Histo-blood group antigens of glycosphingolipids predict susceptibility of human intestinal enteroids to norovirus infection

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ABSTRACT

The molecular mechanisms behind infection and propagation of human restricted pathogens such as human norovirus (HuNoV) have defied interrogation because they were previously unculturable. However, human intestinal enteroids (HIEs) have emerged to offer unique ex vivo models for targeted studies of intestinal biology, including inflammatory and infectious diseases. Carbohydrate-dependent histo-blood group antigens (HBGAs) are known to be critical for clinical infection. To explore whether HBGAs of glycosphingolipids contribute to HuNoV infection, we obtained HIE cultures established from stem cells isolated from jejunal biopsies of six individuals with different ABO, Lewis and secretor genotypes. We analyzed their glycerolipid and sphingolipid compositions and quantified interaction kinetics and the affinity of HuNoV virus-like particles (VLPs) to lipid vesicles produced from the individual HIE-lipid extracts. All HIEs had a similar lipid and glycerolipid composition. Sphingolipids included HBGAs-related type 1 chain glycosphingolipids (GSLs), with HBGAs epitopes corresponding to the geno- and phenotypes of the different HIEs. As revealed by single particle interaction studies of Sydney GII.4 VLPs with glycosphingolipid-containing HIE membranes, both binding kinetics and affinities explain the patterns of susceptibility towards GII.4 infection for individual HIEs. This is the first time norovirus VLPs have been shown to interact specifically with secretor gene dependent GSLs embedded in lipid membranes of HIEs that propagate GII.4 HuNoV ex vivo, highlighting the potential of HIEs for advanced future studies of intestinal glycobiology and host-pathogen interactions.
The successful generation of human mini-guts cultured ex vivo (1), known as human intestinal enteroids (HIEs) or organoids, has boosted a large number of studies of physiology and pathophysiology of the human intestines with high expectations for translational applications (2-9). One remarkable finding is the ability of these non-transformed human cultures to support replication and recapitulate unique aspects of infection of previously noncultivatable pathogens such as human noroviruses (10), cryptosporidium (11,12) and Salmonella Typhi (13). Exploiting this significant advancement, we have conducted a study of lipid and sphingolipid compositions of seven HIE cultures, and characterized the interaction between their lipid membranes and human norovirus (HuNoV) virus-like particles (VLPs). These HIEs uniquely represent individuals with different ABO, Lewis and secretor histo-blood group geno- and phenotypes with varying susceptibilities to human HuNoV infection (10,14).

HuNoVs are the cause of 20% of all gastrointestinal infections worldwide and are responsible for 700 million infections each year (15). The virus is highly contagious and thus constitutes a major societal challenge. In most cases, the infection is self-limiting, but the illness can be chronic, severe or even fatal (16-20). There is a large genetic and phenotypic variation of circulating strains of HuNoV, and the predominant GII.4 strains change through critical (epochal) mutations and appear as new, globally occurring, variants every 2-3 years (21). The epidemiologically dominating GII strains typically infect secretors, persons who carry a functional secretor α1,2-fucosyltransferase coded for by the FUT2 allele (Fig. S1), while non-secretors are homozygous for non-functional secretor (FUT2) alleles and are resistant to infection. The name “secretor” originally refers to the secretion of histo-blood group antigens (HBGAs) in saliva but, as recognized later, also reflects the expression of HBGA in the gastrointestinal epithelium. Surprisingly few studies have successfully addressed the structural characterization of various glycoconjugates, i.e. glycolipids and glycoproteins carrying HBGAs of human intestinal tissues (22-28). Major reasons for this may be difficulties in obtaining representative material and sufficient amounts of tissues for chemical characterization and difficulties in establishing cultures of non-transformed intestinal cells that allow short- and long-term interaction studies with various pathogens and toxins.

The introduction of an ex vivo infection model of HuNoV in HIEs has allowed for detailed studies aimed at revealing the virus-host cell interactions at the molecular and cellular levels, the understanding of which is crucial for the development of novel anti-viral drugs and vaccines (10,29-33). HIEs are derived from stem cells isolated from intestinal crypts in human intestinal tissue obtained from surgery or biopsy samples (1). These cultures recapitulate the natural intestinal epithelium from where they are taken. HIEs are non-transformed, physiologically active, and contain multiple epithelial cell types (enterocytes, goblet cells, enteroendocrine and Paneth cells) and they may be grown as three-dimensional cultures in Matrigel or as monolayer cultures in collagen-coated wells (3,4,6,34). HIEs thus provide excellent model systems to extend our knowledge on microbe host-cell interactions and specifically at the individual level where glycoconjugates uniquely reflect individual genotypes of the donors (14). Although HIEs are susceptible to HuNoV replication, the receptor is unknown and possible attachment factors on HIEs have not been fully characterized. As a result, it is now important to characterize the lipid composition of a series of HIEs and their individual interactions with HuNoV VLPs as a model to compare the differences between susceptible and resistant enteroid cultures.

In this study, we analyzed the lipid and sphingolipid composition of seven HIE lines generated from jejunal biopsies from six individuals. These HIEs were defined by their ABO, Lewis and secretor HBGA geno- and phenotypes with genomic DNA sequencing and ELISA (Table 1), and by their susceptibility to
Enteroid glycosphingolipids relate to norovirus infection (14). We performed lipid analyses of lower and upper phases from Folch partitionings and characterized phospholipids, cholesterol and sphingolipids. We further performed kinetic studies of HuNoV VLPs binding to membrane-embedded glycosphingolipids (GSLs) isolated from individual HIEs. Our results establish that the HBGAs of GSLs match the ABO, Lewis and Secretor genotypes of individual HIEs and that this correlates with susceptibility to HuNoV GII.4 infection ex vivo. This strongly supports the concept that GSLs of human intestinal cells act as important attachment factors in the natural HuNoV infection process.

