCR) complex, several (co-)receptors and cytokines that stimulate differentiation of naïve CD4+ cells into T helper cells, and the iCOSL-iCOS (inducible T-cell co-stimulator ligand/receptor) signaling axis, which contributes to the regulation of activated T cells. In line with T helper cell activation, many genes involved in B cell development were also up-regulated.

GSEA evaluates transcriptome data in the context of predefined gene sets, which are compiled on the basis of previous gene expression analyses. We used it as an independent platform to validate the IPA. Congruent with IPA, GSEA indicated that the hallmark inflammatory response gene set, and the KEGG gene sets representing antigen processing and presentation, and T cell receptor signaling were up-regulated in CF (Fig. 1B–D). Collectively, these data point towards the activation of an innate and adaptive immune response and the recruitment of immune cells in the CF gut.

URA employs prior knowledge of the interactions within signaling cascades to identify molecules that potentially cause the changes in gene expression observed in the data set [10]. Testament to its ability to correctly infer such underlying causes, URA identified CFTR as an upstream regulator that is in an inhibited state in the CF ileum (Table 1). URA indicated that the CF intestinal gene expression profile is most consistent with enhanced exposure to bacterial lipopolysaccharide (LPS). Further, URA identified molecules operating downstream of LPS, like the Toll-like receptor (TLR4) and the T cell receptor complex (TCR), pro-inflammatory transcription factors (nuclear factor κB, NFκB; signal transducer and activator of transcription, STAT1), and pro-inflammatory cytokines, including IFNγ, IL-1β, IL2, IL-6 and TNFα. The transcription regulator c-Myc and cytokines such as CSF and HGF are involved in cell cycle progression and cellular transformation. Apart from CFTR, among the most significantly inactivated molecules identified by the URA are the anti-inflammatory IL-10 receptor and the PPARα/γ nuclear receptors (see below).

GSEA mirrored these findings, showing up-regulation of gene sets marking TLR, IFNα, IFNγ, IL2-STAT5, IL6-JAK-STAT3, TNFα-NFκB signaling, and hematopoietic cell development (Table 2). It also showed activation of the hallmark c-Myc and E2F targets gene sets in the CF ileum. The activation of c-Myc and E2F-type transcription factors may reflect proliferation of lymphoid and myeloid lineages, but can also point towards enhanced proliferation of epithelial cells [15].

Table 1
URA of the CF ileum. URA was applied to assess the activation state of molecules that potentially cause the changes in gene expression observed in the data set. Depicted are molecules with |Z-score| > 4.

Molecule	Category	Z-score	−log(overlap P-value)
Activated in CF			
TNF	Cytokine	8.75	35.3
lipopolysaccharide	Toxicant	8.16	45.2
IFNG	Cytokine	7.51	33.4
CSF2	Cytokine	7.07	18.6
E. coli B5 lipopolysaccharide	Toxicant	6.30	9.7
IL1B	Cytokine	6.24	22.4
CD28	Transmembrane receptor	5.61	5.3
CD3	Complex	5.61	13.8
E. coli B4 lipopolysaccharide	Toxicant	5.41	11.9
IL6	Cytokine	5.39	26.7
CD40LG	Cytokine	5.28	12.4
IL5	Cytokine	5.20	17.2
CD38	Enzyme	5.15	11.8
IL2	Cytokine	5.12	12.5
IL1A	Cytokine	5.11	3.5
ERBB2	Kinase	4.63	22.1
IL27	Cytokine	4.61	6.3
NFkB (complex)	Complex	4.59	10.7
RELA	Transcription regulator	4.49	12.3
IL3	Cytokine	4.46	12.2
STAT1	Transcription regulator	4.45	20.5
IL15	Cytokine	4.41	13.7
HGF	Growth factor	4.25	14.4
TLR4	Transmembrane receptor	4.17	8.7
MYC	Transcription regulator	4.16	19.3
PDGF BB	Complex	4.15	12.3
IL1	Group	4.12	6.2
Interferon alpha	Group	4.12	10.1
CSF1	Cytokine	4.09	7.1
IL4	Cytokine	4.08	21.6
F2	Peptidase	4.06	8.1
TCR	Complex	4.04	12.7
CCND1	Transcription regulator	4.04	3.4
OSM	Cytokine	4.02	13.3
Inactivated in CF			
IL10RA	Transmembrane receptor	−8.97	39.9
CFTR	Ion channel	−6.30	32.1
PPARA	Ligand-dependent NR	−5.57	40.2
POR	Enzyme	−5.11	28.2
PPARGC1A	Transcription regulator	−5.04	6.9
PPARG	Ligand-dependent NR	−4.49	22.7
RICTOR	Other	−4.47	3.1
let-7	Microrna	−4.09	10.3

3.2. LPS represses the activity of type II ligand-dependent nuclear receptors in the CF ileum

The IPA indicated that the release of LPS and IL-1β inhibits the activity of the retinoid X receptors (RXRα/β) in the CF ileum (Fig. 1A; Table S1). Previous studies have shown that LPS leads to transrepression of RXRα and reduces its nuclear localization in hepatocytes [16–18]. Because the RXRs form obligate heterodimers with type II ligand-dependent nuclear receptors, including the farnesoid X receptor (FXR; *NR1H4*), the liver X receptor (LXR; *NR1H2/3*), the

Table 2
GSEA of CF ileum. Enrichment scores of hallmark and KEGG gene sets representing pathways involved in cell cycle control and immunity.

