Role of Heat-Stable Enterotoxins in the Induction of Early Immune Responses in Piglets after Infection with Enterotoxigenic Escherichia coli

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Abstract

Enterotoxigenic Escherichia coli (ETEC) strains that produce heat-stable (ST) and/or heat-labile (LT) enterotoxins are cause of post-weaning diarrhea in piglets. However, the relative importance of the different enterotoxins in host immune responses against ETEC infection has been poorly defined. In the present study, several isogenic mutant strains of an O149:F4ac, LT+ STa+ STb+ ETEC strain were constructed that lack the expression of LT in combination with one or both types of ST enterotoxins (STa and/or STb). The small intestinal segment perfusion (SISP) technique and microarray analysis were used to study host early immune responses induced by these mutant strains 4 h after infection in comparison to the wild type strain and a PBS control. Simultaneously, net fluid absorption of pig small intestinal mucosa was measured 4 h after infection, allowing us to correlate enterotoxin secretion with gene regulation. Microarray analysis showed on the one hand a non-toxic related general antibacterial response comprising genes such as PAP, MMP1 and IL8. On the other hand, results suggest a dominant role for STb in small intestinal secretion early after post-weaning infection, as well as in the induced innate immune response through differential regulation of immune mediators like interleukin 1 and interleukin 17.

Introduction

Enterotoxigenic Escherichia coli (ETEC) are a diverse group of pathogens that are characterized by the ability to colonize the small intestine while producing enterotoxins, which induce severe secretory diarrhea [1,2]. ETEC strains are recognized as one of the major causes of dehydrating diarrhea in children in developing countries and as an important causative agent of traveler’s diarrhea [3,4]. ETEC can also cause diarrhea in newborn calves and in suckling or recently weaned piglets. The apparent similarities between porcine and human ETEC infections [5,6] and between both species, makes the pig an excellent intestinal model.

Virulent ETEC strains produce fimbriae allowing the bacteria to colonize a host expressing the corresponding fimbrial receptors. ETEC that cause porcine post-weaning diarrhea are frequently of the O149 serotype and carry the F4 (K88) adhesin that permits adhesion of the bacteria to pig intestines [7,8]. Furthermore, ETEC strains are known to produce heat-labile enterotoxin (LT) and heat-stable enterotoxins a and b (STa, STb), which induce water and electrolyte loss from the intestine [9]. An individual ETEC strain may produce one or more enterotoxins [10,11,12], which may explain differences in virulence. However, only limited information is available concerning the contribution of the different enterotoxins to the virulence of a strain. The relative importance of LT as a virulence factor compared to STb has been demonstrated in a gnotobiotic piglet infection model, using isogenic deletion mutants of a naturally occurring porcine pathogen or by complementing a non-pathogenic E. coli strain with either STb or LT [13,14,15]. Also, LT has well known adjuvant capacities [16] and is able to down-regulate innate host responses in vitro [17,18]. Additionally, studies with the human epithelial cell line HCT-8 suggest a role for STa in the induction of an IL-8 response [19].

Little is known about the induction of host early immune responses after infection with ETEC and how these innate immune responses relate to the resolution of infection [2,19]. In a recent study, increased fecal IL-8 levels appeared to be important in resolving ETEC infection [20].

In order to investigate the role of the various enterotoxins, in the present study, various mutant strains of the ETEC reference strain GIS26 (O149:F4ac+, LT+ STa+ STb+) lacking one or more enterotoxins were generated. The “in vivo small intestinal segment perfusion” (SISP) technique [21,22] was used to correlate...
pathogen induced gene expression by microarray analysis with a functional response (fluid absorption).

Materials and Methods

Animals

Eight 5-week-old female piglets (Belgian Landrace), weaned on day 28, were purchased from a commercial pigery. The animal experiment was reviewed and approved by the Ethical Committee of the Faculty of Veterinary Medicine at Ghent University, in accordance with the Belgian law on animal experimentation (EC2000/77). The presence of the F4 receptor on the brush border of small intestinal enterocytes was confirmed on intestinal villi of each piglet as described by Van den Broeck et al. [23].

Bacterial Strain and Mutants

The hemolytic E. coli strain GIS26, serotype O149:K91:F4ac (GIS26 WT), producing the heat-labile enterotoxin (LT) and heat-stable enterotoxin types a and b (STa, STb), was used to generate mutant strains, lacking one or more enterotoxins. Mutants were generated using the bacteriophage lambda recombinase system (λ-Red) as described by Datsenko and Wanner [24]. Briefly, L-arabinose induced GIS26 transformants carrying the Red helper plasmid (pKD46) were grown at 30°C and electroporated with PCR products using standard procedures. The PCR products were generated by primers targeting an antibiotic resistance cassette (chloramphenicol or kanamycin) with Flippase recognition target (FRT) sites from a template (pKD3 or pKD4) but flanked by 50 basepairs of either the upstream or downstream region of the gene to be disrupted. Mutants were generated using the bacteriophage lambda recombinase system (λ-Red) as described by Datsenko and Wanner [24]. The PCR products were generated by primers targeting an antibiotic resistance cassette (chloramphenicol or kanamycin) with Flippase recognition target (FRT) sites from a template (pKD3 or pKD4) but flanked by 50 basepairs of either the upstream or downstream region of the gene to be disrupted.

The primers used to disrupt the enterotoxin genes are listed in Table 1. Electroporated cells were spread on Luria-Bertani agar plates containing kanamycin (10 μg/ml) or chloramphenicol (5 μg/ml) to select for antibiotic resistant transformants. Subsequently the antibiotic resistant cassettes were removed from the estA, estB or eltAB mutants by transformation with pCP20. pCP20 shows temperature-sensitive replication and can be thermally induced to generate mutant strains, lacking one or more enterotoxins. Mutants were generated using the bacteriophage lambda recombinase system (λ-Red) as described by Datsenko and Wanner [24].

Functional Characterisation of ETEC Enterotoxins

Toxin Detection and Quantification

Different methods were used to verify absence of toxin production in the different mutant strains. Bacterial strains were grown overnight at 37°C in Casamino Acids-Yeast Extract (CA-YE) medium pH 8.2 while shaking. For the detection of LT 0.25% w/v glucose was added to the growth medium for maximum toxin secretion. Before harvesting the supernatants, OD values at 650 nm of all strains were adjusted to the same value with CA-YE medium. For filtration the supernatants a 0.22 μm low protein-binding filter was used (Millipore, Massachusetts, USA).

LT was detected in filtered supernatant of polymyxin B-treated cultures by the commercial VET-RPLA kit (Oxoid, Hampshire, UK), a reversed passive latex agglutination test and quantified by a GM1 enzyme-linked immunosorbent assay (ELISA), using 100 μl of undiluted filtered supernatant [25]. The detection limit of the GM1 ELISA was 0.1 ng/ml.

STa secretion was demonstrated with two commercial competitive enzyme immunoassays (ELA) (Oxoid, Hampshire, UK and Bachem, Bubendorf, Switzerland), following manufacturer’s instructions. The assay provided by Bachem also allowed for quantification of the toxin (detection limit of 0.6 ng/ml).

STb secretion was detected by immunoblotting using a polyclonal rabbit anti-STb serum (Dr. J. Daniel Dubreuil). Briefly, filtered supernatant of the overnight cultures was boiled for 5 minutes in Laemmli sample buffer. Proteins were separated using a 10–20% Tris-Tricine gel (Bio-Rad, California, USA) and blotted onto a polyvinylidene fluoride membrane. Following overnight blocking the membrane was incubated with a 1/500 dilution of the STb antiserum. The secondary antibody was a swine anti-rabbit Ig labeled with horseradish peroxidase (Dako, Glostrup, Denmark). Enzymatic activity was revealed by enhanced chemiluminescence (ECL) using Pierce ECL Western Blotting Substrate (Thermofisher Scientific, Illinois, USA). A direct STB ELISA was also performed as previously described, using the polyclonal rabbit anti-STb serum from Dr. J. Daniel Dubreuil [26] for quantification (detection limit of ±80 ng/ml). Briefly, supernatant of the overnight cultures was filtered and two-fold dilution series in 0.1 M carbonate buffer pH 9.6 were coated overnight at 4°C on Maxisorp plates (Nunc, New York, USA). Subsequent incubation steps were: incubation for 2 h at 37°C with 3% gelatin, incubation for 1 h at 37°C with a 1/100 dilution in PBS containing 0.05% Tween® 20 of the anti-STb antibody, incubation for 1 h at 37°C with a 1/1000 dilution in PBS containing 0.05% Tween® 20 of swine anti-rabbit Ig labeled with horseradish peroxidase, incubation for 30 minutes at 37°C with a 2.2’-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Roche, Basel, Switzerland) solution containing H2O2. In between each incubation, the plates were washed with 3% gelatin, 0.05% Tween 20, 100 ng/ml of ABTS, and 100 ng/ml of ABTS with 100 μl of 10% H2O2. The ABTS peroxidase solution was mixed with 0.2 ml of 0.6 M phosphoric acid and 0.2 ml of 100 ng/ml of ABTS.OD values were read in a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.
tion step, plates were washed with PBS containing 0.05% Tween® 20. The OD of the wells was measured at 405 nm.

Anesthesia Protocol

Piglets were fasted overnight. The next morning, premedication was administered by intramuscular injection of 40 mg/kg azaperon (Janssen Animal Health, Beerse, Belgium) and 0.1 mg/kg morphine (Sterop, Brussels, Belgium). After 20 minutes, anesthesia was induced by IV injection of 2–4 mg/kg propofol (AST Farma, Oudewater, The Netherlands). After endotrachal intubation the piglets were kept under long-term anesthesia with a mixture of 1.5% isoflurane (Euphar, Oostkamp, Belgium) and 40% oxygen (Air Liquide, Luik, Belgium). Fentanyl (Janssen-Cilag, Beerse, Belgium) at a rate of 5–10 μg/kg/h was given IV as an analgesic. Hematocrit values (Hct) were assessed at regular timepoints and when they exceeded 35, 10–15 ml/kg/h ringer lactate (Baxter, Illinois, USA) was infused via the ear vein to prevent dehydration. Temperature, heart rate, oxygen saturation, expiratory CO₂ and non-invasive blood pressure were monitored continuously.

Surgery

The surgical and experimental procedures have previously been described in detail [21,27]. In brief, the abdomen was opened at the linea alba and five small intestinal segments of 20 cm in length were made in the mid-jejunum, starting at a distance of 200 cm distal to the ligament of Treitz. These segments retained their vascularization and were cannulated with a rubber tube at the proximal and distal ends to inject and collect fluid respectively.

