Gastrointestinal norovirus infections and the development of the next generation of mucosal vaccines

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"Human norovirus interaction with histo-blood group antigens blocked by antibodies"

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Mano šeimai ♥

To my family ♥

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ABSTRACT

Human norovirus (HuNoV) is the causative agent of the winter vomiting disease and the leading cause of outbreaks of gastrointestinal infections across all settings and age groups in the world. The virus is highly contagious making outbreaks difficult or often impossible to control and having a high impact on societal costs and resources. Therefore, there is a high urge for the design and development of a HuNoV vaccine. Since research on HuNoV biology and pathogenesis has been hampered by the inability to infect and efficiently propagate the virus in cell cultures, HuNoV receptor studies that address antibody-mediated protection against HuNoV have not been possible. However, such a model has recently been developed. This thesis has focused on two crucial steps towards the development of a novel mucosal subcomponent HuNoV vaccine. The first was to identify membrane components carrying histo-blood group antigens (HBGAs) that are required for HuNoV infection in the epithelial cells of the human intestine, represented as cultures of human intestinal enteroids (HIEs). The second step was to identify highly immunogenic peptides from the HuNoV capsid for generating a subcomponent vaccine that stimulates strong and long-lasting HuNoV-specific immune responses. The key findings have advanced our basic knowledge on the lipid, glycolipid and glycoprotein composition of HIEs, established from jejunal biopsies of individuals with different ABO, secretor and Lewis status. These components may all be of importance for understanding the pathogenesis of HuNoV gastrointestinal infection, as well as contribute in designing a mucosal subcomponent vaccine against HuNoV effectively preventing future HuNoV disease and outbreaks.

Keywords: human norovirus; gastrointestinal infection; mucosal vaccine; subcomponent vaccine; human intestinal enteroids; histo-blood group antigens; lipidomics; glycoproteomics; glycosphingolipids.

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SAMMANFATTNING PÅ SVENSKA

Humant norovirus (HuNoV) är orsaken till vinterkräksjukan och den största anledningen till utbrott av gastrointestinala infektioner i alla typer av miljöer och bland alla åldersgrupper i världen. Viruset är oerhört smittosamt vilket gör att utbrott är svåra eller ofta omöjliga att kontrollera och har stor påverkan på samhälleliga kostnader och resurser. Det finns därför ett stort behov av att ta fram och utveckla ett vaccin mot HuNoV. Forskningen kring biologin och patogenesen hos HuNoV har hämmats av vår tidigare oförmåga att infektera och på ett effektivt sätt föröka viruset i cellkulturer. Därför har identifiering av receptorerna för HuNoV liksom utveckling av ett fullgott antikroppsmedierat skydd inte tidigare varit möjligt. Dock har en sådan experimentell modell nyligen utvecklats. Den här avhandlingen fokuserar på två avgörande steg mot utvecklandet av ett unikt per oralt delkomponentvaccin mot HuNoV. Det första steget var att identifiera membrankomponenter som bär på vävnads-blodgrupps antigener (HBGAs) som behövs för infektion av HuNoV i epiteliala celler i människans tarm, representerade av *in vitro* kulturer av humana minitarmar s.k. enteroider (HIE:s). Det andra steget var att identifiera starkt immunogena peptider från HuNoV's kapsel för att framställa ett delkomponentsvaccin som stimulerar till ett starkt och långvarigt HuNoV-specifikt immunsvar. Nyckelupptäckterna har fördjupat våra baskunskaper om lipiders, glykolipiders och glykoproteiners sammansättning i HIEs, etablerade från tunntarms biposier från individer med olika ABO, sekretor och Lewis status. De här komponenterna är av stor betydelse för förståelsen av patogenesen av gastrointestinala infektioner orsakade av HuNoV och kommer förhoppningsvis bidraga till att designa ett peroralt komponentvaccin mot HuNoV som effektivt förhindrar framtida HuNoV-infektioner och sjukdomsutbrott.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Parveen N, **Rimkute I**, Lundgren A, Block S, Rydell G, Midtvedt D, Larson G, Hytönen, V, Zhdanov VP, and Höök F.

Membrane Deformation Induces Clustering of Norovirus Bound to Glycosphingolipids in a Supported Cell-Membrane Mimic.

Journal of Physical Chemistry Letters, 2018 May 3;9(9):2278-2284.*

II. **Rimkute I**, Ståhlman M, Tenge V, Lin SC, Haga K, Atmar RL, Estes MK, Lycke N, Thorsteinsson K, Bally M, Nilsson J, and Larson G.

Structural characterization of lipids and sphingolipids in human intestinal enteroids relates histo-blood group antigens of glycosphingolipids to cell permissiveness to Norovirus infection.

Manuscript.

III. Nilsson J, **Rimkute I**, Sihlbom C, Tenge V, Lin SC, Atmar RL, Estes MK, and Larson G.

Glycoproteins of human intestinal enteroids vary in histo-blood group antigen expression in accordance with host genetics and susceptibility to human GII.4 Norovirus infection.

Manuscript.

IV. **Rimkute I**, Nasir W, Schön K, Vorontsov E, Larson G, and Lycke N.

A novel mucosal vaccine against norovirus based on subcomponents from the capsid and a strong mucosal adjuvant.

Manuscript.

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CONTENT

ABBREVIATIONS

$3CL^{pro}$	viral 3C-like protease
ADCC	antibody-dependent cellular cytotoxicity
AFM	atomic force microscopy
Ag	antigen
APC	antigen-presenting cell
ASM	acid sphingomyelinase
BabA	blood group binding adhesion from *H.pylori*
BCR	B cell receptor
BJAB	human Burkitt lymphoma B cell line
C1P	ceramide-1-phosphate
cAMP	cyclic adenosine monophosphate
CBA	chromatogram binding assay
Cer	ceramide
CFA	complete Freund's adjuvant
CTB	cholera toxin B subunit
CTL	cytotoxic T lymphocyte
DAG	diacylglycerol
DC	dendritic cell
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
ER	endoplasmic reticulum
FA	fatty acid
FCV	feline calicivirus
fJAM-A	Feline Junctional Adhesion Molecule A
Fuc	fucose
GAG	glycosaminoglycan
Gal	galactose
GalCer	galactosylceramide
GalNAc	N-Acetylgalactosamine
GALT	gut-associated lymphoid tissue
GC	germinal center
GclCer	glucosylceramide
Glc	glucose
GlcNAc	N-Acetylglucosamine
Gn	gnotobiotic
GPL	glycerophospholipid
GSL	glycosphingolipid
HAI	hemagglutination inhibition
HAT	hypoxanthine-aminopterin-thymidine
HBGA	histo-blood group antigen
HIE	human intestinal enteroid
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HuNoV	human norovirus
i.n.	intranasal
ISCOM	immune stimulating complex
LacCer	lactosylceramide
Le^a	Lewis a

Le^b	Lewis b
Le^x	Lexis x
Le^y	Lewis y
LPA	lysophosphatic acid
LPC	lysophosphatidyl-choline
LPS	lipopolysaccharide
MAb	monoclonal antibody
MHC	major histocompatibility complex
mLN	mediastinal lymph node
MLN	mesenteric lymph node
MPL	monophosphoryl lipid A
MR	mannose receptor
MS	mass spectrometry
MuNoV	murine norovirus
NoV	norovirus
NTZ	nitazoxanide
ODN	oligodeoxynucleotide
ORF	open reading frame
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidyl-ethanolamine
PI	phosphatidylinositol
PRR	pattern recognition receptor
PS	phosphatidylserine
QCM-D	quartz crystal microbalance with dissipation
RHDV	rabbit hemorrhagic disease virus
RT-qPCR	real-time reverse transcription-polymerase chain reaction
S1P	sphingosine-1-phosphate
SA	sialic acid
se	non-secretor
Se	secretor
sIgA	secretory IgA
SL	sphingolipid
SM	sphingomyelin
SMS	sphingomyelin synthase
SPC	sphingosyl-phosphorylcholine
SPR	surface plasmon resonance
STD-NMR	saturation transfer difference nuclear magnetic resonance
SV40	simian virus 40
TAG	triacylglycerol
TCR	T cell receptor
Tfh	T follicular helper cell
TGF	transforming growth factor
Th	T helper cell
TIRFM	total internal reflection fluorescent microscopy
TLC	thin-layer chromatography
TLR	toll-like receptor
Treg	regulatory T cell
VLP	virus-like particle
α2FucT	α1,2-fucosyltransferase
α3/4FucT	α1,3/α1,4-fucosylransferase

1 NOROVIRUS

1.1 HISTORY

Gastrointestinal infections or gastroenteritis of nonbacterial etiology have been studied widely between 1950 and 1960 during the "golden age" of virology. However, attempts to identify an agent causing an infection resembling of "winter vomiting disease" first described by Zahorsky in 1929 (1) were unsuccessful even with the help of latest tissue-culture techniques. As researchers learnt later, it wasn't the first challenge by norovirus (NoV) to scientific community.

The emphasis on defining a viral etiologic agent of acute gastroenteritis was raised 40-50 years ago. It was based on several observations. Most of infectious gastroenteritis occurring among young children and adults did not have any identified etiology (2, 3). Since bacteria were rarely found as causative agents, it was assumed that the main cause should be of viral origin (2, 3). Furthermore, gastroenteritis could be induced in adult volunteers using bacteria-free stool filtrates derived from naturally occurring gastroenteritis outbreaks (4). Using latest techniques, as organ cultures, was promising as an easier way of identification of nonbacterial etiology agents. Though available organ cultures helped to discover such viruses such as the ECHO and coxsackie (2, 3), the cause of "winter vomiting disease" remained unidentified. Thus, there was a need to look for other techniques to resolve the viral etiology of this disease.

The breakthrough came in October 1968 with another outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio. Symptoms were mostly vomiting and nausea with some developing diarrhea (5). Unfortunately, classical laboratory studies and cell or organ cultures did not reveal an etiological agent. However, stool filtrates from the outbreak were used for volunteer studies to prove their infectiosity and to collect convalescent serum from challenged volunteers (6). In 1972 Dr. Albert Kapikian made the first attempt to combine the low titer, but infectious, stool filtrate from the Norwalk outbreak with immune serum from volunteers and could visualize the virus using electron microscopy (the method was called immune electron microscopy). Finally, clear aggregates of non-enveloped and antibody-coated virus-like particles of only 27 nm size were visualized (Fig. 1)(6). These virus particles were confirmed to be the cause of the

Norwalk gastrointestinal infection outbreak and so the name “Norwalk virus” was given to the etiological agent of winter vomiting disease. It was finally settled as NoV by an international committee on taxonomy of viruses in 2002 (ICTVdB2004).

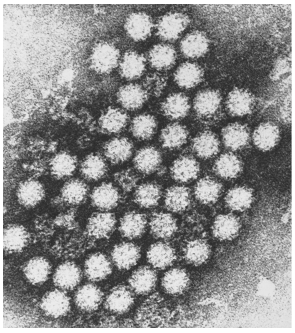


Figure 1. A virus aggregate observed after incubation of stool filtrate with convalescent serum using electron microscopy. It was identified as the cause of the “winter vomiting disease” outbreak in Norwalk and referred to as Norwalk virus. (6)

1.2 CLASSIFICATION

Noroviruses belong to the *Caliciviridae* family, which also comprises four other genera: *Sapovirus, Lagovirus, Vesivirus* and *Nebovirus*. All genera have closely related genome structures, but are genetically and antigenically distinct. They also infect different species. Sapovirus infects porcine, mink, dogs, sea lions, bats and humans. They are the second most common cause of human gastroenteritis after norovirus within thr *Caliciviridae* family. Lagoviruses are well known for causing hemorrhagic disease in lagomorphs, like rabbits and hare. Vesiviruses have feline calicivirus as the major representative of the genus and neboviruses are infectious to cattle.