Results
Pheno- and genotypic histo-blood group status of HIEs

HIE lines were phenotyped by enzyme linked immunoassays and genotyped by PCR amplification of ABO, FUT2 and FUT3 genes followed by DNA sequencing to determine their HBGa status. Phenotyping assays analyzed for the presence of Le^a, Le^b, A, B, and H antigens. Genotyping assessed single nucleotide polymorphisms and deletions at known mutation sites of the ABO, FUT2 and FUT3 genes and the results are summarized in Table 1. Overall phenotyping results were in agreement with the genotyping findings. Out of seven characterized HIEs, four were secretor-positive (SeSe, Sese or Sesese; J1, J2, J6, J4FUT2) and three were secretor-negative (sese; J4, J8, J10), while five were Lewis-positive (LeLe or Lele; J2, J4, J6, J8, J4FUT2) and two were Lewis negative (lele; J1, J10). Only J2 and J6 were both secretor- and Lewis-positive and only J10 was negative for both features. J4FUT2 line was generated from J4 by overexpressing the FUT2 gene (14). As recently shown, only the secretor-positive HIEs are susceptible to ex vivo HuNoV GII.4 Sydney infection (10,14).

Structural characterization of major lipids of HIEs

The TLC and LC-MS analyses of Folch lower phases of the seven HIE lines identified their general lipid compositions (Fig. 1A, B). The lipids were dominated by glycerophospholipids [phosphatidylethanolamine (PE), and phosphatidylserine (PS)], constituting around 80 mol% of the whole lipid composition and making up 49 mol%, 18 mol% and 11 mol% of the total lipid content, respectively. Free cholesterol (FC) constituted about 12 mol% of the lipids, and the rest was made up of various sphingolipids (see below). The composition of all major phospholipids in all HIEs analyzed was essentially the same, including the dominance of PC over PE and PS. The fatty acid distribution of the different glycerolipids of individual HIEs are presented in Figs. S2 and S3 in Supporting Information.

Structural characterization of sphingolipids of HIEs

Sphingolipids constituted around 9 mol% of the measured lipids in all HIEs (Fig. 1C-F). The major sphingolipids were sphingomyelin [SM (d18:1), 2.8 mol%] and dihydrosphingomyelin [DH-SM (d18:0), 1.4 mol%], free ceramides [Cer (d18:1) and DH-Cer (d18:0), 2.4 mol%], sulfatide [SHexCer (d18:1), 1.8 mol%], monohexosyleramide [HexCer (d18:1), 0.2 mol%] and GSLs with 4-7 monosaccharide residues. Some of these sphingolipids could be identified already in the rough lower phase extracts (Fig. 1A), but mild alkaline hydrolysis efficiently removed the glycerolipids and exposed all the sphingolipids on the TLC-plates (Fig. 1E). Using a borate-impregnated TLC plate, and reference GalCer and GlcCer standards, it was possible to identify the major HexCer of all HIEs as GalCer with a complex composition of the ceramide part (Fig. 1F). The only major qualitative difference in sphingolipid composition for the different HIEs was in the region of GSLs with 4-7 glycan residues constituting the HBGa epitopes (Fig. 1A, E). Sialic acid containing GSLs were undetectable using the classical resorcinol staining of lipid extracts on TLC plates (not shown). Detailed structural characterization of fatty acids and long chain bases of free ceramides, mono- and dihexosyleramides and sphingomyelins is given in Figs. S4, S5 and S6 of Supporting Information.

Antibody staining of HBGa epitopes of lipid extracts from HIEs

Anti-HBGa antibody chromatogram-binding assays (CBAs) of lower and upper phases extracted from HIEs were used to detect and characterize GSLs after separation on TLCs (Fig. 2, 3 and S8). When staining for Lewis a
(Leα) and Lewis b (Leβ) four major patterns were obtained (Fig. 2A, B and Fig. S8B, C). The J4 and J8 HIE lower phase lipids were heavily stained for Leα but were negative for Leβ, which matches well with their Lewis-positive, secretor-negative genotypes (Fig. 2A, B and Table 1). The J2 and J6 lower phase lipids were weakly stained for Leα, but strongly stained for Leα(Fig. 2A, B). This confirms their Lewis positive, secretor-positive genotypes (Table 1). The appearance of weak bands of Leα in J2 and J6 is a parallel to the composition of HBGA GSLs found in meconium samples and in fetal gut (35), but is different from the composition found in adult small intestines where Leα is not detected in secretor-positive samples (27). The 1J sample was negative for both antibodies, supporting the Lewis negative genotype of this HIE (Fig. 2A, B and Table 1). The J10 sample was negative for the anti-Leβ antibody (Fig. 2B), but showed, surprisingly in relation to its Lewis and secretor negativity (Table 1), a weak but distinct staining band with the anti-Leα antibody (Fig. 2A). The weak Leα positive bands may be explained by the observation that not only the FUT3 but also the FUT5 gene encodes for an α1,3,1,4-fucosyltransferase that may be expressed in these cells and enable the biosynthesis of small amounts of Leα structures from the type 1 (Galβ1,3GlcNAc) precursor (Fig. S1).

Staining with the anti-A monoclonal antibody (Fig. 2C), reactive to a terminal GalNAcα1,3 residue, showed the presence of an A active hexaglycosylceramide (A6-1) in the 1J extracts and of an A active heptaglycosylceramide (A7-1) in the J6 HIE extracts. This difference between these two HIEs in histo-blood group A expression is in agreement with both being A and secretor-positive but 1J being Lewis negative and J6 being Lewis-positive (Table 1). Surprisingly, the lipid extract of J10, which was typed as Lewis- and secretor-negative, showed a distinct double band stained with the anti-A antibody. To further characterize this GSL, additional antibodies, reactive towards glycans with a terminal GalNAc α1,3 residue, were used. However, the GSL was not reactive when stained with an anti-Forssman antibody (Fig. S9B), nor was it stained when using another anti-A antibody, specific for the A type 2 chain structure (not shown), but it did stain with an anti-A6-1 monoclonal antibody (Fig. S9C). Importantly, this clear reactivity could only be observed after mounting 20 times more of J10 lipid extracts than usually used and indicates that indeed small amounts of the A6-1 GSL was produced by the J10 HIEs. None of the lower phase lipids of the HIEs were reactive with the anti-B antibody used for this study (Fig. 2D). However, only J2 was genotyped as B, and this antibody was reactive only towards the monofucosylated B (B6-1) and not towards the difucosylated B (B7-1; BLεβb). BLeβ is indeed expected to be the B-active HBGA GSL produced by J2 HIE, since this HIE has an OB, Lewis (FUT3; LeLe) and secretor (FUT2; Sese) positive genotype. Thus, there would be no major bands detected with the anti-B antibody.

