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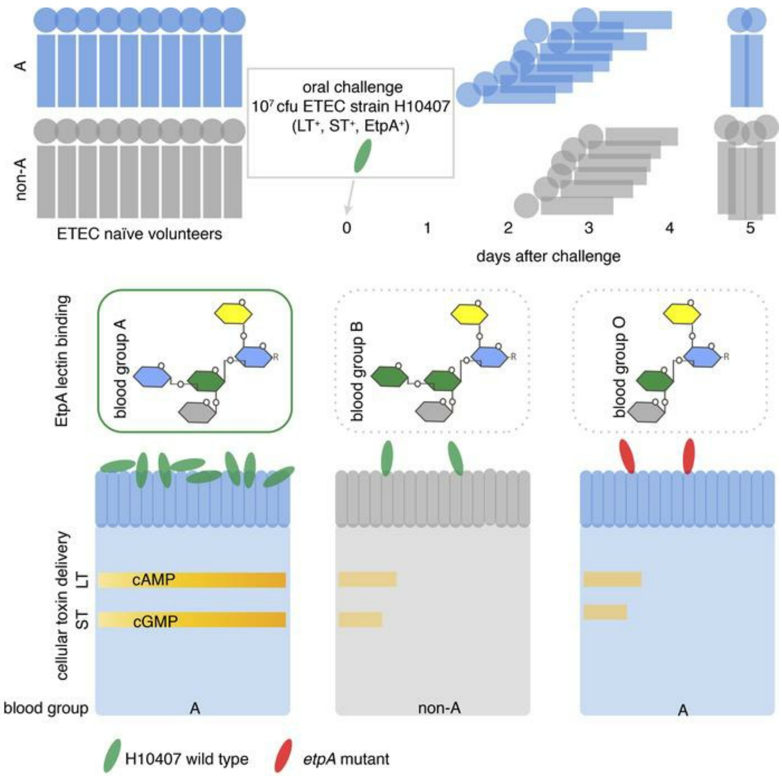
Graphical abstract



Enterotoxigenic *Escherichia coli*- blood group A interactions intensify diarrheal severity

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conflict of interest statement

jmf is listed as the inventor on a US patent 12/079,304 involving the EtpA adhesin.

Abstract:

Enterotoxigenic Escherichia coli (ETEC) infections are highly prevalent in developing countries where clinical presentations range from asymptomatic colonization to severe cholera-like illness. The molecular basis for these varied presentations, that may involve strain-specific virulence features as well as host factors, have not been elucidated. We demonstrated that when challenged with ETEC strain H10407, originally isolated from a case of cholera-like illness, blood group A human volunteers developed severe diarrhea more frequently than individuals from other blood groups. Interestingly, a diverse population of ETEC strains, including H10407, secrete the EtpA adhesin molecule. As many bacterial adhesins also agglutinate red blood cells, we combined the use of glycan arrays, biolayer inferometry, and non-canonical amino acid labeling with hemagglutination studies to demonstrate that EtpA is a dominant ETEC blood group A specific lectin/hemagglutinin. Importantly, we have also shown that EtpA interacts specifically with glycans expressed on intestinal epithelial cells from blood group A individuals, and that EtpA-mediated bacterial-host interactions accelerate bacterial adhesion and effective delivery of both the heat-labile and heat-stable toxins of ETEC. Collectively, these data provide additional insight into the complex molecular basis of severe ETEC diarrheal illness that may inform rational design of vaccines to protect those at highest risk.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is major cause of infectious diarrhea in the developing world(1, 2). These organisms are estimated to be responsible for millions of infections and hundreds of thousands of deaths, particularly in young children (3). Likewise, these pathogens are a principal cause of diarrhea in travelers to endemic areas and an occasional cause of illness in developed countries(4-7). Despite significant declines in deaths from acute diarrhea(8), enteric infections caused by ETEC and other pathogens remain inextricably associated with malnutrition, stunted growth, and impaired intellectual development(9), poorly understood sequelae that appear to result from gut dysfunction that follows repeated episodes of diarrhea in childhood(10). The ultimate impact of these illnesses is further amplified by poor responses to vaccines, increased mortality from other infections, and emerging evidence that early gut dysfunction may be reflected in subsequent development of obesity and its attendant metabolic complications(11, 12). Consequently, these infections continue to exact an extraordinary toll on global health.

The ETEC pathovar is defined by the production of plasmid-encoded heat-labile (LT) and/or heat-stable (ST) enterotoxins(13). Effective delivery of these toxins to cognate receptors on the surface of intestinal epithelial cells is ultimately required to drive net salt and water efflux into the lumen of the small intestine that results in diarrhea. Although ETEC was originally identified in individuals suffering from severe cholera-like diarrhea(14), the manifestations of infection may range from asymptomatic colonization to fulminant illness clinically indistinguishable from that caused by *V. cholerae(15-17)*. The molecular basis for these varied clinical presentations is presently unclear.

In the classic paradigm for ETEC virulence, these organisms adhere to small intestinal epithelia *via* plasmid-encoded fimbrial colonization factors to effect toxin delivery. However, recent studies suggest that the molecular pathogenesis of this diverse group of pathogens may involve a repertoire of additional virulence features (18-20) including a variety of different adhesins to interact with the intestinal epithelium(21).

Because of their impact on global health, these pathogens are a principal target for vaccine development(22). Although there is currently no vaccine based on the classical virulence paradigm that affords significant broad-based protection against ETEC (23), more recently discovered virulence factors and an improved understanding of ETEC microbial pathogenesis could offer additional avenues to protect those at highest risk for severe disease and uncover novel molecular targets for future vaccine development (24).

Among the more recently described putative virulence molecules not targeted in the current generation of ETEC vaccines is EtpA, a novel high molecular weight secreted adhesin (25) bridge between ETEC and intestinal epithelia (26). EtpA appears to be conserved among a phylogenetically and geographically diverse population of ETEC strains (27-29), and has shown promise as a protective antigen in preclinical vaccine studies (30-33). Nevertheless, many questions remain to be answered about the contribution of this antigen and other molecules to disease pathogenesis, and their potential use in vaccines relative to canonical ETEC pathovar specific adhesins.

One approach that has been used to explore ETEC pathogen biology is the controlled human infection model, in which healthy immunologically naïve volunteers are challenged by ingestion of enterotoxigenic *E. coli*. Notably, this model was used initially to establish these toxin-producing *E. coli* as a cause of diarrheal illness in humans(34), and subsequently to examine virulence features of ETEC(35), immune responses to experimental infection(36) and to conduct vaccine studies(37-40). The majority of ETEC human challenge studies conducted to date have involved H10407, a prototype strain originally isolated from an adult with severe, cholera like illness in Bangladesh(41). While diarrheal illness following H10407 challenge tends to be significantly more severe than that associated with other ETEC strains(42, 43), the severity of diarrhea can vary substantially among volunteers within the same cohort implicating the involvement of one or more host factors in disease outcome.

Here, we demonstrate that when challenged with H10407, naïve human volunteers with blood group A are at significantly increased risk for severe diarrhea relative to other blood groups. In addition, we found that the EtpA adhesin is a lectin that selectively promotes interaction of ETEC with human small intestinal epithelial cells expressing blood group A glycans. These EtpA-dependent ETEC-host interactions potentially provide a molecular basis for selection, and an accelerated course of illness in blood group A positive hosts.

Results

Blood group A volunteers challenged with ETEC have more severe disease.

Because pathogen interactions with human blood group antigens can influence the outcome of infection by other gastrointestinal pathogens(44, 45), we questioned whether diarrheal severity following ETEC infection was determined by blood group. To examine the impact of ABO blood groups on the development of diarrheal illness, we analyzed samples from 106 adult human volunteers who had participated in one of four earlier controlled human infection model studies in which they were challenged with ETEC strain H10407 at the Johns Hopkins Center for Immunization Research, and for whom prospectively collected metadata on diarrheal severity were available. Each volunteer was challenged with 1-2 x 10^7 colony forming units of ETEC H10407, was ETEC-naïve prior to experimental challenge, and all subjects were treated with antibiotics upon meeting early treatment criteria (table s1) or at 120 hours post inoculation to clear their infections.

Interestingly, we found that development of severe diarrheal illness was significantly (p=0.015) accelerated in individuals with blood group A relative to those in non-A blood groups (Figure 1A), with volunteers in the A blood group requiring early initiation of antibiotic therapy more frequently (72%) than those in blood groups B or O (43%). Despite earlier antibiotic administration, maximal diarrheal volumes over 24 hours, total diarrheal volume prior to treatment, and the frequency of diarrheal episodes tended to be higher in A vs non-A individuals (Figure S1 A-D). Notably, individuals with blood groups B or O were considerably more likely to remain free of diarrhea or have mild diarrhea (44%) following challenge than volunteers expressing the A blood group (19%) (table 1). Conversely, of the 106 subjects, 81% of those expressing the A blood group antigen (type A or AB) had moderate to severe diarrhea (MSD) compared to 56% of individuals with non-A (B or O) blood types ($p=0.017$), and the overall relative risk of MSD among individuals with A blood group compared to the non-A blood groups is 1.44 (95% CI=1.1-1.9, $p=0.009$) (Figure 1B, table s2). These data therefore suggest that upon challenge with H10407, blood group A volunteers (tables S3, S4) are significantly more likely to manifest the severe, cholera-like spectrum of illness originally associated with this isolate.