Bacterial Inoculum

The GIS26 strain or its isogenic mutant strains were cultured for 16 h in Trypsone Soy Broth (Difco Laboratories, Bierbeek, Belgium), and bacteria were collected by spinning at 5000 x g for 15 minutes. Subsequently, the bacteria were washed and resuspended in PBS at a concentration of 5 x 10⁸ bacteria per ml (OD₆₅₀ of 0.5), as confirmed by counting CFU.

Perfusion

SISP experiments were performed essentially as described by Nabuurs et al. [21]. Three piglets were used to compare the effect of GIS26 WT and four mutant strains on net absorption and host early immune responses (microarray analysis). In addition, five piglets were used to further investigate the role of STb on net absorption. In brief, fifteen minutes before perfusion, segments were injected with 5 ml bacterial inoculum (2.5 x 10⁸ CFU) or with PBS only (control). The position of the GIS26 mutants, the GIS26 wild type strain and PBS was randomized. Intestinal segments were perfused with 0.9% NaCl, supplemented with 0.1% glucose and 0.1% casamino acids. Each segment was perfused with 32 ml over 4 h by injecting 2 ml of perfusion fluid every 15 minutes whereafter piglets were euthanized with an overdose sodium pentobarbital (Kela Laboratoria, Hoogstraten, Belgium). Residual fluids in the segments were collected and in the three pigs used for microarray analysis, a small piece of tissue of each segment was sampled and frozen for RNA isolation. Net fluid absorption was calculated from the difference between the inflow and outflow divided by the surface area (length x circumference) of each segment.

Isolation of Total RNA

Approximately 100 mg of frozen intestine was homogenized in 1 ml TRIzol® Reagent (Invitrogen, Merelbeke, Belgium) to extract total RNA. These homogenates were further purified using the RNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands) with an on column DNase treatment (RNase-free DNase set, Qiagen Benelux). Spectrophotometric RNA quality control was done using Nanodrop® ND-1000 (Isogen Life Science, De Meern, The Netherlands) using only samples with a 260/230 ratio between 1.8-2.1 and 260/230 ratio between 1.3-2.0. RNA integrity was assessed using a Bioanalyser 2100 (Agilent, California, USA). A part of the isolated RNA was used for microarray analysis, and another part was for expression analysis of selected genes by PCR.

Microarray Analysis

The Porcine Genome Array (Affymetrix, California, USA) was used containing 23,937 probe sets to interrogate 23,256 transcripts in pig, which represents 20,201 Sus scrofa genes. Per sample, an amount of 100 ng of total RNA spiked with bacterial RNA transcript positive controls (Affymetrix) was converted to double stranded cDNA in a reverse transcription reaction. Subsequently, the sample was converted and amplified to antisense cRNA and labeled with biotin in an in vitro transcription reaction. All steps were carried out according to the manufacturer's protocol (Affymetrix). A mixture of purified and fragmented biotinylated cRNA and hybridisation controls (Affymetrix) was hybridized on Affymetrix GeneChip® Porcine Genome Arrays followed by staining and washing in a GeneChip® fluids station 450 (Affymetrix) according to the manufacturer's instructions. To assess the raw probe signal intensities, chips were scanned using a GeneChip® scanner 3000 (Affymetrix).

Analysis of Microarray Data

R (version 2.11.1), a free software environment for statistical computing and graphics, was used in combination with the affy library [version 1.26.1] of BioConductor (www.bioconductor.org) to calculate the MAS 5.0 detection calls and the RMA [28] expression values. The MAS 5.0 detection calls were used to decide whether a signal was significantly above background.

For 5,781 probe sets, none of the signals had a present detection call and these were omitted from further analysis. Also the spot controls were removed prior to the analysis. A set of 18,246 probe sets was retained. The normalized intensity values of the different conditions were compared with the limma package [version 3.4.3, [29]] of BioConductor. Hereto, a linear model with pig and treatment as factors was fitted. With this design, estimates for all effects of interest were obtained. These contrasts of interest were estimated and tested whether they were significantly deviating from 0 with a moderated t-statistic. Transcripts were selected based on the more stringent cut-off of the uncorrected P-values, i.e. P<0.001. This cut-off on the P-values was combined with a cut-off on the fold-change of two (i.e., an absolute log2 ratio larger than 1).

To annotate the probes, the latest annotation (NetAffx annotation date 2008-12-01) was used [http://www4.ncsu.edu/~stsai2/annotation/][30].

Quantitative Real-time PCR Analysis

Two μg of total RNA of each sample was converted to single stranded complementary DNA by reverse transcription (AMV-Reverse Transcriptase, Promega Benelux) with random priming. Nine genes from the microarray analysis were selected for confirmation by quantitative real-time PCR. Intestinal housekeeping genes ribosomal protein L4 (RPL4) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were chosen after checking the expression
stability of a set of five housekeeping genes using the Genorm software [31]. Primers for RPL4, GAPDH, IL8, PAP and FABP2 (Table 2) were from a previous study [32]. The primers for IL1A, IL17A, TLR4, MMP1, MMP3 and CYP1A1 (Table 2) were designed using the Beacon Designer software (PREMIER Biosoft International, California, USA). To avoid contamination of genomic DNA the primers were chosen in different exons. Primer concentrations were tested during optimization reactions using pooled cDNA.

Subsequently, quantitative real-time PCR (qRT-PCR) was performed for each primer set using the SYBR Green PCR Master Mix (Applied Biosystems, California, USA) and 100 ng of template cDNA. A two-step program was run on the StepOnePlus real-time PCR system (Applied Biosystems, California, USA). Thermal cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis confirmed primer specificities. All reactions were run in triplicate and a standard curve for all genes, including housekeeping genes, was generated using serial dilutions of a pooled sample. PCR efficiency of 90-110% (3.2 < slope > 3.8) together with a correlation coefficient of >0.99 were accepted. Values for each target gene were normalized using the geometric mean of the expression of RPL4 and GAPDH, according to the standard curve method for the analysis of the expression of the genes [33].

Statistical Analysis

Graphpad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA) was used to analyze STa EIA and perfusion experiments. STa EIA results and net absorption data of perfusion experiments were analyzed using one-way analysis of variance and the Bonferroni post-hoc test. Analysis on STb ELISA results was performed using Dehsaft Microplate analysis software (BioMetallics Incorporated, New York, USA).

The relationship between the levels of gene expression of selected genes, qRT-PCR versus microarray data, was determined by linear regression.

Results

In vitro Toxin Phenotype of Mutant Strains Differences from their Toxin Genotype

Absence of the targeted toxin genes was first verified by PCR and sequencing of the toxin genes for each of the generated mutant strains. Simultaneously the presence of the other wild type toxin gene(s) was also verified for each mutant (Table 3: genotype). Next, we compared in vitro production of the toxins between wild type and mutant strains. Both GIS26 AbdA and GIS26 AbdA AbdB:KAN lack production of STa as compared to GIS26 wild type and GIS26 AbdB:KAN (P<0.001) (Figure 1A). However, unexpectedly no STa was detectable in the GIS26 AbdB:KAN strain (P<0.001). This was confirmed with two different kits. The presence or absence of LT in supernatant of polymyxin B-lysed bacteria was verified five times with a non-quantitative method. Subsequently, these results were confirmed through detection of LT in the normal culture supernatant by a quantitative method (Figure 1B). Only the wild type GIS26 produced detectable amounts of LT. In Figure 2A, the results of STb detection in culture supernatant are presented. Purified STb was used as a positive control. The wild type strain and both GIS26 AbdA and GIS26 AbdA AbdB mutant strains showed a clear band for STb. In contrast, no STb could be detected in the supernatant of GIS26 AbdB:KAN and GIS26 AbdB:KAN mutants. The supernatant of these negative strains was 10x concentrated by trichloroacetic acid precipitation but also in these samples there was no detection of STb (data not shown). Quantifying the amount of STb by direct ELISA (Figure 2B) revealed a 3-fold reduction in amount of STb for GIS26 AbdA as compared to the wild type strain. This was confirmed by Western blot when equal amounts of the supernatant of both the wild type strain and the GIS26 AbdA mutant were diluted 4 times (Figure 2C).

Conclusions from these data are summarized in Table 3 in which a new strain designation for every mutant is introduced based on the phenotype. To avoid confusion, this new designation was used throughout the rest of this manuscript.

Table 2. Primer sequences used for qRT-PCR.

| Symbol | Name | Probe set ID | Forward primer | Reverse primer |
|--------|------|--------------|----------------|----------------|
| RPL4   | Ribosomal protein L4 | Ssc.12277.1.S1_at | GAGAAACGGTGCGGCAAT | GCCACCAAGAGCAGAT |
| GAPDH  | Glyceraldehyde-3-phosphate dehydrogenase | Ssc.14942.1.S1_at | GGTCGGAGGTGAGGATTTG | ACTGTCGCGGAAATTGC |
| IL1A   | Interleukin-1, alpha | Ssc.113.1.S1_at | TCTGTTGACTCTACAGAATCT | CACGAAAGAAGAGAAGCT |
| IL8    | Interleukin-8 | Ssc.658.1.S1_at | TCAGGAAGGCTGACGAGTA | CAGAAGAAGAAGAGAAGCT |
| IL17A  | Interleukin-17, alpha | SscAffx.23.1.S1_at | CCTTCAGTTACCTCCTTCAAA | CCTTCAGACGTTACAG |
| PAP    | Pancreatitis-associated protein | Ssc.16470.1.S1_at | GGGAGAAGCCGCAACAGAC | AGGAGCAAGAAGGATCCT |
| FABP2  | Intestinal fatty acid binding protein | Ssc.16525.1.S1_at | TGCACGTCCTATTAGAGTACC | TTACGAGATCTAACAG |
| TLR4   | Toll-like receptor 4 | Ssc.12781.1.A1_at | TGGAGTATTAGGAGGAGTAC | CACTCGGAGGTGGAAA |
| MMP1   | Matrix metalloproteinase 1 | Ssc.16013.1.S1_at | GAGTTTGGCAGTAGATGAGTGAAG | ACTAGGGAGAAGGAGAT |
| MMP3   | Matrix metalloproteinase 3 | Ssc.15927.1.A1_at | GAGATTGAGGAGGAGTAC | CACTCGGAGGTGGAAA |
| CYP1A1 | Cytochrome P450 1A1 | Ssc.208.1.S1_at | TGGGAACAGTGGGCAAGAT | CATCGGAGGTGAGGAA |

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STb Seems to Play an Important Role in the Induction of Small Intestinal Secretion

Strains were administered in vivo in small intestinal segments using the SISP technique and the capacity of the mutants to induce a secretory response was compared to the wild type strain. All used piglets were F4 receptor positive as determined by an in vitro villous adhesion assay [23]. In each piglet, an uninfected (PBS control) segment, a wild type GIS26-infected segment and segments infected with the different mutant strains were present. The segments were perfused during 4 h and net absorption was calculated.