The Folch upper and lower phase lipids of the HIEs J4 and J4FUT2, being the secretor-negative J4 line transduced with a functional FUT2 construct, were compared in more detail using the anti-Leα and anti-Leβ antibodies (Fig. 3). As can be seen already with the chemical staining, the upper phase lipids of both HIE cultures are devoid of ceramides, cholesterol, phosphoglycerolipids and sphingomyelin but contain HBGA GSLs (Fig. 3A). CBA with anti-Leα reveals that a minor portion of the Leα pentaglycosylceramide (Leα-5), especially the more polar (slower moving) ceramide bands, appears also in the upper phase of both HIEs (Fig. 3B). Overlay with anti-Leβ does not stain the J4 extracts, but the J4FUT2 extracts are stained (Fig. 3C). The appearance of Leβ in J4FUT2 extracts was expected since a functional FUT2 gene was transduced into the original J4 HIE culture, enabling the biosynthesis of the Leβ epitope. A specific study of Leα, Leβ and A HBGA structures of the upper phase GSL of all HIEs (Fig. S8) corroborated the results of the lower phase CBAs (Fig. 2) and supported the almost perfect match between genotypes and HBGA phenotypes of the HIE GSLs.

GII.4 Sydney VLPs bind to pure GSLs and lipid extracts of HIEs

Sydney VLPs, representing one of the more recent human GII.4 HuNoV strains shown to replicate in HIEs (10), were used for assaying its binding pattern to reference type 1 chain GSLs (Fig. 4A, B). As can be seen in Fig. 4B this VLP is rather promiscuous in its binding to most of the HBGA structures tested. Although showing a preference for α1,2-fucosylated structures (H5-1, A6-1, B6-1, Leα, ALεβ, BLεβ), the VLPs also showed some binding to
non-fucosylated LeaCer and to the α1,4-fucosylated Leb GSL.

With this in mind, we tested whether the Sydney VLP would show binding to vesicles made from the Folch lower phase lipids of the HIEs using a total internal reflection fluorescence (TIRF) microscopy-based assay. This assay relies on immobilizing VLPs at the bottom of a glass-bottom well and on imaging the binding and release of single fluorescent glycosphingolipid-containing liposomes to individual VLPs under equilibrium conditions (Fig. 5A). Analysis of the arrival rate of the vesicles yields information on the association behavior, where the rate of arrival is directly proportional to the association rate constant \( k_{on} \). On the other hand, analysis of the vesicle’s residence time can be used to estimate the dissociation rate constant \( k_{off} \). Taken together, this assay therefore allows for the semi-quantitative determination of the dissociation constant \( K_D = k_{off}/k_{on} \) by independently measuring \( k_{on} \) and \( k_{off} \) (36). Here, this assay was carried out by forming supported lipid bilayers of pure B6-1 GSL in POPC lipids on a glass surface to immobilize the VLPs. To detect the interaction between surface-immobilized VLPs and Folch lower phase lipids from HIEs, we used vesicles made of such lipids and containing small amounts of fluorescent lipids for visualization (Fig. 5A).

As shown in Fig. 5B, vesicles from J2 and J6 HIEs exhibited excellent binding to the GII.4 Sydney VLPs. In comparison, binding to J4 and J8 vesicles was greatly reduced while no VLP binding to J10 was observed. Negative controls, i.e. assays without VLPs, showed very little background binding and were similar in counts to the J10 experiments (Fig. S10). These results agree well with the binding pattern of the GII.4 Sydney strain deduced from the HBGA GSL content of the HIEs as well as the infection susceptibility of J2 and J6 and resistance of J4, J8 and J10. The weak interaction with J4 and J8 is in line with the weak binding of these VLPs to reference Lea (Fig. 4), the dominating HBGA GSL present in large amounts in these cells (Fig 1A). These binding data further support the concept that HBGAs of the type 1 chain GSLs of human small intestinal cells may indeed be important attachment factors for human GII.4 HuNoV infection.

To further investigate the relationship between virus binding kinetics, affinity to the membranes and susceptibility to virus infection, we characterized in detail the interaction kinetics of HuNoV VLPs to the various HIE membranes. Representative association and dissociation plots are shown in Figs. 5C and 5D, while the resulting \( k_{on} \), \( k_{off} \) and \( K_D \) are compared semi-quantitatively in Figs. 5E, 5F and 5G, respectively. Noteworthy, dissociation rate constants were significantly lower for the two HIEs susceptible to HuNoV infection (J2 and J6), indicating that the ability of the virus to remain attached to the cell surface may be key in determining its susceptibility. The association rate only moderately correlates with susceptibility to virus infection. While the association rate constant is clearly the highest for the J6 HIE, and accordingly \( K_D \) the lowest for this case, it appears that J2, also susceptible to infection, is not distinctly different from the infection resistant HIEs J4 and J8 in terms of their association behavior, indicating that the recruitment of the virus to the ligands may not be the major determinant in the context of virus infection. In line with their ability to replicate the virus, the two susceptible and permissive HIEs, J2 and J6, exhibit the lowest \( K_D \), values, indicating that the affinity is the strongest for those cells that can be infected. This effect is however less pronounced for J2, suggesting that in this case, factors other than virus-glycolipid ligand interaction may be determining their ability to replicate the virus. In agreement with the very low vesicle surface coverage reported for J10 (Fig. 5B), the association rate for this HIE membranes was reduced to 0.01% of the value for J6. The dissociation events were thus too few to allow for data fitting and \( k_{off} \) determination.

Discussion
The \textit{ex vivo} culture system of HIEs has established a new niche towards understanding the physiology of the human gastrointestinal tract and the pathophysiology of human gastrointestinal diseases. Our aim was to link the biochemical nature of these cultures with \textit{in vivo} pathophysiology of the digestive tract specifically related to HuNoV infection and propagation. Since individuals vary in their clinical susceptibility to different HuNoV strains depending on their HBGA status, we analyzed the composition of lipids and glycosphingolipids of seven HIE lines established from individuals, varying in their ABO, Lewis and secretor genes and correlated HuNoV GII.4 Sydney VLP binding to lipid extracts of such HIE cultures to
their ex vivo permissiveness to Sydney virus infection.