EtpA interacts specifically with blood group A glycans

Pathogens use adhesins possessing glycan specific lectin activity(46) to engage blood group antigens expressed on mucosal surfaces, including the gastrointestinal tract (47). The apical surface of intestinal epithelial cells is heavily decorated with a variety of glycoproteins that form the glycocalyx (48) and glycosylation patterns of these proteins are in part determined by blood group (47, 49). Because earlier studies had suggested that EtpA, a secreted adhesin molecule discovered in H10407(25), engages the surface of gastrointestinal epithelia (26), we conducted glycan array screening to further elucidate the nature of EtpA-mediated bacterial adhesion. Using rEtpA to probe arrays containing more than 400 unique glycan species, we found that rEtpA interacted almost exclusively with glycans related to blood group A. In contrast, we observed little or no binding to other glycans including those of blood group B, or blood group O (Figure 2A, supplemental dataset 1). Next, in biolayer interferometry experiments we found that EtpA interacted with blood group A related glycans with high affinity, when the glycan is presented as a multivalent trisaccharide conjugated to polyacrylamide(PAA): GalNAcα1-3(Fucα1,2)Galß-PAA; kD~9 x 10^{-8} M) (Figure 2B).

Similarly, we demonstrated that EtpA can engage N-Acetylgalactosamine (GalNAc) (kD~1.6 x 10^{-8} M), the terminal sugar in blood group A, with higher affinity than galactose (Gal) (kD~1.9 x 10^{-7}), the terminal sugar of blood group B (Figure 2C). Collectively, these studies suggested that the EtpA adhesin is a blood group A specific lectin.

Many bacterial adhesins possess the ability to agglutinate red blood cells (46). Included among these adhesins is filamentous hemagglutinin from *Bordetella* pertussis(50), a component of acellular pertussis vaccines(51), that like EtpA belongs to the two-partner family of bacterial exoproteins(25). We found that EtpA could decorate the surface of human RBCs belonging to blood group A1 (Figure 3A) and to a lesser extent cells from those from individuals from blood group A2 that exhibit lower levels of the glycosyltransferase enzyme activity required for addition of terminal GalNAc residues that determine the A blood group(52); however, only a small percentage of blood group B, or O erythrocytes had detectable EtpA on the surface. Similarly, in RBC pull-down assays, more rEtpA remained associated with erythrocytes from A1 and A2 blood groups (Figure 3B), than those from B, or O blood groups. In addition, incubation of EtpA with RBCs from blood group A1 resulted in agglutination but yielded poor agglutination activity with other RBCs (Figure 3C). In addition, pre-treatment of target A1 cells with α-N-Acetylgalactosaminidase, completely abrogated the hemagglutinin activity of EtpA (Figure 3D), as did addition of excess GalNAc, but not GlcNAc, or galactose, the terminal sugar of blood group B (Figure s2). We also demonstrated that latex microparticles coated with EtpA specifically agglutinated A1 RBCs (Figure 3E). EtpA therefore belongs to a large family of bacterial adhesins that possess lectin/hemagglutinin activity(46), and this protein preferentially targets GalNac residues presented in the context of the A blood group.

EtpA is a predominant blood group A binding partner in ETEC

Next, to determine whether EtpA is the predominant blood group A binding partner in ETEC, we examined interaction of A blood group glycans with subcellular protein fractions isolated from

H10407. Blood group A glycans interacted only with a high molecular weight species (in excess of 150 kD) in culture supernatants, with no detectable interaction with preparations from the inner or outer membranes (Figure 4A). Because the EtpA adhesin is the only known ETEC-specific virulence molecule in the ETEC secretome of this size(53), we next compared binding of blood group A glycans to secreted proteins from the wild type H10407 strain and isogenic *etpA* mutants. Binding occurred only in supernatants prepared from wild type ETEC, or the complemented *etpA* mutant strain, but not the *etpA* isogenic deletion mutant (Figure 4, B-D). Interestingly, blood group A erythrocytes have been traditionally used to screen for the presence of CFA/I fimbriae in ETEC(54). However, we found that CFA/I mediated non-specific hemagglutination of RBCs from all blood types tested consistent with the idea that CFA/I interactions are mediated via sialic acid(55) (Figure S3). To further confirm the role of EtpA in mediating interaction with the A blood group, we next tagged the ETEC proteome using the noncanonical amino acid azidonorleucine (ANL)(56), and incubated tagged supernatant and outer membrane protein (OMP) fractions with erythrocytes. Again, we found that a single band from tagged wild type supernatant, corresponding to EtpA bound significantly only to A1 erythrocytes (Figure 4E). However, testing of ANL-labeled outer membrane proteins (OMPs) purified from H10407 failed to detect any specific interactions of ETEC OMPs with RBCs of any major blood group (Figure S4), further suggesting that the secreted EtpA molecule is the dominant blood group A-specific lectin expressed on the surface of ETEC. We also demonstrated that EtpA expression by a geographically diverse group of ETEC conferred the ability to bind blood group A, suggesting that these findings are not limited to the H10407 prototype strain (Figure S5A-D).

EtpA directs ETEC interaction with blood group A on host intestinal epithelial cells.

We questioned whether blood group A related glycans on the surface of intestinal enterocytes might therefore direct EtpA-mediated interactions required for effective bacterial adhesion and toxin delivery. Using HT-29 intestinal cells, derived from a blood group A positive host, we found that EtpA bound to the surface of these cells and co-localized with blood group A glycans (Figure 5A).

Conversely, recombinant EtpA bound very poorly to the surface of HT-29A$^{-/-}$ cells, a HT-29-derived line CRISPR-Cas9-engineered to eliminate the gene encoding the glycosyltransferase responsible for addition of terminal GalNac residues to the core H (blood group O) antigen(45). Likewise, we found that EtpA from ANL-tagged wild type ETEC interacted preferentially with HT-29 parental cells compared to the mutant HT-29 A-/- cells (Figure 5B). Interestingly, EtpA expressing ETEC localized to regions of blood group A expression on the surface of HT-29 cells (Figure 5C), and EtpA-expressing ETEC adhered more readily to parental cells expressing blood group A (Figure 5D,E, Fig. S6A). Importantly, we found that optimal delivery of heat-labile toxin (LT) required both EtpA (Figure S6B) and the presence of the A blood group antigen (Figure 5F). Similarly, we noted that addition of exogenous GalNAc impaired both EtpA-mediated adhesion and toxin delivery to HT29 parental cells (Figure S7A,B), suggesting that this terminal glycan on blood group A is important for EtpA pathogen-epithelial cell interactions. Notably, while purified LT was significantly less potent than cholera toxin (CT) in activating cAMP in target cells, direct toxin mediated activation of this cyclic nucleotide was actually enhanced in cells lacking the A blood group (Figure s8), recapitulating recent observations with CT(45). Collectively, these data suggest that the presence of blood group A effectively promotes bacterial adhesion and ETEC-host interactions that culminate in enhanced toxin delivery.

HT-29 cells are derived from a metastatic colorectal carcinoma, and glycan distribution can vary significantly with location within the gastrointestinal tract(57). In addition, ETEC are thought to preferentially target the small intestine, where the bulk of fluid secretion occurs following colonization, rather than the colon.

We sought, therefore, to confirm these interactions using small intestinal enteroids(58, 59) derived from human stem cells obtained from individuals belonging to each of the major ABO blood groups. Using ileal enteroids, we found that while EtpA clearly bound to cells from individuals who are blood group A, the purified adhesin bound very poorly to cells expressing either B or O blood groups

(Figure 6A,B). We also found EtpA-expressing bacteria within areas of blood group A expression on the epithelial surface of polarized enteroids (Figure 6C), and interestingly, we also observed apparent rearrangement or “footprints’ of the A blood group at sites of bacterial attachment suggesting an intimate interaction of ETEC with these cell surface glycans (Figure 6D). Finally, we found that ETEC adhered much more readily to polarized intestinal monolayers derived from individuals with blood group A compared to other blood groups (Figure 6E), and that adhesion of wild type ETEC to A blood group enteroids was significantly accelerated relative to bacteria lacking *etpA* (Figure 6F), culminating in enhanced toxin delivery by the EtpA expressing wild type (Figure 6G, H). EtpA therefore appears to be a dominant pathovar-specific lectin uniquely capable of preferentially engaging A blood group glycans expressed on small intestinal epithelial surfaces to accelerate effective ETEC pathogen-host interactions.