In a first set of experiments, the effect of the wild type GIS26 strain on net absorption was compared to the effects of mutant strains GIS26 (STa⁺ STb⁺ LT⁺), GIS26 (STa⁺ STb⁺LT⁻), GIS26 (STa⁺ STb⁻ LT⁻) and GIS26 (STa⁻ STb⁻ LT⁻) (Figure 3). For all three piglets the PBS segments showed net absorption while the wild type GIS26 (STa⁺ STb⁺ LT⁺)-infected segments all showed net secretion (P<0.001). As expected, the mutant strain GIS26 (STa⁺ STb⁻ LT⁻) that did not express enterotoxins was no longer able to reduce net absorption and values were comparable with the PBS group. All other mutant strains significantly reduced net absorption when compared to the PBS

| GIS26 strain       | Genotype   | Phenotype   | new strain designation |
|--------------------|------------|-------------|------------------------|
| wild type          | estA⁺ estB⁺ eltA⁺B⁺ | STa⁺ STb⁺ LT⁺ | GIS26 (STa⁺ STb⁺ LT⁺) |
| ΔestB,ΔeltAB       | estA⁺ estB⁻ eltA⁺B⁻ | STa⁺ STb⁻ LT⁻ | GIS26 (STa⁺ STb⁻ LT⁻) |
| ΔestA              | estA⁻ estB⁺ eltA⁻B⁺ | STa⁻ STb⁺ LT⁻ | GIS26 (STa⁻ STb⁺ LT⁻) |
| ΔestA,ΔestB:KAN    | estA⁻ estB⁻ eltA⁻B⁻ | STa⁻ STb⁻ LT⁻ | GIS26 (STa⁻ STb⁻ LT⁻) |

aGenotype was assessed by PCR and sequencing.
bPhenotype was determined by detection of the different enterotoxins in culture supernatant as shown in Figure 1 and Figure 2.
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STa and LT expression by different isogenic ETEC strains following in vitro culture. Both toxins were detected by enzyme immunoassays. Mean values ± SD are shown. (A) Samples for STa were tested in triplicate in three independent experiments. (B) LT results are representative for 2 independent experiments. ND = below detection limit of 10 ng/ml. WT = wild type strain. doi:10.1371/journal.pone.0041041.g001
The amount of STb, whereas a significantly lower effect occurred on a similar effect on fluid secretion as the strain secreting only a low dilution of the supernatant of both strains (20\%)

The strain producing only STa, GIS26 (STa

compared to the GIS26 (STa

by the GIS26 ETEC strain we performed five additional SISP

To further confirm the role for STb in the induction of secretory responses by the GIS26 ETEC strain in intestinal segments of 5-week-old piglets but that presence of STa and LT may be able to compensate for the lack of STb, especially in piglets where the wild type ETEC strain has a strong secretory effect.

Microarray Analysis

ETEC regulates gene expression of several porcine genes important in inflammatory responses. Intestinal cDNA isolated from mock (PBS)-infected segments was compared with cDNA from wild type ETEC-infected segments, to detect differences in gene expression, 4 h after infection. The difference in gene expression between mock-infected and ETEC-infected segments was determined as the statistical mean of three piglets, indicating the average differential expression. In the latter comparison, 153 transcripts were down-regulated and 157 up-regulated (Table 4). Because of the large number of differentially expressed transcripts, a more stringent cut-off, log-ratio < -2 and log-ratio > 2 was used, after which 15 ETEC down-regulated (PBS vs. ETEC) and 23 ETEC up-regulated (ETEC vs. PBS) transcripts remained (Table 5 and Table 6).

Most transcripts down-regulated by ETEC (Table 5) are not associated with immune responses, the majority of genes has a specific function in the intestinal metabolism (PKC\(\beta\), PTPRR, SLIC25A27, PRR15, PPARG1A, ATP10D, CDC10, KIAA1468, GPT2, PHLPPE) or in transport of fluids and electrolytes (KCNJ13, AQP8, ATG10, APOC3). Another transporter SLIC26A3 is up-regulated by ETEC (Table 6). This protein is functionally coupled to CFTR (cystic fibrosis transmembrane regulator) and to NHE3 (Na/H exchanger-isoform 3), which are both involved in the secretory pathway of LT, STa and STb [34,35].

In contrast, immunomodulatory genes are abundantly present in the list of 23 transcripts up-regulated after ETEC infection (Table 6). Among them, interleukin 1 (IL1A and IL1B), the interleukin 1 receptor antagonist (IL1RN) and interleukin 17 (IL17A), three cytokines with a known function in inflammatory responses, and DUOX2 that plays a role in the signaling pathway of these cytokines. Furthermore, the genes MMP1 and MMP3 belonging to the family of matrix metalloproteinases (MMP) have been described to regulate various aspects of inflammation and immunity by acting on pro-inflammatory cytokines, chemokines and other proteins [36]. Also interesting is pancreatitis associated protein (PAP), alias REG3A, which has anti-bacterial and anti-inflammatory properties [37,38,39]. PAP, a marker for pancreatitis, is also expressed in Paneth cells [40], pig small intestine [41], and human colon where it is up-regulated after inflammation [42]. Another up-regulated gene, with a central role in the activation of inflammation, is the ectoderm-neural cortex-1 protein (ENC1), involved in the ubiquitin 1 conjugation pathway [43].

Microarray analysis of mutant ETEC - versus wild type ETEC-infected jejunum suggests a role for STb in ETEC-
induced immune responses. To reveal an influence of the different enterotoxins on innate immune responses, the differential transcriptional regulation between wild type ETEC strain GIS26 and its isogenic mutants was analyzed in the microarray study. This study was performed with RNA of the three pigs in Figure 3.

The mutant strain, GIS26 (STa STb LT), produced no STa and LT but showed normal STb levels as compared to the wild strain GIS26 (STa STb LT) (Figure 1 and Figure 2). When microarray results from this mutant were compared to GIS26 (STa STb LT) no differential transcripts were reported (Table 4). This result is in agreement with the secretory responses, where no significant difference could be detected between this mutant and the wild type strain (Figure 3 and Figure 4).

Another mutant, GIS26 (STa STb LT) expressing only STa (Figure 1 and Figure 2), was also compared to GIS26 (STa STb LT). Again no differential gene expression was revealed (Table 4). As this mutant strain showed a significant loss in capacity to reduce net absorption as compared to the wild type strain (Figure 3), this...
result indicates that STa can compensate for the absence of STb (and LT) in the activation of innate immune responses but not for the induction of secretion.

The third mutant GIS26 (STa\(^+\) STb\(^{low}\) LT\(^+\)) only expressed reduced amounts of STb in vitro (Figure 1 and Figure 2), and when compared to the wild type strain, 43 transcripts were found to be differentially regulated of which 20 down-regulated (up-regulated in the wild type ETEC strain), and 23 up-regulated (Table 4).

When the mutant strain GIS26 (STa\(^+\) STb\(^-\) LT\(^+\)) was compared to GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) only expressed no enterotoxins at all, was compared to GIS26 (STa\(^+\) STb\(^{low}\) LT\(^+\)), which expressed no enterotoxins at all, was compared to GIS26 (STa\(^-\) STb\(^+\) LT\(^-\)), in total 54 transcripts were differentially regulated. Twenty-seven genes were down-regulated (up-regulated in the wild type ETEC strain), and 27 up-regulated (Table 4).

The differentially expressed transcripts were subdivided into five groups based on the presence or absence (up-regulated or down-regulated) in each of the three comparisons listed in Tables 7, 8, 9, 10 and 11.

### Table 4. Summary of differentially expressed probe sets of all mutant strains and control versus wild type ETEC strain.

| PBS vs. GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) | log-ratio <−1 | log-ratio >1 | log-ratio <−2 | log-ratio >2 |
|-------------------------------------------|--------------|--------------|--------------|--------------|
| GIS26 (STa\(^-\) STb\(^-\) LT\(^+\)) vs. GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) | 0 | 0 | 23 | 15 |
| GIS26 (STa\(^-\) STb\(^+\) LT\(^+\)) vs. GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) | 0 | 0 | 20 | 23 |
| GIS26 (STa\(^-\) STb\(^{low}\) LT\(^+\)) vs. GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) | 20 | 23 | 27 | 27 |

In the first group, transcripts present in all three of the comparisons listed were found. The genes of group I (Table 7), up-regulated by ETEC, are probably regulated by the heat-stable enterotoxins expressed, since no differences in gene expression are found with mutants that still express STa or normal levels of STb but that lack LT. Among these SLC26A3, IL1A and MMP3 were found. It can be speculated that high levels of STb or STa are important in the induction of these immune genes.

In group II (Table 8) the retrieved genes were differentially regulated by both mutant strains with no LT, no STa and no or weak STb expression but not found in the PBS versus wild type strain comparison. Only three genes were left, namely SERPINE1, TLR4, and SLC2A14 (down-regulated). Of these three, the serine protease inhibitor SERPINE1 and the Toll-like receptor for LPS (TLR4) have a well-known function in the immune/inflammatory response.

### Table 5. Transcripts down-regulated by ETEC. Microarray data expressed as a log2 fold-change of PBS versus ETEC-infected small intestinal segments at 4 h (for full list see Table S1).

| Probe Set ID | Log2 ratio | Gene symbol | Gene title | Tentative function (UniprotKB) |
|--------------|------------|-------------|------------|--------------------------------|
| Ssc.2299.1.51_at | 3.96 | PKC1 | Phosphoentpyruvate carboxykinase | Gluconeogenesis |
| Ssc.2114.1.51_at | 3.46 | PTPRR | Receptor-type protein-tyrosine phosphatase R precursor | Hydrolase, protein phosphatase, receptor |
| Ssc.1828.1.51_at | 3.41 | KCNJ13 | Inward rectifier potassium channel 13 | Voltage-gated channel, potassium transport |
| Ssc.2041.1.51_at | 2.71 | SLC25A27 | Mitochondrial uncoupling protein 4 | Transport (transmembrane), binding |
| Ssc.1148.1.51_at | 2.66 | PRRT3 | Proline-rich protein 15 | Developmental protein |
| Ssc.1848.1.51_at | 2.59 | AQP8 | Aquaporin 8 | Transport |
| Ssc.2952.1.51_at | 2.37 | ATG10 | APG10 autophagy 10-like | Ligase, autophagy, protein transport, transport, Ubl conjugation pathway |
| Ssc.168.1.51_at | 2.37 | PPARC1 | Peroxisome proliferator activated receptor gamma coactivator 1 alpha | Transcription, transcription activator |
| Ssc.923.1.51_at | 2.27 | ATP1D | Potential phospholipid-transporting ATPase VD | Hydrolase |
| Ssc.159.1.51_at | 2.22 | CDC10 | Septin 7 (CDC10 protein homolog) | Cytokinesis, mitosis, cell cycle |
| Ssc.179.1.51_at | 2.16 | KIAA1468 | Protein KIAA1468 | Binding |
| Ssc.7458.1.51_at | 2.15 | GPT2 | Alanine aminotransferase 2 | Aminotransferase, transferase |
| Ssc.4724.1.51_at | 2.14 | PHLPPL | PH domain leucine-rich repeat-containing protein phosphatase 2 | Protein binding, catalytic activity |
| Ssc.2041.2.51_at | 2.02 | SLC25A27 | Mitochondrial uncoupling protein 4 | Transport (transmembrane), binding |
| Ssc.1039.1.51_at | 2.00 | APOC3 | Apolipoprotein C-III precursor | Transport, G-protein coupled receptor protein signaling pathway |

The selection criteria to define a transcript as differentially regulated were: an absolute log2 ratio equal or larger than 2, a MAS 5.0 present detection call and an uncorrected P value of <0.001.