Only a few attempts to characterize the lipid composition of human intestines have so far been reported (22-25,27,37) and to the best of our knowledge, this is the first to report on both the detailed glycerol- and sphingolipid compositions of human intestinal epithelia and definitely the first comparing epithelia of so many individuals. Our studies are based on enteroids derived selectively from jejunal stem cells. Thus, differences between different parts of the intestines are not addressed in this study, but are likely to appear (25). Additionally, the culture conditions, as well as the lack of innervation and naturally surrounding cells and matrices, are not identical to the in vivo situation, which may affect the lipid composition of these cultures. For improving the general conclusions from the lipid analysis, however, we analyzed the composition of differentiated jejunal enteroids established from six different individuals and grown under identical, strictly controlled conditions. This means that the cultures are sterile and under no influence from any gut microflora, bile, gastric or pancreatic juices or subjected to any shear forces caused by peristaltic movements. Finally, the progenitor stem cells, from which these cultures are established, were taken from biopsies of adult patients undergoing diagnostic or therapeutic enteroscopies, and may not completely reflect the cellular composition of intestines of healthy newborns, children or even healthy adults. The glycoprotein composition of the various HIEs will be reported separately (Nilsson et al. Manuscript), and here we focus strictly on membrane-associated lipids and their interactions with HuNoV GII.4 infection.

The major lipid components were essentially identical and quantitatively in the same range for all HIEs and also similar to what has been reported before for human meconium, which represents shed fetal intestinal epithelia (35). The complete cellular lipid composition of adult intestinal epithelia has not been studied before in molecular detail although it is known to change during enterocyte differentiation and postnatal maturation, which probably affects the physical mucosal barrier, its selective permeability and functionality (38-41). Since we studied the general lipid compositions of the HIE cultures we could not define differences in asymmetries over basolateral or apical parts of the membranes nor differentiate between lipids originating from the cellular plasma membranes, from endosomes or from other subcellular organelles. However, since HIEs have been shown to support replication, and recapitulate unique aspects of infection, of previously non-cultivatable pathogens other than HuNoV, such as cryptosporidium (11,12) and Salmonella Typhi (13), knowing the lipid composition of HIEs will probably be useful and of general significance for the field of host-pathogen interactions. Of special interest is the binding of enterotoxigenic E. coli to histo-blood group A antigens on the apical surfaces of polarized small intestinal enteroid monolayers (42) which appears similar to the binding of HuNoV to fucosylated HBGAs of our HIEs (14).

For sphingolipids there is biologically an asymmetric distribution over membrane bilayers, with a natural accumulation in the (apical) plasma membrane and with a majority, if not all, of the glycosphingolipids appearing in the outer leaflet of the bilayer (43,44). This distribution is related both to the chemical properties of the sphingolipid long chain bases, but also to the hydrophilic glycan parts of the GSLs extending towards the exterior water milieu. For GSLs lateral clusters or microdomains within the plane of the plasma membrane have been called “glycosynapses” (45), indicating a functional role for such microdomains. Both sphingomyelin and free ceramides as well as GSLs have been associated with the formation of microdomains (46), and with a prominent accumulation of sphingolipids in the outer layer of epithelial membranes, a structured organization is likely needed.

We identified SM as the major sphingolipid, also being the dominant sphingolipid in isolated human epithelial cells (27). Indeed, sphingomyelin is often the most abundant sphingolipid in membranes and is highly interactive with cholesterol, another major component of the HIEs, setting the basic prerequisites for microdomain formations, and possibly playing functional roles in cell signaling, trafficking, sorting, polarization and apoptosis (47,48). Most tissues contain sphingomyelin with 16:0, 18:0, 22:0, 24:0 and 24:1 acyl chains (49). We also found C16:0 to be the dominant non hydroxy fatty acid of sphingomyelin (SM d18:1) and dihydro sphingomyelin (DH-SM d18:0) in all HIEs (Fig. S4, S6). In bilayer membranes, cholesterol favorably interacts with DH-SM (d18:0/16:0) and such membranes are more
condensed than the ones with SM (d18:1/16:0) (50).

Another major component of the sphingolipids in the HIEs was the free ceramides showing a very complex composition of both long chain bases (d18:0, d18:1 and t18:0) and of hydroxy and non-hydroxy C16:0-24:1 fatty acids (Figs. S4-S6). The biosynthesis of these different structures is highly regulated by different serine palmitoyltransferases, synthases and desaturases as well as by sphingomyelinase (Fig. S7) coded for by the corresponding genes SPTLC1, SPTLC2, CERS3, SGMS1, DES1, DES2, and ASM. Interestingly, the receptor protein for murine NoV (MNV) CD300if is a type 1 integral membrane protein. It has been shown to bind to ceramide (and phosphatidylserine) and, by doing so, its protein epitopes are altered, suggesting a conformational change of the receptor complex and facilitating MNV infection (51). Ceramide has also been shown to affect porcine calcivirus infection (same family of Caliciviridae as HuNoV), although through another mechanism (33,52). Of particular interest is the recent finding that ceramide in the apical cell membrane plays an important role for HuNoV GI.3 replication in HIE cultures (33).

Ceramides are additionally important as biosynthetic precursors of all GSLs (Fig. S7), which, through the action of specific glycosyltransferases, are extended in a step-by-step process to acidic or neutral GSLs in linear or branched glycan chains The HBGAs epitopes of human intestines are typically found on neutral GSLs with 5-12 monosaccharide residues as the result of α-glycosyltransferase activities, coded for mainly by the ABO, Lewis (FUT3) and secretor (FUT2) genes (24,27) (Fig. S1), also being responsible for the biosynthesis of HBGA epitopes on intestinal glycoproteins.

After mild alkaline hydrolysis of lower phase lipids and using an appropriate solvent for borate-impregnated TLC separation, we could establish GalCer as the major component of HexCers in all HIEs (Fig. 1F). The presence of GalCer in the HIE cultures may reflect a potentially important role in the cell membrane, as GalCer has been shown to bind to HuNoV VLPs, particularly in membrane domains of solid supported lipid bilayers (53). However, the presence of GalCer also in the J10 cultures, which are not permissive to the virus, possibly indicates that GalCer may function as an additional attachment factor of importance for endosomal uptake and transfer but not as an individually selective plasma membrane factor. GalCer also serves as the natural precursor to sulfatide (3-O-Sulfo GalCer, SHexCer), which is another major sphingolipid component of HIEs and of human intestines, with both tissues showing a very heterogeneous ceramide part (23,24) (Figs. S4 and S5). The significance of the structural heterogeneities of the ceramides of GSLs has to await further functional studies of the HIEs. The glycan parts of the diHexCers were not resolved in this study and thus the diHexCers could theoretically be lactosylceramide (GalGlcCer) as seen in adult intestine (22) or digalactosylceramide (GalGalCer) (Fig. S7), or a combination of both, as seen in human meconium (23).