Discussion

Blood group dependent microbial-host interactions can dictate clinical outcomes of infection with important gastrointestinal pathogens. In the case of *Helicobacter pylori*, the BabA adhesin facilitates attachment to the gastric mucosae by engaging ABO(60) and Le^b, blood group antigens potentiating the delivery of other secreted virulence factors that lead to development of gastric ulcers and cancer(44, 60, 61). Similarly, the clear epidemiologic associations between severe *Vibrio cholerae* infections and O blood group(62-66), are reflected in recent observations that cholera toxin (CT) preferentially activates enterocytes of blood group O lineage(45). Both CT and closely related heat-labile toxin (LT) of enterotoxigenic *E. coli* appear to share in a differential binding of blood group antigens that favors intoxication of blood group O enterocytes(67, 68). Interestingly, because of molecular similarity between CT and LT, some earlier field studies examined ETEC outcomes relative to blood group with the implicit assumption that LT-producing ETEC would lead to more severe disease following infection of O blood group hosts, however these studies yielded conflicting results(69, 70).

By contrast, from the human volunteer ETEC challenge studies a surprising but clear relationship between A blood group and disease severity begins to emerge. The ETEC controlled human infection model has a number of potential advantages over prior field studies(3, 62, 69, 70) in defining a molecular basis for disease. First, the present studies were confined to ETEC-naïve adults challenged with a defined inoculum of a single strain (H10407) isolated from an individual with severe cholera like illness(41). The controlled human infection model may have therefore permitted us to unmask somewhat subtle molecular associations that might have otherwise been hidden by confounding variables common to field studies of enteric infections in developing countries including malnutrition, breast feeding, altered microbiota, co-infection with other enteric pathogens, as well as the genetic variability inherent in the infecting ETEC strains.

The studies presented here provide an expanded view of ETEC pathogen-host interactions beyond a classical paradigm in which these organisms engage the intestinal surface via fimbrial colonization factors and deliver heat-labile and/or heat-stable enterotoxins. The present studies of ETEC paint a more complex picture in which A blood group expression potentially facilitates EtpA-mediated lectin specific bacterial interactions with intestinal epithelia, that culminate in effective toxin delivery and symptomatic diarrheal illness.

Our studies suggest that the many ETEC strains that produce the EtpA adhesin(27, 29) may be particularly well-equipped to preferentially infect hosts who express A blood group antigen on mucosal surfaces. When infected with H10407, an EtpA-producing strain, immunologically naïve subjects with blood group A appear to be at significantly increased risk for diarrheal illness, and the ensuing diarrhea tends to be more severe relative to the other major blood groups. Intriguingly, the

ETEC H10407 strain, isolated from a case of severe cholera-like diarrheal illness more than 40 years ago, is typically among the most virulent strains to be tested in human challenge studies(43, 71).

It is likely however, that while EtpA leads to enhanced virulence in blood group A hosts, other putative virulence factors identified in H10407, including the mucin-degrading EatA protease(19) collectively augment virulence by accelerating toxin delivery that culminates in severe diarrhea.

Several additional features of our studies deserve comment. We were not able to examine "secretor status", *i.e.,* expression of the FUT2 α1,2 fucosyltransferase required to synthesize ABO blood group antigens on intestinal epithelia(52). Because approximately 20% of the population are non-secretors due to defects in FUT2(72), it is likely that some of the A blood group positive individuals did not express the A antigen on their gastrointestinal epithelia. Theoretically, this might account for some individuals in the A blood group that failed to become ill following challenge. Conversely, while A blood group is a risk factor in this model, the development of severe diarrheal illness among non-A volunteers demonstrates that expression of A glycans is not an absolute requirement for disease. *Vibrio cholerae* infections provide an important analogous paradigm for severity of illness related to blood group. While severe *cholera* is significantly associated with O blood group, it is not exclusively observed in blood group O individuals(66). We should also note that the ETEC challenge studies were done with a relatively high inoculum of bacteria (~10^7) designed to assure a reasonable attack rate(73), a feature that may not be reflected in naturally occurring infections in young children where A blood group also appears to be a risk for development of symptomatic infections(3). It is also worth noting that volunteers in these studies were treated with antibiotics to clear the infection once they met the definition of severe diarrheal illness, a feature that might also have impacted the ultimate outcome.

The secreted EtpA lectin likely acts in concert(21) with at least two additional glycan binding tip adhesins of ETEC fimbriae with different substrate specificities. These include FimH of the chromosomally-encoded type 1 pili that binds to mannosylated surface proteins on epithelial cells(74), and the CfaE adhesin located at the tips of canonical plasmid-encoded CFA/I pili that putatively binds to sialylated glycoproteins(55, 75). The latter is consistent with the observation that ETEC expressing CFA/I mediate mannose-resistant(54) and neuraminidase-sensitive(75, 76) hemagglutination with all major blood groups and across species to include chicken and bovine erythrocytes. Therefore, it appears that EtpA is the only blood group A specific lectin identified in ETEC to date. Nevertheless, we cannot rule out the possibility that our studies failed to identify another blood group A lectin that was not efficiently expressed under laboratory culture conditions.

Interestingly, findings presented here are in keeping with earlier birth cohort studies of children under the age of 2 years in Bangladesh, that also demonstrated an association between A blood group and symptomatic ETEC infection*(3)*. Therefore, while our recent studies have potentially important clinical and vaccinology implications, further study of the relationship between blood group, disease severity, and antigen expression could guide and inform use of these antigens in vaccines.

These additional molecular details regarding the nature of ETEC pathogen-host interactions could promote an improved understanding of the virulence elements and host determinants related to the most severe forms of illness that are clinically indistinguishable from severe cholera(15-17). Consequently, identification of EtpA as blood group A lectin could focus antigen development and inform vaccine design to specifically protect individuals that are highest risk for severe cholera-like diarrheal illness.

Methods

ETEC controlled human infection volunteer challenge studies

Samples for blood group analysis were obtained from a convenience sample of 106 human volunteers from four prior independent controlled human infection model ETEC challenge studies conducted at the Johns Hopkins Center for Immunization Research (ClinicalTrials.gov identifiers NCT01739231, NCT01060748, NCT00844493, and NCT01922856). All volunteers were experimentally infected with 1-2 x 10^7 colony forming units of the EtpA-expressing ETEC strain H10407. Subjects in these studies were treated with antibiotics once they met previously defined endpoint criteria (summarized in table S1) or at 120 hours post inoculation to clear their infections. Additional details of the of the study design are included in the supplemental Materials and Methods.

glycan array screening

Glycan arrays containing 411 separate features were fabricated as previously reported (77) except for the addition of a washable fluorescent dye, the free acid of DyLight™649 (0.7 µg/mL, ThermoScientific), to the print buffer as an indicator of successful liquid deposition and spot morphology. The array format and assay have been described previously(77), along with analysis of reproducibility (78) and validation with numerous antibodies and lectins (79, 80). Slides were blocked overnight at 4 °C with 3% BSA (w/v; 200 µL/well) in PBS and then washed 6 times with PBST (PBS with 0.05% tween 20; 200 µL/well). Polyhistidine-tagged rEtpA-6His was diluted to final concentrations of 20 and 200 µg/ml in PBST buffer containing 3% bovine serum albumin (BSA, Sigma) and 1% human serum albumin (HSA, Sigma), and then 50 µL of each sample was added into two different wells on different slides. Incubation buffer alone served as the negative control for samples analyzed in duplicate. After incubation with rEtpA for 2 h at 37 °C (100 rpm), arrays were washed and then probed with mouse monoclonal IgG anti-His_5 antibody (Qiagen, 34660) diluted 1:500 to a final concentration of 0.4 µg/ml (1 h at 37 °C; 100 rpm), followed by Dylight 549-conjugated affinity purified Goat anti-mouse IgG (Jackson ImmunoResearch, 115-505-071; 1 h at 37 °C; 100 rpm). After washing 7 times with PBST (200 µL/well), slides were immersed in wash buffer for 5 min, and centrifuged at 1000 rpm for 5 min. Slides were scanned at 10 µm resolution with a Genepix 4000B microarray scanner (Molecular Devices Corporation) with excitation/emission wavelengths of 532/575nm and analyzed with Genepix Pro 6.0 software as previously reported(77). Heatmaps were assembled in R version 3.2.2, The R Foundation for Statistical Computing, using the gplots and RColorBrewer packages made available through the Comprehensive R Archive Network (CRAN) at http://cran.wustl.edu/. Additional details of the glycan array screening can be found in the supplementary materials.

bacterial strains and culture conditions

ETEC strain H10407 was provided from GMP stock produced at the Walter Reed Army Institute of Research, USA. *etpA* mutant H10407 (jf1668) and complemented (jf1697) strains were generated in previous studies (26). ETEC strains were routinely grown in Luria broth (LB) or LB agar (2% agar) at 37 °C from glycerol stocks preserved at -80 °C. Bacterial strains and plasmids used in this study are listed in Table S5. Strains 100137, 200145, 30051, 400643, 500632, 601142, 700241 were provided by David Rasko of the Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, MD. Strain 12005000131-1 was provided by Elizabeth Cebelinski of the Molecular Epidemiology section of the Infectious Diseases Laboratory, Minnesota Department of Health, St. Paul, MN.

protein expression and purification

The two-partner secreted EtpA glycoprotein was purified from culture supernatants of *E. coli* Top10 co-expressing plasmids pJL017 and pJL030(81). Briefly, *E. coli* Top10 carrying pJL017 and pJL030 (Table S5) was grown in Luria broth with ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml) at 37 °C, 230 rpm until OD 600nm reached ~0.5-0.6. Recombinant protein expression was then induced with 0.0002% arabinose for 6h at 37 °C, culture supernatant concentrated using a 100 kD Molecular-Weight-Cut-Off (MWCO) filter (Millipore) and rEtpA-myc-6His protein was purified using immobilized metal affinity chromatography as described previously(81).