Gene set	Size	NES	FDR
Hallmark Myc targets v1	190	3.04	< 0.001
Hallmark E2F targets	181	3.02	< 0.001
Hallmark IFNγ response	170	2.95	< 0.001
Hallmark Myc targets v2	56	2.56	< 0.001
Hallmark IL6-Jak-Stat3 signaling	63	2.52	< 0.001
KEGG Hematopoietic cell lineage	49	2.38	< 0.001
Hallmark IFNα response	81	2.21	< 0.001
KEGG Toll-like receptor signaling	68	2.05	< 0.001
Hallmark TNFα signaling via NFκB	152	1.83	0.002
Hallmark IL2-Stat5 signaling	150	1.82	0.002

pregnane X receptor (PXR; *NR1I2*) and PPARα/δ/γ, this markedly affects the hepatic expression of genes involved in carbohydrate and lipid metabolism. Several of the top canonical pathways identified by IPA are linked to these functions (Fig. 1A). In the CF ileum, among the most strongly down-regulated genes are the PXR-induced carboxylesterase *Ces2a* (*CES-*), the FXR targets *Fgf15* (human orthologue: *FGF19*) and *Nr0b2*, involved in intestinal bile acid transport and hepatic bile acid synthesis, and the PPARα target *Pdk4*, which encodes a pyruvate dehydrogenase kinase that, by repression of the pyruvate dehydrogenase complex, restricts glycolysis and enhances fatty acid oxidation (Fig. 2A) [19]. The set of RXRα/β-controlled genes identified by IPA further includes members of the ATP binding cassette family (*Abca1*, *Abcb1a*, *Abcg5*, *Abcg8*), which encode proteins that facilitate transport of sterols and xenobiotics to the intestinal lumen, and the fatty acid binding proteins involved in the transport of bile acids and fatty acids [17,20–22]. Also down-regulated are genes of the aldehyde dehydrogenase, and the cytochrome P450 and the flavin-containing monooxygenase families, involved in xenobiotic and lipid metabolism. GSEA corroborated these findings, demonstrating down-regulation of the hallmark gene sets representing bile acid, fatty acid and xenobiotic metabolism (Fig. 2B).

3.3. Differential regulation of genes involved in intestinal barrier function

Inflammation of the gut wall has been shown to increase both the permeability of the paracellular route to ions and water and to affect active, transcellular transport of solutes across the epithelium [23–25].

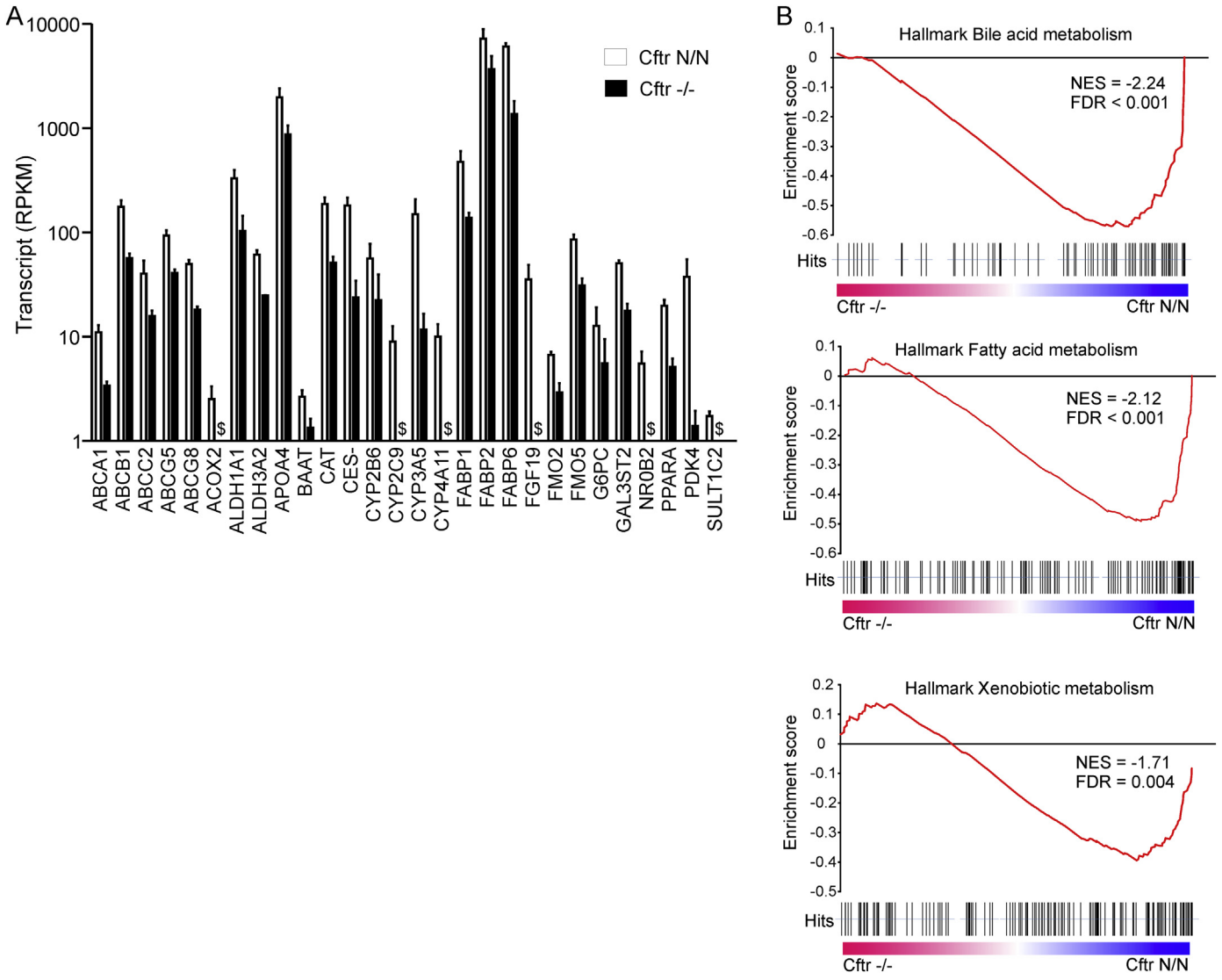

Fig. 2. Impaired type II ligand-dependent nuclear receptor function. A. Transcript levels of IPA-defined genes that are regulated by type II ligand-dependent nuclear receptors and involved in the metabolism and transport of fatty acids, sterols, bile acids and xenobiotics. Gene symbols refer to the human orthologues. $: < 1 RPKM. Data shown are mean ± SE of 3 animals per group (2F/1M). B. Enrichment plots of gene sets representing bile acid, fatty acid and xenobiotic metabolism.