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Table 6. Transcripts up-regulated by ETEC. Microarray data expressed as a log2 fold-change of ETEC-infected versus PBS treated small intestinal segments at 4 h (for full list see Table S1).

| Probe Set ID | Log2 ratio | Gene symbol | Gene title | Tentative function (UniprotKB) |
|--------------|------------|-------------|------------|-------------------------------|
| Ssc.15927.1.S1_at | 4.18 | MMP3 | Stromelysin-1 precursor; Matrix metalloproteinase-3 | Proteolysis, metalloendopeptidase activity |
| Ssc.15927.2.S1_at | 4.16 | MMP3 | Stromelysin-1 precursor; Matrix metalloproteinase-3 | Proteolysis, metalloendopeptidase activity |
| SscAffx.23.1.S1_at | 3.68 | IL17A | Interleukin-17 precursor; Cytotoxic T lymphocyte-associated antigen 8 | Cytokine, inflammatory response |
| Ssc.16470.1.S1_a_at | 3.10 | PAP (REG3A) | Pancreatitis-associated protein 1 precursor | Acute phase response, inflammatory response |
| Ssc.6180.1_A1_at | 2.90 | SLCA11 | Cystine/glutamate transporter | Response to toxin, transport |
| Ssc.15927.2_A1_at | 2.90 | MMP3 | Stromelysin-1 precursor; Matrix metalloproteinase-3 | Proteolysis, metalloendopeptidase activity |
| Ssc.17573.1_S1_at | 2.72 | IL1B | Interleukin-1 beta precursor | Inflammatory response, cytokine, pyrogen |
| Ssc.29329.1_A1_at | 2.71 | DCHS2 | Dachsous 2 isoform 1 [H. sapiens] | Cell adhesion, calcium ion binding |
| Ssc.113.1_S2_at | 2.68 | IL1A | Interleukin-1 alpha precursor | Inflammatory response, cytokine, pyrogen |
| Ssc.15601.1_s_A1 | 2.65 | IL1B | Interleukin-1 beta precursor | Inflammatory response, cytokine, pyrogen |
| Ssc.24966.1.S1_at | 2.47 | NP | Purine nucleoside phosphorylase | Glycosyltransferase, transferase |
| Ssc.30277.1.A1_at | 2.46 | SLC26A3 | Chloride anion exchanger | Antiport, transport (excretion) |
| Ssc.18918.1_A1_at | 2.43 | GPX2 | Glutathione peroxidase-gastrointestinal | Oxido-reductase, peroxidase, response to oxidative stress |
| Ssc.29281.1_A1_at | 2.35 | SLCA11 | Cystine/glutamate transporter | Response to toxin, transport |
| Ssc.33.1_S1_at | 2.33 | DUOX2 | Dual oxidase 2 precursor [H. sapiens] | Oxido-reductase, peroxidase, cytokine-mediated signaling pathway |
| Ssc.113.1_S1_at | 2.30 | IL1A | Interleukin-1 alpha precursor | Inflammatory response, cytokine, pyrogen |
| Ssc.11609.1_A1_at | 2.28 | ASNS | Asparagine synthetase | Ligase |
| Ssc.19907.1_S1_at | 2.22 | F3 | Tissue factor precursor | Blood coagulation |
| Ssc.12431.1_A1_at | 2.11 | MYOSB | Myosin Vb | Protein transport |
| Ssc.18603.1_A1_at | 2.07 | GOS2 | Putative lymphocyte G0/G1 switch protein 2 | Cell cycle |
| Ssc.16013.1_S1_at | 2.05 | MMP1 | Interstitial collagenase precursor; Matrix metalloproteinase-1 | Metalloprotease activity |
| Ssc.30857.1_S1_at | 2.05 | ENC1 | Ectoderm-neural cortex-1 protein | Ubl conjugation pathway |
| Ssc.16250.1_S2_at | 2.01 | IL1RN | Interleukin-1 receptor antagonist protein precursor | Cytokine activity, interleukin-1 receptor antagonist activity, immune response, inflammatory response |

The selection criteria to define a transcript as differentially regulated were: an absolute log2 ratio equal or larger than 2, a MAS 5.0 present detection call and an uncorrected P value of <0.001.

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Group III consists of those transcripts present in PBS versus wild type but differentially regulated in only one of both mutant strains. Group III (Table 9) therefore can be subdivided into two categories. The first one includes six transcripts present both in GIS26 (STa STb LT) versus wild type and PBS versus wild type comparisons. Most notable is the 2-fold down-regulation of MMP3 in two transcripts absent from GIS26 (STa STb LT) versus wild type, although MMP3 seemed equally down-regulated in both mutants when comparing another transcript (Ssc.15927.1.S1_at) (Group I, Table 7). In mutant GIS26 (STa STb LT) the P-values for the other two transcripts (Ssc.15927.2.A1_at and Ssc.15927.2.S1_at) were at the borderline (Table S1). However, when less stringent P-values were applied these transcripts were also retrieved as differential expressed in mutant GIS26 (STa STb LT) compared to the wild type strain. The second category within Group III (Table 9), represents 24 transcripts in common for both GIS26 (STa STb LT) versus wild type and PBS versus wild type strain. The larger number of genes in common can be explained by the complete absence of enterotoxins in the mutant strain, by which its effect on net absorption is quite similar to PBS. There is no clear difference in the log2 fold change between these two groups except the fact that transcripts in GIS26 (STa STb LT) versus wild type ETEC have a lower expression as compared to PBS versus the wild type strain. This may be due to the presence of LPS and/or other metabolites in the mutant-infected segments. However, the fact that some genes involved in immune regulation like IL1B and IL17A, are listed here and not with the GIS26 (STa STb LT) mutant is interesting. It suggests that STb can regulate these genes, since this toxin is completely absent in the mutant strain GIS26 (STa STb LT).
### Table 7

Microarray data expressed as a log2 ratio of PBS and mutant ETEC-infected versus wild type ETEC-infected (WT) small intestinal segments at 4 h (Group I, transcripts in common for the three comparisons where differential regulation was found).

| Probe Set ID     | Log2 ratio | Gene symbol | Gene title                                           | Tentative function (UniprotKB)                  |
|------------------|------------|-------------|------------------------------------------------------|-------------------------------------------------|
| Ssc.22959.1.S1_at| 2.83       | GIS26 STa   | Phosphoenolpyruvate carboxykinase                    | Gluconeogenesis                                  |
|                  | 3.24       | STblow LT   |                                                       |                                                 |
|                  | 3.96       | WT          |                                                       |                                                 |
| Ssc.20419.1.S1_at| 1.52       | SLC25A27    | Mitochondrial uncoupling protein 4                    | Transport (transmembrane), binding               |
|                  | 1.86       |             |                                                       |                                                 |
|                  | 2.71       |             |                                                       |                                                 |
| Ssc.29525.1.A1_at| 1.62       | ATG10       | APA10 autophagy 10-like [H. sapiens]                  | Ligase, autophagy, protein transport, transport, Ub conjugation pathway |
|                  | 1.88       |             |                                                       |                                                 |
|                  | 2.37       |             |                                                       |                                                 |
| Ssc.16864.1.S1_at| 1.42       | PPARGC1A    | Peroxisome proliferator activated receptor gamma coactivator 1 alpha | Transcription, transcription activator           |
|                  | 1.44       |             |                                                       |                                                 |
|                  | 2.37       |             |                                                       |                                                 |
| Ssc.7301.1.A1_at | 1.75       | CDC10       | Septin 7                                             | Cytokinesis, mitosis, cell cycle                 |
|                  | 1.81       |             |                                                       |                                                 |
|                  | 2.22       |             |                                                       |                                                 |
| Ssc.5000.1.A1_at | 1.23       | ERBB2       | Receptor protein-tyrosine kinase erbB-2 precursor    | Activator, kinase, receptor, transferase, tyrosine-protein kinase |
|                  | 1.32       |             |                                                       |                                                 |
|                  | 1.85       |             |                                                       |                                                 |
| Ssc.298.1.S1_at  | 1.78       | PRSS7       | Enteropeptidase precursor (Enterokinase)             | Hydrodase, protease, serine protease             |
|                  | 1.53       |             |                                                       |                                                 |
|                  | 1.85       |             |                                                       |                                                 |
| Ssc.14573.1.S1_at| 1.01       | EYA2        | Eyes absent homolog 2                                 | Activator, chromatin regulator, developmental protein, hydrodase, protein phosphatase, transcription regulation |
|                  | 1.08       |             |                                                       |                                                 |
|                  | 1.77       |             |                                                       |                                                 |
| Ssc.10602.1.A1_at| 1.08       | FLRT3       | Leucine-rich repeat transmembrane protein FLRT3 precursor | Cell adhesion                                   |
|                  | 1.15       |             |                                                       |                                                 |
|                  | 1.59       |             |                                                       |                                                 |
| Ssc.20832.1.S1_at| 1.26       | SCTR        | Secretin receptor precursor                          | G-protein coupled receptor, receptor, transducer |
|                  | 1.24       |             |                                                       |                                                 |
|                  | 1.55       |             |                                                       |                                                 |
| Ssc.16538.1.S1_at| 1.37       | C1orf168    | –                                                     | –                                               |
|                  | 1.21       |             |                                                       |                                                 |
|                  | 1.55       |             |                                                       |                                                 |
| Ssc.18915.1.A1_at| 1.23       | ZC3H11A     | Zinc finger CCCH domain-containing protein 11A       | Nucleic acid-, zinc ion-, protein binding        |
|                  | 1.3        |             |                                                       |                                                 |
|                  | 1.54       |             |                                                       |                                                 |
| Ssc.27422.1.A1_at| 1.19       | ACBD5       | acyl-Coenzyme A binding domain containing 5 [H. sapiens] | Transport                                       |
|                  | 1.19       |             |                                                       |                                                 |
|                  | 1.52       |             |                                                       |                                                 |
| Ssc.17849.1.A1_at| 1.44       | SLC30A10    | Solute carrier family 30; zinc transporter 8 [H. sapiens] | Ion transport, transport, zinc transport       |
|                  | 1.2        |             |                                                       |                                                 |
|                  | 1.51       |             |                                                       |                                                 |
| Ssc.208.1.S1_at  | 1.32       | CYP1A1      | Cytochrome P450 1A                                    | Monoxygenase, oxidoreductase                     |
|                  | 1.3        |             |                                                       |                                                 |
|                  | 1.46       |             |                                                       |                                                 |
| Ssc.7116.1.A1_at | 1.18       | NT5C3       | 5-nucleotidase; pyrimidine 5-nucleotidase [H. sapiens] | Hydrolase, transerase                           |
|                  | 1.25       |             |                                                       |                                                 |
|                  | 1.39       |             |                                                       |                                                 |
| Ssc.10703.1.A1_at| 1.52       | SLC25A27    | Mitochondrial uncoupling protein 4                    | Transport (transmembrane), binding               |
|                  | 1.4        |             |                                                       |                                                 |
|                  | 1.02       |             |                                                       |                                                 |
| Ssc.26709.1.S1_at| −1.11      | GPR183      | EBV-induced G protein-coupled receptor 2              | Adaptive immunity, immunity, humoral immune response |
|                  | −1.22      |             |                                                       |                                                 |
|                  | −1.12      |             |                                                       |                                                 |
| Ssc.3509.1.S1_at | −1.18      | HK2         | Hexokinase, type II                                   | Kinase, transference                            |
|                  | −1.14      |             |                                                       |                                                 |
|                  | −1.3       |             |                                                       |                                                 |
| Ssc.11194.1.S1_at| −1.18      | PLAU        | Urokinase-type plasminogen activator precursor       | Blood coagulation, fibrinolysis, plasminogen activation |
|                  | −1.28      |             |                                                       |                                                 |
|                  | −1.32      |             |                                                       |                                                 |
| Ssc.18603.1.A1_at| −1.57      | G0S2        | Putative lymphocyte G0/G1 switch protein 2           | Cell cycle                                      |
|                  | −1.80      |             |                                                       |                                                 |
|                  | −2.07      |             |                                                       |                                                 |
| Ssc.12431.1.A1_at| −2.4       | MYO5B       | Myosin Vb                                            | Protein transport                               |
|                  | −2.46      |             |                                                       |                                                 |
|                  | −2.11      |             |                                                       |                                                 |
Table 7. Cont.