Since GSLs bearing HBGAs are enriched in the human intestinal epithelium, we hypothesized that individual differences of HIE GSL subsets would be related to the HBGAs genotype of the individuals from which these cultures were established. Overall, this was also true (Table 1, Figs. 2-3, Figs. S1, S8-S9). Thus, the J1 HIE, being secretor-positive, Lewis-negative and of blood group A, strongly expressed A-6 type 1 chain. The J2 HIE, being secretor-positive, Lewis-positive and of blood group B, expressed Leα and BLeβ (chemical staining). The J4 HIE, being secretor-negative, Lewis-positive and of blood group O, strongly expressed Leα. When transduced with a functional FUT2 the J4FUT2 HIE additionally expressed Leβ. The J6 HIE, being secretor-positive, Lewis-positive and of blood group A, expressed Leα and ALeβ (A7-1). The J8 HIE, being secretor-negative, Lewis-negative and of blood group B, strongly expressed Leβ but not Leα. Finally, the J10 HIE, being secretor-negative, Lewis-negative and of blood group A, did not express Leα but did surprisingly show reactivity with the general anti-A antibody. Using the same antibody, the phenotyping of the supernatant of J10 cultures, using the ELISA assay, also occasionally showed some “A” reactivity. This reactivity varied in intensity among different lipid preparations of the J10 HIEs. We could exclude that the GSL was the Forssman antigen or an A type 2 chain GSL; it was confirmed as an A type 1 chain structure using an anti-A6-1 specific monoclonal antibody (Fig. S9). Interestingly, the expression of this GSL in the J10 line is very low and cannot be directly related to the FUT2 or FUT3 genotype of these cells. Clearly, the major HBGA reactive
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Importantly, we did not find any reactivity with two anti-Leα antibodies, indicating the lack of an α1,2-fucosylation of the terminal Gal of any type 2 chain GSLs.

Our recent studies have shown that overexpression of FUT2 in the J4 secretor-negative HIE cells allows for HuNoV GII.4 infection (14) and, as shown here, leads to a secretor-positive phenotype (Fig. 3C). We have shown in earlier studies that HuNoV VLPs bind to HBGAs of reference GSLs in a strain-specific manner (54-57) and we now add GII.4 Sydney to this list (Fig. 4B). It is clear from HuNoV infections in HIEs that the GII.4 genotype prefers to replicate in secretor-positive cells (19).

Our findings from overlay and TIRF microscopy assays confirm that GII.4 VLPs prefer to bind to type 1 chain GSL structures dependent on secretor status (FUT2 expression), which further supports GSLs as one of the important attachment factors for this virus.

Beyond this, we further characterized the interaction kinetics between individual HuNoV VLPs and lipid bilayers made from lipids extracted from the different HIEs in an attempt at better understanding how affinity and interaction kinetics relate to susceptibility to virus infection. While many efforts have been put into the identification of viral receptors, the interplay between the characteristics of the interaction between the virus and the receptor-containing membrane and infection is only poorly understood. It remains widely unknown how binding and release of the virus at the cell surface is modulated and whether the interaction should be strong or weak, fast or slow. The availability of lipid material from HIEs and the advent of methods allowing for the dynamic studies of interaction kinetics on a single particle level, such as the TIRF assay used here, open new avenues to the study of such processes. Our study suggests that slower dissociation may correlate better with susceptibility while the role of virus association to the membrane is less critical. It was also shown that generally, the apparent affinity between the virus and the membrane is the highest (lowest K0) for membranes made of lipids of susceptible cells, although intermediate levels of binding was also observed for the extracts of non-susceptible cells containing Leα. In the future, kinetic studies of virus-HIE membrane interactions, like the one presented here are expected to extend our understanding on the role of cholesterol, glycerolipids and sphingolipids (including HBGAs active GSLs) in HuNoV infection. HIEs offer, in combination with the methods described here, a new model of looking at the human gut as a model system not only for HuNoV infection, but also for other pathogens and for dissecting various membrane components of the gut epithelium, to study other gastrointestinal diseases as well as the physiology and biochemistry of the intestines.

Experimental procedures

Establishment of human intestinal enteroids

Establishment of HIE cells from patient tissues was originally described by Dr. Hans Clevers’ group (1,58,59), and maintenance, expansion and differentiation of cell cultures, resistant or susceptible for HuNoV replication, has already been reported (8,32). Cell pellets of differentiated jejunal HIEs from six separate individuals (named 1J, J2, J4, J6, J8, J10) and J4 HIEs transduced with the FUT2 gene were collected from individual cultures and stored at -80°C (10,14). Ethical approvals were obtained from the Institutional Review Board of Baylor College of Medicine and Affiliated Hospitals.

Production and characterization of secretor-negative J4 HIE cultures transduced to express functional FUT2

The secretor-negative J4 line was genetically modified to express functional FUT2 following lentivirus transduction. The cDNA of FUT2 was obtained from mRNA of J2 by using the forward primer 5'-ATGGCCCACTTCATCCT-3’ and the reverse primer 5’-TTAGTGCTTGAGTAAGGGGGAC -3’. The cDNA was cloned into the lentiviral expression vector pLNSIN-IRE-puro or pLNSIN-IRE-hygromycin (60) using an In-Fusin cloning kit (Takara-Clontech) according to the manufacturer’s protocol. Lentiviruses were produced in HEK293T cells by transfecting four plasmids (pMDLg/pRRE, pMD2.G, pRSV-Rev, and pLVSIN-IRE-puro-FUT2) using Polyethylenimine HCL Max MW 40000 (Polysciences) as a transfection reagent. The culture supernatant was harvested 60-72 h post transfection. The supernatant was concentrated by LentiX-concentrator (Takara-Clontech) according to the manufacturer’s protocol. Lentiviruses were produced in HEK293T cells by transfecting four plasmids (pMDLg/pRRE, pMD2.G, pRSV-Rev, and pLVSIN-IRE-puro-FUT2) using Polyethylenimine HCL Max MW 40000 (Polysciences) as a transfection reagent. The culture supernatant was harvested 60-72 h post transfection. The supernatant was concentrated by LentiX-concentrator (Takara-Clontech) according to the manufacturer’s protocol. HIEs were trypsinized for 10 min at 37 °C and the trypsin was inactivated by CMGF/-10% FBS.
After pelleting the cells by centrifugation (300Xg), the cells were plated with the lentivirus solution and the plate was incubated with centrifugation (300Xg) for 1 h. After incubation, lentivirus solution was removed and cells were washed in CMGF- and plated in CMGF+/ Y-27632 (10 µM, Sigma) with Matrigel (Corning). Five days after lentivirus infection, cells were treated with puromycin (2 µg/mL) or hygromycin (300 µg/mL) and they were maintained until mock-treated cells were completely dead. Colonies that survived the selection were then cultivated as a modified HIE line (14).