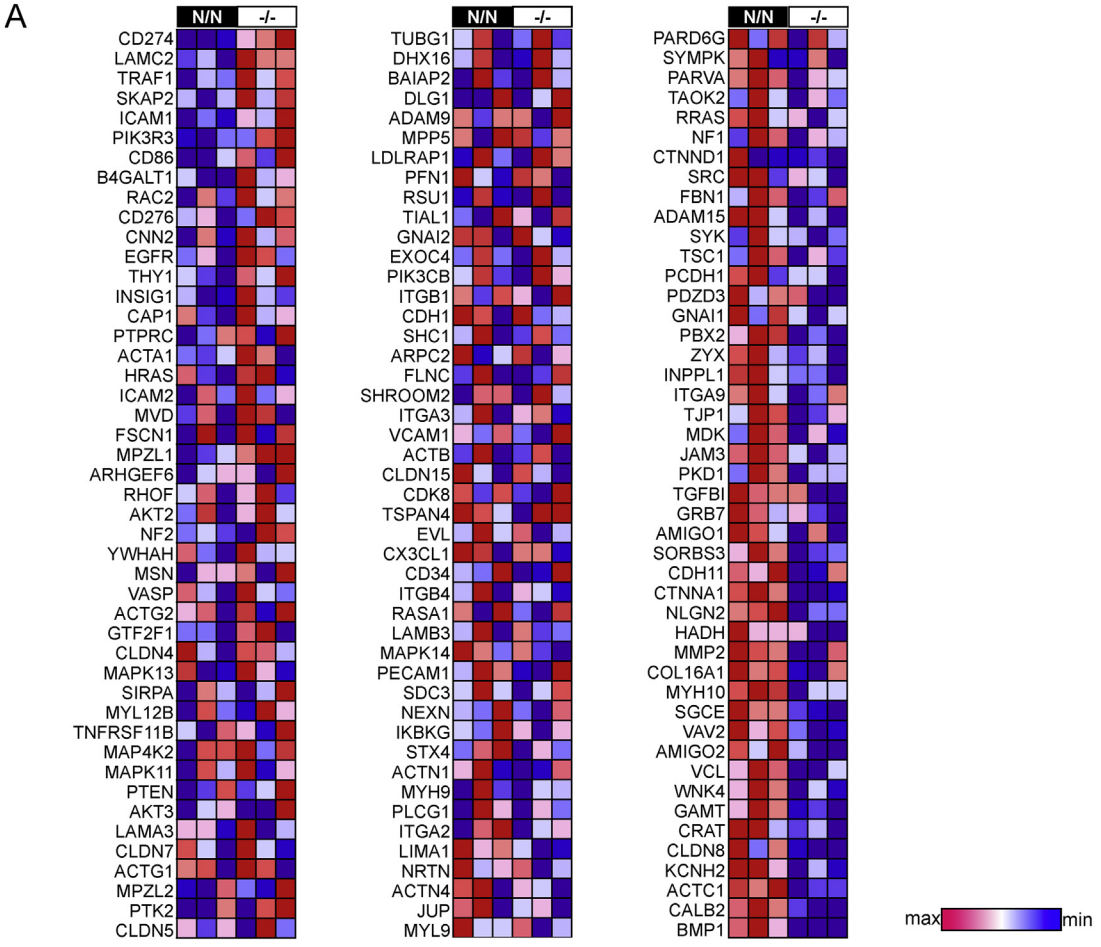

Fig. 3. Differential regulation of genes involved in intestinal barrier function. A. Heat map depicting relative transcript levels of genes comprised in the GSEA hallmark apical junction set, in CF (−/−) and wildtype (N/N) mice. B. Enrichment plot of the gene set representing the apical junction complex. C. Transcript levels of genes that encode proteins that make up the tight junctions and are expressed at > 1 RPKM. Data shown are mean ± SE of 3 animals per group (2F/1M).*P < .05; **P < .01 (Paired *t*-test). Gene symbols refer to the human orthologues.

Inflammation modulates the expression and localization of proteins that form the tight junctions, and is typically associated with up-regulation of the pore-forming claudin-2, whereas the expression of barrier-forming claudins is reduced [23]. Correspondingly, in CF mice, it was shown previously that small intestinal expression of *Cldn2* was increased, whereas the expression of some barrier-forming claudins (*Cldn1*, *Cldn8*) was reduced [26]. However, it has also been argued that, in CF patients, paracellular chloride absorption in the jejunum is reduced, which implies a reduction in chloride permeability of the tight junctions [27].

Presently, IPA did not signal overt barrier dysfunction in CF mice, but GSEA did indicate differential expression of genes in the hallmark

set that represents the apical junction complex (Fig. 3A, B). However, the leading edge of the enrichment plot contained numerous genes involved in the formation of focal adhesions, which are not restricted to epithelia (e.g. *Itga9*, *Pkd1*, *Sgce*, *Vcl*, *Zyx*), and the differential regulation of these genes may point to remodeling of non-epithelial tissues. Nevertheless, the set also included genes encoding bona fide epithelial markers, like *Cldn8* and *Tjp1* (ZO-1). Transcript levels of *Cldn8*, which is expressed at comparatively low levels in the ileum, were lowered circa 5-fold in the CF ileum, compared to controls, whereas transcript levels of the barrier-forming *Cldn3* were marginally (< 1.5-fold), albeit consistently, reduced (Fig. 3C). We observed a modest increase (ca. 1.5-fold) in *Cldn2* transcript levels in only two out of the three couples analyzed. Transcript levels of the other major cation-selective pore-forming claudin, *Cldn15*, which serves as a sodium shunt required for the operation of sodium-coupled solute transporters, was unaffected [28]. Next to the claudins, occludin (*Ocln*), tricellulin (*Marveld2*) and marvelD3 (*Marveld3*) form a distinct family of tight junction proteins. In CF ileum, trancript levels of *Ocln* were marginally reduced (< 1.5-fold), whereas levels of *Marveld3* were increased circa 1.5-fold. *Marveld2* transcript levels were not affected. Also, transcript levels of genes encoding proteases involved in the dynamic regulation of intercellular junctions (*Adam19*, *St14*, *Ctsk*, *F11r*) were similar between genotypes (not shown). However, we did observe a consistent reduction (> 2-fold in all 3 couples) in *Wnk4* transcript levels in the CF ileum (1.3 ± 0.3 vs. 2.8 ± 0.6 RPKM in CF and control mice, respectively; $P < .01$, $N = 3$). In renal epithelia, this protein kinase is localized to tight junctions and its activation enhances paracellular chloride permeability, conceivably through phosphorylation of specific claudins that form anion-selective pores [29,30].