| Probe Set ID       | Log2 ratio | Gene symbol | Gene title                  | Tentative function   |
|--------------------|------------|-------------|-----------------------------|----------------------|
|                    | GIS26 (STa† STb† LT†/ WT | GIS26 (STa† STb† LT†/ WT | PBS/WT |                     |
| Ssc.113.1.S1_at    | −1.79      | −1.77       | −2.3                        | IL1A                 | Interleukin-1 alpha precursor | Inflammatory response, cytokine, pyrogen |
| Ssc.30277.1.A1_at  | −1.79      | −1.88       | −2.46                       | SLC26A3              | Chloride anion exchanger       | Antiport, transport (excretion) |
| Ssc.113.1.S2_at    | −1.69      | −1.82       | −2.68                       | IL1A                 | Interleukin-1 alpha precursor | Inflammatory response, cytokine, pyrogen |
| Ssc.15927.1.S1_at  | −2.3       | −2.28       | −4.18                       | MMP3                 | Stromelysin-1 precursor; matrix metalloproteinase-3 | Proteolysis, metalloendopeptidase activity |

The selection criteria to define a transcript as differentially regulated were: an absolute log2 ratio equal or larger than 1, a MAS 5.0 present detection call and an uncorrected P value of <0.001. doi:10.1371/journal.pone.0041041.t007

Validation of the Microarray with qRT-PCR Analysis
Validation of expression differences measured with microarrays using an alternative method is essential [44]. This was done through quantifying the expression with RT-PCR on nine selected genes, eight differentially regulated immune response genes IL1A, IL2, IL7A, PAP, TLR4, MMP1, MMP3, CYP1A1, and a presumed constitutive reference gene, FABP2 (Table 2). FABP2, also named intestinal fatty acid-binding protein (I-FABP), is a specific marker for the relative amount of epithelium [45], and its constitutive expression should be unaffected by ETEC infection, which is the case here. No expression differences were found with qRT-PCR consistent with the microarray data.

Linear regression analysis showed that the correlation between the values of the microarray and qRT-PCR data was highly significant for IL1A, IL7A, PAP, TLR4, MMP1, MMP3, and CYP1A1 and significant for MMP1 and IL2 (Figure 5).

Table 8. Microarray data expressed as a log2 fold-change of PBS and mutant ETEC-infected versus wild type ETEC-infected (WT) small intestinal segments (Group II, transcripts differentially regulated by both mutant ETEC strains).

| Probe Set ID       | Log2 ratio | Gene symbol | Gene title                  | Tentative function   |
|--------------------|------------|-------------|-----------------------------|----------------------|
|                    | GIS26 (STa† STb† LT†/ WT | GIS26 (STa† STb† LT†/ WT | PBS/WT |                     |
| Ssc.9781.1.S1_at   | −1.36      | −1.25       | −1.11                       | SERPINE1             | Plasminogen activator inhibitor-1 precursor | Plasminogen activation, cellular response to LPS, defense response to Gram-negative bacterium, positive regulation of IL-8 production, positive regulation of leukotriene production involved in inflammatory response |
| Ssc.12781.1.A1_at  | −1.16      | −1.26       | −0.94                       | TLR4                 | Toll-like receptor 4 precursor | Immunity, inflammatory response, innate immunity, lipopolysaccharide receptor activity |
| Ssc.1674.1.A1_at   | −1.09      | −1.08       | −0.74                       | SLC2A14              | Glucose transporter 14 (H. sapiens) | Developmental protein, glucose transmembrane transporter activity |

The selection criteria to define a transcript as differentially regulated (indicated in bold) were: an absolute log2 ratio equal or larger than 1, a MAS 5.0 present detection call and an uncorrected P value of <0.001. Transcripts not in bold are not differentially regulated as they do not meet these strict requirements. doi:10.1371/journal.pone.0041041.t008

Discussion
The contributions of different enterotoxins of an F4+ ETEC strain to the induction of small intestinal secretion and early innate immune responses were studied in weaned piglets by use of isogenic deletion mutants. To our surprise, we were not able to obtain a mutant strain with an LT only phenotype. We have no direct explanation for the effect of deletion of one toxin gene (eltAB or estA) on the expression of other toxins. The methodology used is very gene specific and we always confirmed by PCR that only the target toxin gene was deleted. In addition, genome sequencing of the wild type GIS26 ETEC strain revealed that eltAB and estA are present on different virulence plasmids and therefore polar effects of the deletion of eltAB on estA and vice versa can be excluded. However, differences in toxin expression might be regulated at the level of transcription where one toxin controls the expression of another toxin but this requires further investigation. Therefore, due to the discrepancy between genotype and phenotype in some of the mutants, conclusions on toxin knockout in the present study...
Table 9. Microarray data expressed as a log2 fold-change of PBS and mutant ETEC-infected versus wild type ETEC-infected (WT) small intestinal segments at 4 h (Group III, transcripts differentially regulated in the PBS/WT comparison and in one of both mutant strains).

| Probe Set ID | Log2 ratio  | Gene symbol | Gene title                                      | Tentative function (UniprotKB)                                      |
|--------------|-------------|-------------|------------------------------------------------|---------------------------------------------------------------------|
| Ssc.7991.1A1_at | 1.84        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | KIAA1468 -- Binding                                                  |
| Ssc.1039.1S1_at | 1.74        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | APOC3 Apolipoprotein C-III precursor Transport, G-protein coupled receptor protein signaling pathway |
| Ssc.26348.1S1_at | 1.42        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | PRLR Prolactin receptor precursor Receptor, T cell activation       |
| Ssc.1147.1A1_at | -1.18       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | LPL Lipoprotein lipase precursor Lipid degradation, hydrolase       |
| Ssc.15927.2A1_at | -1.72       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | MMP3 Stromelysin-1 precursor; matrix metalloproteinase-3 Proteolysis, metalloendopeptidase activity |
| Ssc.15927.2A1_at | -2.28       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | MMP3 Stromelysin-1 precursor; matrix metalloproteinase-3 Proteolysis, metalloendopeptidase activity |
| Ssc.18284.1A1_at | 2.45        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | KCN13 Inward rectifier potassium channel 13 Voltage-gated channel, potassium transport |
| Ssc.9238.1A1_at | 1.08        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | ATP10D Potential phospholipid-transporting ATPase VD                |
| Ssc.20419.2S1_at | 1.11        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | SLC25A27 Mitochondrial uncoupling protein 4 Transport (transmembrane), binding |
| Ssc.22210.2S1_at | 1.34        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | MTHFD2L Similar to bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase [H. sapiens] Hydrolase, oxireductase |
| Ssc.2342.1S1_at | 1.11        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | ORE2C2 Transcriptional activator                                     |
| Ssc.27502.1S1_at | 1.38        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | ITGB8 Integrin beta-8 Integrin, receptor, cell adhesion             |
| Ssc.24037.1S1_at | 1.14        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | UBE2J2 Ubl conjugation pathway                                       |
| Ssc.28515.1S1_at | 1.13        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | USP2 Ubiquitin carboxyl-terminal hydrolase 2 Cell cycle, Ubl conjugation pathway |
| Ssc.2132.1S1_at | 1.03        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | RPS6KAS Ribosomal protein S6 kinase alpha 5 Serine/threonine-protein kinase, transfease, response to stress and external stimulus |
| Ssc.30861.1A1_at | 0.93        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | FLRT3 Leucine-rich repeat transmembrane protein FLRT3 precursor Cell adhesion |
| Ssc.4664.1S1_at | 0.96        | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | PPP2R2C Serine/threonine protein phosphatase 2A Signal transduction  |
| Ssc.13849.1S1_at | -0.92       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | TNAGL1 Androgen-regulated gene 1 [H. sapiens] Immune response, polysaccharide binding |
| Ssc.2722.1A1_at | -0.96       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | SERPINB2 Plasminogen activator inhibitor-2 precursor Protease inhibitor, anti-apoptosis |
| Ssc.9334.1S1_at | -0.93       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | RPA Ribose-5-phosphate isomerase Isomerase                          |
| Ssc.30734.1S1_at | -0.64       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | TSPAN7 Transmembrane 4 superfamily member 2 Cell proliferation, cell motility |
| Ssc.3012.1S1_at | -0.83       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | UPP1 Uridine phosphorylase 1 Glycosyltransferase, immune response   |
| Ssc.9461.1A1_at | -0.60       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | ERRF1 Mitogen-inducible gene 6 protein Response to stress, protein kinase binding |
| Ssc.2165.2S1_at | -0.72       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | SFN Stat5; Epithelial cell marker protein 1 DNA damage response, apoptosis |
| Ssc.12463.1A1_at | -0.97       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | TRIM16 Tripartite motif protein 16 Interleukin-1 binding            |
| Ssc.24966.1S1_at | -1.10       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | NP Purine nucleoside phosphorylase Glycosyltransferase, transferase |
| Ssc.15601.1A1_s_at | -1.44       | GIS26      | GIS26 (STa STb WT / STblow STb LT WT)           | IL1B Interleukin-1 beta precursor Inflammatory response, cytokine, pyrogen |
Table 9. Cont.