**Characterization of HIE pheno- and genotypes**

Individual enteroid monolayers were propagated as described previously (8,10). Media was replaced with PBS, and the cells were heated for 5 min. After clarification, the supernatants were analyzed by enzyme immunoassay for the presence of Leα, Leβ, A, B, and H antigens, as described previously (61). For genotyping, DNA was extracted from the monolayers and amplified by PCR to determine FUT2, FUT3 and ABO genotypes using primers specified in Table S1. The amplicons were purified using the GeneJET PCR Purification Kit (Thermo Scientific) and sequenced by GeneWiz (South Plainfield). The resulting chromatograms were assessed for single nucleotide polymorphisms and deletions at known mutations sites (FUT2: A385T, G428A, C571T, C628T; FUT3: T59G, T202C, C314T, G484A, G508A, G667A, G808A, T1067A; and ABO: nucleotides 261 and 297 in exon 6 and 657, 703, 771, 796, 803, 829 and 930 in exon 7).

**Lipid extracts from HIEs**

The lipid extraction was performed using the Folch partition method, which has been described elsewhere (62) and is well established. Frozen cell pellets (1-2 x10⁶ cells) of HIEs were vigorously mixed with 3 mL distilled water (dH₂O) until the cell pellets dissolved. The cell suspensions were then transferred to Kimax tubes and mixed vigorously with 12 mL chloroform:methanol (C:M) solution (2:1, by vol.), so that the final proportions of chloroform:methanol:water (C:M:W) would be 8:4:3 (by vol.). The extraction of lipids was conducted at 60 °C for 1 h. Following centrifugation at 400Xg for 10 min, the lower and upper phases were collected separately, solvents evaporated under nitrogen, and extracts weighed and dissolved in C:M (2:1, by vol.). Lipid lower phases were used for lipidomics LC-MS and TLC analyses and stored at 4 °C. The upper phases of Folch partitions of lipids were desalted using Isolute Spe columns MFC18 (Biotage) under the Vac-Elut 15 mm Hg vacuum. Each column was first wetted with practical upper phase of Folch partitions from C:M:W (8:4:3, by vol.), then the upper phases from lipid extracts were applied once onto the column without drying. The washing step was performed using the practical upper phase fraction and then by drying the column under vacuum. Five mL of C:M (2:1, by vol.) was applied to elute the lipids. Finally, the desalted upper phase lipids from HIEs were dried under nitrogen and dissolved in C:M (2:1, by vol.), used for TLC analyses and stored at 4 °C.

**Mild alkaline hydrolysis**

Folch partition lower phase lipids were treated with 0.2M KOH in methanol for 3 h at room temperature. The hydrolysis was stopped by neutralization with 1 drop of acetic acid and diluted with chloroform and water, so that only one phase persists. Samples were desalted using Isolute Spe columns MFC18 applying the protocol described above.

**Reference GSLs**

This study used the reference GSLs lactotetraosylceramide (Le4Cer), H5-1, A6-1, A7-1, B6-1, Leα-5, Leβ-6 from type 1 chain HBGAs of pooled human meconia and three fractions of a blood group A, B and O type meconia (AIII, BIII and OIII) containing GSLs with 4 to 7 monosaccharides in the glycan chains. The GSLs were purified either from the meconium samples of single ABO blood group typed individuals or from the pooled meconium samples of individuals with identical ABO blood groups and were previously structurally characterized by MS and ¹H-NMR (23,63,64). The reference Leα and Forssman GSLs were prepared from dog intestines and have been equally characterized (37,65).

**Production of HuNoV VLPs**

The HuNoV VLPs used in this study were produced in SF-9 insect cells using the baculovirus construct from the GII.4 Sydney strain as described elsewhere (66,67).
TLC separation and detection of lipids and GSLs

Pure GSLs (2 μg), meconium extracts (14 μg) or HIE lipid extracts (100 μg) were applied to alumina backed silica gel 60 HPTLC plates (Merck) and chromatographed at room temperature with chloroform:methanol:water (C:M:W, 60:35:8 by vol.) for 20 min. Two to three sets of GSLs were run in parallel on the same TLC-plate, and the dried plate was then cut in two to three parts. GSLs on one of the plates were visualized by spraying with anisaldehyde:sulfuric acid:acetic acid (1:2:97, by vol.) and heated at 180 °C for color development. The other plate parts were used for the chromatogram-binding assay (CBA).

For optimal separation and characterization of the monohexosylceramides (HexCer) GlcCer and GalCer, glass backed silica gel 60 HPTLC plates were sprayed with sodium tetraborate, 1% solution in water, activated at 150 °C overnight, eluted with C:M:W 100:30:4 by vol. (68) and stained with the anisaldehyde reagent.

To chemically detect sialic acid -containing GSLs, HIE lipid extracts (upper and MAH treated lower phases) were applied on glass backed silica gel 60 HPTLC plates and chromatographed at room temperature with chloroform:methanol:water (C:M:W, 60:35:8 by vol.) for 20 min. Dry plates were visualized by spraying with resorcinol-HCl reagent (10 mL of 2 % resorcinol in water, 40 mL concentrated HCl, 0.125 mL 0.1M cupric sulfate) and heated at 150 °C for color development.

Chromatogram binding assay with antibodies (TLC-CBA)

GSLs and lipid extracts were separated by TLC using C:M:W (60:35:8, by vol.) as eluent. After chromatography, the plates were dried and immersed into a solution of 0.3% polyisobutylmethacrylate in diethyl ether:n-hexane (1:1, by vol.) for 60 s, dried, and incubated in PBS, containing 3% bovine serum albumin (BSA) and 0.05% Tween-20 for 1 h as described before (69).