ETEC subcellular fractionation and A blood group Far Western analysis

ETEC H10407 was grown overnight in 10 ml cultures of Luria broth at 37 °C as described above, diluted into 500ml cultures the next morning and grown for 3 hours at 37 °C. Cells were harvested by centrifugation at 5000 x *g* for 10 min at 4 °C and the pellet re-suspended in 8ml 50mM Tris buffer pH 7.8 containing 1mM EDTA and 1mg DNAse. Subcellular fractions were then prepared as previously described(82). Briefly, cells were disrupted using a French Press, followed by the addition of

2mM $MgCl_2$ to *E. coli* lysate and centrifugation at 5000 *g* for 10 min at 4 ºC to remove large debris/intact cells. Supernatant was collected, supplemented with phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1mM and ultracentrifuged at 100,000 *g* for 1hr at 4ºC. Supernatant from this step was saved as cytosolic fraction. The remaining cell pellet was re-suspended in ice-cold 10mM HEPES pH 7.4, layered over a gradient of 69, 52.8 and 26.4% sucrose in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, and ultracentrifuged at 113,000 *g* for 16hr at 4ºC. The visible upper layer containing inner membranes (im), and lower high-density layer with outer membranes (om) were collected. Membrane proteins were reconstituted ice-cold HEPES pH 7.4 and centrifuged for 1h at 100,000 *g*, 4ºC. Finally, the protein pellets from inner and outer membranes were re-suspended in 0.25 and 0.5ml dH_2O, respectively.

Far-Western blotting was used to test blood group A glycan interaction with ETEC proteins. Briefly, ~10 µg of ETEC proteins was resolved by SDS-PAGE on NuPAGE® 4-20% Tris-Glycine gel (Life Technologies) and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in TBS-T (Tris Buffered Saline, 0.01% Tween-20) for 1 h and then incubated with a final concentration of 5 µg/ml of biotinylated multivalent blood group A tri-saccharide (Glycotech; 01-032) overnight at 4 °C. Biotinylated glycans were detected using streptavidin-HRP conjugate (1:20,000) and EtpA was detected by immunoblotting using anti-EtpA rabbit polyclonal antibody.

hemagglutination studies

We examined the ability of EtpA to agglutinate human erythrocytes belonging to blood groups A1, A2, B and O (Immucor, Inc. Cat no. 0002338). Briefly, 2-fold dilutions of rEtpA in PBS in a final volume of 10 µl were mixed with 40 µl of ~ 1% cell suspensions (in PBS) of red blood cells (RBCs) expressing the A1, A2, B and O antigens in 96-well round bottom plates, then incubated for 30min at 4 ºC. Plates were then imaged to show agglutinated versus non-agglutinated (settled) RBCs. In parallel experiments, recombinant polyhistidine-tagged EtpA was immobilized onto cobalt impregnated super paramagnetic polystyrene beads (Dynabeads® TALON®, Thermofisher; Cat. no. 101.01D) that were then incubated with RBCs.

To examine the specificity of EtpA induced agglutination of RBCs expressing the A antigen, agglutination assays were performed in the presence of excess terminal sugars specific to individual blood types: GalNAc (blood group A), Gal (blood group B) or control GlcNAc at 50mM final concentration. α-*N*-Acetylgalactosaminidase (P0734S; New England Biolabs) was used to remove terminal GalNAc from the A blood group antigen by incubating 50µl of a 1% RBC suspension with 20 U of α-*N*-Acetylgalactosaminidase for 4h at 37 °C.

To examine the potential contribution of the CFA/I colonization factor to hemagglutination, we followed a protocol similar to that previously described by Evans(54) Briefly, bacteria were grown overnight on CYE agar (1% Casamino Acids (Becton Dickinson, Franklin Lakes, NJ), 0.15% yeast extract (Becton Dickinson), 2% agar, 0.005% $MgSO_4$, and 0.0005% $MnCl_2$). Bacteria were re-suspended in PBS (A_{600} 3.0) and diluted 4-fold prior to use. Human erythrocytes (Immucor, Norcross, GA, 2-4% suspension) were diluted 1:4 in PBS. 25 µl of each bacterial and RBC suspension were added to a flat-bottomed 96 well plate and rotated at 240 rpm at room temperature for 15 minutes after which agglutination was visualized. Control anti-A IgM antibodies (Immucor) were used at 1:32 dilution.

Fluorescence Activated Cell Sorting

Red Blood cell ghosts were prepared at 4°C. 800 µl of a 2-4% suspension of RBCs was washed once in PBS and re-suspended in 1.5 ml of ghost buffer (50mM $NaPO_4$, pH 8.0) and incubated on ice for 1 minute. RBCs were pelleted at 25,000 x g for 10 minutes then washed twice in ghost buffer. Ghosts were re-suspended in 400 ul PBS.

For the FACS analysis, 15 µg of biotinylated EtpA or BSA was added to 190 µl of RBC ghosts and rotated at RT for 1.5 hours. Cells were washed twice in PBS and pelleted by spinning at 25,000 x g for 5 minutes, then rotated for 30 minutes with a 1:200 dilution of streptavidin-conjugate nanocrystals (Qdot 525, Q10141MP, ThermoFisher), then washed twice in PBS and re-suspended in 200 ul of PBS. Fluorescence was measured using FACSCalibur (BD Biosciences) and data was analyzed using FlowJo software, v10.

molecular cloning of mutant Met-RS

To permit noncanonical amino acid labeling of ETEC proteins with azidonorlucine (Anl), a mutant form of the methionyl-tRNA synthetase (NLL-MetRS) gene from pAM1(56) was cloned (In-Fusion, Clontech) into the *NcoI* and *EcoRI* sites of pBAD/*Myc*-His B (table S5) using primers jf091715.1 and jf091715.2 (table S6). The resulting plasmid pBAD-MetRS was transformed into wild-type H10407 or the jf1668 *etpA* mutant to generate jf3422 and jf3904 respectively.

protein interaction studies using noncanonical amino acid labeling of ETEC

Proteomes of strains jf3422 and jf3904 bearing the MetRS expression plasmid were labeled by growth with azidonorleucine as previously described (56). Briefly, strains were grown overnight in Luria broth containing ampicillin 100 µg/ml, 0.2% arabinose, and 1 mM azidonorleucine (HAA1625, Iris Biotech, GMBH). Cell-free supernatants were concentrated 10-fold using a 10Kd MWCO centrifugal concentrators and dialyzed against PBS prior to use.

Outer membrane proteins were prepared from ANL-tagged bacteria grown in the same fashion using an established protocol(83). Briefly, labeled bacteria were re-suspended in 20 ml ice-cold lysis buffer (50 mM Tris, 1 mM EDTZ, pH 7.8) and lysed by sonication (4 x 20 sec bursts). Approximately 1 mg of DNase I (Sigma) was added to each tube and the extracts adjusted to 2 mM $MgCl_2$ and 100 µM PMSF. Intact cells and large debris were removed by low-speed centrifugation at 3,000 g for 10 minutes, and membranes were pelleted from clarified lysates by ultracentrifugation for one hour at 100,000 x g. Membrane pellets were then re-suspended in 2 ml 10 mM HEPES pH 7.4, and layered onto discontinuous sucrose gradients consisting of a top layer of 0.77 M sucrose, a middle layer of 1.44 M sucrose, and a bottom layer of 2.02 M sucrose. After centrifugation at 113,000 g for 16 hours at 4°C, the

OMPs were recovered from the interface between the 1.44 and 2.02 M layers. OMP pellets were washed by resuspension in 14 ml 10 mM HEPES pH 7.4, collected by centrifugation at 100,000 g for one hour, and re-suspended in 0.5 ml PBS.