3.4. CF is associated with down-regulation of genes involved in the absorption of monosaccharides, amino acids and B vitamins

Apart from a reduced activity of ligand-dependent nuclear receptors that form heterodimers with RXRα/β, URA signaled a marked inactivation of the transcription factor hepatocyte nuclear factor 4α (HNF4α; Z-score: −3.76, overlap P-value: $1.13 \cdot 10^{-17}$). In the liver, HNF4α controls many genes involved in glucose and lipid metabolism, in part through crosstalk with ligand-dependent nuclear receptors such as PPARα. In the intestine, HNF4α function is associated with enterocyte maturation as many of the genes induced by HNF4α are involved in nutrient absorption and processing [15,31,32]. Thus, the set of established intestinal HNF4α targets includes genes involved in lipid metabolism also targeted by type II ligand-dependent nuclear receptors (*Apoa4*, *Fabp1*), as well as genes encoding brush border ectoenzymes such as alkaline phosphatase (*Alpi*), alanyl aminopeptidase (*Anpep*), lactase (*Lct*), meprin 1 (*Mep1a*) and trehalase (*Treh*) [15,32].

Consistent with repression of HNF4α, we detected low *Alpi* and *Mep1a* transcript levels in the ileum of CF mice. Also, we observed a strong reduction in the transcript levels of lactase (*Lct*), whereas trehalase (*Treh*) transcript levels were reduced to a more moderate extent (Fig. 4A). In contrast, expression of other brush border disaccharidases, i.e. maltase-glucoamylase (*Mgam*) and sucrose-isomaltase (*Sis*), was similar in CF and wildtype mice. Further, we found that the expression of the major monosaccharide transporters located in the brush border membrane, GLUT5 (*Slc2a5*) and SGLT1 (*Slc5a1*), was similar in both genotypes.

Of the major aminopeptidases, only glutamyl aminopeptidase (*Enpep*) transcript levels showed a moderate reduction in the CF ileum, compared to controls, whereas the expression of alanyl aminopeptidase (*Anpep*) was not significantly affected (Fig. 4B). The expression of the sodium-dependent amino acid transporters *Slc6a19* and *Slc6a20a* (*SLC6A20*) was modestly reduced in CF mice. No statistically significant difference was observed in the expression of the PEPT1 dipeptide transporter (*Slc15a1*).

Intriguingly, we noted strong induction of *Slc6a14*, encoding a broad-specificity amino acid transporter (ATB0,+), in the CF intestine: whereas expression was negligible (< 1 RPKM) in ileum of controls, this gene was robustly expressed in the ileum of CF mice (Fig. 4B). It has been proposed that ATB0,+ is required for nitric oxide production in inflamed tissue, as it facilitates cellular uptake of arginine, a substrate of the inducible nitric oxide synthase (*Nos2*) [33]. Commensurate with this hypothesis, we found that *Nos2* transcript levels were also elevated in CF mice, compared to controls (79.6 ± 9.9 vs. 22.3 ± 4.6 RPKM in CF and control mice, respectively; $P < .05$, $N = 3$). Consistent with an altered host-microbe interaction, we also observed a strong induction of *Fut2* in CF ileum (7.4 ± 2.3 vs. 0.3 ± 0.04 RPKM in CF and control mice, respectively; $P < .05$, $N = 3$). This epithelial fucosyltransferase plays a key role in host-microbe interactions, and is induced by bacterial colonization of the gut and, in particular, by LPS [34,35]. Increased *Fut2* expression has been observed before in CF mice [36].

The ileum plays a crucial role in the enterohepatic circulation of bile acids and cobalamin (vitamin B12). Therefore, we also investigated the expression of plasma membrane proteins involved in the uptake of this and other water-soluble vitamins, and of the apical sodium-dependent bile acid transporter (*Slc10a2*). We found that transcript levels of *Cubn*, which encodes the cobalamin receptor cubilin, were strongly (> 10-fold) reduced in the CF ileum, compared to control tissue, whereas the transcript levels of amnionless (*Amn*), which is also thought to be involved in cobalamin uptake, were more moderately reduced (Fig. 4C). Further, we observed a moderate reduction in the expression of the sodium-dependent multivitamin transporter (*Slc5a6*), principally involved in biotin and panthothenic acid uptake. The expression of the pantetheine hydrolase ectoenzyme vanin-1 (*Vnn1*), a PPARα target required for intraluminal production of panthothenic acid from its non-absorbable precursors, was also markedly (> 3-fold) lower in the CF ileum than in control tissue [37]. The expression of ascorbic and folic acid transporters (*Slc23a1* and *Slc46a1*, respectively) and of *Slc10a2* was not affected.

Finally, we assessed the expression of some major intestinal solute transporters. These facilitate either sodium and chloride uptake via proton (*Slc9a3*) and bicarbonate (*Slc26a3*, *Slc26a6*) antiport, respectively, or mediate sulfate/citrate/succinate (*Slc13a1/2*), carnitine (*Slc22a5*), nucleoside (*Slc28a1/2*) and phosphate (*Slc34a2*) uptake via sodium symport. This analysis showed that transcript levels of the organic cation/carnitine transporter OCTN2 (*Slc22a5*) and the concentrative nucleoside transporter 1 (CNT1; *Slc28a1*) were significantly (ca. 2- and 20-fold, respectively) lower in CF than in control tissue (Fig. 4D). The expression of the other solute transporters in this category was not notably affected, but expression of *Pdzk1*, encoding the adaptor protein PDZK1 (NHERF3), was 4-fold lower in CF than in control tissue. PDZK1 interacts with several solute transporters and other proteins in (or at) the plasma membrane of intestinal epithelial cells [38]. Most prominently, it is required for regulation of the sodium-proton exchanger type 3 (NHE3/*SLC9A3*) and, consequently, sodium absorption [38,39]. Transcript levels of another adaptor protein involved in NHE3 regulation, *Slc9a3r1* (NHERF1), were also lowered in the CF ileum, albeit only to a moderate extent (< 2-fold). Similarly modest reductions in transcript level were observed for *Slc9a3r2* (NHERF2) and *Pdzd3* (NHERF4).