| Probe Set ID  | Log2 ratio | Gene title | Tentative function (UniprotKB) |
|---------------|------------|------------|--------------------------------|
| Ssc.29329.1.A1_at | 0.97       | DCHS2      | Protocadherin protein CDHJ [H. sapiens] Cell adhesion, calcium ion binding |
| Ssc.17573.1.S1_at | 1.52       | IL1B       | Interleukin-1 beta precursor Inflammatory response, cytokine, pyrogen |
| SscAffx.23.1.S1_at | 2.87       | IL17A      | Interleukin-17 precursor Cytokine, inflammatory response |

The selection criteria to define a transcript as differentially regulated (indicated in bold) were: an absolute log2 ratio equal or larger than 1, a MAS 5.0 present detection call and an uncorrected P value of $<0.001$. Transcripts not in bold are not differentially regulated as they do not meet these strict requirements.

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GIS26 (STa susceptible to STa induced diarrhea [46]. However, results with the piglets could be of importance since neonatal animals are more important role for STb in the early secretory response. This difference could also be explained by a difference in age of the piglets used. Whereas previous studies used piglets less than two weeks old, piglets in our study were five weeks old. It has been described that presence of STb is more often associated with ETEC isolates from post-weaning diarrhea than from neonatal diarrhea [12,50]. This could also explain why the number of STa in the induction of secretion by the wild type GIS26 E. coli strain, since no significant difference was found between wild type and GIS26 (STa STb LT) strains. Also, the relative unimportance of STa is further confirmed by the limited effect of GIS26 (STa STb LT) on net absorption. Here, the age of the piglets could be of importance since neonatal animals are more susceptible to STa induced diarrhea [46]. However, results with GIS26 (STa STb LT) also suggest that the combined effects of LT and STa on net absorption can lead to secretion in some pigs. This effect seems however variable and might be dependent on an underlying infection. One candidate is rotavirus. Several publications already suggested that diarrhea due to ETEC could be aggravated by a concurrent infection with rotavirus [47,48,49].

Previous studies with isogenic deletion mutants in a gnotobiotic infection model highlighted the importance of LT as a virulence factor compared to STb [13,14,15]. Our results suggest an important role for STb in the early secretory response. This difference could also be explained by a difference in age of the piglets used. Whereas previous studies used piglets less than two weeks old, piglets in our study were five weeks old. It has been described that presence of STb is more often associated with ETEC isolates from post-weaning diarrhea than from neonatal diarrhea [12,50]. This could also explain why the number of isolates in which STb is present increases with the age of the animal [51]. For LT on the other hand, it has been shown in vitro that binding to its receptor GM1 on brush border vesicles is stronger in neonatal piglets compared to 4-week-old piglets [52]. Furthermore, the difference in sampling time and model used may also explain the conflicting results between this study and others.

In the above mentioned studies [13,14,15] pigs were orally infected and clinical signs of diarrhea where recorded until 96 h after infection. Alternatively, 4 h could be too early for LT to note any appreciable effect. In a mouse intestinal loop model, secretory effects of STa and STb were already visible 30 minutes after administration, and any effect for LT on secretion was only noted 3 h after incubation with a maximal effect at 8 h [53]. Furthermore, unpublished studies of our lab could not demonstrate net fluid secretion via the intestinal segment perfusion technique the first 4 hours after incubation with 3 μg LT, whereas after 6 hours decreased absorption and sometimes secretion could be seen (unpublished data).

Having established that STb seems to be the most significant enterotoxin responsible for secretory responses, the correlation with gene expression was explored. The microarray analysis data were validated through the quantitative RT-PCR on eight selected immune genes and a reference gene (FABP2). A good correlation was obtained for the immune genes and for FABP2 a constitutive expression was measured in both data sets.

First, a comparison was made between normal (PBS control) versus ETEC-infected small intestinal segments. The number of differentially expressed transcripts, 310 in total (38 transcripts when using an absolute log2-ratio larger than 2), was similar to an earlier study examining the influence of ETEC on gene expression, also using the SISP technique [54], paralleling the drastic change in fluid absorption. As expected, genes with a function in transport of fluids and electrolytes, such as KCNJ13, AQP8, ATG10, and APOC3, were significant differential down-
regulated in ETEC infected segments. The apical chloride anion exchanger DRA (SLC26A3) is functionally coupled to CFTR (cystic fibrosis transmembrane regulator) and NHE3 (Na/H exchanger-isomorph 3), which are both involved in the secretory pathways of LT, STa and STb [34,35]. The transcript for this gene was significantly up-regulated in ETEC infected segments, clearly demonstrating its involvement in the disturbance of water and electrolyte transport after ETEC infection.

The physiological response to ETEC is also accompanied by a marked change in mucosal expression of innate immune genes. From the 38 transcripts (absolute log2-ratio larger than 2), 15 genes including PAP and MMP1 appeared to be associated with ETEC infection irrespective of the enterotoxins produced (Table 11, group V). Niewold et al. [34] already suggested a possible role for PAP and MMP1 in ETEC infection, and they were also found in reaction to Salmonella typhimurium [55] and Lactobacillus plantarum [56], suggesting them to be important in a general antibacterial response. This is probably consistent with the established function for PAP as serum marker for Crohn’s disease [40], which may be also applicable for ETEC infection. IL-8 was found in the same general response group as PAP en MMP1, but with a lower expression level (absolute log2-ratio between 1 and 2) (Table S1). Its induction by ETEC may be in agreement with its apparent important role in infection resolution of ETEC [20]. Indeed, when piglets are infected with the F4’ GIS26 (WT) strain it results in a rapid colonization and a fast F4 specific mucosal immune response [57]. In vitro results, with the same ETEC strain, indicated that flagellin is involved in the induction of IL-8 [58], regardless of F4. This is in agreement with the absence of a differential regulation of IL-8 in our mutant strains, being all flagellin positive.

Further comparisons were done to establish gene expression associated with specific enterotoxins produced by ETEC. The comparisons GIS26 (STa STb’ LT’) versus wild type and GIS26 (STa’ STb LT’) versus WT showed no differential expression, showing that the presence of LT had no influence on the early gene expression following ETEC infection and indicating that presence of either one of the heat stable enterotoxins is sufficient to activate the early immune responses. Comparison GIS26 (STa STb’ LT’) versus WT showed the difference in gene expression (43 transcripts) due to the 3-fold lower STb concentration as in comparison GIS26 (STa’ STb LT’) versus WT (Figure 2B). Comparison GIS26 (STa’ STb’ LT’) versus GIS26 (STa STb’ LT’) showed 54 transcripts associated with presence or absence of all three toxins. Subsequently, transcripts were grouped according to genes in common between the three comparisons in which differential expression was found. This is not necessarily a functional grouping, and in fact only groups I and V could be related to specific factors. Group I represents genes related to STb, group V represents genes unrelated to enterotoxins (see above). The other groups cannot be easily related to specific factors, however, they allow for comparison between strains.

Table 10. Microarray data expressed as a log2 fold-change of PBS and mutant ETEC-infected versus wild type ETEC-infected (WT) small intestinal segments at 4 h (Group IV, differentially regulated transcripts exclusively found in one of the mutant strain comparisons).

| Probe Set ID       | Log2 ratio GIS26 (STa STb’ LT’) / WT | Log2 ratio GIS26 (STa’ STb LT’) / WT | Gene symbol | Gene title                      | Tentative function                  |
|-------------------|--------------------------------------|--------------------------------------|-------------|----------------------------------|-------------------------------------|
| Ssc.26516.1.A1_at | 1.00                                 | 0.87                                 | ABCG8       | ATP-binding cassette, sub-family G, member 8 | Transport                           |
| Ssc.16332.1.S1_at | 1.01                                 | 0.71                                 | ABCC2       | Canalicul multiospecific organic anion transporter 1 | Transport                           |
| Ssc.17339.1.S1_at | 1.01                                 | 0.81                                 | SLC15A1     | Oligopeptide transporter, small intestine isomorph (Intestinal H+ / peptide cotransporter) | Digestion, protein transport        |
| Ssc.5656.1.S1_at | 1.14                                 | 0.87                                 | TLL2        | Toll-like 2 [H. sapiens]          | Developmental protein, hydrolase, protease, metalloprotease |
| Ssc.196.1.S1_at  | −1.55                                | −1.21                                | PLAT        | Tissue-type plasminogen activator precursor | Serine-type endopeptidase activity |
| Ssc.9311.1.A1_at | −1.22                                | −0.92                                | PHLDA1      | Pleckstrin homology-like domain, family A, member 1 [H. sapiens] | Apoptosis, protein binding |
| Ssc.10552.1.A1_at| −1.14                                | −0.86                                | PTPRG       | Protein-tyrosine phosphatase gamma precursor | Hydrolase, protein phosphatase |
| Ssc.3139.1.A1_at | −1.13                                | −0.91                                | RGS2        | Regulator of G-protein signaling 2 | Signal transduction inhibitor |
| Ssc.11076.1.S1_at| −1.57                                | −2.07                                | SDS         | L-serine dehydratase              | Gluconeogenesis, lyase             |
| Ssc.2464.1.S1_at | −1.35                                | −1.80                                | STC1        | Stanniocalcin 1 precursor         | Hormone activity, response to nutrient |

The selection criteria to define a transcript as differentially regulated (indicated in bold) were: an absolute log2 ratio equal or larger than 1, a MAS 5.0 present detection call and an uncorrected P value of < 0.001. Transcripts not in bold are not differentially regulated as they do not meet these strict requirements.

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which have shown an increase in prostaglandin E2 (PGE2) enterotoxins expressed. This is in agreement with previous studies.

Results with the mutant strains suggest that bacteria that cause severe secretory diarrhea. Furthermore, our response (see above), MMP3 may be specific for Gram-negative bacteria.