Erythrocyte membranes were prepared using the method previously described by Steck, *et al* (84). Briefly, suspensions of red blood cells (RBCs) were sedimented at 2300 x g for 10 minutes at 4°C, the pellet recovered, and then re-suspended in 5 volumes of cold phosphate buffered saline (PBS), pH 8.0. After centrifugation, packed RBCs were hemolyzed by mixing with 5 mM sodium phosphate, pH 8.0, and incubating on ice for 1 minute. The resulting membranes then harvested by centrifugation at 22,000 x g for 10 minutes, and the supernatant removed by aspiration.

HT-29 membranes or membranes from the HT-29-A$^{-/-}$ line were prepared as follows. Cells grown in monolayers on a T25 flask were washed with PBS, and the supernatant decanted. After addition of 4 ml of fresh PBS, cells were recovered by scraping, and the suspension centrifuged for 5 minutes at 6000 rpm. Cell pellets were re-suspended in 1 ml of ice cold lysis buffer (5mM PO4, pH8, 0.5 mg/ml DNase I, containing Pierce Protease Inhibitor ThermoFisher 88665), transferred to a microfuge tube spun for 5 minutes at 6000 rpm, supernatant removed and replaced with 1 ml of fresh lysis buffer. Cells were then incubated on ice for 30 minutes, flash-frozen in an ethanol-dry ice bath, and thawed at 37°C. After repeating the freeze-thaw cycles twice, the extract was passed through a 26-gauge needle 4-5 times to sheer DNA and spun at 25,000 x g for 10 minutes at 4°C. Resulting translucent membrane pellets were then re-suspended in PBS.

Erythrocyte ghosts or HT-29 membrane preparations were incubated with ANL-labeled bacterial proteins at 4°C overnight, pelleted by centrifugation, washed in 1 ml of ice-cold PBS, then re-suspended and boiled in click buffer (50 mM HEPES, 0.5% (w/v) SDS, pH 7.5). ANL-containing proteins were labelled for 1 h at 37°C in the dark with 5 µM tetramethylrhodamine (TAMRA)-alkyne (Molecular Probes) in the presence of 25 mM ascorbate, 1.5 mM $CuSO_4$ and 2.5 mM Tris(3-

hydroxypropyltriazolylmethyl)amine. Labelled proteins were separated by SDS-PAGE in the dark and the gel scanned on a Typhoon FLA 9500 phosphorimager (GE Healthcare Life Sciences) using excitation at 532 nm and a 575 nm long-pass filter. Positive control TAMRA-labelled rEtpA was produced by reacting EtpA with 120 µM azidobutyric acid NHS ester (Lumiprobe), unreacted label was then removed by dialysis, and the azide-labelled EtpA reacted with TAMRA-alkyne, as before.

Bio-Layer Interferometry

Bio-Layer Interferometry was used to determine affinity of EtpA-blood group binding using OctetRed96 (Pall ForteBio Corporation, USA). Briefly, biotinylated blood group A antigens conjugated to polyacrylamide (either as the disaccharide GalNAcα1-3Galß-PAA-biotin; GlycoTech, Gaithersburg, MD, cat. no. 01-017; or the trisaccharide GalNAcα1-3(Fucα1,2)Galß-PAA-biotin, GlycoTech 01-032), or related target sugars α-D-galactose-PAA-biotin, GlycoTech 01-003; GalNAcα1,3GalNAcß-PAA-biotin, GlycoTech 01-026; αGalNAc-PAA-biotin, GlycoTech 08-010; were immobilized onto the streptavidin (SA) biosensors (18-5019; Pall ForteBio Corporation). These were then incubated with two-fold serial dilutions (2000-500nM) of purified rEtpA or control BSA (2000 nM) prepared in 1X PBS as analyte. Affinity constant (kD) values were calculated using Octet software version 8.1.

growth and differentiation of human enteroids from different blood groups

Enteroid experiments were conducted at either John Hopkins University School of Medicine (JHU), or at Washington University School of Medicine in Saint Louis (WUSTL), and detailed experimental protocols for both institutions are included in the supplementary methods. In brief, biopsy samples were obtained from adult patients undergoing routine endoscopy at each institution with patient consent and approval of the respective Institutional Review Board. Small intestinal epithelial (enteroid) cell lines were then generated from dissociated crypts as previously described (85-87), stored and then accessed from the Washington University Digestive Diseases Research Core Center (DDRCC) Biospecimens Core or the respective aligned program at JHU. Purified cell lines of each of the described ABO types were thawed and re-suspended in Matrigel (BD Biosciences, San Jose, California, USA) and then propagated at 37°C, 5% CO_2 with Advanced DEM/F12, media (Invitrogen) supplemented with Wnt3a, R-spondin 1, and Noggin (WRN). Protocols for cellular differentiation and growth of polarized epithelial monolayers were performed as described in detail in the supplementary methods. Blood typing by PCR and immunofluorescence microscopy was performed as previously described (45).

α1-3-N-acetylgalactosaminyltransferase deficient cell line

Cells lacking the α1-3-N-acetylgalactosaminyltransferase (accessions GI: 55773627, GenBank: D82843.2) were constructed from HT-29 cells (ATCC HTB-38) by CRISPR/Cas9 genome editing in the Genome Engineering and iPSC Center at Washington University (http://geic.wustl.edu) as previously reported(45). Both parental HT-29 cells and the resulting HT-29 $A^{-/-}$ line were propagated in McCoy's-5A medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum.

ETEC adhesion and toxin delivery assays

To examine delivery of the heat-labile toxin (LT), cultures of HT-29 or HT-29A-/- were differentiated by growing cultures at confluence for 1 week prior to use in 96 well plates. Where indicated, phosphodiesterase (PDE) inhibitors vardenafil HCL·3H20 (Sigma Y0001647), cilostazol (Sigma PHR1503), and rolipram (Sigma R6520), were added to cell cultures 1 hour prior to infections at a final concentration of 6.125 μM (88). ETEC (H10407) or the *etpA* mutant (jf1668) were grown overnight in 1 ml of LB media. The following morning, bacteria were diluted 1:100 for 90 minutes to mid-log growth phase. HT-29 and HT-29A-/- were infected with bacteria at a Multiplicity of Infection (MOI) of ~100. MOI was determined by plating serial dilutions of bacterial suspensions and counting HT-29 cells on a hemocytometer after trypsinization of adherent cells. Infection of HT-29 cells by H10407 or the *etpA* mutant proceeded for 2 hours at 37°C and 5% CO2 in media containing PDE inhibitors. After 2 hours, the cells were washed once in pre-warmed media and the infection continued for an additional hour in media with PDE inhibitors. Cellular cyclic AMP (cAMP) (Arbor Assays, Ann Arbor, MI) levels were used to assess the efficiency of LT delivery.

Adhesion assays were performed as previously described using mid-log phase bacterial cultures (26). After 30 minutes, the monolayers were washed 3 times with pre-warmed media then treated with 0.1% Triton X-100 in PBS for 5 min. Ten-fold dilutions of the Triton X-100 lysates were plated on Luria agar and bacterial adherence was calculated as the percentage of organisms recovered per colony forming unit of inoculum. Adherence assays for enteroids were performed in the same fashion but washed at different time points as indicated in the figure legends.

confocal laser scanning microscopy

HT-29 cells were seeded onto glass coverslips pre-treated with poly-L-lysine in 24 well tissue culture plates. Enteroids grown on Transwell filters were processed by removing the filter from well, and then treated as below. Cells were blocked with 1% BSA in PBS and incubated with biotinylated EtpA (50 µg/ml final concentration) for 2 hours at 37 ºC or overnight at 4 ºC. After washing with PBS three times, EtpA was detected using Qdot 605 streptavidin conjugate (Life Technologies). A blood group antigen was detected using mouse monoclonal antibody (IgM, Santa Cruz sc-69951) against human blood group A, followed by Alexa Fluor 647 conjugated goat anti-mouse IgM heavy chain Molecular Probes A21238. CellMask deep red plasma membrane stain (ThermoFisher Scientific C10046) 1:2,000, and DAPI (4',6-diamidino-2-phenylindole, dihydrochloride 1:6,000 were used to stain cellular membranes and nuclei respectively. ETEC H10407 (serotype O78:H11) was visualized with polyclonal rabbit antibodies against O78 obtained from the Penn State University E. coli Reference Center, followed by detection with Goat anti-rabbit IgG (H&L) cross-absorbed AlexaFluor (488 or 594) secondary antibody conjugates (Invitrogen A11070, A11072) .