3.5. Effect of antibiotic treatment on intestinal gene expression in CF mice

Assuming that increased exposure of the intestinal epithelium to bacterial endotoxins does indeed lead to repression of signaling through RXR and other ligand-dependent nuclear receptors, we reasoned that measures to counteract SIBO would restore their activity. To test this, we treated animals with an antibiotic regimen that was previously shown to reduce the bacterial load in CF while increasing community diversity, leading to a convergence of the microbiome composition of CF and wildtype mice [13]. While this regimen had a marked effect on ileal gene expression in both CF and wildtype mice, judging from the

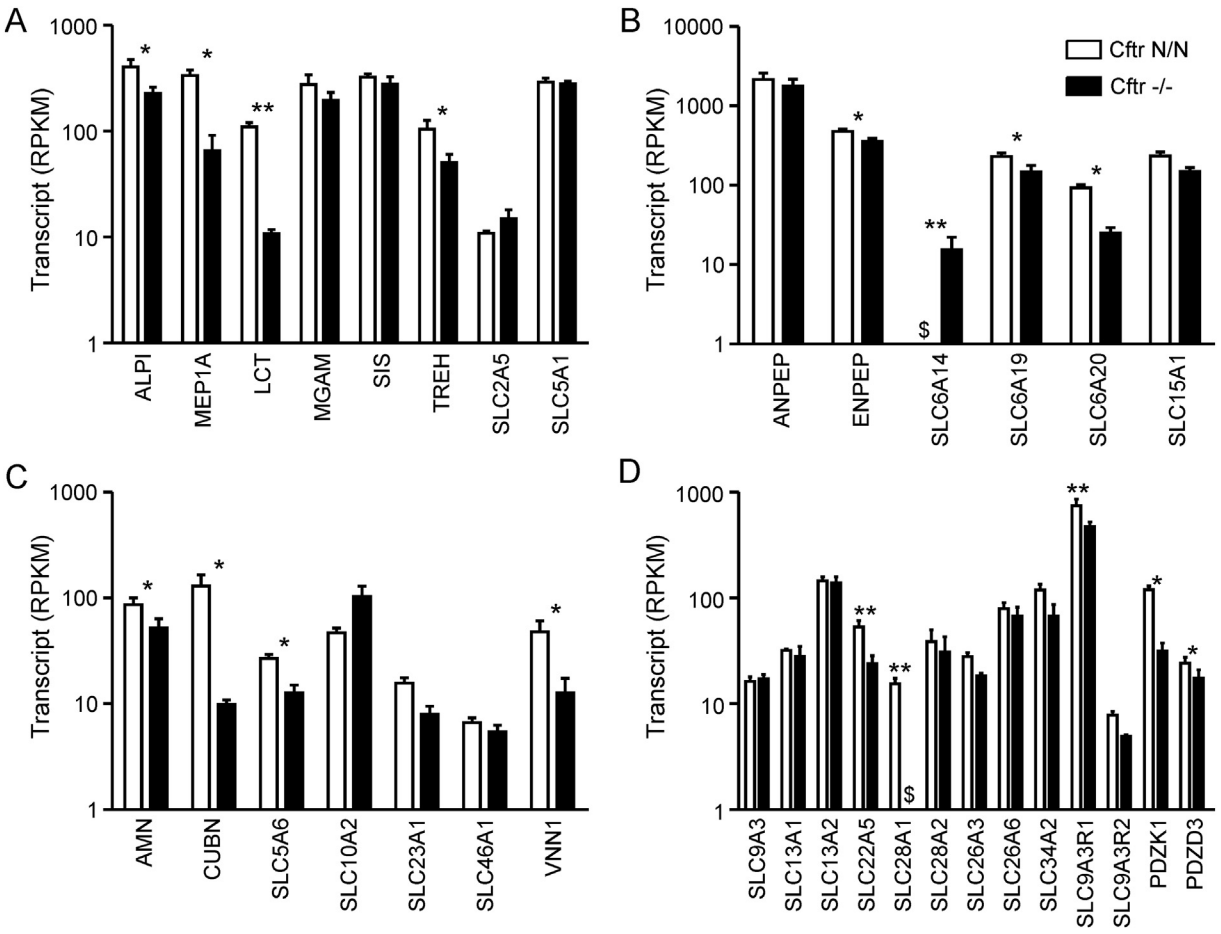

Fig. 4. Transcript levels of genes encoding proteins located at the apical plasma membrane of intestinal epithelial cells in the ileum of CF mice and controls. A. Transcript level of genes encoding alkaline phosphatase, meprin1 and membrane proteins involved in the enzymatic release and uptake of monosaccharides. B. Transcript level of genes encoding membrane proteins involved in the enzymatic release and uptake of oligopeptides and amino acids. C. Transcript level of genes encoding membrane proteins involved in the uptake of vitamins and bile acids. D. Transcript level of genes encoding solute transporters and adaptor proteins involved in the uptake of sodium, chloride, nucleosides, phosphate and sulfate. Data shown are mean ± SE of 3 animals per group (2F/1M). $: < 1 RPKM. *P < .05;**P < .01 (Paired *t*-test). Gene symbols refer to the human orthologues.