Table 11. Microarray data expressed as a log2 fold-change of PBS and mutant ETEC-infected versus wild type ETEC-infected (WT) small intestinal segments (Group V, differentially regulated transcripts only found in the comparison WT/PBS).

| Probe Set ID       | Log2 ratio | Gene symbol | Gene title                        | Tentative function                      |
|--------------------|------------|-------------|-----------------------------------|-----------------------------------------|
| Ssc.21194.1.S1_at  | 1.92       | PTPRR       | Receptor-type protein-tyrosine phosphatase R precursor | Hydrolase, protein phosphatase, receptor |
| Ssc.11487.1.A1_at  | 1.57       | PRR15       | –                                 | Developmental protein                   |
| Ssc.18488.1.S1_at  | 1.69       | AQP8        | Aquaporin 8                        | Transport                               |
| Ssc.7458.1.A1_at   | 1.31       | GPT2        | Alanine aminotransferase           | Aminotransferase, transferase           |
| Ssc.4724.1.S1_at   | 1.18       | PHLPP       | –                                 | Protein binding, catalytic activity     |
| Ssc.16250.1.S2_at  | −1.37      | IL1RN       | Interleukin-1 receptor antagonist protein precursor | Cytokine activity, interleukin-1 receptor antagonist activity, immune response, inflammatory response |
| Ssc.16013.1.S1_at  | −1.00      | MMP1        | Intestinal collagenase precursor; matrix metalloproteinase-1 | Proteolysis, metalloendopeptidase activity |
| Ssc.30857.1.S1_at  | −0.80      | ENC1        | Ectoderm-neural cortex-1 protein   | Ubl conjugation pathway                 |
| Ssc.19907.1.S1_at  | −1.55      | F3          | Tissue factor precursor            | Blood coagulation                       |
| Ssc.11609.1.A1_at  | −1.10      | ASNS        | Asparagine synthetase              | Ligase                                  |
| Ssc.33.1.S1_at     | −1.22      | DUOX2       | Dual oxidase 2 precursor; [H. sapiens] | Oxidoreductase, peroxidase, cytokine-mediated signaling pathway |
| Ssc.29281.1.A1_at  | −1.47      | SLC7A11     | Cystine/glutamate transporter      | Response to toxin, transport            |
| Ssc.18918.1.A1_at  | −0.92      | GPX2        | Glutathione peroxidase-gastrointestinal | Oxidoreductase, peroxidase, response to oxidative stress |
| Ssc.6180.1.A1_at   | −1.64      | SLC7A11     | Cystine/glutamate transporter      | Response to toxin, transport            |
| Ssc.16470.1.S1_at  | −0.12      | REG3A (PAP) | Pancreatitis-associated protein 1 precursor | Acute phase response, inflammatory response |

The selection criteria to define a transcript as differentially regulated (indicated in bold) were: an absolute log2 ratio equal or larger than 2, a MAS 5.0 present detection call and an uncorrected P value of <0.001. Transcripts not in bold are not differentially regulated as they do not meet these strict requirements.

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From the long list (Tables 7, 8, 9, 10, 11 and Table S1), only the most prominent genes and those earlier implicated in secretory bacterial pathogenesis are discussed in this paper. The matrix-metalloproteinase, MMP3, reported as critical for CD4+ T lymphocyte migration in the intestinal mucosa [59], was significantly up-regulated in the wild type strain. A previous study on acute cholera also demonstrated the expression of matrix metalloproteinases (MMP1 and MMP3) in duodenal mucosa [60]. Whereas MMP1 seems to be part of the general antibacterial response (see above), MMP3 may be specific for Gram-negative bacteria that cause severe secretory diarrhea. Furthermore, our results with the mutant strains suggest that MMP3, as all genes of group I (Table 7), is at least partially regulated by the heat stable enterotoxins expressed. This is in agreement with previous studies which have shown an increase in prostaglandin E2 (PGE2) synthesis by STb in vivo [34]. MMP production has been shown to be PGE2-regulated in various cell types [61,62,63,64]. We therefore speculate that high levels of STb are important in the induction of these immune genes and that STa might be able to compensate for the loss of STb.

The genes SERPINE1 and TLR4, both involved in immune/inflammatory responses, are inducible by lipopolysaccharide, present in the outer layer of Gram-negative bacteria [65,66,67,68]. For the porcine TLR4 gene this has also been confirmed in LPS-stimulated porcine dendritic cells and an intestinal epithelial cell line [69,70]. In addition Vibrio cholerae, secreting cholera toxin (an enterotoxin homologous to LT), induced TLR4 expression in vitro in a human IEC [71]. Our results showed no differential regulation of these genes in segments infected with the Gram-negative GIS26 ETEC strain (comparison 3, Table 8). For TLR4, this is in agreement with an in vitro study on porcine epithelial cells where an STa secreting ETEC strain...
even seemed to down-regulate TLR4 expression at very high concentrations [69]. A previous microarray study on the porcine intestinal epithelial cell line IPEC-J2 also lacked induction of TLR4 after co-incubation with a LT\(^+\) STb\(^+\) F4ac ETEC strain, compared to mock-infected cells [32]. A down-regulated expression of TLR4 in the segments infected with mutant strains GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) and GIS26 (STa\(^+\) STb\(^-\) LT\(^+\)) was found here. Taken together, it is suggested that TLR4 and SERPINE1 are not solely regulated by LPS from ETEC but are rather down-regulated in the absence of LT, STa and (most of) STb.

In group III of Table 9, strongly regulated immune genes are IL1B and IL17A. IL-17 is generally thought to increase inflammation by recruiting other immune cells. CD4\(^+\) Th17 cells, characterized by the production of IL-17 [72], are probably involved in clearance of extracellular pathogens [73,74,75]. They have also been shown to play an important role in the pathogenesis of colitis and several other autoimmune diseases (reviewed in [76,77]). Furthermore, much of the IL-17 released during an inflammatory response is produced by innate immune cells including granulocytes and monocytes [78]. These early responses have a central role in the initiation of IL-17-dependent immune responses, even before the activation of Th17 cells (reviewed in [79]). Here, IL17A was found to be upregulated by ETEC (PBS versus WT comparison), and the GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) mutant lacked this upregulation (GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) versus WT comparison) (Table 7). This suggests the IL17A response to be enterotoxin specific. Since LT does not seem to have an influence on gene expression (Table 4), it is suggested that STa or STb are responsible. From the comparisons GIS26 (STa\(^+\) STb\(^+\) LT\(^+\)) and GIS26 (STa\(^-\) STb\(^-\) LT\(^-\)) versus the wild type strain, it can be concluded that already limited amounts of STb (STb\(^+\)) are sufficient to elicit an IL17A response. A similar

![Figure 5. Linear regression of qRT-PCR CT ratios versus log2 expression ratios as obtained by microarray analysis for IL1A, IL8, IL17A, PAP, FABP2, TLR4, MMP1, MMP3 and CYP1A1.](image-url)
reaction is seen with IL-1β. Since there is no difference in gene expression between the wild type strain and GIS26 (STa - STb- LT+), presence of only STa also seems sufficient to induce these responses.

In summary, our data suggest that the wild type ETEC strain used in this study can influence immune responses by a variety of pathways. Results from this study can be useful to select either targets for intervention or parameters to measure severity of intestinal diseases. This is also the first study to investigate both the functional role of ETEC enterotoxins and their possible influence on ETEC induced innate immune responses. Our data show the existence of at least two different responses; first what appears to be a general antibacterial response, comprising genes such as PAPI, MMP1 and IL-8 and second, a heat-stable enterotoxin specific response, comprising genes such as IL-17A and IL-1β.

**Supporting Information**

**Table S1** Microarray data expressed as a log2 fold change of PBS and mutant ETEC-infected versus wild type-infected (WT) small intestinal segments.

**References**

1. Nagy B, Fekete PZ (2005) Enterotoxigenic Escherichia coli in veterinary medicine. Int J Med Microbiol 295: 443–454.
2. Qadri F, Swaroop AM, Farsouq AS, Sack RB (2005) Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 18: 465–483.
3. Guerrant RL, Oria R, Bushen OY, Patrick PD, Houpt E, et al. (2005) Global impact of diarrheal diseases that are sampled by travelers: the rest of the lipopolysaccharin. Clin Infect Dis 41 Suppl 8: S524–S530.
4. Thapar N, Sanderson IR (2004) Diarrhoea in children: an interface between developing and developed countries. Lancet 363: 641–653.
5. van der Meulen J, Hulst MM, Smits MA, Schuurman T (2010) Small intestinal segment perfusion test in piglets: future applications in studying probiotics-gut crosstalk in infectious diarrhoea? Benef Microbes 1: 439–445.
6. Zhang W, Robertson DC, Zhang C, Bai W, Zhao M, et al. (2008) Escherichia coli constructs expressing human or porcine enterotoxins induce identical diarrheal diseases in a piglet infection model. Appl Environ Microbiol 74: 5832–5837.
7. Imberechts H, Bertschinger HU, Stamm M, Syddler T, Pohl P, et al. (1994) Prevalence of F107 imbriae on Escherichia coli isolated from pigs with oedema disease or postweaning diarrhoea. Vet Microbiol 40: 219–230.
8. Salajka E, Salajkova Z, Alexa P, Horreich M (1992) Colonization factor different from K88, K99, F41 and 982P in enterotoxigenic Escherichia coli strains isolated from postweaning diarrhoea in pigs. Vet Microbiol 32: 163–175.
9. Nataro JP, Kaper JB (1998) Diarrheagenic Escherichia coli. Clin Microbiol Rev 11: 141–190.
10. Frydenhal K (2002) Prevalence of serogroups and virulence genes in the porcine diarrheagenic E. coli. Vet Microbiol 85: 169–182.
11. Nagy B, Casey TA, Moon HW (1990) Phenotype and genotype of Escherichia coli isolated from pigs with postweaning diarrhoea in Hong Kong. J Vet Res 47: 210–212.
12. Berberov EM, Zhou Y, Francis DH, Scott MA, Kachman SD, et al. (2004) Relative importance of heat-labile enterotoxin in the causation of severe diarrheal disease in the gnotobiotic piglet model by a strain of enterotoxigenic Escherichia coli that produces multiple enterotoxins. Infect Immun 72: 3914–3924.
13. Erume J, Berberov EM, Kachman SD, Scott MA, Zhou Y, et al. (2008) Comparison of the contributions of heat-labile enterotoxin and heat-stable enterotoxin b to the virulence of enterotoxigenic Escherichia coli in Peyer’s positive young pigs. Infect Immun 76: 3141–3149.
14. Zhang W, Berberov EM, Feeeling J, He D, Mosley RA, et al. (2006) Significance of heat-stable and heat-labile enterotoxins in porcine colibacillosis in an additive model for pathogenicity studies. Infect Immun 74: 3107–3114.
15. Rappuoli R, Pizza M, Douce G, Dougan G (1999) Structure and mucosal adjuvanticity of cholera and Escherichia coli heat-labile enterotoxins. Immunol Today 20: 493–500.
16. Chikaratmy K, Ghosh S, Koley H, Mukhopadhyay AK, Ramamurthy T, et al. (2008) Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (βDEF-1) expression in the intestinal epithelial cells. Cell Microbiol 10: 2520–2537.
17. Johnson AM, Kausik RS, Francis DH, Fleckenstein JM, Hardwidge PR (2009) Heat-labile enterotoxin promotes Escherichia coli adherence to intestinal epithelial cells. J Bacteriol 191: 178–186.
18. Huang DB, DuPont HL, Jung ZD, Carlin L, Olkhuysen PC (2004) Interleukin-6 response in an intestinal HCT-8 cell line infected with enteroaggregative and enterotoxigenic Escherichia coli. Clin Diagn Lab Immunol 11: 548–551.
19. Long KZ, Rosado JL, Santos JJ, Haa M, Al Mamun A, et al. (2010) Associations between mucosal innate and adaptive immune responses and resolution of diarrheal pathogen infections. Infect Immun 78: 1221–1228.
20. Naburu MJ, Hoogendoom A, van Zijderveld FG, van der Kin JD (1993) A long-term perfusion test to measure net absorption in the small intestine of weaned pigs. Res Vet Sci 55: 108–114.
21. Niewold TA, Kristsen HL, van der Meulen J, Smits MA, Hulst MM (2005) Development of a porcine small intestinal cDNA microarray: characterization and functional analysis of the response to enterotoxigenic E. coli. Vet Immunol Immunopathol 103: 317–329.
22. Van den Broeck W, Cox E, Godderis BM (1999) Receptor-specific binding of purified F4 to isolated villi. Vet Microbiol 68: 253–263.
23. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.
24. Verhelst R, Schroyen M, Buys N, Niewold TA (2010) The effects of plant polyphenols on enterotoxigenic Escherichia coli adhesion and toxin binding. Livestock Science 133: 101–103.
25. Takeda T, Nair GB, Suzuki K, Zhe HX, Yokoo Y, et al. (1993) Epitope mapping and characterization of antigenic determinants of heat-stable enterotoxin (STa) of enterotoxigenic Escherichia coli by using monoclonal antibodies. Infect Immun 61: 289–294.
26. Naburu MJ, Hoogendoom A, van Zijderveld FG (1994) Effects of weaning and enterotoxigenic Escherichia coli on net absorption in the small intestine of pigs. Res Vet Sci 56: 379–385.
27. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, et al. (2003) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31: e15.
28. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: 1–16.
29. Tsai S, Cassidy JP, Freking BA, Nonneman DJ, Rohrer GA, et al. (2006) Annotation of the Affymetrix porcine genome microarray. Anim Genet 37: 423–429.
30. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.
31. Geens MM, Niewold TA (2010) Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enteroaggregative Escherichia coli, Escherichia coli, and E. coli lipopolysaccharide. Comparative and Functional Genomics doi:10.1155/2010/469583.
32. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.
33. Dubreuil JD (2008) Escherichia coli STb toxin and colibacillosis: knowing is half the battle. FEMS Microbiol Lett 278: 137–145.