Noroviruses are genetically classified into 10 genogroups (GI to GX) or could also be divided into 60 P-types (14 GI, 37 GII, 2 GIII, 1 GIV, 2 GV, 2 GVI, 1 GVII and 1 GX) based on their nucleotide diversity of the RNA-dependent RNA polymerase (7). GI, GII, GIV, GVIII and GIX (re-classified GII.15) viruses can infect humans, where GI and GII are responsible for the majority of gastrointestinal infections caused by noroviruses. GIII viruses infect cows and sheep, GIV.2 can infect dogs and cats, GV – mice and rats, GVI and GVII – dogs, and GX – bats. Two more new genogroups are in the process of being assigned, GNA1 (found in harbor porpoise) and GNA2 (found in sea lions). Genogroups are further divided into genotypes based on phylogenetic clustering of the complete VP1 capsid protein amino acid

sequence. There are nine genotypes in the GI group, where GI.1 is the Norwalk virus. The group causes one tenth of all NoV outbreaks (8). Genogroup GIV comprises only two genotypes with GIV.1 being the only one infecting humans, and GIV.NA1 to be confirmed. Epidemics caused by GIV.1 are rarely detected. The GII genogroup is the largest and has 26 genotypes along with two tentative genotypes, GII.NA1 and GII.NA2. Genotypes GII.11, GII.18 and GII.19 are specific to porcine only. Though genetic diversity among noroviruses is wide, a single genotype appears to cause the majority of norovirus outbreaks around the world. Specifically, GII.4 genotype has learnt to evolve genetically giving rise to new strains every 2-3 years and replacing previous strains. GII.4 genotype is responsible for around 80% of all outbreaks caused by human norovirus (9). The global GII.4 strains to mention include the US95/96 (emerged in 1995), Farmington Hills (2002), Hunter (2004), Den Haag (2006), New Orleans (2009) and Sydney (2012). Many other strains of GII.4, e.g. GII.4 Dijon171/96 (1996) or GII.4 Ast6139 (2000), which were also used in our studies, might not have spread globally, but were identified as unique and named after the location where cases occurred sporadically (10). Lately, emergence of rare genotypes (GII.17 (11, 12)) and new recombinant genotypes (GII.P16-GII.4 and GII.P16-GII.2 (13)) causing outbreaks and sporadic cases (14) suggests that evolution of NoV is not complete and the classification might be extended in the future.

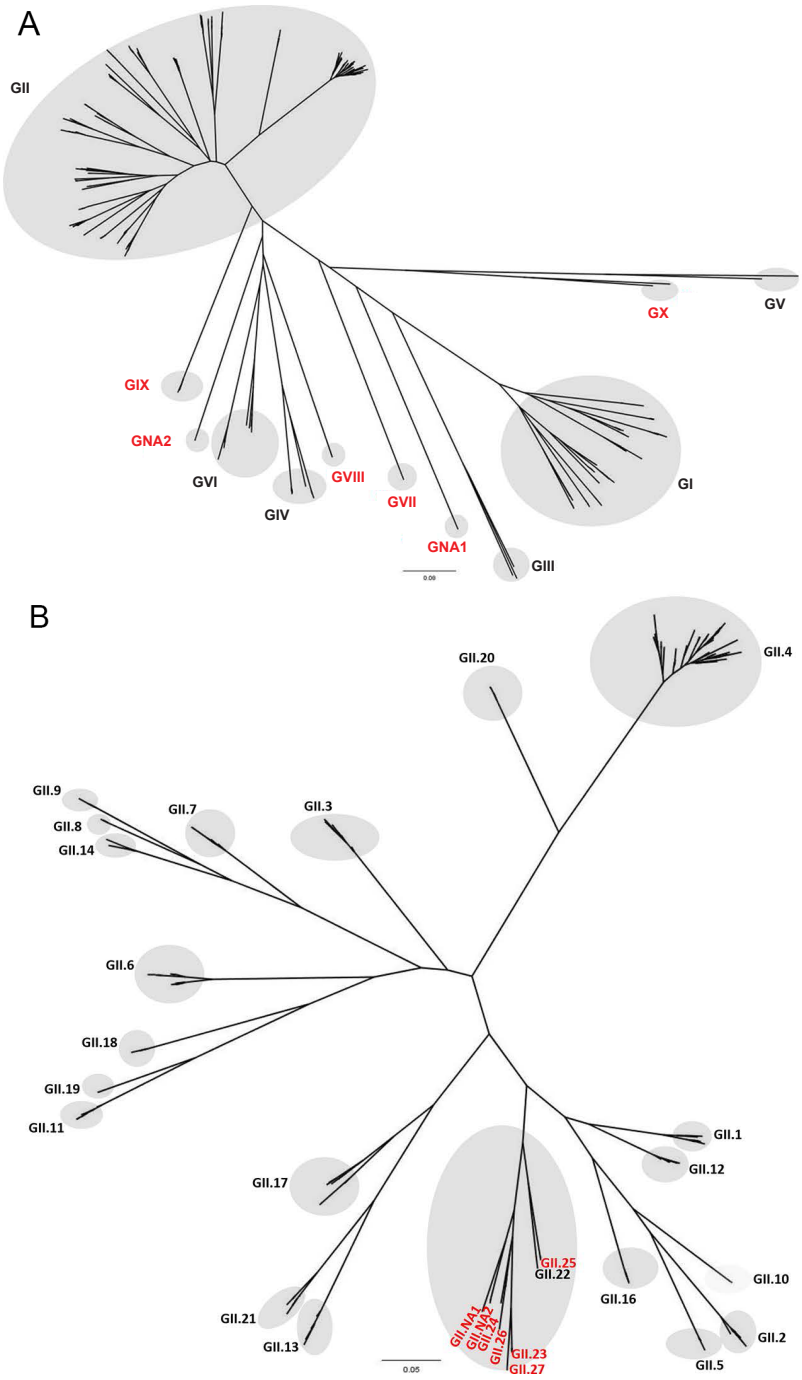


Figure 2. The diversity of norovirus genus (A) and GII genogroup (B). Genogroups or genotypes in red have been designated recently (7).

1.3 STRUCTURE

Norovirus is a positive-sense single stranded RNA virus. Its' genome length is 7.7 kb and it is organized into 3 open reading frames (ORFs) (Fig. 3a). ORF1 encodes a non-structural polyprotein, which is processed post-translationally by the viral 3C-like protease ($3CL^{pro}$) into at least 6 proteins (15). The identified proteins begin from N terminal in the following order: p48, which has been associated with cellular trafficking (16); NTPase, the nucleoside triphosphatase; p22, which been related to Golgi disruption and inhibition of protein secretion (17); VPg, viral genome-linked protein, which is proposed to interact with translational initiation factors (18); $3CL^{pro}$ is the viral protease; and RdRp, the RNA-dependent RNA polymerase. ORF2 encodes the capsid protein, the major structural component VP1, which also mediates viral entry into target cells. VP1 ranges between 530 and 555 amino acids and is 58-60 kDa molecular weight. The structure of GI.1 norovirus capsid has been resolved by X-ray crystallography, which has revealed that the norovirus capsid is formed by 180 copies of VP1 (19). Single VP1 is constituted by the shell domain designated to S and the protruding domain designated as P (Fig. 3b-d). The S domain spans VP1 from N terminal to amino acid 225. The P domain is made of the remaining amino acids and is divided into P1 and P2 subdomains. The P2 domain is an insertion between amino acids 279 and 405, and is the most variable and most exposed region of the VP1 protein on the virus. The P domain is also important for histo-blood group antigen (HBGA) attachment, specifically interacting with fucose. It has been shown that depending on the concentration of fucose there are possibly up to four binding sites on the dimer of the P domain (20). ORF3 encodes a small basic protein VP2 that is associated with virions and is required for capsid assembly. The VP2 is important for the stability, as well as functional change and nuclear localization of the VP1 (21-24).

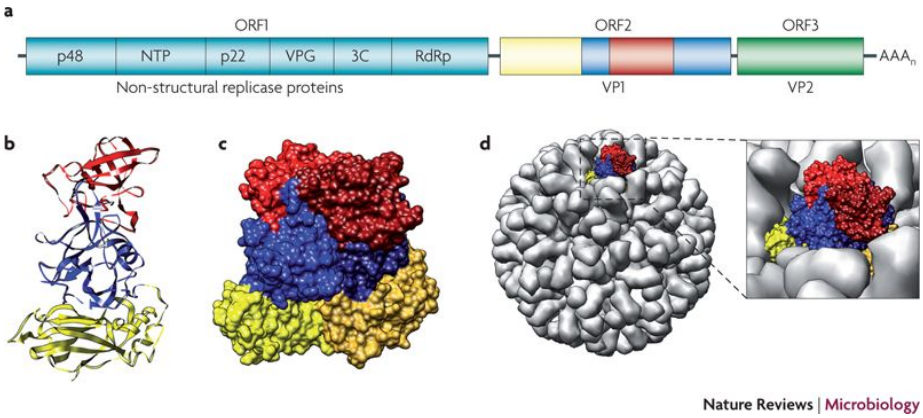


Figure 3. Genome and capsid structure of human norovirus. A – human norovirus genome composition; B – VP1 structure; C – VP1 dimer, where one monomer is colored darker than another; D – virus-like particle formed of 180 VP1 monomers. Yellow – shell domain; blue – P1 domain; red – P2 domain (25).

1.4 EPIDEMIOLOGY

1.4.1 TRANSMISSION

Norovirus is a highly contagious agent. It has been estimated that as little as 18 particles could infect a person (26). Despite the low infecting dose, the yield of virus particles from subsequent vomitus and feces is significantly higher (27). Moreover, one recent publication has also revealed that the virus can be shed in stools as viral clusters enveloped within vesicles, which also increases the inoculum dose to the receiving host (28). This all increases chances for a person to person transmission route, where contaminated fluids from infected individuals may reach the next person directly through fecal-oral route or ingestion of aerosolized vomitus route. The virus can also be transmitted through fomites or contaminated surfaces.

A large threat of norovirus transmission is contaminated food. This includes food handled by infected individuals or regular food sources like leafy greens (29, 30), seafood (31-33) and fruits (34, 35) washed or irrigated in contaminated water. Recreational and drinking water can also be a source of NoV (36). Outbreaks of the virus are most often related to long-term care facilities, but can also appear at restaurants, parties and events, cruise ships, military forces, schools and communities (37-44).

1.4.2 SEASONALITY

Norovirus infections are also called “winter vomiting disease” due to their peak appearance in colder seasons. Indeed, GII.4 genotype virus caused outbreaks are related to winter season in Northern hemisphere and spring season in Southern hemisphere, which spans the months of September through February (14, 45, 46). However, one should also keep in mind that non-GII.4 viruses related infections are constantly present throughout the year (14), which also reflects differences in epidemiology of and/or susceptibility in populations to different NoV genotypes.

1.5 SYMPTOMS

1.5.1 ACUTE INFECTION

The incubation time for human norovirus (HuNoV) is around 48 h and the disease lasts around 24 h (5). However, symptoms might be prolonged, especially, in very young and elderly people. One study has reported a 30-day mortality rate of 7% in a group of patients of average age of 77 years (47). Major symptoms are nausea, vomiting, abdominal cramps and malaise, but it can also be followed with diarrhea, anorexia, headache, myalgia, fever and chills (27). Though symptoms last only a few days, shedding of the virus in feces remains for around 29 days (27) and introduces challenges to control the spread of HuNoV.

1.5.2 CHRONIC INFECTION

There is a number of studies relating chronic norovirus infections (symptoms lasting >30 days) to immunodeficiencies, either primary congenital or secondary acquired. The chronic infection has been reported to last up to 7 years (48) and can be difficult to control even under reduced treatment by immunosuppressive drugs. The most vulnerable immunodeficient groups to the virus are solid organ transplant recipients, such as heart (49, 50), kidney (51-53), lung (54), pancreas (48) and intestine (55), as well as after bone marrow transplantations (56-59). The chronic infection diagnosis in the latter group is more challenging since symptoms might be confused with another common condition in such patients, i.e. graft-versus-host disease, where treatment of infection becomes difficult. Common variable immunodeficiency is the most common symptomatic antibody deficiency in Europeans and it is often accompanied with enteropathy (60-66). Norovirus has been suggested as a dominant cause of these enteropathies (67). The infection is also responsible for greater symptoms and histopathological findings in this group of patients, such as malabsorption and subtotal villous

atrophy (67), which can also be confused with coeliac disease resulting in delayed treatment. Such infections in primary immune deficient patients lead to protracted diarrhea, weight loss and a need for parenteral treatment (68). An extended study of solid organ transplant recipients revealed that chronic norovirus infections could lead to shedding of the virus for months to years (between 32-1164 days) (69). It has also been reported that during the prolonged viral shedding (898 days) an intra-host evolution of norovirus has occurred, where at least 25 capsid protein amino acids in the virus have mutated (52, 70).