For anti-HBGA CBA the TLC plates were overlaid with mouse monoclonal antibodies specific for either Lea (1:200) CatNo Ab00492-1, Absolute Antibody) or Leb (1:100) CatNo 912501, BioLegend) (70). The antibodies were all diluted in PBS with 0.5% BSA and left on the plate for 1h 30 min. After removal of the primary antibody, the polyclonal goat anti-mouse detection antibody conjugated with alkaline phosphatase (ALP) (CatNo A0162, Sigma), diluted 1:500 in PBS with 0.5% BSA, was added for 1h 30 min. After washing the color reagent BCIP/NBT Fast (CatNo B5665, Sigma) was applied. After washing, plates were dried and photographed. Plates were always washed three times with PBS and 0.05% Tween-20 and all steps performed at room temperature.

Lipid analysis

Lower phase lipids, extracted using the Folch procedure (62), were analyzed using a combination of direct infusion mass spectrometry and ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

Phospholipids, i.e. phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserine (PS) and sphingomyelins (SM) were analyzed using a QTRAP 5500 mass spectrometer (Sciex) equipped with a robotic nanoflow ion source, TriVersa NanoMate (Advion BioSciences, Ithaca, NJ). The analysis was made using precursor ion scanning in negative (PC, PE and PS) and positive (SM) ion mode according to previous work (72-74) and the lipid species were quantified using.
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Sphingolipids, i.e. ceramides (Cer), monohexosylceramides (HexCer) and dihexosylceramides (diHexCer), were analyzed using UPLC-MS/MS on a QTRAP 5500 mass spectrometer. Lipid species were separated using an Acquity BEH C8 column (2.1 × 100 mm with 1.7 μm particles; Waters) with water, acetonitrile and isopropanol as mobile phases (75). Quantification was made using external calibration curves with available reference substances.

Free cholesterol was quantified using straight-phase HPLC coupled to evaporative light scattering detection according to a previous work (76).

**Lipid vesicle preparation**

Bilayer and detection vesicles to be used in the TIRF microscopy-based assay were prepared by lipid hydration and extrusion. In brief, appropriate amounts of lipids dissolved in chloroform:methanol (2:1, by vol.) were added to a round-bottom flask. The lipid solution was then dried under a light stream of nitrogen and subjected to vacuum for at least 90 min. After drying, the lipid film was hydrated in PBS buffer and vortexed several times before extruding the suspension by passing it several times through a polycarbonate membrane of appropriate pore size (Whatman) using a mini-extruder (Avanti Polar Lipids).

Bilayer vesicles were prepared by extruding a vesicle suspension, consisting of 95% (w/w) 1-palmitoyl-2-oleoyl-SN-glycero-3-phosphocholine (POPC) and 5% (w/w) B6-1 GSL, several times through a polycarbonate membrane with a pore size of 50 nm at room temperature. Passivation vesicles were prepared by extruding a POPC vesicle suspension through a polycarbonate membrane with pore size of 100 nm at room temperature. Detection vesicles were prepared by extruding a vesicle suspension consisting of 99% (w/w) Folch lower phase lipids and 1% (w/w) N-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rh) through a polycarbonate membrane with a pore size of 100 nm. Lipid hydration and extrusion was carried out at 60 °C. The so-obtained vesicles all had similar size distributions as verified by nanoparticle tracking analysis (see Supporting Information Figure S11).

**HuNoV VLP binding to HIE lipids vesicles**

The surface of glass-bottom microtiter wells (96 well-plate) (Mat Tek) was cleaned using 3.5% Hellmanex II (Hellma Analytics) in ultrapure water (18 MΩ) for 2 h and then thoroughly rinsed with ultrapure water. To form supported lipid bilayers containing 5% (w/w) of B6-1 GSL, 50 μL vesicle suspension (total lipid concentration 0.1 mg/mL) was incubated for 30 min at room temperature. The wells were then washed several times with PBS, keeping the surface hydrated. HuNoV GI.4 Sydney VLPs were then added to the microtiter wells coated with the B6-1 GSL-presenting supported lipid bilayer and incubated for 45 min at room temperature. After an extensive washing with PBS, POPC vesicles were added to reach the final concentration of 10 μg/mL. The fluorescent vesicles for detection of VLP binding were added to the well at the final concentration of 1,3x10^11 particles/mL in PBS maintaining a total volume of 100 μL in the well. The number of vesicles in each sample was quantified using nanoparticle tracking analysis (Nanosight NS 300) and following the manufacturer’s instructions for particle quantification. Time-lapse movies were recorded at room temperature with a TIRF microscope, 45 min after the injection of fluorescent vesicles.

A Nikon Eclipse Ti2-E inverted microscope with a 60X magnification (NA = 1.49) oil immersion objective (Nikon Corporation) was used to acquire the time-lapse movies at a frame rate of 1 FPS. The microscope was equipped with a solid-state light source (Spectra III light engine, Lumencor), a multibandpass filter cube 86012v2 Dapi/FTC/TxRed/Cy5 (Nikon Corporation) and a Prime 95B sCMOS camera (Teledyne Photometrics). The images were further analysed using an in house Matlab (Mathworks) script (36) allowing for the determination of the vesicle surface coverage over time, the rate of arrival of the vesicles and their residence time. The number of bound particles was measured in three separate spots in each well, with each spot being recorded for 350 frames. Here, any vesicle staying for three frames or more was considered. The number of bound particles was averaged over each time-lapse, and the surface coverage was evaluated as the average number of bound particles in each spot. The association rate for each measurement was determined by summing together the arrival events on three different spots on the well followed by a linear fit.
excluding the first 46 frames from it. The dissociation behavior was determined by fitting the dissociation curves obtained from the residence time (see (36) for further details). Although vesicle binding to surface-immobilized VLPs has been previously reported to exhibit a multiexponential decay (36,56), we here applied a single exponential fit as an approximation of the dissociation behavior (see Supporting Information Fig. S12).