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**Author Contributions**

Critical revision of the manuscript: JvDM MG FG JDD. Conceived and designed the experiments: ML SS FG JvDM TN EC. Performed the experiments: ML MG. Analyzed the data: ML MG JDD BMG TN EC. Contributed reagents/materials/analysis tools: SS FG JvDM JDD. Wrote the paper: ML MG TN EC.
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35. Lamprecht G, Gaco V, Turner JR, Natour D, Gregor M (2009) Regulation of the intestinal anion exchanger DRA (downregulated in adenoma). Ann N Y Acad Sci 1165: 261–266.

36. Parks WC, Wilson CL, Lopez-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4: 617–629.

37. Iovanna J, Orell B, Keim V, Dagon JC (1991) Messenger RNA sequence and expression of rat pancreatic-associated protein, a lectin-related protein overexpressed during acute experimental pancreatitis. J Biol Chem 266: 24664–24669.

38. Mouaedd V, Soubeyran P, Vasseur S, Duseti NJ, Dagon JC, et al. (2001) Cdx1 promotes cellular growth of epithelial intestinal cells through induction of the secretory protein PAP1. Eur J Cell Biol 80: 156–163.

39. Vasseur S, Folch-Puy E, Hlouchev V, Garcia S, Fiedler F, et al. (2004) pI improves pancreatic response to acute pancreatitis by enhancing the expression of the anti-inflammatory protein pancreatitis-associated protein I. J Biol Chem 279: 7199–7207.

40. Desjeux A, Barbet M, Barthellemys S, Dagon JC, Hastier P, et al. (2002) Serum measurements of pancreatitis associated protein in active Crohn’s disease with ileal location. Gastroenterol Clin Biol 26: 23–29.

41. Wintero AK, Fredholm M, Davies W (1996) Evaluation and characterization of a porcine small intestine cDNA library: analysis of 939 clones. Mamm Genome 7: 509–517.

42. Dieckgraefe BK, Stover WF, Kowalski JK, Swanson PE, Harrington CA (2000) Analysis of mucosal gene expression in inflammatory bowel disease by parallel oligonucleotide arrays. Physiol Genomics 4: 1–11.

43. Collins CA, Brown EJ (2010) Cytosol as battleground: ubiquitin as a weapon for both host and pathogen. Trends Cell Biol 20: 205–213.

44. Chuaqui RF, Bonnet RF, Best CJ, Gillespie JW, Thag MJ, et al. (2002) Post- analysis follow-up and validation of microarray experiments. Nat Genet 32: 509–514.

45. Pellec MM, Namietz J, Kierlewski W, Namietz J, Ainsulakiwicz M, et al. (2003) Intestinal-type and liver-type fatty acid-binding protein in the intestine. Tissue distribution and clinical utility. Clin Biochem 36: 529–533.

46. Giannella MA, Mann EA (2003) E.coli heat-stable enterotoxin and guanylyl cyclase C: new functions and unsuspected actions. Trans Am Clin Climatol Assoc 114: 67–85, discussion 85–86.

47. Lecej GB, Bablauk RK, Clare DA, King MW (1982) Rotavirus and hemolytic enteropathogenic Escherichia coli in weanling diarrhoea of pigs. J Clin Microbiol 16: 715–723.

48. Tzipori S, Chandler D, Makin T, Smith M (1980) Escherichia coli and rotavirus infection of different ages. Vet Res Commun 30: 57–71.

49. Chapman TA, Wu XY, Barchia I, Bettelheim KA, Driesen S, et al. (2006) Expression of putative Escherichia coli heat-labile enterotoxin (LT) receptors on intestinal brush borders from pigs and healthy and diarrheic swine. Appl Environ Microbiol 72: 4782–4795.

50. Flach CF, Qadri F, Bhuiyan TR, Alam NH, Jennische E, et al. (2007) Broad up-regulation of innate defense factors during acute cholera. Infect Immun 75: 2341–2350.

51. Corcoran ML, Steeler-Stevenson WG, DeWitt DL, Wilt LM (1994) Effect of cholera toxin and pertussis toxin on prostaglandin H synthase-2, prostaglandin E2, and matrix metalloproteinase production by human monocytes. Arch Biochem Biophys 310: 481–488.

52. Goretti M, Piguet A, Salvat C, Berenbaum F, Jacquet C (2010) Inhibition of matrix metalloproteinase −3 and −13 synthesis induced by IL-ibeta in chondrocytes from mice lacking microsomal prostaglandin E synthase-1. J Immunol 185: 6244–6252.

53. Lee J, Banu SK, Subbarao T, Starzinski-Powitz A, Arboh JA (2011) Selective inhibition of prostaglandin E2 receptors EP2 and EP4 inhibits invasion of human immortalized endometrial epithelial and stromal cells through suppression of metalloproteinases. Mol Cell Endocrinol 332: 306–313.

54. Shapiro SD, Kobayashi DK, Perlman AP, Welpus HG (1993) Induction of macrophage metalloproteinases by extracellular matrix. Evidence for enzyme- and substrate-specific responses involving prostaglandin-dependent mechanisms. J Biol Chem 268: 8170–8175.

55. Halili MA, Andrews MR, Labzin LI, Schroeder K, Matthias G, et al. (2010) Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS. J Leukoc Biol 87: 1103–1114.

56. Muzzo M, Bossoio D, Polecatanu N, D’Amico G, Stopazzio A, et al. (2000) Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol 164: 5984–6004.

57. Niu QM, Cuesta N, Vogel SN (2006) Transcriptional regulation of lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) expression in murine macrophages: role of interferon regulatory factors 1 (IRF-1) and 2 (IRF-2). J Endotoxin Res 12: 285–295.

58. Visintin A, Mazzoni A, Spitzer JH, Wylle DH, Dower SK, et al. (2001) Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol 166: 249–255.

59. Moue M, Tohno M, Shimazu T, Kido T, Aso H, et al. (2000) Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epithelial cell line. Biochim Biophys Acta 1700: 134–144.

60. Raymond CR, Wilkie BM (2005) Toll-like receptor, MHC II, B7 and cytokine expression by porcine monocytes and monocyte-derived dendritic cells in response to microbial pathogen-associated molecular patterns. Vet Immunol Immunopathol 107: 235–247.

61. Nandakumar NS, Pugazhendi S, Ramakrishna BS (2009) Effects of enteropathogenic bacteria & lactobacilli on chemokine secretion & Toll-like receptor gene expression in two human colonic epithelial cell lines. Indian J Med Res 130: 170–178.

62. Aggarwal S, Gharalit N, Eirik M, de Sauvage FJ, Garney AL (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 278: 1910–1914.

63. Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, et al. (2003) Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. J Exp Med 202: 761–769.

64. Kelly MN, Kolls JK, Happel K, Schwartmann KD, Schwarzenberger P, et al. (2005) Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against Toxoplasma gondii infection. Infect Immun 73: 617–621.

65. McKenzie BS, Kastelein RA, Cua DJ (2006) Understanding the IL-23-IL-17 immune pathway. Trends Immunol 27: 17–23.

66. Doveu C, McGeorge MJ, Cua DJ (2008) Cytokines that regulate autostimulatory. Curr Opin Immunol 20: 663–668.

67. Dong C (2008) IL-23/IL-17 biology and therapeutic considerations. J Immunother 3: 43–46.

68. Hsu S, Alcorn P, Buonocore S, Kulberg MG, Cua DJ, et al. (2006) Interleukin-23 drives innate and T cell-mediated intestinal inflammation. J Exp Med 203: 2473–2483.

69. Cua DJ, Tato CM (2010) Innate IL-17-producing cells: the sentinel of the mucosal immune system. Nat Rev Immunol 10: 479–489.

70. Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30: 207–210.