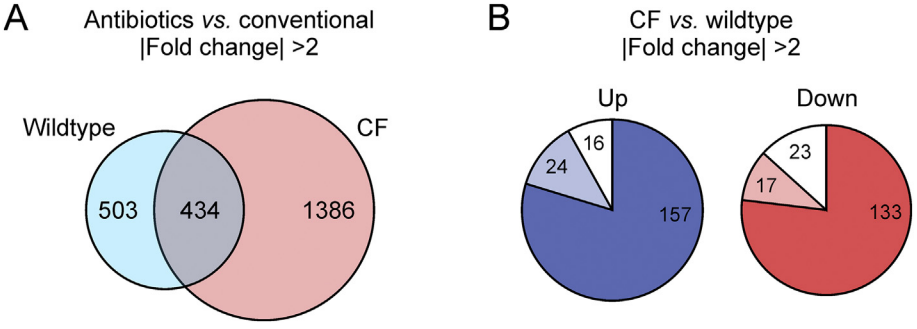

Fig. 5. Effect of antibiotic treatment on intestinal gene expression in CF and wildtype mice. A. Venn diagram representing the set of genes of which transcript levels were increased or decreased > 2-fold by antibiotic treatment in CF and/or wildtype mice. B. Effect of antibiotic treatment on the set of genes that are up- or down-regulated in CF ileum by a factor of > 2 in all 3 couples. Colored segments of the pie charts represent the fraction of these differentially regulated genes that are up- or down-regulated by antibiotic treatment by a factor > 1.5. The deep-colored segments represent genes up- or down-regulated by a factor > 2.

number of genes that were up- or down-regulated in the respective genotypes, it had the largest impact in CF mice (Fig. 5A). Antibiotic treatment (partially) corrected the expression of 331 genes out of a total set of 370 that were consistently up- or down-regulated by a factor > 2 in CF compared to wildtype mice (Fig. 5B).

The URA was used to assess the effect of antibiotic treatment on the activation state of key inflammation modulators in the ileum of CF and wildtype mice. This analysis showed that antibiotic treatment reduced the activation state of typical inflammation modulators in both genotypes, which indicates that these immune modulators also shape the normal host response to the gut microbiota (Fig. 6A). In the CF ileum, the antibiotic treatment reduced their activity to (or below) the levels observed in conventionally reared wildtype mice (Fig. 6B). This suggests that antibiotic treatment effectively reduces bacterial numbers and exposure to LPS, and attenuates the attendant immune response to a level normally observed in the mouse intestine. Congruently, using conventional RT-qPCR to assess gene expression in a larger cohort, we found that after antibiotic treatment, transcript levels of typical inflammation markers in CF mice and controls converge (Fig. S1).

Consistent with reduced LPS exposure, IPA indicated that the activity of the LPS/IL1-mediated inhibition of RXR pathway was reduced after antibiotic treatment (Z-score: −2.50, overlap P-value: $5.77 \cdot 10^{-5}$), and URA showed that the activation state of type II ligand-dependent nuclear receptors tended to improve (Fig. 6C). This was also reflected

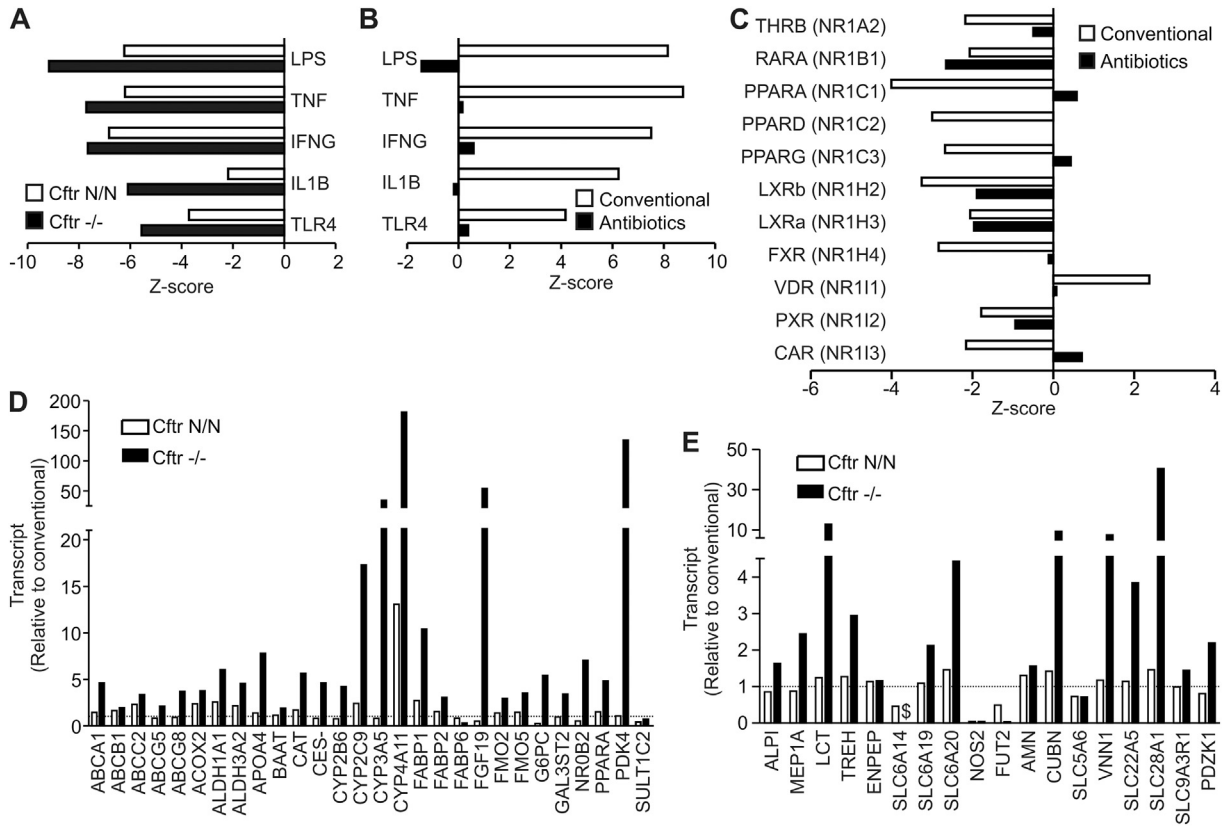

Fig. 6. Effect of antibiotic treatment on the expression of genes involved in the inflammatory response, lipid metabolism, and solute transport. A. The URA was used to assess the activation state of inflammation modulators in antibiotic-treated CF and control mice, relative to conventionally reared animals of the same genotype. B. The activation state of inflammation modulators in antibiotic-treated CF mice, relative to conventionally reared wildtype mice. For comparison, the activation state in conventionally reared CF mice is included (data as in Table 1). C. Activation state of ligand-dependent nuclear receptors in CF mice, before and after antibiotic treatment, compared to conventionally reared wildtype mice. D. Effect of antibiotic treatment on the expression of genes involved in the metabolism and transport of fatty acids, sterols, bile acids and xenobiotics (see Fig. 2A). Data depict transcript levels relative to conventionally reared animals of the same genotype. Gene symbols refer to the human orthologues The horizontal, dotted line denotes a relative transcript level of 1 (no change). E. Effect of antibiotic treatment on transcript levels of genes encoding enzymes, solute transporters, receptors or adaptor proteins that are differentially regulated in CF mice and controls. Data depict transcript levels relative to conventionally reared animals of the same genotype. Gene symbols refer to the human orthologues. The horizontal, dotted line denotes a relative transcript level of 1 (no change). $: No transcript detected.

by the fact that expression of most of the genes involved in lipid metabolism that are controlled by such nuclear receptors was restored (Fig. 6D).