1.5.3 UNUSUAL MANIFESTATION

It has been described that norovirus infections can also be related to unusual set of symptom. For instance, infants have been reported to be at risk of central nervous system involvement (71, 72), necrotizing enterocolitis (73) and ileal perforation (74) related to norovirus infection. One report has related HuNoV infection in children with obstructive ureteral stone appearing as elevation of blood or urinary uric acid in urine and leading to nephrolithiasis (75). Other clinical reports have identified rhabdomyolysis (muscle breakdown) (76) and Stevens-Johnson syndrome (77) accompanying norovirus gastroenteritis in young children. Moreover, an outbreak among British soldiers in Afghanistan caused headache, neck stiffness and photophobia. One patient has even developed intravascular coagulation (Center for Disease and Prevention, 2002). Post-infectious functional gastrointestinal symptoms (dyspepsia, constipation and gastroesophageal reflux disease) have also been described as possible complications of the infection (78).

1.5.4 ASYMPTOMATIC INFECTION

Even if a person does not develop symptoms, one can still be affected with HuNoV. Early studies of HuNoV challenged volunteers have indicated that even asymptomatic individuals can develop mucosal lesions, typical to the gastrointestinal infection (79). Only much later after the virus discovery, it has been noticed that even asymptomatic individuals can be continuously shedding the virus (80). Asymptomatic infections can also appear among immunosuppressed patients (51, 52). Since it is difficult to follow asymptomatic infections in immunodeficient and especially in immunocompetent individuals, the prevalence of such infections and the length of shedding remain to be uncovered.

1.6 PATHOGENESIS

1.6.1 HUMAN BIOPSIES

Right after the discovery of NoV in 1972, human challenge studies helped to learn about the target site in human intestine upon the infection. Comparison of intestinal biopsies from volunteers inoculated with either Norwalk or Hawaii NoV revealed that, although immunologically distant strains, they both resulted in identical intestinal mucosal lesions of the proximal small intestine, which can be found already 4 hours before clinical symptoms (79, 81). The observed mucosal lesions included altered mucosal architecture, mucosal inflammation and absorptive cell abnormalities. Other challenge studies indicated blunted villi, shortened and distorted microvilli, swollen mitochondria and intercellular edema with decreased activities of alkaline phosphatase, sucrase and trehalase (82, 83). Intestinal lesions were resolved in most volunteers after two weeks. Human biopsy findings were also consistent with HuNoV studies in gnotobiotic piglets, which have been found replicating HuNoV at early time points in enterocytes on the tips of the small intestine (84). The latest study of biopsies from immunodeficient transplant patients chronically infected with NoV detected viral antigen only in duodenal and jejunal enterocytes, but also some in the lamina propria, where inflammation was prevalent (85). Major capsid protein was detected in all segments of the small intestine. This protein was also found in macrophages, which was related to possible phagocytosis of infected epithelial. Finally, a small number of T cells and dendritic cells were found containing major capsid protein too. However, the significance of these finding remains unclear due to the limited number of sections from patient biopsies, and, hopefully, will be resolved in the nearest future.

1.6.2 ANIMAL MODELS

Chimpanzees were used to study Norwalk virus infection and immunizations with VLPs of GI.1 Norwalk and GII MD145 strains (86). Inoculations were done intravenously, which does not represent the natural transmission route of the virus, and immunizations were given intramuscularly. Though chimpanzees did not develop symptoms during the infection, it was possible to detect both shedding of the virus in stool and increasing antibody titers. Viral genome was detected in biopsies from duodenum and jejunum and, surprisingly, also in liver – though no histological changes could be observed there. Viral antigen was found in the lamina propria and related to the presence in dendritic cells (DC) and B cells. However, the study could be applied only to Norwalk virus, but not the GII genogroup virus. A lack of

symptomatic disease presentation is limiting further studies of HuNoV in chimpanzees.

Another study was conducted in newborn pigtail macaques (*Macaca nemestrina*) that were inoculated with Toronto Norwalk-like virus (87). These animals developed diarrhea, vomiting and became dehydrated. Symptoms were accompanied with viral shedding in feces, which was transmitted to other newborn pigtail macaques. No other non-human primate studies were successful in developing a HuNoV animal model (88).

Gnotobiotic (Gn) piglets have been extensively used for HuNoV studies and specifically for GII.4 genotype (84, 89-94). After oral inoculation these animals developed diarrhea, shed virus, develop low serum and mucosal antibodies, and have detectable virus genome levels in the intestine. The virus clearly replicates in enterocytes on the tips of small intestinal villi at an early time point, but also some virus antigen could be found in the lamina propria at later time points of the infection (84). Gn piglets have also been used for immunization studies using P particles, though not fully successful due to a lack of complete protection against the virus (90). RAG2/IL2RG double knockout Gn piglets experienced prolonged viral antigen retention in the intestines and asymptomatic virus shedding (91). One study with simvastatin, a cholesterol reducing statin, has shown that the drug can increase the infectivity and the severity disease of the virus (89, 95). The Gn piglet model has also been used to study effects of commensal bacteria to HuNoV replication *in vivo*. The HBGA antigen expressing *Enterobacter cloacae* inhibited HuNoV replication and virus shedding, suggesting a protective role exhibited by bacteria (96). In contrast, Gn calves haven't been studied as broadly, but they also develop diarrhea, shed the virus in stools and have detectable viral antigen in enterocytes and lamina propria, accompanied with mild histological changes in the small intestine (97). Serum and mucosal IgA/IgG production can also be followed in these animals. Unfortunately, despite all the important findings in the described animal models, further studies are lagging due to difficulties of getting ethical permissions for handling larger animals and high costs to cover.

Currently, the only small animal model described is a recombinant activation gene (Rag-/-) and common gamma chain (γc-/-) deficient BALB/c mouse (98). The model can support GII.4 HuNoV cultures injected intraperitoneally, but not orally. Viral genome and antigen is present in liver and spleen, which, perhaps, supports the injection route, but fails to be used for further HuNoV pathogenesis studies. Overall, the importance to develop an animal model to study HuNoV infection remains necessary.

1.6.3 CELL CULTURES

Notably, most developed cell culture models are to date applied to study murine norovirus (MuNoV) and feline calicivirus (FCV). MuNoV does not cause symptoms in mice, can be easily replicated in DCs and macrophages, binds to terminal sialic acid (both α2,3- and α2,6- linked) moieties on gangliosides, requires receptor CD300lf for its infection and the entry is cholesterol- and dynamin-dependent, but pH independent (99-103). Further, *in vitro* infection of MuNoV can also be enhanced by bile acids. Secondary bile acids (GCDCA and LCA) have been shown to bind to MuNoV P domain and enhance its' ability to bind to cells in a CD300lf receptor-dependent manner (104). Interestingly, ceramide has been shown to modify distinct CD300lf antibody epitope, which could lead to altered conformation and/or clustering of CD300lf on the cell membrane and promote viral entry (105). Moreover, the discovery of CD300lf receptor helped to identify a small population of intestinal cells called tuft cells, which can also be infected with MuNoV (106). FCV while coming from the norovirus family demonstrates a tropism and pathogenesis that differs from MuNoV. For instance, FCV causes respiratory infection in cats, is cultured in Crandall Reese feline kidney cells and binds to Feline Junctional Adhesion Molecule A (fjAM-A) (107, 108). The P2 domain of FCV binds the membrane domain of fjAM-A, which induces the conformational change in the FCV capsid and facilitates viral genome escape (109). FCV entry strictly requires the acidic environment of the endosome for uncoating, so it is clathrin-, cathepsin L- and pH- dependent (110, 111). Clearly, noroviruses have broadly adapted to infect various hosts. Hopefully, these basic discoveries will contribute to understanding novel and critical aspects of HuNoV pathogenesis.

Despite clear indications of HuNoV infection targeting the small intestine, specifically the epithelium (79, 81-83, 85), the field has been struggling to get more significant evidence for the pathogenesis of the virus. Particularly, the reason has been a lack of success to culture the virus in classical primary and immortalized cells (5, 112, 113). In addition, culture systems were not robust or reproducible (114-119). However, recent studies have introduced two different cell systems to support the replication of HuNoV *in vitro*. Each of them utilizes a different cell type to support replication of the virus. The first one uses a transformed murine B cell line (BJAB) and the second one employs intestinal epithelial cells derived from human stem cells, also known as human intestinal enteroids (HIEs) (120, 121). These culture systems also differ in the way they facilitate the infection. The BJAB system requires commensal bacteria producing HBGA (*Enterobacter cloacae*) for the virus to infect. Interestingly, an opposite finding has been published based on studies

in Gn piglets, where HuNoV infection decreased upon the exposure to the bacterium (96). Meanwhile, HIEs, depending on the infecting strain, necessitate or facilitate the infection in the presence of bile acids (121, 122) (Murakami et al. under review). A recent study using X-ray crystallography has shown that bile acids bind at partially conserved pockets on the P domain of some genotypes of HuNoV and stabilize P domain loops in HBGA non-binders, as well as augment binding of GII.1, GII.10 and GII.19 (not GI.1, GII.3 and GII.4) with HBGA (123). However, the possibility of bile acids having a direct effect on the target cell under the infection of other genotypes of HuNoV awaits further investigations. It was also noted that NoV as a non-enveloped virion might have adapted another form of egress than by cell lysis instead forming viral clusters inside extracellular vesicles (28). Such vesicles protect viruses from the outer exposure, still requiring the cellular receptor for targeting of new cells (as was shown with murine norovirus and CD300lf) and increasing not only the delivered infectious dose, but also the production of virus in infected cells. This discovery might provide some insights to the current cell culture models, which still need to improve the yield of viral titers and extensive passaging. Further work in the field is required to determine the cell types targeted by HuNoV under different conditions of host immune status and times of infection. In this way, hopefully, future studies of HuNoV culturing will facilitate the discovery of attachment factors and/or entrance receptors required by the virus.

1.7 DIAGNOSIS

Irrespective of presence of accompanying symptoms, infections can be diagnosed by laboratory analyses. Such methods focus on viral antigen and viral genetic material (RNA) detection. Viral particles can be detected in stools, vomitus, water, food and environmental specimens. Most diagnostic laboratories use real-time reverse transcription-polymerase chain reaction (RT-qPCR) to detect NoV. As diagnostic tools evolve, RT-qPCR is being replaced by TaqMan-based RT-qPCR, which makes the assay more sensitive and rapid, as well as provides both confirmation and quantitation in a single assay (124-126). The technique is so sensitive that it can detect as few as 10-100 NoV copies per gram of sample. Different primer sets allow distinguishing between GI and GII genogroups. Furthermore, recent advancements in laboratory diagnostics introduced commercial platforms to detect multiple gastrointestinal pathogens, including GI and GII genogroups of NoV, which can be a distinct advantage when a fast diagnosis of the cause of gastroenteritis is needed, especially in immunodeficient patients (127-129). Rapid enzyme immunoassays (EIAs) detecting NoV antigen in stool

samples are also worth mentioning. Unfortunately, the disadvantage of EIAs is their poor sensitivity, but the test may serve to rapidly generate preliminary results by detection of NoV during outbreaks (130). One should then keep in mind that negative EIA results should still be confirmed by another technique, as RT-qPCR. Lastly, though not a routine method, but to be acknowledged as an important tool in epidemiologic studies of NoV, is genotyping. Those laboratories, which choose to participate in CaliciNet, a national laboratory surveillance network for norovirus outbreaks coordinated by CDC in the US, submit genetic sequences of norovirus strains to the CaliciNet database. Thus, the database contributes to monitoring circulating and newly emerging NoV strains in the US.