Confocal microscopy images were captured using a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope. Image files were saved in LSM format and processed using Fiji(89), Image J v 2.0.0., and Volocity three-dimensional image analysis software (version 6.3, PerkinElmer).

blood group typing

Coded, de-identified samples forwarded to Washington University were used in a blinded fashion to type for the major ABO blood group antigens. Plasma or serum were used in reverse immunotyping for antibodies directed at the ABO antigens was performed using standard methods. Briefly 2 µl of red blood cells (Immucor, Norcross, Georgia, USA) from each blood group were incubated with 4 µl of patient sera or plasma at room temperature for 5 minutes in a 96 well plate, then examined microscopically for agglutination. For available saliva, DNA was extracted from 50 µl of sample (Qiagen 69504), and genotyping by PCR was performed as previously described(90). Salivary genotyping results were confirmed by dot immunoblotting. Briefly 0.5 µl of saliva diluted 1:4 in PBS was dotted onto nitrocellulose membranes that were then incubated in a solution of H_2O_2 (3%) for 5 minutes. Membranes were then incubated with 1:20 dilutions of anti-A, anti-B, or anti-H antibodies (Sigma) for 30 minutes, washed three times in PBS with 0.05% Tween, and probed with goat anti-mouse IgM HRP-conjugate (1:5,000 dilution, Santa Cruz, sc-2064) and developed using ECL Western Blotting substrate (ThermoFisher).

ABO determinations were then matched to diarrheal outcomes in which the severity of illness was dichotomized into none (no or mild disease) and moderate to severe diarrhea (MSD) using established criteria(71).

statistics

Log-rank (Mantel-Cox) testing was used to compare time-to-treatment outcomes with $p<0.05$ considered significant. Meta-analysis (pooled fixed effects) was used to compare the relative risk of moderated to severe diarrheal illness. Pearson chi-square testing was used to compare diarrheal illness and severity by blood group. Mann-Whitney testing was used to compare non-parametric data . More than two groups of data were analyzed using Kruskal-Wallis testing for multiple comparisons of non-parametric data. p values <0.05 were considered to be significant. p values or adjusted p values and represented as **** of <0.0001, ***p<0.001 ** <0.01, *<0.05 throughout.

study approval

Institutional Review Board approval was obtained at each of participating study site (Washington University, Naval Medical Research Center, and Johns Hopkins University) prior to sample interrogation and analysis. Written informed consent was received from all participants prior to inclusion in the studies.

Author contributions:

JMV, MK, MD were involved in study design, data curation, manuscript preparation and project administration. TJV, AP, and PK conducted cloning of metRS and ANL labeling experiments. SC, ALB, DAS, BD, CDH, RLG, CKP, MGP, MSR designed and conducted ETEC Controlled Human Infection Model studies in volunteers, and processed the data and samples. WSW, and JCG conducted glycan array experiments; PK FMK, JFA, BT were involved in protein interaction, hemagglutination, microscopy, adhesion and toxin delivery studies. AS and MK conducted flow cytometry experiments. MC, SS, JFA, MD, MK, and BT acquired, maintained and propagated small intestinal stem-cell banks or intestinal epithelial cells. PK and TJV were responsible for recombinant protein production.

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figure legends

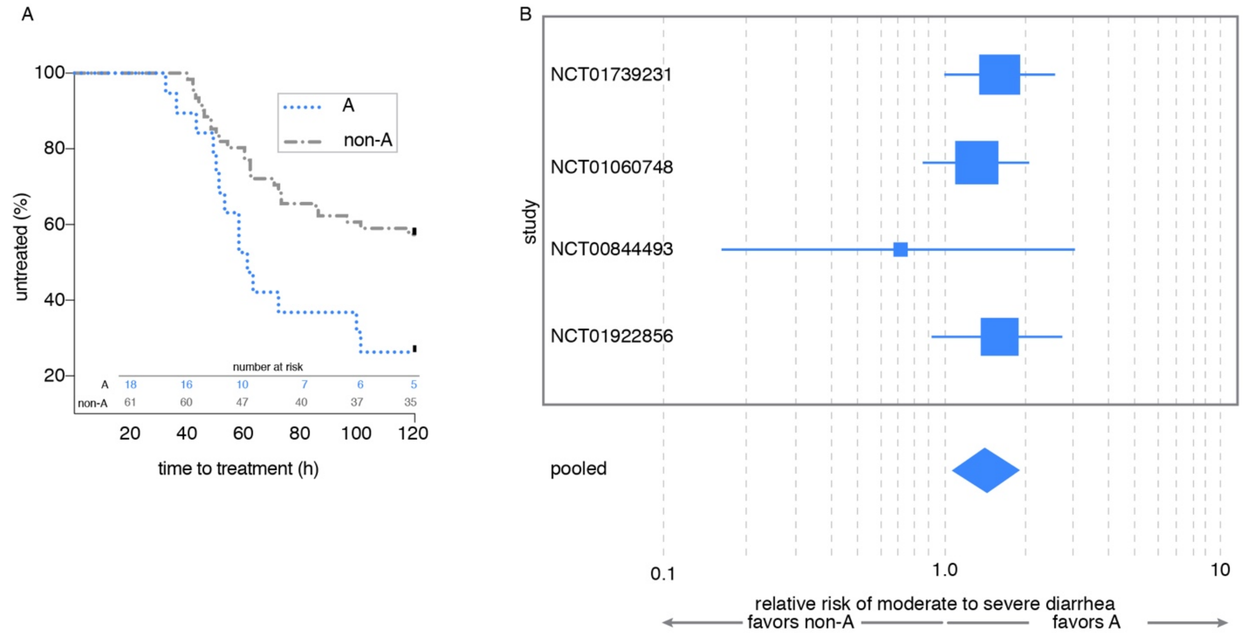

Figure 1. Diarrheal severity is increased in blood group A positive volunteers relative to non-A blood groups.

A. Development of severe diarrheal illness is accelerated in A blood group hosts. Kaplan-Meier curves indicate the proportion of subjects treated following the development of severe diarrheal illness in volunteers belonging to A and non-A blood groups. Time-to-treatment data was available for 79 of the 106 subjects (18 subjects in the A blood group and 61 subjects in the non-A group). Data were censored at 120 hours when all remaining untreated individuals received antibiotics to clear their infection. p=0.015 by log-rank (Mantel-Cox) testing. **B.** Individuals of A blood group are more likely to develop moderate-severe diarrhea. Shown are relative risk data from four independent controlled human infection model challenge studies of H10407 involving a total of 106 volunteers. Study numbers at left refer to ClinicalTrials.gov designations. Box size indicates relative study size and lines represent 95% confidence intervals for the relative risk of severe diarrhea. The width of the diamond indicates the 95% confidence intervals for the pooled effect. The relative risk (pooled fixed effects) of moderate to severe diarrheal illness dichotomized by A (blood groups A, and AB) vs non A

(O and B) was 1.441 (95% CI, 1.097 to 1.893), (p=0.009). Cochran's Q test of heterogeneity was insignificant (p=0.667, Q=1.5682, df = 3).