We also noted that antibiotic treatment reversed the observed changes in the expression of genes involved in the absorption of monosaccharides, amino acids, nucleosides and B vitamins. Antibiotic treatment strongly stimulated expression of *Cubn*, *Lct*, *Slc6a20*, *Slc28a1* and *Vnn1* in CF mice, and led to a more moderate induction of *Alpi*, *Mep1a*, *Pdzk1*, *Slc6a19*, *Slc9a3r1* and *Treh* (Fig. 6E). In contrast, *Fut2*, *Nos2* and *Slc6a14* transcript levels were strongly reduced by antibiotic treatment. In wildtype mice, antibiotic treatment had comparatively little effect on the expression of these genes, except for *Nos2*. Thus, these data show that antibiotic treatment corrects many aspects of anomalous gene expression in the CF ileum.

4. Discussion

In CF, intestinal dysbiosis is thought to be a key factor in the onset of intestinal inflammation [2]. Presently, transcriptome analysis showed unequivocal evidence for altered host-microbe interactions and the activation of an innate and adaptive immune response in the distal small intestine of a CF mouse model that is triggered by bacterial endotoxins. Gut inflammation was associated with a marked overall reduction in the activity of type II ligand-dependent nuclear receptors, which is predicted to strongly affect fatty acid, sterol, bile acid and xenobiotic metabolism and transport. Apart from these marked effects on lipid handling, we noted that CF is associated with reduced expression of genes involved in the absorption of other nutrients. Most of these changes in gene expression could be corrected by antibiotic treatment, suggesting that dysbiosis markedly affects enterocyte maturation in CF.

For the transcriptome analysis, congenic, sex-matched littermate couples (*Cftr* N/N, *Cftr* −/−) were used, and each pair of animals was housed together, to minimize bias due to environmental factors. Conceivably because of this pairing strategy, the differences in gene expression observed between CF and wildtype mice were remarkably consistent between couples, albeit that the analysis is based on only a limited number of animals. To further validate the data, we assessed expression of a select number of genes by RT-qPCR in a larger cohort and found the results to be in line with the RNAseq data (e.g. Fig. S1). However, as both female and male couples were analyzed jointly, potential sex-related differences in the response to *Cftr* deletion were not accounted for.

Both IPA and GSEA indicated activation of the LPS receptor complex, and T and B cell responses in the CF ileum. This signals the recruitment and differentiation of immune cells in response to a microbial challenge. These findings are in line with previous studies demonstrating immune cell infiltration and SIBO in the CF gut [3–5]. A previous microarray analysis has also shown up-regulation of genes associated with the innate immune response, but did not yield clear signs

for the development of an adaptive immune response [3]. Methodological differences may underlie this disparity. Foremost, we exclusively sampled the ileum (instead of the entire small intestine), which generally contains more bacteria than the proximal sections of the small intestine and, consequently, may be more susceptible to inflammation. Further, we employed a dietary regimen that avoided the use of a liquid elemental diet and its inherent effects on small intestinal bacterial counts [8]. For similar reasons, mice were kept on normal drinking water (without added osmolytes) as of several days before experimentation. It has previously been shown that bacterial overgrowth is readily diminished by application of a polyethylene glycol/electrolyte drinking solution, probably because it accelerates intestinal transit [40]. Of note, we found that this regimen did not fully normalize gene expression in CF mice: the down-regulation of *Cubn*, the striking up-regulation of *Fut2* and *Slc6a14*, as well as the induction of inflammation markers (e.g *Ccl8*, *Saa1*, *Ubd*) evident from the transcriptome dataset, was also observed in CF mice that were not withheld laxative before sampling (Fig. S2). This suggests that administration of polyethylene glycol/electrolyte drinking solution does not correct dysbiosis completely. We speculate that in particular mucosa-attached communities persist and modulate the host transcriptome [41].

The IPA indicated that signaling through type II ligand-dependent nuclear receptors is markedly repressed in the CF intestine, as a consequence of LPS/TLR-mediated activation of pro-inflammatory signaling pathways that lead to repression of RXRα/β. LPS, a component of the outer membrane of gram-negative bacteria, triggers the production of pro-inflammatory cytokines by macrophages and other immune cells by activation of the LPS receptor complex [42]. In liver, this produces an acute phase response, which is characterized by distinct changes in several major metabolic pathways that are largely controlled by type II ligand-dependent nuclear receptors [18,43]. LPS and pro-inflammatory cytokines were shown to cause NFκB-mediated transrepression of hepatic RXRα, and to decrease its nuclear levels [16–18]. In addition, NFκB may tether FXR, leading to a comprehensive repression of FXR-RXRα target genes [44,45]. The gut microbiota is known to modulate the activity of FXR by intra-luminal conversion of primary bile acids, which limits the level of FXR-antagonistic species, but may also reduce uptake of the FXR-activating type [46,47]. Our data indicate that dysbiosis, in addition, represses FXR through its effects on the host immune response.