1.8 TREATMENT

To date there is no specific treatment to NoV infection. However, it is important to replace lost fluids and electrolytes, which can be done orally or intravenously in special cases. It might be advisable to take antimotility and antisecretory agents in case of critical performance. Though no specific antiviral agent to control NoV infection has yet been developed, a number of studies focus on different antiviral strategies (131). Those studies include targeting of viral attachment and entry, polymerase inhibitors using nucleoside analogs or non-nucleoside inhibitors, protease inhibitors, and host-factor drugs specific to host proteins and immunomodulators. Nitazoxanide (NTZ), a broad-spectrum antiparasitic and antiviral drug, is undergoing clinical trials with controversial results. Though the drug has been shown to result in a broad antiviral response (132), the mechanism responsible for its antiviral effect is unknown. In clinical trials the drug has demonstrated successful treatment of both immunocompetent and immunodeficient patients infected with NoV. Studies have shown reduction in longevity of symptoms, with a complete resolution of symptoms and clearance of norovirus from stool samples of immunodeficient patients even without a reduction of the immunosuppressive drugs (133-135). In contrast, some case reports claim NTZ being ineffective (48, 50, 136) and the drug has also failed to inhibit MuNoV infection (137). Other treatment options have shown some positive outcomes in immunodeficient patients using oral immunoglobulin (138, 139) or treatment with ribavirin (140).

1.9 PREVENTION

It is difficult to prevent norovirus outbreaks, because a single NoV exposure results in a rapid infection and subsequent rapid spread from person to

person. Thus, one method is primary prevention, which chases back the source of NoV contamination to food and water. For this, knowing the specific sequence of the P domain of the virus that caused the outbreak helps to find a common exposure source (141). Next, preventing spread from secondary sources, such as person-to-person transmission and contaminated surfaces, has been one of the challenges due to relative resistance of the virus to inactivation by disinfectants. The chemical resistance has been demonstrated in other noroviruses in the past (142) and has also been recently confirmed in HuNoV HIE cultures (143). The latter study implicated that bleaching could completely inactivate GII.4 viruses, while such a popular disinfectant as alcohol showed very poor affectivity in HuNoV inactivation. Future studies should be initiated to test other viral inactivation methods, such as heat and high hydrostatic pressure, to HuNoV in HIEs. Finally, though there are two NoV vaccine candidates undergoing clinical studies, no vaccine has yet been commercially approved (144-146). It is a clear challenge in the field to develop an effective and protective vaccine. Once the challenge is overcome, the most reliable tool in prevention and control of the virus will be introduced.

2 HOST-PATHOGEN INTERACTIONS

The role of various membrane components in viral attachment and entry to the host cell has been explored in a number of studies (147-151). Viruses can be divided into enveloped (e.g. Adenoviridae, Herpesviridae, Retroviridae) and non-enveloped (e.g. Polyomaviridae, Caliciviridae). In general, the cycle of viral infection is divided into four steps: entry, translation, replication and assembly/release (Fig. 4). The early stage of viral entry into the host cell involves the binding of the virus to one or more cell-surface receptors followed by entry into the cell. Most enveloped viruses enter through endocytosis and/or direct penetration into the cytoplasm, while non-enveloped use either clathrin-mediated endocytosis or clathrin-independent/caveolin-mediated endocytic pathway (Fig. 4). Some of these mechanisms, such as clathrin-mediated endocytosis are ongoing, whereas others, such as caveolae, are ligand and cargo induced. As it will be discussed in this chapter, different membrane components play critical roles in generating the initial host-pathogen interactions contributing to the viral pathogenesis.

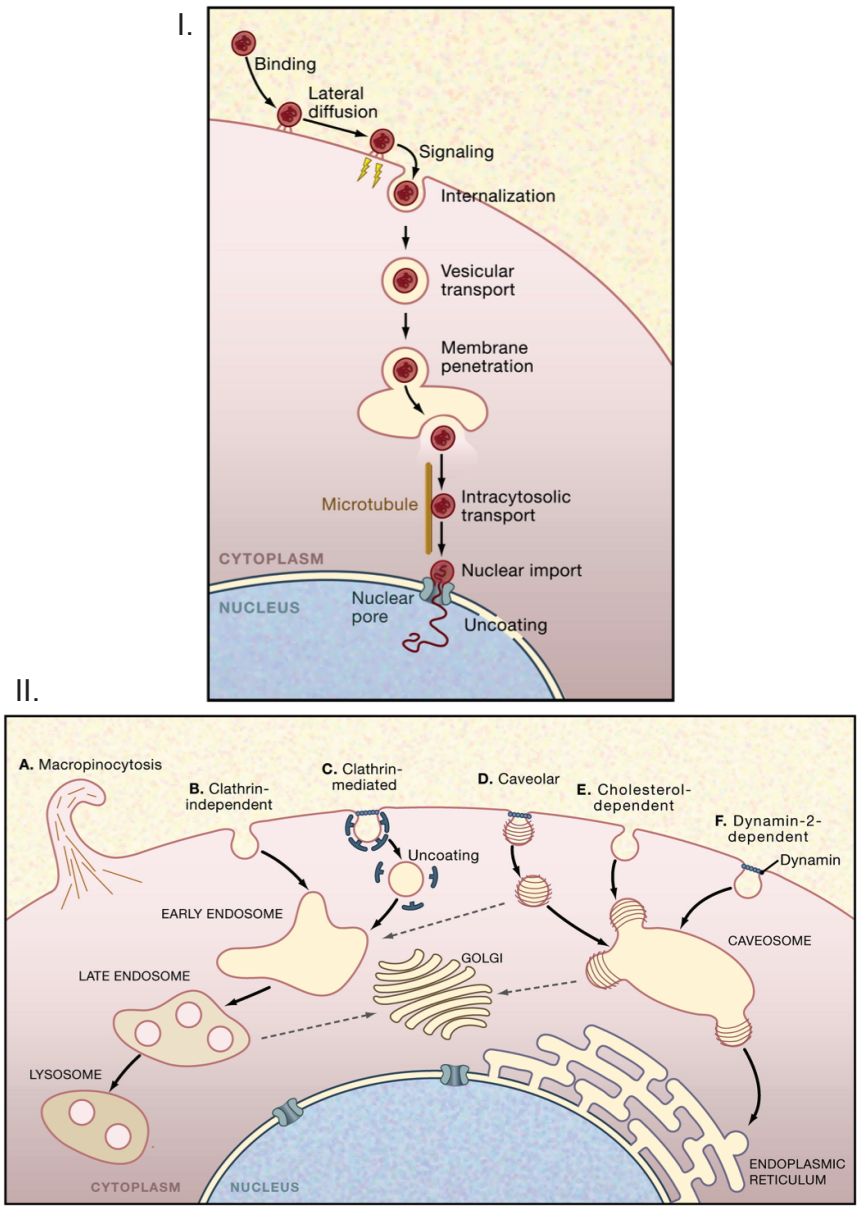


Figure 4. Virus cycle of infection (I) and different endocytic pathways (II) (150). Adenoviruses use the micropinocytosis entry (IIA), influenza virus and arenaviruses use clathrin-independent pathway (IIB), clathrin-mediated pathway is the most common uptake pathway for viruses (IIC), caveolar pathway is cholesterol-dependent and brings viruses including SV40, coxsackie B, mouse polyoma and Echo 1 (IID), another cholesterol-dependent pathway devoid of clathrin and caveolin-1 is used by polyoma and SV40 (IIE), while dynamin-2 dependent pathway is used by Echo virus 1 (IIF).

2.1 MEMBRANE LIPIDS

Cellular membranes are formed from a chemically diverse set of lipids. A high lipid diversity is universal to eukaryotes and is seen from a lipid bilayer to a whole organism, highlighting its' importance and suggesting that membrane lipids fulfill many functions. Indeed, the correct composition and structure of cell membranes define key physiological and pathophysiogical aspects of cells. Therefore, even small changes in lipid structures and their composition have significant effects on essential biological functions.

2.1.1 CLASSIFICATION

Membrane lipids are classified into glycerophospholipids (GPLs), sphingolipids (SLs) and sterols (dominantly cholesterol in mammals) (152). Combinations of various structural components shape the chemical variety of GPLs and SLs. Fatty acid (FA) is one of such structural components and they differ in chain lengths, double bond numbers, configurations, positions and in hydroxylation. In fact, combinations of the two FAs, the linkage between the two and the head group shapes the chemical diversity of GPLs. Thus, there are four major GPL types: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). Phosphatidic acid (PA) also belongs to GPL group, but constitutes the smallest portion of GPLs. Their hydrophobic portion is diacylglycerol, which contains saturated (without double bonds) on unsaturated (with double bonds) FAs chains with varying lengths etc. PCs are distinguished by carrying the choline head group. PCs contribute to >50% of the phospholipids in most mammalian membranes, and have a nearly cylindrical molecular geometry, which forms a spontaneous bilayer with the lipidic tails facing each other and the polar head groups interacting with the aqueous phase outside of the bilayer. PEs have a relatively small head group – ethanolamine. PSs have a serine and tend to incorporate at least one stearic acid (C18:0; in the nomenclature XX:Y XX stands for the number of carbons in the chains and Y indicates the number of double bonds). PIs are characterized by their inositol head group and in most tissues predominantly integrate stearic (C18:0) and arachidonic (C20:4) acids.

SLs are constituted of one sphingoid long chain base and one FA, and their chemical difference is determined in the length and type of sphingoid base, FA and head group. The major SL components in mammalian cells are sphingomyelin (SM), free ceramides (Cer) and glycosphingolipids (GSLs). While ceramide is the main backbone of SLs, the head groups differ. SM has a phosphorylcholine head group and GSLs contain mono-, di-,

oligosaccharides for their head groups based on glucosylceramides (GlcCer) and sometimes galactosylceramides (GalCer). Monosaccharide ceramides can be decorated with sulfate and, thus, compose sulfatides. Gangliosides are also synthesized from GlcCer and typically contain sialic acids. Sphingolipids have ceramides as their backbone, which vary in sphingoid bases such as sphinganine (d18:0), sphingosine (d18:1) or phytosphingosine (t18:0) dependent on ceramide synthases involved in the biosynthesis. The diversity of sphingoid bases contributes to the physicochemical characteristics of various SLs. Fatty acid part is mostly saturated in SLs and can be longer than in GPLs. These features allow SLs to form taller, narrower cylinders than PCs, which contributes to tight packing in the membrane.

Signaling lipids are produced from hydrolysis of GPLs and SLs. Examples of such lipids from GPL hydrolysis are lysophosphatidylcholine (LPC), lysoPA (LPA), PA and diacylglycerol (DAG), and from SLs sphingosylphosphorylcholine (SPC), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P) and Cer.

Cholesterol is an important lipid component of the mammalian cell membranes. It is composed of steroid backbone (four fused rings) and a small branched hydrophobic tail. The rigid steroid backbone favors its' interaction with SLs in membranes, hence, contributing to lipid rafts formation. Chemical features of sphingolipids allow cholesterol to get incorporated close to them and, thus, contribute to the integrity and dynamics of the membrane. The cholesterol and sphingolipid interactions in the membrane are explained by "umbrella model". It says that cholesterol positions into regions of membranes with strongly hydrated large head groups as those found in sphingolipids. This way sterol rings are protected from the aqueous environment. In addition, the packing of cholesterol with sphingolipids is more likely when there are saturated FAs in sphingolipids (153). Together these lipids form microdomains or lipid rafts in the membrane likely functioning to support the physical structure of membranes and generate areas in the membrane specialized in lipid influx and efflux, protein trafficking, signal transduction and viral entry (154).

2.1.2 SYNTHESIS

The diversity of head groups and FAs creates the variation of more than 1000 different lipid species (155). The synthesis of structural lipids is restricted to specific compartments in mammal cells. The main lipid biosynthetic organelle is the endoplasmic reticulum (ER). It produces the majority of structural lipids, including phospholipids, cholesterol and Cer. In addition, in

myelinating and epithelial cells the ER synthesizes GalCer, which stabilizes myelin and apical membranes (156). Also, ER produces triacylglycerol (TAG) and cholesteryl esters, but they are non-structural molecules and we will leave them out. However, the ER displays low concentrations of cholesterol and complex sphingolipids, because these synthesized lipids are rapidly transported to other organelles. Indeed, significant levels of lipid synthesis appear in Golgi. Specifically, the later synthesis steps of SLs, which involves production of SM, GlcCer, lactosylceramide (LacCer) and oligosaccharide GSLs (157). The majority of SLs leave Golgi to the plasma membrane. Thus, it is thought that the production of SLs might also have a role in the sorting of membrane proteins and lipids between the ER, the plasma membrane and endosomes through lipid rafts. Interestingly, though the plasma membrane is not responsible for the autonomous synthesis of its' structural lipids, there is evidence provided for synthesis and degradation of lipids involved in signaling pathways in the plasma membrane (158). For instance, sphingomyelin synthase (SMS) can contribute to the total cellular SM through its' synthesis from Cer on the plasma membrane (159, 160). Likewise, acid sphingomyelinase (ASM) can mediate the formation of Cer from SM (161).