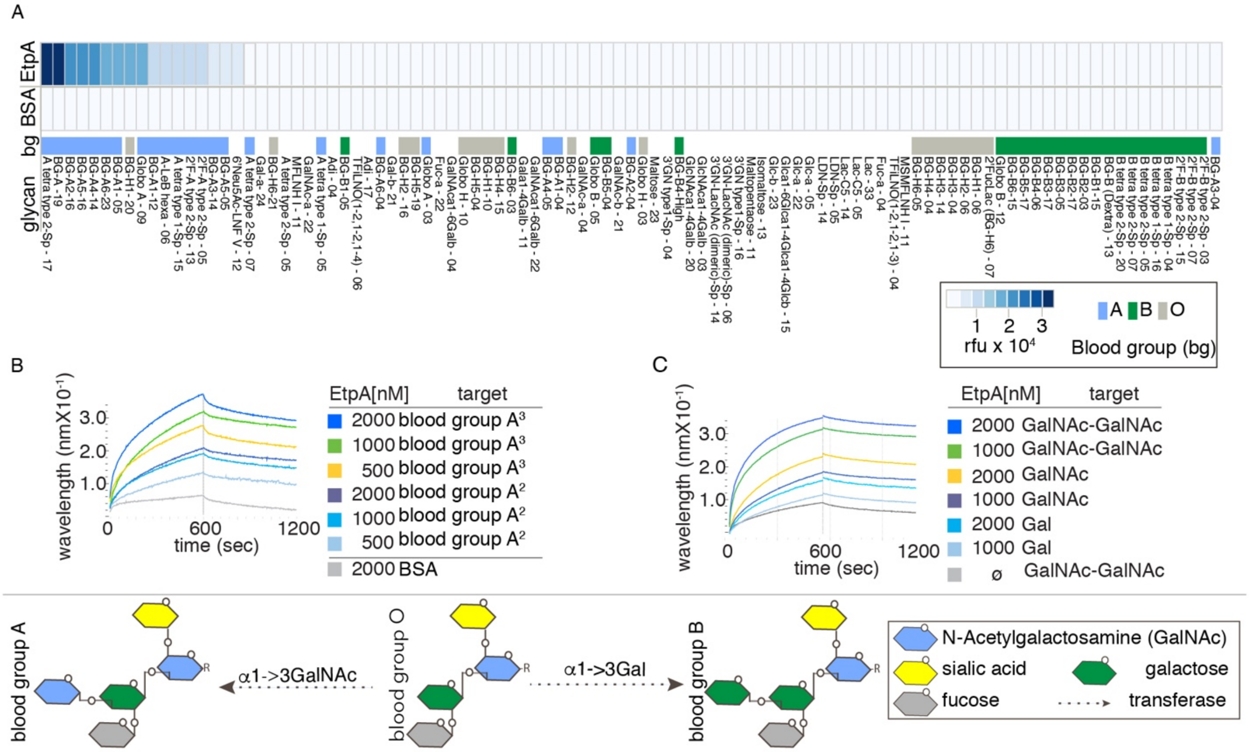

Figure. 2. EtpA interacts preferentially with blood group A glycans

A. glycan array data (top row) demonstrating EtpA binding predominately to blood group A glycans. The top 100 of 411 glycans are ranked in order of diminishing binding activity from left to right. BSA (middle row) is shown as a control. Values represent background-corrected median data from two experimental replicates. Distribution of blood group (bg) binding is shown in the bottom row of the figure. Heatmap key (lower right) shows relative fluorescence units (rfu), and coding for blood group antigens. Names for glycans correspond to supplemental data table 1. **B.** EtpA binding to blood group A glycans assessed by Bio-Layer Interferometery assays. Data are representative of three independent experiments. Blood group A^3 refers to the biotinylated trisaccharide-polyacrylamide (PAA) conjugate GalNAcα1-3(Fucα1,2)Galß-PAA-biotin; blood group A^2 refers to the disaccharide conjugate GalNAcα1-3Galß-PAA-biotin. **C.** EtpA binding to terminal GalNAc residues relative to galactose. Schematic (bottom) shows structures of blood groups A and B terminating in GalNAc and Gal sugars, respectively. Blood group O (core H) blood group lacks either terminal sugar residue.

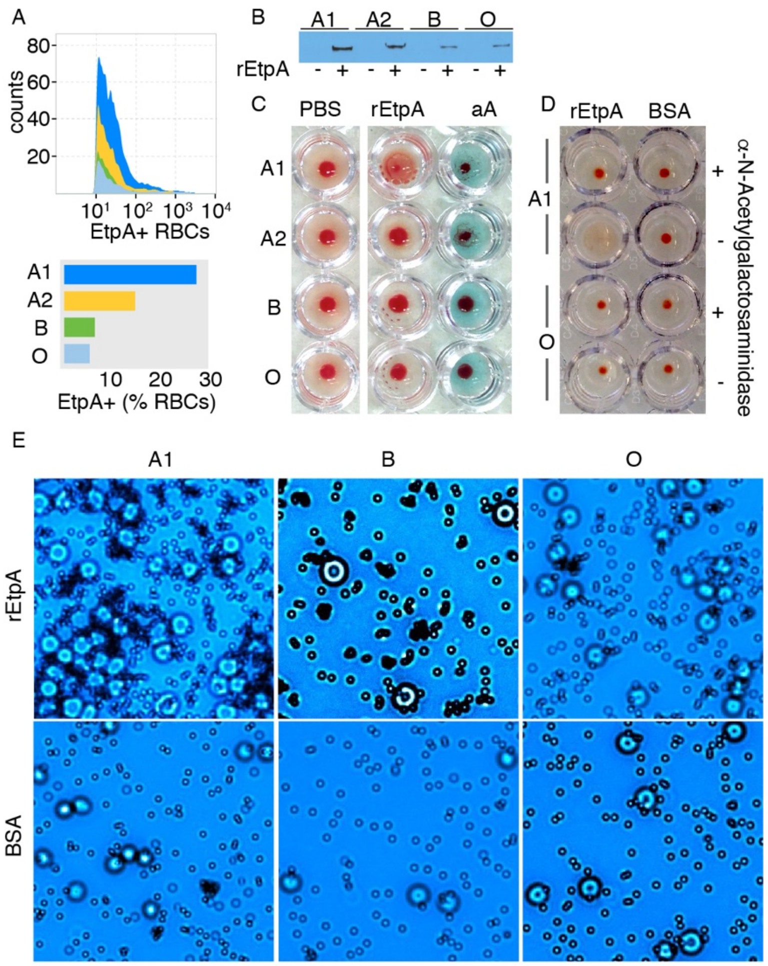

Figure 3. EtpA is a blood group A specific hemagglutinin

A. binding of recombinant EtpA (rEtpA) to the surface of group A1 and A2 red blood cell ghosts (RBCs) relative to B, and O RBCs is shown in FACS data (top) and the graph (bottom). **B.** RBC pull-down of rEtpA with different blood group ghost erythrocytes. Shown in the immunoblot is rEtpA-myc-his identified by anti-myc antibodies. **C.** EtpA agglutinates A1 RBCs but exhibits minimal or no hemagglutination activity with RBCs from those with A2, B, or O blood groups. **D.** EtpA-mediated hemagglutination is dependent on terminal GalNac residues. A1 RBCS pretreated with α–N-Acetylagalactosaminidase (top row) fail to agglutinate in the presence of rEtpA. Shown (row 2, left) are positive controls (no enzyme pretreatment) and blood group O negative controls (bottom rows). **E.** EtpA-coated latex microspheres (top row) agglutinate RBCs from blood group A, but not B, or O.

Bottom row shows BSA-coated particles as controls. Each of the images are representative of three experimental replicates.

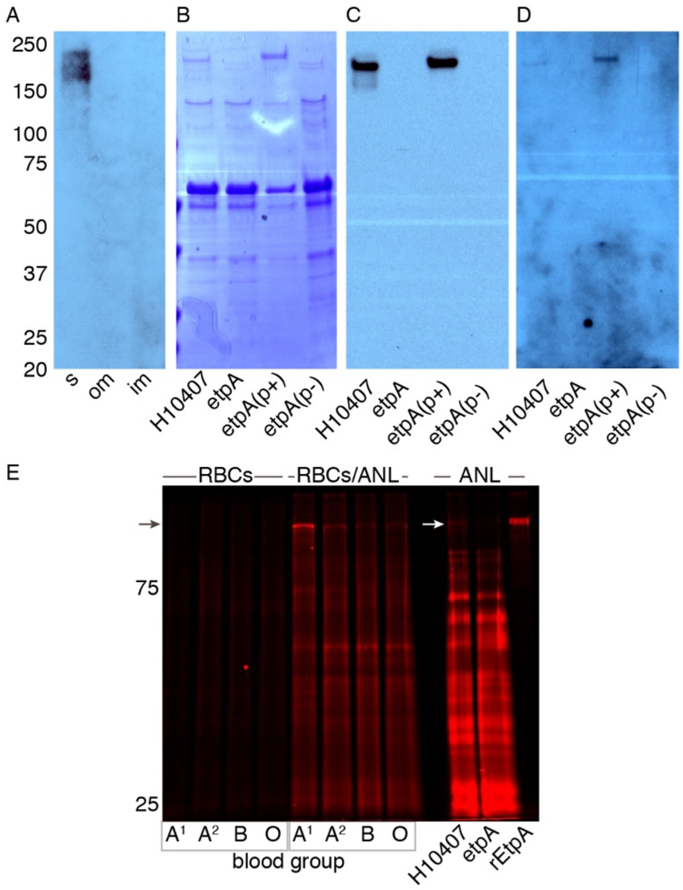

Figure 4. EtpA is a dominant blood group A binding partner of ETEC H10407

A. Blood group A antigen far-Western blot with subcellular fractions of ETEC H10407 including supernatant (s), outer membrane (om) and inner membrane (im) demonstrates binding of biotinylated blood group A to high molecular weight protein in concentrated culture supernatant. **B.** Coomassie-stained gel of supernatant proteins from H10407 (wild type), *etpA* mutant jf1668, *etpA* mutant complemented with the *etpBAC* locus plasmid, pJY019 (*etpA*(p+); etpA(p-) equals mutant complemented with cloning vector alone. **C.** EtpA immunoblot confirming the presence of EtpA in culture supernatants from H10407 and the complemented mutant. **D.** Far-Western blot of culture supernatants shown in B,C shows binding of biotinylated blood group A only in the presence of EtpA. **E.** EtpA is the dominant A blood group specific interacting partner among ETEC proteins from ANL-labeled bacteria. Shown is a fluorescence image of ANL-labeled proteins from ETEC H10407 that interact with erythrocyte ghosts from A1, A2, B, and O blood groups (middle, RBCs/ANL). RBC ghosts alone are shown at left, and input protein (ANL) from H10407 and the *etpA deletion mutant* (jf1668) are shown at right. The migration of EtpA is indicated by the arrows, and TAMRA-labeled recombinant EtpA (rEtpA) is shown at far right as a positive control. Each image is representative of three experimental replicates.