**Data availability**

Lipidomics data are available upon request to Göran Larson, University of Gothenburg, Gothenburg, Sweden, goran.larson@clinchem.gu.se and VLP binding data are available upon request to Marta Bally, University of Umeå, Umeå, Sweden, marta.bally@umu.se. All other data are contained within the manuscript.
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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
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The abbreviations used are: HIE, human intestinal enteroid; HBGA, histo-blood group antigen; GSL, glycosphingolipid; VLP, virus-like particle; HuNoV, human norovirus; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylyserine; FA, fatty acid; FC, free cholesterol; LPC, lyso-phosphatidylcholine; SM, sphingomyelin; DH-SM, dihydrosphingomyelin; Cer, Ceramide; DH-Cer, dihydrosphingosine (d18:0) ceramide; SHexCer, sulfatide; HexCer, monohexosylceramide; diHexCer, dihexosylceramide; GalCer, galactosylceramide; GlcCer, glucosylceramide; d18:0, sphinganine base; d18:1, sphingosine base; t18:1, phytosphingosine base; CBA, chromatogram-binding assay; TIRF, total internal reflection fluorescence.

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Table 1. Jejunal enteroid geno- and phenotypic HBGA characteristics.

| HIE ID | Secretor | Lewis | HIE HBGA | HuNoV propagation |
|--------|----------|-------|----------|-------------------|
| FUT2   | Genotype | Phenotype | Genotype | Phenotype | Genotype | Phenotype | Genotype | Phenotype | Summary | GII.4 SYD |
| 1J     | Se, se   | Positive | le<sup>202,314</sup>, le<sup>202,314</sup> | Negative | OA | A | A | +** |
| J2     | Se, se   | Positive | Le, Le | Positive | OB | B Le<sup>b</sup> | B Le<sup>b</sup> | + |
| J4     | se<sup>428</sup>, se<sup>428</sup> | Negative | Le, le<sup>202,314</sup> | Positive | OO | Le<sup>a</sup> | Le<sup>a</sup> | - |
| J6     | Se, Se   | Positive | Le, le<sup>59,508</sup> | Positive | OA | A Le<sup>b</sup> | A Le<sup>b</sup> | + |
| J8     | se<sup>428</sup>, se<sup>428</sup> | Negative | Le, le<sup>202,314</sup> | Positive | OO | Le<sup>a</sup> | Le<sup>a</sup> | - |
| J10    | se<sup>428</sup>, se<sup>428</sup> | Negative | le<sup>484,667</sup>, le<sup>59,508</sup> | Negative | OA | A* | A* | - |
| J4 FUT2| Se, se<sup>428</sup> | Positive | Le, le<sup>202,314</sup> | Positive | OO | Le<sup>b</sup> | Le<sup>b</sup> | + |

*FUT2, FUT3 and ABO genotypes were assessed for single nucleotide polymorphisms and deletions at known mutations sites as follows: FUT2: A385T, G428A, C571T, C628T; FUT3: T59G, T202C, C314T, G484A, G508A, G667A, G808A, T1067A; and ABO: nucleotides 261 and 297 in exon 6 and 657, 703, 771, 796, 803, 829 and 930 in exon 7. Identified mutation sites are indicated by smaller case letters and in the superscript (ex., se<sup>385</sup>). Secretor, Lewis and HBGA phenotypes were obtained from ELISA. Se/se – secretor; Le/le – Lewis; HIE – human intestinal enteroid; HBGA – histo-blood group antigen; HuNoV – human norovirus.* see Discussion. **Unpublished.
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Figures

Figure 1. Lipid and sphingolipid content of HIEs.
A) TLC of HIEs Folch lower phase lipids with reference glycolipids enriched from an OSeLe meconium sample (63,64,77). B) Molar percentages of cholesterol and glycerolipid and (C, D) sphingolipids of HIEs. E) TLC of alkaline stable fractions of Folch lower phase lipids of HIEs. F) TLC of alkaline stable fractions of Folch lower phase lipids of HIEs using borate impregnated plate. GalCer, GlcCer, HexCer from meconium (OLeSe), case 1 (ALeβ) and 3 (OLeα) represent references and previously published glycolipid extracts of human intestinal epithelium (23,24).

Single data points represent the raw data, horizontal lines the means, and error bars the standard deviation for J2 (n=5) and J10 (n=3); J4 (n=2), J1, J6, J8 and J4FUT2 (n=1). Numbers next to TLCs correspond to the number of monosaccharides in GSLs; a star sign (*) labels unknown structure colored pink with anisaldehyde staining. Sphingolipids marked with OH in brackets represent sphingolipids with hydroxy fatty acids.
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Figure 2. TLC–CBA of HBGAs of GSLs from HIEs.
HBGA epitopes were detected with A) anti-Le^a B) anti-Le^b C) anti-A and D) anti-B antibodies. AAS - Anisaldehyde staining shows migration of reference GSLs also mounted on first lane of all TLC-CBAs.
Figure 3. TLC-CBAs of Folch upper and lower phase lipids from J4 and J4FUT2 HIEs. A) Anisaldehyde staining. B) anti-Le^a antibody. C) anti-Le^b antibody. Reference glycolipids are run on the first lanes and annotated in the margins. up = upper phase; lp = lower phase lipids.
Figure 4. Binding of GII.4 Sydney VLP to reference GSLs.
A-B) TLC-CBA with GII.4 Syd NoV VLPs binding to type 1 chain reference GSLs of human meconium.
Figure 5. Kinetic analysis of the binding of GII.4 Sydney VLP to HIE–vesicles. A) Total internal reflection fluorescence microscopy (TIRFM) assay to probe the interaction between surface-
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immobilized norovirus virus-like particles (NoV-VLPs) and HIE-vesicles. GII.4 Sydney VLPs were immobilized on the bilayer and binding to VLPs was detected with 1% (w/w) rhodamine (Rh) labeled vesicles made of lower phase lipid extracts. B) Vesicle surface coverage. C) Representative association curves displaying the number of newly arrived vesicles as a function of time for the different HIE vesicles. D) Representative normalized dissociation curves displaying the number of vesicles still bound with time. No dissociation events were recorded for J10. E) Arrival rate (n’) obtained by fitting association data with a linear function. F) Dissociation rate constant (koff) values obtained from fitting the decay functions with a monoexponential function. See Supporting Information Fig. S12 for the fits. G) koff/ n’ values. These values are proportional to the dissociation constant K_D. In (B, E-G), n=6 for B6-1, n=7 for J2 and J6, and n=3 for all others. Single data points represent the raw data, horizontal lines the means, and error bars the standard deviation. The statistical significance was determined using Welch’s t-test: *p < 0.05, **p < 0.01.
Histo-blood group antigens of glycosphingolipids predict susceptibility of human intestinal enteroids to norovirus infection

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