2.1.3 FUNCTION

Lipids have several major functions in cells, such as membrane structural composition, source of heat and energy, signaling molecules, protein recruitment platforms and substrates for post-translational protein-lipid modification. The major function of lipids in membranes is compartmentalization giving unique integrity to cells and organelles.

Multiple functions of the plasma membrane depend on the lipid composition. Head group and hydrophobic tails of lipids affect the spontaneous curvature of the membrane. Lipids with long and saturated fatty acids, such as sphingolipids, make membranes thicker and less fluid owing to the tight packing of hydrophobic tails and stronger lipid-lipid interactions (162, 163), while unsaturated lipids do the opposite. It has been shown that saturated, unsaturated lipids and cholesterol form separate regions with high lipid packaging and less in artificial lipid membranes (162). This is underlying the lipid raft hypothesis that shows high lipid order and the ability to concentrate proteins (163, 164). For example, proteins like B cell receptors or cytoskeletal components initiate the formation of membrane heterogeneities on the nanoscale level and lipids have roles in stabilization of these microdomains and their expansion (165, 166). Also, microdomain formation can be supported by lipid-lipid interactions when SLs interact with

cholesterol through saturated hydrophobic chains and stabilize microdomains (163). Thus, it should be reasonable to assume that if the lipid environment is altered, a protein bound to the same environment may also be affected by this difference and thereby operate in a different manner. Furthermore, both leaflets of the plasma membrane contain specific lipid compositions. The outer leaflet contains mostly PC and SL, while the inner involves PE, PS and PI (167). If PS gets externalized, then its effect on many cell activities appears, for instance, the phagocytic clearance of apoptotic bodies (168). In addition, ceramide, C1P, sphingosine and S1P also act as signaling molecules themselves and, therefore, the dynamic alterations directly regulate many cellular responses raging from development and expansion to autophagy and apoptosis (169). Indeed, lipids have a broad spectrum of effects on cellular functions, but we would like to keep our focus on lipids from host-pathogen interaction angle.

2.1.4 LIPID RAFTS

Simons and Ikonen have initiated the concept of lipid rafts in 1997 (170). However, the biological significance of it has been debated for long time. Indeed, lipid raft studies have been hampered by their size, which is too small to resolve using conventional microscopy. Moreover, the morphology of lipid rafts is poorly known. Studies have been relying on chemical properties defining these microdomains such as insolubility in cold non-ionic detergents followed by flotation on sucrose-density gradients, high density and resistance to mechanical stress. Nevertheless, it is largely agreed that the presence of both cholesterol and sphingolipids is essential for the formation of lipid rafts in the plasma membrane. Removal of raft cholesterol with β-methylcyclodextrin or hydrolyzing membrane sphingolipids with sphingomyelinase results in dissociation and inactivation of most lipid raft proteins (171-173). Certainly, lipid rafts control many protein-protein, lipid-protein, protein-carbohydrate and carbohydrate-carbohydrate interactions at the cell surface. This is determined by the capacity of lipid rafts to incorporate or exclude proteins selectively, and the ability to fuse into larger domains. Lipid rafts are involved in protein sorting, membrane trafficking and signaling leading to proliferation, apoptosis, migration or adhesion (174, 175). Therefore, it is not surprising that lipid rafts are exploited by different pathogens to infect host cells (147-149, 176, 177).

The involvement of lipid rafts in caveolin-mediated pathway is indicated by requirement of cholesterol. This has been demonstrated using simian virus 40 (SV40) (178-180). Indeed, SV40 requires to bind MHC class I molecules to enter the host cells, which is followed by internalization through the flask-

shaped caveolae. When cholesterol is removed by chelators' inhibitors from these cells, SV40 fails to facilitate the entrance. In addition, MHC class I molecules are hardly detected in lipid rafts of resting cells, but this membrane area becomes highly enriched in MHC class I molecules after the binding of virus. Clearly, the formation and dynamics of lipid rafts play a role in pathogenesis of SV40. Interestingly, enveloped viruses have also been reported to be dependent on lipid rafts at the cell surface. The fusion of such viruses' membrane with cellular membranes requires a conformational change of the virus envelope glycoprotein. It was shown that for alphaviruses this fusion is dependent on the presence of both cholesterol and sphingolipids in the plasma membrane, indicating the involvement of lipid rafts (181-183). Similarly, the complex binding of HIV to the cell surface appears dependent on lipid rafts. HIV glycoprotein gp120 first binds to CD4, which subsequently gathers lipid rafts enriched in co-receptors necessary for the entry - CCR5 and/or CXCR4 (184, 185). Furthermore, gp120 has been shown to directly interact with gangliosides in lipid rafts (186). Moreover, sequestration of cholesterol or inhibition of the GSLs synthesis prevents the infection by HIV-1 *in vitro* and *in vivo* (187-191). Overall, though only a fraction of viral proteins have been found associated with lipid rafts, it should be kept in mind that more extensive identifications can be limited by the poor biochemical characterization of lipid raft subsets and the transient nature of the association (192). Hence, our detailed analysis of cellular lipid, such as GPLs and SLs, and protein components together with dissected glycan architecture of a fraction of those components supports the architecture of plasma membranes in humans and will contribute to future studies of lipid rafts and their essential roles to pathogenesis of infections, including HuNoV.

2.2 GLYCOCONJUGATES

Glycobiology studies structure, biosynthesis, function and evolution of saccharides/ carbohydrates/ sugar chains/ glycans largely distributed in nature and involved in numerous biological processes. The field greatly contributed to the structure and biochemistry of simple and complex glycans found in nature at the beginning of 20th century. Indeed, a number of glycobiologist received a Nobel prize for their achievements at that time (193). However, glycobiology was left in a shadow for many years and now it is gaining back more interest due to the development of better technologies to study the complexity of glycans. Apart of their well-known function in energy generation and metabolism, glycans inevitably have many biophysical and structural roles. After all glycans form a dense coating of complex and diverse carbohydrates found on all cell surfaces and even extracellular

molecules (the coating is also called “glycocalyx”). It shouldn’t be a surprise that, indeed, many infectious agents or symbiotic organisms tend to exploit it and mediate interactions with the host (194). In addition, pathogens also express glycans on their surfaces modulating their antigenicity. Furthermore, through expression of different glycosidases they can shape the glycan surface of the host (195). Biological roles of glycans have a broad classification and include a long list of functions covering such sections as physical structure, protection, tissue elasticity, glycoprotein folding, degradation and trafficking, adhesins for pathogens, pathogen recognition, immune modulation, antigen recognition, uptake and processing, cellular signaling and many more, which are nicely dissected in the latest review from Dr. Varki A. (196). Our contribution to glycobiology science was to explore binding factors on lipids and proteins possibly playing a role in the latest HuNoV GII.4 strain infection in the human intestinal cell cultures, as well as the first global lipid and proteomic analysis of human intestinal enteroids.

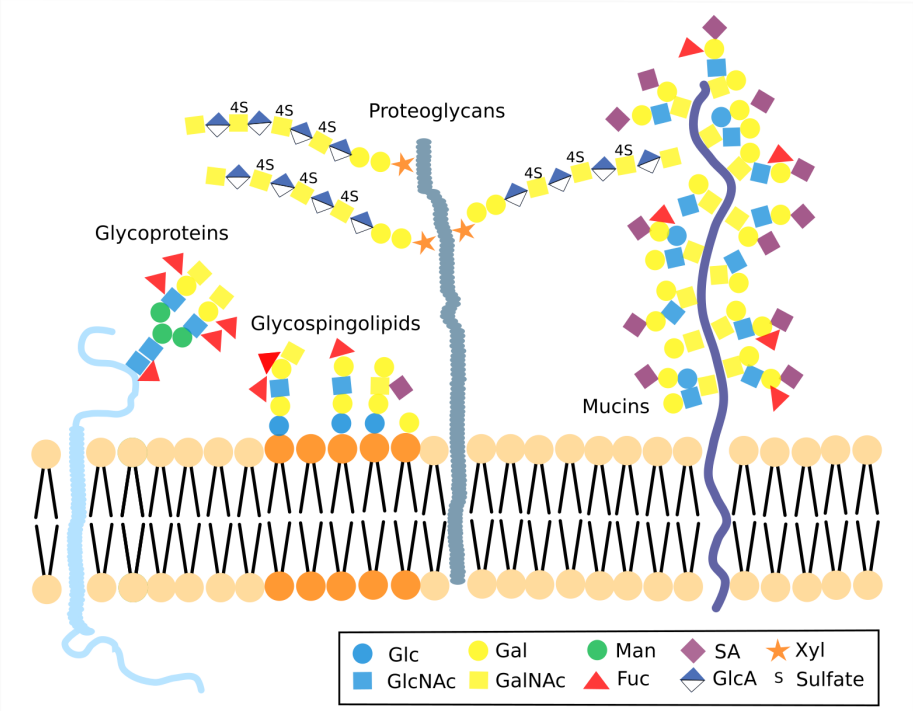


Figure 5. Glycan classes in animals. Glc – glucose, GlcNAc – N-Acetylglucosamine, Gal – galactose, GalNAc – N-Acetylgalactosamine, Man – mannose, Fuc – fucose, SA – sialic acid, GlcA – glucuronic acid, Xyl – xylose.

2.2.1 GLYCAN BIOSYNTHESIS

Glycosylation is catalyzed by multiple glycosyltransferases, which add glycans to specific substrates, specifically, proteins and lipids and other glycans. This process is not a template-driven process, which means that glycan structures are not directly encoded in the genome. Instead the glycosyltransferases are the primary gene products and the glycans the secondary gene products. Typically, glycosyltransferase activities are determined through their cell specific expression, specificity towards the substrate nucleotide donors and to the substrate acceptors. In many cases, glycosyltransferases deliver monosaccharides to oligosaccharides, proteins or ceramides in a step-by-step manner but in other cases, such as the N-glycosylation of proteins a single large oligosaccharide is added, modifying the proteins just synthesized in the endoplasmic reticulum. In contrast to glycosyltransferases, glycosidases can remove glycans from specific glycosidic linkages for recycling purposes or for generating intermediates for other glycan synthetic steps. Hence, the overall product of the glycosylation depends on the availability and specificity of glycosyltransferases and glycosidases, and the competition between them in each cell. Furthermore, the biosynthesis is also affected by the availability of substrate and acceptor structures for different glycosyltransferases. Indeed, the regulation of glycan synthesis is a complex process, which generates and modulates the diversity of biological structures, and at the same time provides many challenges to glycobiology studies (193, 197-199). Glycoengineering of cells allows the destruction and rebuilding of glycosylation machineries in various cells. With now available a highly specific gene editing by clustered regularly interspaced short palindromic repeat/ targeted Cas9 endonuclease (CRISPR/Cas9) technique it is possible to probe and dissect the roles of glycosylation in cell biology, pathogen-host interaction, and glycoengineering of therapeutic glycoproteins and biologics (200).

2.2.2 GLYCOCONJUGATES IN BIOLOGY

Glycoconjugates are very important compounds in biology consisting of glycans of varying size and complexity, covalently linked to non-glycan moieties like proteins and lipids. The synthesis of glycoconjugates is initiated in the ER, finalized in Golgi and the end product is secreted to body fluids or transported to the plasma membrane, which is enriched in glycolipids, glycoproteins and proteoglycans. Glycoconjugates can be linear or branched polymeric structures, where monosaccharides are usually coupled by stereochemical linkages, α and β. The complexity of glycoconjugate structures plays an important role in biological processes encoding many

specific biological messages in a single carbohydrate structure (196, 201, 202).