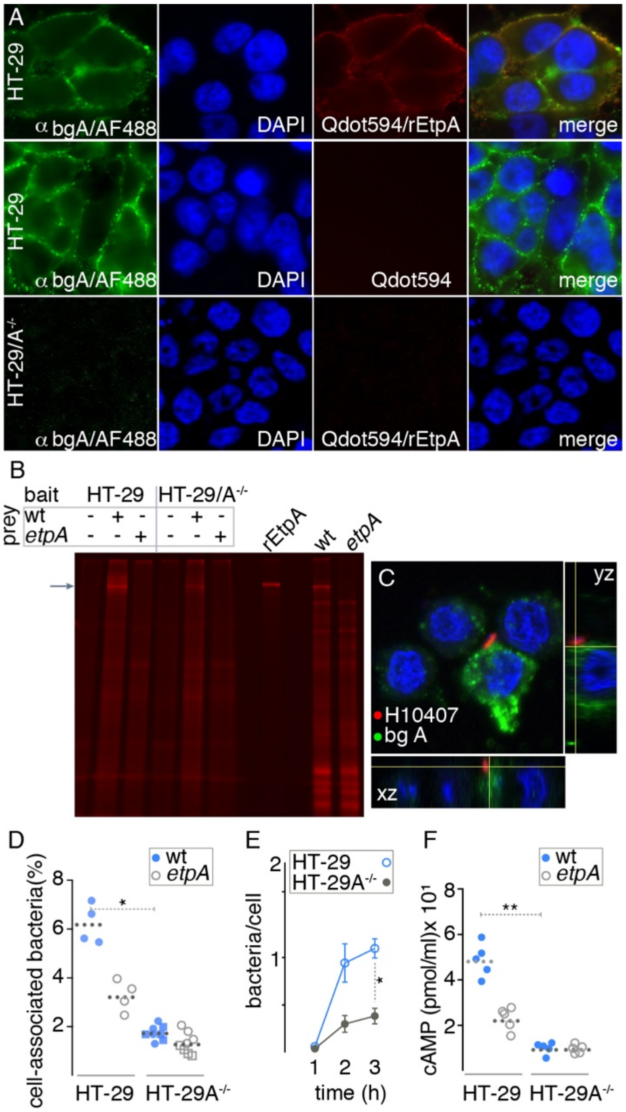

Figure 5. A blood group dependent interactions of EtpA and ETEC with intestinal epithelia

A. EtpA binds to regions of blood group A expression on the surface of HT-29 epithelial cells. Shown in columns from left to right are blood group A (green-antibodies against blood group A, and fluorescent conjugate αbgA/AF488); nuclei (blue, DAPI); biotinylated EtpA (red- streptavidin-coated Qdots594); and merged image. Middle row: no EtpA control. Bottom row: no EtpA binding to the surface of HT29/A$^{-/-}$ cells engineered to remove the α1->3 GalNac glycosyltransferase required for A antigen expression. (100x magnification). **B.** EtpA preferentially engages cells expressing A blood group. Key, upper left indicates the target (bait) cell lines used to attract ANL-labeled bacterial (prey) proteins from with H10407 (wt) or the *etpA* mutant. Labeled recombinant EtpA (rEtpA) and the arrow at left are shown to indicate the predicted migration of EtpA. Lanes at far right show input proteins from the EtpA-expressing H10407 wild type (wt) and the *etpA* mutant. **C.** Localization of EtpA-expressing ETEC to areas of blood group A expression on the surface of HT-29 cells. (original magnification 63x). Images in A, B, C represent one of three experimental replicates. **D.** EtpA and blood group A are required for optimal adhesion. Data are representative of four independent experiments. HT-29 A-/- data include results from two independently generated CRISPR-generated blood group A glycosyl transferase mutant lines (1e6, circular symbols; and 1g10, square symbols; n=8 technical replicates in total). **E.** Blood group A accelerates adhesion of ETEC to target intestinal epithelial cells (n=8 technical replicates for HT-29 cells and n=6 technical replicates for HT-29-/- cells; representative of two independent experiments); symbols represent mean ± SEM. **F.** Presence of A blood group, and EtpA are required for optimal delivery of heat labile toxin by ETEC (n=5 technical replicates representative of three independent experiments).

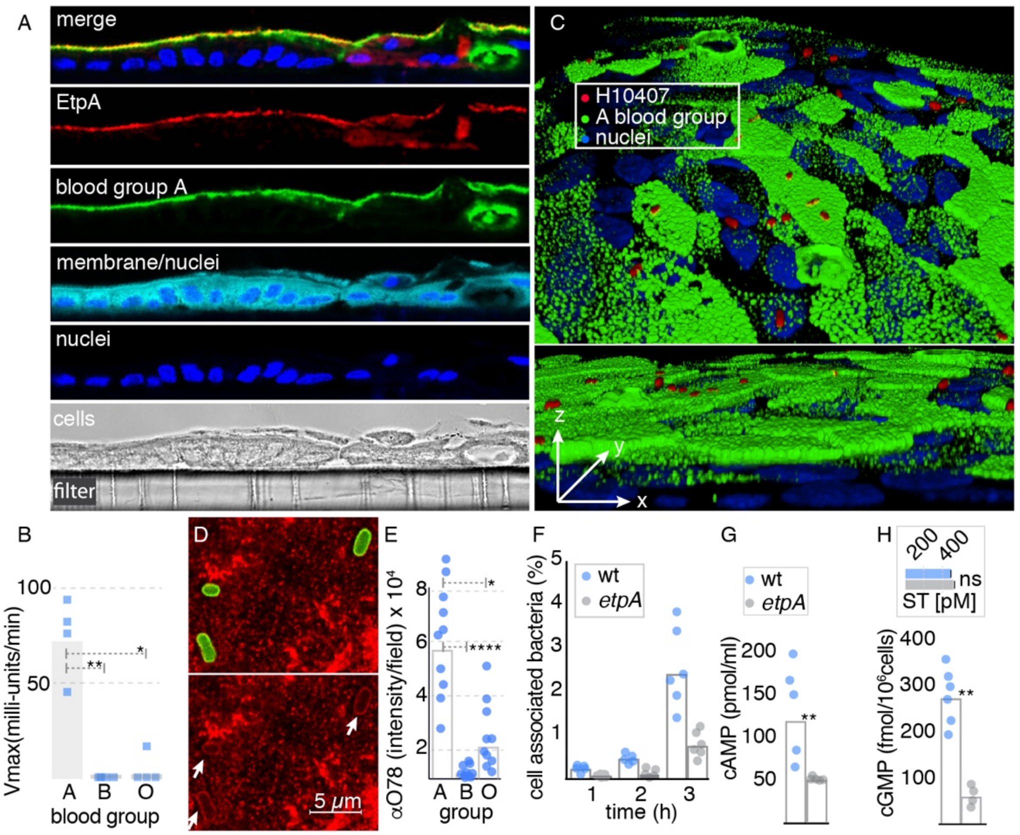

Figure 6. EtpA, and EtpA-expressing ETEC interact preferentially with the surface of blood group A enteroids.

A. Confocal imaging of EtpA binding to surface of polarized small intestinal enteroid monolayers from a blood group A subject. DIC image at bottom shows architecture of polarized enteroids on Transwell filter. (63x magnification) **B.** Kinetic ELISA quantification of biotinylated EtpA binding to surface of small intestinal enteroid cells from blood groups A, B, and O (n=4 technical replicates representative of 3 separate experiments). **C.** Association of EtpA-expressing ETEC H10407 with A blood group on surface of small intestinal cells. Images represent Volocity-processed confocal laser scanning microscopy (CLSM) data of ETEC H10407 (red) to blood group A (green), and nuclei (blue). Lower panel shows polarized orientation of cells with bacteria adherent to the apical surfaces of enterocytes. (63x magnification) **D.** (top)CLSM of ETEC (green), and A blood group (red) on jejunal enteroids. (bottom panel) A blood group “footprints” (arrows) at sites of bacterial attachment. C, and D representative of three experimental replicates. **E.** Bacterial density on surface of small intestinal enteroids determined by CLSM quantification of H10407 (serotype O78). Quantitation based on imaging 10 fields per blood group, at 20x magnification. **F.** Adhesion of wild type H10407 and *etpA* mutant bacteria to blood group A small intestinal enteroids (n= 5 technical replicates; representative of 3 biological replicates); bars: geometric mean values). **G.** production of cAMP by blood group A target enteroids following infection by wild type (wt, H10407) or the *etpA* mutant.. Data represent total of 5 technical replicates from 2 separate experiments. **H.** production of cGMP by blood group A small intestinal enteroids following infection with wild type (n=6) or the *etpA* mutant (n=4); data representative of 3 independent biological replicates.. Inset graph indicates relative (mean± sd, n=6 technical replicates) production of heat stable toxin (ST) by the wild type (blue bars) vs the mutant (grey bars); ns=non-significant.

Tables

table 1

table 1. diarrheal illness and severity by blood group

	blood group		
diarrheal severity	O or B	A or AB	total subjects (%)
none-mild	35 (44)	5 (19)	40 (38)
moderate-severe	44 (56)	22 (81)*	66 (62)
total subjects	79	27	106

*Pearson χ^2 5.694, 1 df, p=0.017