RXRα/β heterodimer nuclear receptors play a key role in the metabolism of fatty acids, sterols, bile acids and xenobiotics. Altered fatty acid, sterol and bile acid transport and metabolism are typical of CF, suggesting that intestinal repression of RXRα/β heterodimer nuclear receptors contributes to the development of CF-related pathologies. For instance, a deficiency of the intestinal FXR target FGF15/FGF19, or loss of the receptor for this hormone (FGFR4), was shown to enhance fecal excretion of bile acids, and to deplete the gall bladder, respectively [48–51]. Similarly, the changes in the expression of genes involved in intestinal lipid handling we present here may play a role in aberrant fatty acid and cholesterol transport and the changes in tissue lipid composition observed in CF patients and mouse models [52–57]. Repression of RXRα/β may also contribute to the previously reported loss of intestinal PPARγ activity in CF mice [7,58]. Interestingly, a reduction in butyrate-producing obligate anaerobes was shown to attenuate PPARγ signaling in mouse colon, leading to induction of *Nos2* and repression of fatty acid β-oxidation, i.e. metabolic changes that parallel those observed presently in CF ileum [59]. The consequent increase in nitrate production and decrease in oxygen consumption was proposed to create a niche for facultative anaerobes, including pathogenic *Enterobacteriaceae*.

Apart from these wide-ranging effects on the expression of genes involved in lipid handling, we also observed discrete changes in the expression of genes involved in the absorption of monosaccharides, amino acids, nucleosides and B vitamins. The most significant changes were observed in the transcript levels of *Cubn*, *Lct*, *Pdzk1*, *Slc28a1* (all markedly reduced in CF ileum). Lowered intestinal expression of *Cubn*, *Lct* and *Pdzk1* has been found in earlier transcriptome analyses on CF mice [3,5], and lowered intestinal lactase, as well as alkaline phosphatase, levels have previously been reported for CF patients [60]. We noted that the down-regulation of *Cubn*, *Lct* and *Pdzk1* observed in the CF ileum is reversed by antibiotic treatment, suggesting that the CF microbiome also modulates the expression of these genes. It has been shown that LPS-provoked acute endotoxemia reduces *Cubn* expression in the kidney [61]. In CF mice, cubilin protein levels in the proximal tubules were also reduced, resulting in a mild (low molecular weight) proteinuria, but *Cubn* expression was apparently not affected [62]. Down-regulation of *PDZK1* (NHERF3) has been observed before in CF mice, and in intestinal tissue of ulcerative colitis patients and murine colitis models, suggesting that the expression of this gene is also controlled by inflammation mediators [5,63]. Indeed, in Caco2-BBE cells, IL-1β repressed *PDZK1* expression [64]. Consistent with our present data, the down-regulation of *PDZK1* in this intestinal cell model may result from repression of ligand-dependent nuclear receptor signaling, as PPARα or RXRα activation was shown to ameliorate the effect of IL-1β on *PDZK1* expression.

Up-regulation of intestinal *SLC6A14* expression has previously been reported for patients suffering from inflammatory bowel disease and for *Vibrio cholerae* infected subjects, which suggests that the regulation of this gene is also linked to host-microbe interactions and inflammation [65,66]. In line with his notion, we found that antibiotic treatment strongly repressed *Slc6a14* expression in the CF ileum. Genome-wide association studies on CF cohorts have shown that single nucleotide polymorphisms at loci near the *SLC6A14* gene are associated with meconium ileus [67]. Although the functional consequences of this polymorphism are unknown, it has been proposed that ATB0,+-mediated uptake of arginine increases nitric oxide production in intestinal epithelial cells and ameliorates the CF-typical fluid secretory defect [68]. This seems to imply that, perhaps contra intuitively, SIBO and a consequent up-regulation of *SLC6A14* improve luminal hydration in CF.

Expression of genes that encode proteins involved in nutrient processing/uptake is particularly high in fully matured enterocytes, located at or near the tips of the villi. Therefore, a reduction in the transcript levels of these genes may signal an underlying defect in enterocyte maturation [6]. Key aspects of enterocyte maturation, like formation of the apical brush border and the expression of nutrient transporters, were shown to be controlled by HNF4α in conjunction with CDX2 [69]. Consistent with impaired enterocyte maturation, URA indicated that HNF4α is in an inactive state in the CF ileum. The set of down-regulated HNF4α targets not only included genes encoding nutrient transporters, but also those encoding proteins involved in host defense, like *Alpi* and *Mep1a* [70,71]. Juxtaposed to HNF4α inactivation, URA and GSEA indicated activation of c-Myc and E2F-type transcription factors, which are linked to stem cell maintenance and cell cycle progression. While this activation is indicative of immune cell infiltration, it is also consistent with the observation that CF is associated with enhanced proliferation of intestinal epithelial cells and expansion of the crypt compartment, as the expression of c-Myc and E2F target genes in epithelial cells is reduced during crypt to villus differentiation [5,15,72,73]. In mice, deletion of *Hnf4a*, similarly, leads to a subtle increase in intestinal epithelial cell proliferation and crypt depth, suggesting that HNF4α inactivation is inexorably linked to protracted activation of E2F-type transcription factors [31]. Interestingly, *Hnf4a null* mice also display a marked increase in small intestinal goblet cell numbers and a thickening of the submucosae, i.e. a phenotype that mirrors aspects of the intestinal CF pathology [5,31,72].

5. Conclusion

Transcriptome analysis indicated the activation of an innate and adaptive immune response in the distal small intestine of a CF mouse model. Inflammation was associated with differential regulation of

numerous genes involved in the transport and metabolism of nutrients and, in particular, lipids, that are targeted by type II ligand-dependent nuclear receptors and/or HNF4α. Most changes in gene expression were reversed by bacterial containment. These findings indicate that CF-related dysbiosis and the attendant immune response shape the enterocyte maturation program, providing new insights into the impact of CF on intestinal epithelial cell dynamics, lipid metabolism and nutrient absorption. Because these data indicate that dysbiosis and the attendant intestinal inflammation contribute to the pathogenesis of CF-associated defects in lipid metabolism and nutrient uptake, therapeutic interventions aimed at containing or reversing SIBO and/or restoring the activity of ligand-dependent nuclear receptors (e.g. by pharmacological agonists) warrant consideration.

Competing interests

The authors declare that they have no competing interests.

Author contributions

PTI Performed animal procedures, tissue collection and RNA isolation, analyzed the data, and drafted the manuscript. KFM: Performed animal procedures and tissue collection. NDAN: Performed animal procedures and tissue collection. KD: Performed data analysis. HRDJ: Obtained funding, designed the study and drafted the manuscript. MJCB: Designed and supervised the study, performed animal procedures and tissue collection, analyzed the data and drafted the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2019.06.028.

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