Glycoproteins

More than half of all proteins are found glycosylated (203). Often glycoproteins carry complex mixtures of glycans, which possess the same protein backbone, but can differ in the glycan components and their attachment sites (204). Glycans are covalently attached to proteins through the carboxamide group of asparagine (N-linked) or hydroxyl group of serine, threonine or tyrosine (O-linked). The glycosylated asparagine is usually present in the consensus sequence Asn-X-Ser/Thr/Cys (X not Pro). N-linked glycans present on glycoproteins in plasma or on the cell surface affect protein clearance, cell adhesion, immunity and infection, which are processes mediated by glycan-protein interactions (205). Furthermore, they may serve as biomarkers and in design of monoclonal antibody production (206-210). N-linked glycans are also important intracellularly by promoting protein folding, quality control and targeting events mediated by lectins (211). The N-glycan structures of mature glycoproteins differ by species, cell type, protein and subcellular localization (212). For instance, N-linked glycoproteins in the ER carry high-mannose glycans and are essential in protein quality control (213). In contrast, O-linked glycoproteins have not been studied as extensively due to challenges to study their much more heterogeneous structures. O-linked glycosylation is categorized into mucin (the attached monosaccharide residue is N-Acetylgalactosamine (GalNAc)) and non-mucin (the attached residue can be N-Acetylglucosamine (GlcNAc), fucose (Fuc), mannose, glucose (Glc), xylose or galactose (Gal)) (214). Mucins are the most commonly found O-linked glycoproteins in membranes and in secretions (215). Another common type of O-glycosylation with large structural diversity involves the glycosaminoglycans (GAGs). They are attached to proteoglycans and are long, unbranched polysaccharides. Some known GAG structures are dermatan sulfate, chondroitin sulfate, heparin/heparan sulfate and keratin sulfate. Various functions have been described for O-linked glycoproteins, such as importance in protein structure and stability, immunity, receptor-mediated signaling, nonspecific protein interactions, modulation of the activity of enzymes and signaling molecules, and protein expression and processing (216, 217). In our study we characterized glycoproteins from human intestinal enteroids of different sensitivity to HuNoV infection. N-linked glycoproteins constituted the largest fraction of extracted membrane proteins.

Glycosphingolipids

Eukaryotic cell surfaces are enriched in GSLs, which are ceramide-linked glycans that can be branched and capped with Fuc and sialic acid (SA). The length of glycan chain can vary from one up to 60 monosaccharides in complex polyglycosylceramides (218, 219). More than 300 known species of GSLs have been characterized based on their chemical composition. In fact, 90% of mammalian GSLs are based on glucosyl-ceramides and the rest of galactosyl-ceramides (220). GSLs are defined by their internal core carbohydrate sequence, which can be of the ganglio-, globo-, lacto- or neolacto- series. Combinations of ceramide sphingoid and fatty acid parts add up to the diversity of GSLs. This disparate population of lipids has been shown to play an important role in the plasma membrane, where they are involved in many molecular level processes such as signal transduction, cell adhesion, modulating growth factor/hormone receptor, antigen recognition and protein trafficking. All these functions or their dysfunctions may contribute to pathogenesis of different illnesses like infectious diseases, lysosomal degradation and storage disorders (GSL storage disorders), neurodegenerative diseases, multiple sclerosis, cancer and diabetes (221, 222). To conduct our norovirus binding studies, we focused on intestinal GSLs decorated with histo-blood group antigens, which might be important attachment factors for the virus infection.

2.2.3 THE ABO(H) AND LEWIS HISTO-BLOOD GROUP SYSTEMS

Karl Landsteiner discovered the ABO blood group system on erythrocytes in 1900 and the structure of these carbohydrate based antigens was defined by Walter Morgan after 50 years (223). Later, it was realized that these antigens are also found in epithelium of gastrointestinal, respiratory and urogenital tracts, as well as in body fluids like saliva. Thus, the terminal glycan epitopes are produced through tissue specific glycosylation of glycosphingolipids and glycoproteins are termed as histo-blood group antigens (HBGAs) (224). Genes encoding specific glycosyltransferases determine the composition of these antigens. Therefore, all individuals present HBGAs on their cell surfaces depending on the activity of the responsible glycosyltransferases.

Biosynthesis of HBGAs

The synthesis of HBGAs is coordinated by a set of glycosyltransferases specific to the respective blood groups (225, 226). The ABH and Lewis HBGAs are closely related and presented as terminal carbohydrate structures

on glycolipids and glycoproteins. Importantly, the HBGAs vary based on the precursor such as type 1 (Galβ1,3GlcNAcβ1-R), type 2 (Galβ1,4GlcNAcβ1-R), type 3 (Galβ1,3GalNAcα1-R) and type 4 (Galβ1,3GalNAcβ1-R). The type 1-3 chains are common among both glycolipids and glycoproteins, while type 4 chains can be found only in glycolipids. Although the ABO epitopes can be expressed on all 4 precursors, the Lewis epitopes are found only on type 1 and type 2 lacto- and neolactoseries of glycans.

ABH antigens

It is important to define ABO as a histo-blood group system, where individuals can be grouped into O, A, B and AB blood groups based on the antigen presentation. These individuals would carry H, A, and B antigens composed of distinct oligosaccharides. The synthesis of A and B antigens is determined by A and B transferases encoded by the ABO gene. Polymorphism of the ABO gene explains the differences of the ABO blood groups.

The H antigen is defined by a terminal α-1,2-fucose residue formed by the action of α-1,2-fucosyltransferases 1 (α2FucT1) and 2 (α2FucT2). The α2FucT1 is found active in erythroid and vascular endothelial cells, and predominantly on the type 2 and 4 chains. The α2FucT2 is found active in secretory cells and epithelia of gastrointestinal, respiratory and urogenital tracts with higher preference to the type 1 and 3 chains (227). Interestingly, some type 2 chain activity has also been reported for the α2FucT2 (226, 228). These transferases are encoded by the *FUT1* and *FUT2* genes, respectively. Complete lack of H is very rare, but is commonly known as the Bombay phenotype and is determined by inactivity of both *FUT1* and *FUT2* genes. There can also be a weak expression of H, referred to as the para-Bombay phenotype, and resulted by *FUT1* mutations. Since the *FUT2* gene is still active in para-Bombay individuals the H antigen is found in secreted fluids and other locations than the red blood cells (229-232). The H is found in high amounts in group O individuals and in low amounts in group A, B and AB individuals since the H antigen is then converted into A and/or B antigens.

The A and B antigens are constructed by glycosyltransferases encoded in *ABO* gene. The allele at *ABO* locus encodes α1,3-GalNAc transferase, which adds α1,3-GalNAc to the Galβ on the H epitope resulting in the A antigen. Similarly, the B allele encodes α1,3-Gal transferase and gives rise to the B antigen. The O allele encodes for an inactivated/truncated form of the A or B glycosyltransferase. Thus, individuals of blood group A express at least one

A allele at the *ABO* locus (genotype *AA* or *AO*) and B blood group individuals express at least one B allele (genotype *BB* or *BO*). Individuals of genotype *AB* are classified into AB blood group. Those who belong to the blood group O have both alleles of ABO locus inactive (genotype *OO*) and do not express neither A nor B antigens. A and B glycosyltransferases are specific to the H precursor structure of type 1, type 2, type 3 or type 4 (233). The biosynthesis of A/B antigens on type 1 chains depends on the expression of *FUT2* gene and the biosynthesis on type 2 chains requires the cellular expression of either *FUT1* or *FUT2* genes (225, 226, 228, 234).

The *FUT1* and *FUT2* genes are closely linked and located on chromosome 19q13.3 (235). Mutations in *FUT2* gene result in the absence of ABH antigens in secretory fluids and such individuals are called non-secretors (se). However, when the *FUT2* gene is expressed, the ABH antigens is produced and individuals are called secretors (Se). The most common mutation in *FUT2* among Europeans is the G428A non-sense mutation and every fifth individual of the Caucasian population is estimated to be a non-secretor, while the G571T mutation is more likely to be found in Asia and Pacific Islands (236, 237). Several other mutations among various ethnic populations have been also identified, but are considered to be rare (227). However, the frequency of non-secretors differs significantly among different populations and could be related to the genetic diversity of various pathogens adapted to infect the host. For instance, the P[6] rotavirus strain has been shown to adapt to a predominantly Lewis negative population in Africa and be responsible for the majority of rotavirus infections there, while the P[8] strain does not infect Lewis negative individuals and is targeting the secretor and Lewis positive population in the Northern hemisphere (238).

Lewis antigens

Lewis antigens are named after a clinical case of Mrs. Lewis first reported by Arthur Mourant in 1946 (239). Soon it was suggested that the new Lewis antigen group is the result of the interaction of two genetically independent loci, Lewis (Le/le) and secretor (Se/se) (240). The final product is determined by the presence of α1,4- or α1,3-fucose on the GlcNAcβ of type1 or 2 glycan chain, respectively, on glycolipids and glycoproteins (225, 226, 241).

The biosynthesis of type 1 Lewis antigens is carried out by an α1,3/α1,4-fucosylransferase 3 (α3/4FucT3) encoded by *FUT3* gene. This enzyme adds α1,4-Fuc to GlcNAcβ on either the type 1 chain precursor or the type 1 H antigen resulting in Lewis a (Le^a) or Lewis b (Le^b) antigens, respectively. Le^a and Le^b antigen biosynthesis is mainly restricted to the epithelia, which

express both the α3/4FucT3 and the α2FucT2. This means that these type 1 chain antigens can also be found in soluble form in secretions and body fluids. However, erythrocytes do not synthesize these antigens themselves, but they gain Lewis antigens through passive adsorption of Lewis positive glycosphingolipids (242), which circulate in lipoprotein complexes in the plasma. Historically, only glycan structures on the type 1 chains were called Lewis antigens, but later the term was adapted for other type chain structures too. Adding to the complexity of the Lewis type of antigens is that the type 1 chain precursor is sometimes called Lewis c and the H type 1 chain is sometimes called Lewis d.

Nevertheless, important to note that individuals who lack the active fucosyltransferase encoded by *FUT3* gene (lele) are called as Lewis negative and express phenotype Le(a-b-). There is a number of point mutations leading to inactive *FUT3* gene, among those the most common are C314T or T202C (243, 244). Individuals who have at least one functional allele at *FUT3* locus (Lele or LeLe) are known as Lewis positive with the phenotype Le(a+b-) or Le(a-b+), corresponding to non-secretors or secretors, respectively. A third group of individuals with the phenotype Le(a+b+) is common in Asian populations and are called weak-secretors/partial secretors due to partially inactivating mutations (245).

When the α3/4FucT3 adds α1,3-Fuc to the type 2 chains precursor or the type 2 H determinant, isomers of Lewis structures are formed such as Lexis x (Le^x) and Lewis y (Le^y), respectively. Other fucosyltransferases than α3/4FucT3 can also add the α1,3-Fuc and, thus, generate the type 2 Lewis antigens. In addition to the *FUT3* gene, they are encoded by *FUT4*, *FUT5*, *FUT6*, *FUT7*, *FUT9*, *FUT10* and *FUT11* genes. The fucosyltransferases encoded by *FUT3* and *FUT5* genes show activity for both α1,3- and α1,4-fucosylation, but the gene product of *FUT3* preferentially fucosylates type 1 chains and the product of *FUT5* has the preference to the type 2 chains (246-250). Interestingly, other fucosyltransferases encoded by *FUT4*, *FUT6*, *FUT7*, *FUT9*, *FUT10* and *FUT11* are restricted to type 2 chains, but they also possess unique acceptor specificities (251-255). The *FUT7* encoded product would transfer α1,3-fucose only on the α2,3-sialylated type 2 precursor resulting in sialyl Lewis x structure, while the *FUT4* coded enzyme would add α1,3-fucose only on the internal GlcNAc in polylactosamine chains (256, 257).

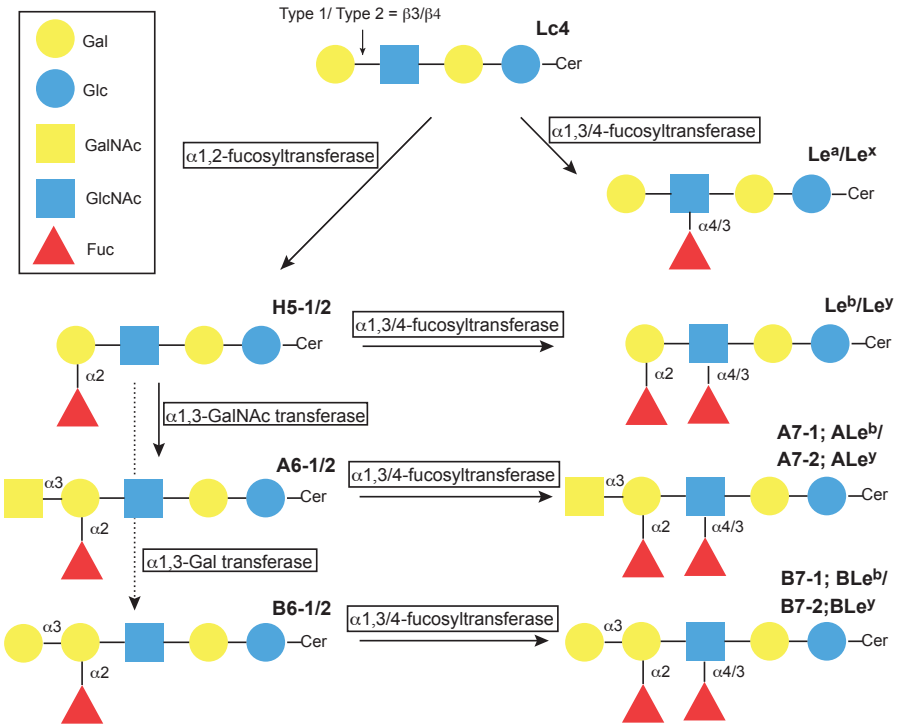


Figure 6. Biosynthesis of HBGA antigens. GalNAc – N-Acetylgalactosamine; Gal – galactose.

Function of HBGAs

Traditionally, majority of research of blood group antigens has focused on understanding blood transfusions. However, HBGAs are primarily tissue antigens and are widely distributed throughout the body. Antibodies to these tissue antigens can cause rejections of transplants and can cause spontaneous abortions (258). Furthermore, growing evidence points to the involvement of HBGAs in infectious diseases, tumor immunology and membrane chemistry (258, 259). Unfortunately, some challenges in the field hamper its' faster development. For instance, most studies have been related to only the ABO phenotype, but seldom to the combination of *ABO*, secretor (*FUT2*) and Lewis (*FUT3*) geno- and phenotypes. Moreover, animal models differ from humans by their antigen glycosylation structures, which have turned researchers into strenuous efforts of humanizing these model systems attempting to overcome the species barrier. This is particularly difficult when studying epithelial cells facing the exterior milieu of various infectious agents. Other strategies have turned into molecular biology techniques or computer modeling. Our study has also taken a step forward in HBGA

research field by analyzing pure GSLs and their binding kinetics to norovirus virus-like particles in artificial bilayer membranes. Furthermore, we also studied human intestinal enteroids, which can grow *in vitro* and resemble small intestine epithelium individually. Indeed, these cells are well suitable for host-pathogen interaction studies too and should add more to the understanding of the HBGA function on the human cell surface in the future.

Epithelial cells express ABH and Lewis antigens, which are effectively used by parasites, bacteria and viruses as receptors for attachment resulting in different susceptibilities depending on the antigen profile of an individual (260). Thus the polymorphism of *ABO, FUT2* and *FUT3* genes in humans ensures the protection from certain infections of part of the population. Some individual could be susceptible to one type of pathogen, yet would be resistant for another type of pathogen that uses a distinct carbohydrate receptor. For example, *Helicobacter pylori* adhesin (Blood group binding Adhesin: BabA) binds to Le^b antigen present on human gastric and duodenal mucosa. However, the binding is lost upon the addition of terminal GalNAcα (the blood group determinant) suggesting that the availability of *H.pylori* attachment factors might be reduced in blood group A and B phenotype individuals, and opposite in blood group O (261, 262). Similarly, rotavirus P[11] VP4 has been observed to exhibit tropism for neonates. This virus has been shown preferentially bind to the H type 2 HBGAs indicating that glycan modification during neonatal development may mediate the age-restricted infectivity of neonatal viruses (263). Importantly, and also the focus of this thesis, *FUT1* and *FUT2* polymorphisms have also been shown to play a role in viral infections, including caused by caliciviruses (264-269). The diversity of *FUT3* gene is also important in the development of some infectious diseases (270).

2.3 HUMAN NOROVIRUS AND ATTACHMENT FACTORS

2.3.1 NOROVIRUS-GLYCAN INTERACTIONS

Noroviruses engage glycans as essential attachment factors to promote infection of host cells. In the last years cell cultures, animal models, structural biology and biophysics have significantly contributed to our understanding of norovirus-glycan interactions. Structural work and biophysical techniques targeting membrane-attached glycans have indicated that a static picture of glycan recognition is not enough. However, it remains a challenge to link

biophysical observations to biological nature of norovirus entrance into the host cell.

Binding studies to HBGAs

It was early observed that GI.1 HuNoV VLPs bind to gastroduodenal epithelial cells carrying H antigen on the type 1, but not the type 2 chains (271). Later, norovirus VLP binding studies have been based on classical enzyme-linked immunosorbent assays (ELISA), where the plate surface would typically be coated with pig gastric mucins, saliva or neo-glycoproteins carrying definite HBGA structures (272-275). These simplistic studies elucidated the distinct binding preferences between the GI and GII genogroups. Specifically, the GI strains preferentially bind to the A antigen, while the binding patterns of the GII genogroup are more complex and are specific for specific strains. For instance, the GII.4 strains require secretor gene dependent HBGA structures, while the GII.3 genotypes exhibit less clear patterns with a better recognition of A and B antigens (275, 276). Interestingly, evolutionary analyses of GII.4 norovirus strains over the period of 1987 to 2005 could identify different binding properties for the same GII.4 genocluster over the period of 20 years (274). The study employed GII.4 VLPs from 5 representative evolutionary clusters and evaluated their binding to saliva from secretors and non-secretors. Similarly, another group conducted HBGA binding studies with 6 different GII.4 norovirus strains from 1987 to 2007 and also found binding patterns differing among the GII.4 strains over the time (277). Interestingly, the GII.4 variants identified after 2002 have evolved the ability to bind non-secretor Lewis HBGA, which could explain the high prevalence of this genotype (278). Clearly, GII.4 noroviruses and, possibly, other noroviruses have undergone an evolution, which has allowed them to change amino acid composition of their binding site in order to escape host immunity under the selection pressure from different HBGAs in different human populations.

Although strong evidence has been implicating ABH antigens in human norovirus infection, additional evidence has been reported suggesting that other co-factors may exist, at least for specific strains (279). In addition, some reports have indicated that some norovirus strains (GI.3 and GII.1) fail to bind to any of the available synthetic HBGAs or saliva glycoproteins, which suggests that additional factors may be involved in norovirus binding (25, 276, 280). In fact, heparin sulfate, citrate, sialic acid and GalCer have all been shown to bind HuNoV (281-284). Furthermore, human milk oligosaccharides, which are naturally occurring in human milk and usually carry HBGAs, have been shown to bind human norovirus (285, 286). All the

additionally identified co-factors, such as bile acid in GII.3 infection (121)(Murakami et al., manuscript in preparation), represent a complicated norovirus cell entry process and could possibly contribute to the future treatment, detection and control strategies against specific norovirus strains.

Crystal structure analysis has been the most important method for delivering information about norovirus-glycan attachment at atomic resolution. However, so far only the intact capsid of GI.1 HuNoV virus has been determined with crystallography (19). The other crystallography studies have been based on P domains. Nevertheless, one recent study used P domains of three different GII.4 variants to co-crystallize them with a panel of HBGAs, including both type 1 and type 2 structures (287). Many HBGA-P domain interactions involved complex binding mechanisms to HBGAs that were not observed before with identical P domains in ELISA. Indeed, these new data provide new insights for improving HBGA binding assays in order to increase our understanding about norovirus and HBGA interactions.

Other assays have been used to investigate the binding of HBGAs to noroviruses, particularly, the dynamics between glycan-norovirus capsid protein to answer how glycan attachment modulates molecular protein dynamics (288). Such affinity data can be extracted from saturation transfer difference nuclear magnetic resonance (STD-NMR) experiments, native mass spectrometry (MS), surface plasmon resonance (SPR), atomic force microscopy (AFM), quartz crystal microbalance with dissipation (QCM-D), and total internal reflection fluorescence microscopy (TIRFM). STD-NMR experiments have emerged as a very robust tool to identify and characterize the ligand binding. This technique is able to discriminate between the binding and non-binding ligand and to deliver the ligands' binding epitope. Recently, these experiments have been extensively employed to characterize binding of HBGAs to noroviruses and other caliciviruses. For instance, it's been suggested that the binding of HBGAs to GII.4 (Saga, Ast6139) and to GII.10 (Vietnam026) capsid proteins is a more complex process involving cooperative binding events (289). The native MS method has been used to determine the stoichiometry of complexes of HBGAs with norovirus P dimer showing that the inner fucose binding pockets may not be available for the B antigen because the binding to these sites would lead to steric clashes with the protein (290). Thus, there might be lower affinity HBGA binding sites yet to be discovered. However, not many studies address glycan-norovirus interactions using the SPR, but a recent study has reported binding of sialyllactose to P-dimers, which was complimented in NMR experiments (291).

Additionally, more emphasis should be given to the presentation of HBGAs as part of membrane components. QCM-D is useful to determine the binding affinity of VLP to glycolipid in the model membrane, as shown before (292) and in one of the papers included in this thesis. Recent studies highlight that membrane embedding of glycans has a huge impact on norovirus attachment (284, 293-296). A study by Rydell et al. also demonstrated the formation of membrane invaginations of giant unilamellar vesicles containing glycosphingolipids with different HBGAs induced by GII.4 VLP binding (296). Furthermore, studies using a model membrane system containing pure GSLs and employing TIRF microscopy allow VLP binding analyses in more complex systems of membranes and cells (284, 293-295, 297).

Carbohydrate binding sites

Noroviruses bind HBGAs in the P domain of their capsid protein VP1 (276, 278, 298). The binding sites of HBGAs to P domain structures from GI and GII noroviruses are distinct in the location and structural characteristics (Fig. 7) (298, 299). This shouldn't be a surprise since GI strains primarily bind to the galactose of the disaccharide precursor and the GII strains bind to the fucose residue of the HBGA. Furthermore, the HBGA binding pocket could be divided into two sites, where site 1 is binding the glycan residue and site 2 is constantly evolving due to its' susceptibility to sequence and structural alterations, thus allowing the HBGA binding to be strain-specific (278, 300). For example, sequence changes in site 2 of the GII.4 variants after 2002 have modulated the ability to bind also to the Le^a antigen (278). In addition, several studies have shown that depending on the fucose concentration, the P dimers of GII.4 (MI001), GII.4 (Saga) and GII.10 can increase the number of bound fucoses from two to four (20, 291, 301). Interestingly, crystal structures revealed that both the secretor and Lewis fucoses can bind to the same canonical fucose binding site, but the genotype determines if the secretor (as for GII.10) or Lewis fucose (as for GII.4 Saga) will bind to the pocket (287). Moreover, flexible loop residues from 391 to 395 on the GII.4 P dimer can also help the Lewis antigen to bind (287). Finally, lately it was reported that the GII.4 P domain can be affected by a post-translational modification, which causes a transformation of amino acid asparagine in an antigenic loop adjoining the binding site, thus, resulting in decreased affinity to HBGA structures (302). This likely demonstrates the multistep evolution of noroviruses to adjust to the diversity of hosts and to escape from immune responses, and may be a critical part in the infectious process after adhesion.

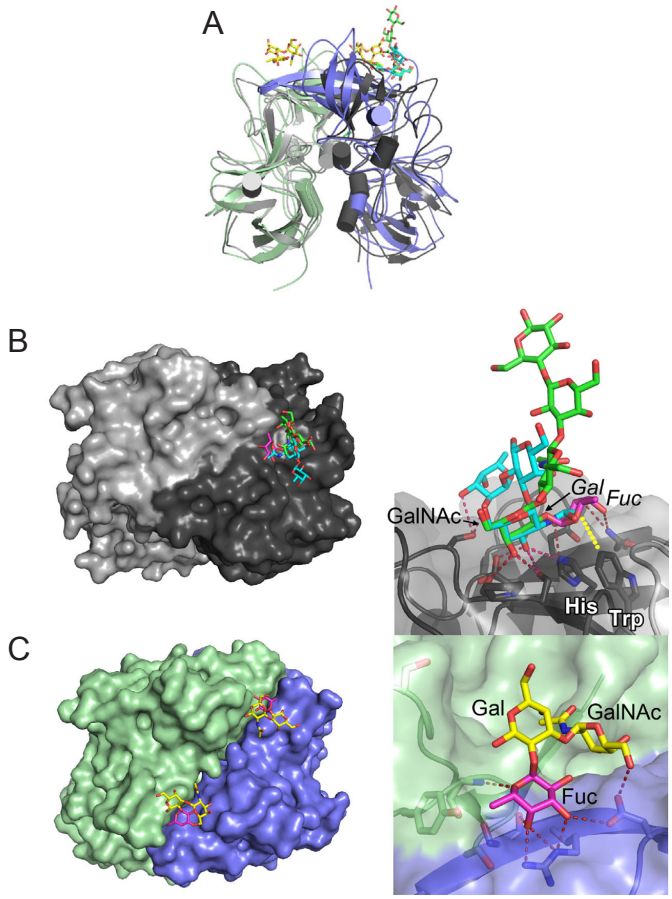


Figure 7. HBGA recognition by GI and GII noroviruses. A, Superimposed GI (grey) and GII (green/slate) P domains in complexes with H-pentasaccharide (green sticks) and A-trisaccharide (cyan sticks), and A-trisaccharide (yellow sticks), respectively. Close-up interaction views of GI NoV P domain with H type 1 pentasaccharide (green sticks) and A-trisaccharide (cyan sticks) in B. H-1 residues are labeled in italics, H bonds are indicted by red dashed lines, and the hydrophobic interaction to Trp with yellow circles. The GalNAc of A antigen partially mimics the interactions of the H-1 fucose. C, close-up view of GII NoV P domain interacting with A-trisaccharide (yellow sticks). The domain is anchored to fucose, while the GalNAc residue provides only limited contacts (151).

2.3.2 VIRAL TROPISM

There is a collected knowledge upon the relationship between histo-blood group antigens (HBGA) and norovirus and rotavirus (271, 296, 299, 303-309). The latter two pathogens are responsible for the largest numbers of non-

bacterial gastroenteritis worldwide. NoV may serve as the most suitable example of viral-host cell interaction based on ligand-HBGA binding. Very early observations from NoV challenged individuals pointed to different populations prone to develop the disease (310, 311). A part of challenged individuals would develop clinical symptoms of gastrointestinal inflammation, while another part would remain asymptomatic. Antibody response between two groups would differ too. Symptomatic ones would have higher levels of serum antibody titers than asymptomatic, but those would not protect from re-challenges after 1-2 years. Clearly a key factor allowing some individuals to remain NoV infection resistant was missing. Finally, a hint appeared in the literature when it was published that NoV-like particles induce hemagglutination of red blood cells from different blood groups (312). Then the first report of NoV disease association with ABO HBGA type followed, where it was stated that individuals with O phenotype are more likely to be infected with NoV (303). That study was based on individuals challenged with NoV GI.1 Norwalk strain and gave rise to many other studies about the relationships between NoVs and HBGAs. Finally, the publication came pointing to the importance of secretor status to NoV binding on gastrointestinal epithelial cells (271). After one year 4 distinct-strain specific patterns of NoV binding to HBGA family could be described (313), which brought a wider picture of a complicated complexity among NoV strains leading to infection. Finally, a study conducted by Harrington and colleagues confirmed NoV attachment to HBGAs (305). Thus, the importance of this virus-ligand interaction became evident. Having a fast development of NoV-like particles production, a greater variety of VLPs binding studies could be performed. Four binding patterns were extended to six and today there are 7 binding patterns identified (Table 1)(276). For instance, we have a group of viruses preferably binding to A and O secretors only (NoV GI.1). Another group would bind to A, B and O secretors (GII.4, VA387) or A, B secretors binders (GII.5, MOH), or even Lewis positive secretors and non-secretors (GII.9, VA207). The viral attachment ability has been developed so well that we would not find any individual HBGA pattern unrecognized by any of NoV strains. To top up a beautiful diverse attachment pattern of NoV, there are some strains identified as non-recognizers of any fucosylated HBGAs (GI.3 (Desert Shield virus; VA115), GII.1) (25, 276, 280). For a long time the field lacked a direct experimental system to test whether the infections caused by NoV were indeed dependent on host cell HBGAs. Since 2016 human NoV infection is possible to propagate in tissue cultures in laboratory settings (121), which opened a door for advanced studies upon HuNoV and host cell interactions.

Table 1. Norovirus binding patterns to HBGAs.

Norovirus variant	A/B	H	Le
GII.4 VA387	+	+	-
GII.4 GrV	+	+	-
GI.1 Norwalk	+	+	-
GI.2 C59	+	+	-
GII.3 PIV	+	+*	-
GII.3 MxV	+	+*	-
GII.1 Hawaii	+	+*	-
GII.5 MOH	+	-	-
GII.2 BUDS	+	-	-
GII.2 SMV	+	-	-
GI.8 Boxer	-	+	+
GII.9 VA207	-	+*	+
GII.13 OIF	-	-	+
GI.3 DSV	-	-	-
GI.3 VA115	-	-	-

*Binding groups are coded in colors. * Positive signal for binding, but not strong (276).*

FUT2 polymorphism in susceptibility

Earlier studies have shown that some individuals after challenge with high doses of virus remained resistant to the disease (303). Whether these individuals were just symptom-free or uninfected due to an innate resistance or pre-existing immunity to NoV remained questionable. There could be several factors determining the susceptibility to infection with enteric pathogen such as genes encoding molecules mediating attachment, recognition of pathogen, inflammatory cytokine responses, innate and acquired immunity (314). Similar resistance patterns were detected in wild rabbits, where rabbit hemorrhagic disease virus (RHDV) is a highly virulent calicivirus that has become a major cause of mortality in wild rabbit populations (315). Early challenge studies on GI.1 virus have implicated that non-secretor individuals were resistant to norovirus infection, while most of the secretor individuals got infected (316-318). Similar secretor driven susceptibility was observed in the GII.4 challenged volunteers, where all non-secretor individuals remained symptom-free, thus, protected from the disease (319) Moreover, outbreak studies from Sweden, Denmark and China following norovirus GI.6, GII.6 and GII.4 caused infections, could all report

that, indeed, symptomatic individuals were restricted to the secretor status (320-322). These studies, as well as the analyses of serum antibody prevalence and titers of individuals with different ABO, Lewis and secretor status (323), clearly demonstrated that the secretor status, dependent on the *FUT2* gene polymorphism, is a factor determining the susceptibility to the virus (324, 325). The latest genome wide association meta-analysis by Bustamante and co-workers additionally suggested a new *FUT2* allele mutation variant W152X significantly associated with diarrhea under age of 1 (325). It remains unclear how this variant would be related to NoV infection, but the study has implicated the importance of *FUT2* gene in diarrhea in young children from any population. However, there is still a lack of evidence for a full protection to NoV infection related to any one of the *FUT2* mutations (266, 316, 324, 326-330), especially considering the big diversity of NoV strains that has developed over the years. Study by Larson and colleagues extended the previous knowledge focused on secretors and showed that ABO phenotype and Lewis genotype does not correlate with antibody prevalence and titers against NoV GII (267), which still does not exclude the importance of the latter in the viral-host attachment pathogenicity for single strains. The secretor status has lately been shown to be of major importance in susceptibility to GII.4 human norovirus (HuNoV) replication in human intestinal enteroid cultures (HIE) (121).

3 VACCINE DEVELOPMENT

3.1 PROTECTIVE IMMUNITY

Vaccines represent one of the greatest achievements of modern medicine. Despite their success, a majority of vaccines have been developed empirically, with little or no understanding of the detailed mechanisms by which they convey resistance against infection. The most important function of the immune system is to protect us from pathogens. Immune protection rests on both innate and adaptive immune responses, and the interaction between the two. Since adaptive immunity is epitope specific and has long-term memory, it is the ultimate target to be achieved by an effective novel vaccine. Recent advances in innate immunity have offered new insights about the mechanisms of vaccine-induced immunity and have facilitated a more rational approach to vaccine design. Immune protection can be achieved through stimulation of neutralizing antibodies (331). In fact, antibodies are good correlates of protection against many pathogens, including viruses, such as influenza (332, 333), rotavirus (334), and norovirus (316, 335-337). However, also cell-mediated immunity against these virus infections have recently been ascribed critical roles. For example, there is growing evidence that a key protective role is played by tissue-resident memory T cells. Both cytotoxic CD8 T cells and effector CD4 T cells can develop from these tissue-resident memory cells as found in protection against influenza infection (338). Indeed, even in the complete absence of antibodies lung memory CD4 T cells could be broadly protective as observed in an experimental model of vaccination against influenza. Also, protection against HIV infection or malaria relies not only on specific antibodies, but requires strong T-cell responses (339, 340). Therefore, vaccine development today is aimed at stimulating antigen-specific antibodies as well as tissue resident memory T cells that persist long time after vaccination.

Naïve T cells are activated by dendritic cells (DCs) in lymphoid organs. DCs are the professional antigen-presenting cells (APCs) for priming of T cells. Upon antigen (Ag) exposure, the DC will internalize the Ag by phagocytosis, pinocytosis or receptor-mediated endocytosis, and subsequently the Ag is digested into peptides, which will be presented on human leukocyte antigen (HLA) -DP, -DQ, -DR/ major histocompatibility complex (MHC) class II molecules expressed on the surface of the DCs, B cells and other APCs. It is known that mostly peptides with a high affinity for the MHC class II molecules can effectively prime T cell responses. Virus proteins, produced inside the infected cell, are displayed on HLA-A, -B, -C/ MHC class I

molecules, which are recycled to the surface of the cell, and, thereby, can prime CD8 T cell responses. The HLA/MHC molecules are capable of binding many different peptides and since each individual can carry 3 different HLA /MHC molecules of the class I and class II types, this leads to an impressive combination of isoforms, which contribute to increasing the diversity of HLA molecules of each individual. These divergent and complex panels of HLA/MHC molecules are responsible for the broad ability to always find epitopes that can trigger an immune response against the many diverse pathogens we can encounter. In this thesis work we have focused on MHC class II molecules, and presentation of peptide Ags that result in priming of CD4 T cells and antibody responses.

CD4 T cells or T helper cells (Th) express the CD4 co-receptor and the T cell receptor (TCR), which recognizes complexes of peptides and MHC class II molecules on the antigen-presenting cells (APCs). When the T cell is activated in the draining lymph node, it can differentiate into one of several effector subsets of Th cells dependent on the microenvironment at the inductive site. The most important CD4 T cell subsets include Th1, Th2, Th17, T follicular helper cells (Tfh) or regulatory T cells (Treg). Each of these subsets is functionally distinct and defined by their cytokine production (341). Th1 development is dependent on IFN-γ and IL-12. These cells are producers of IL-2 and the most important producers of IFN-γ. Their function is to provide cell-mediated immunity against viruses and intracellular bacteria, as well as support B cell differentiation to antibody production. For Th2 development we require the IL-4 cytokine, which plays a critical role against helminthic parasites infections through secretion of IL-4, IL-5 and IL-13. Th17 cells develop when IL-6, IL-23, IL-21 and transforming growth factor (TGF)-β are present in the microenvironment at the inductive site. Th17 cells can make IL-17, IL-21 and IL-22, and they protect against virus, bacterial and fungal infections. Tregs differentiate in the presence of TGF-β, which is a cytokine they also produce along with IL-10. The Tfh subset is involved in regulation of the B cell response in the germinal center (GC) of the draining lymph node. These cells produce cytokines that are critical for B cell isotype differentiation also called immunoglobulin class-switch recombination (CSR) and IL-4, TGF- β or IFN-γ can drive B cell IgG1, IgE, or IgA production. IL-21 is influencing differentiation and maturation of activated B cells into B memory cells and long-lived plasma cells in the GC.

CD8 T cells or cytotoxic T lymphocytes (CTLs) have the CD8 co-receptor and react to peptides presented by MHC I class molecules, which are expressed by all nucleated cells in the body. These cells are essential for cell-mediated immunity against intracellular pathogens and produce IFN-γ, TNF-

α, perforins and granzymes.

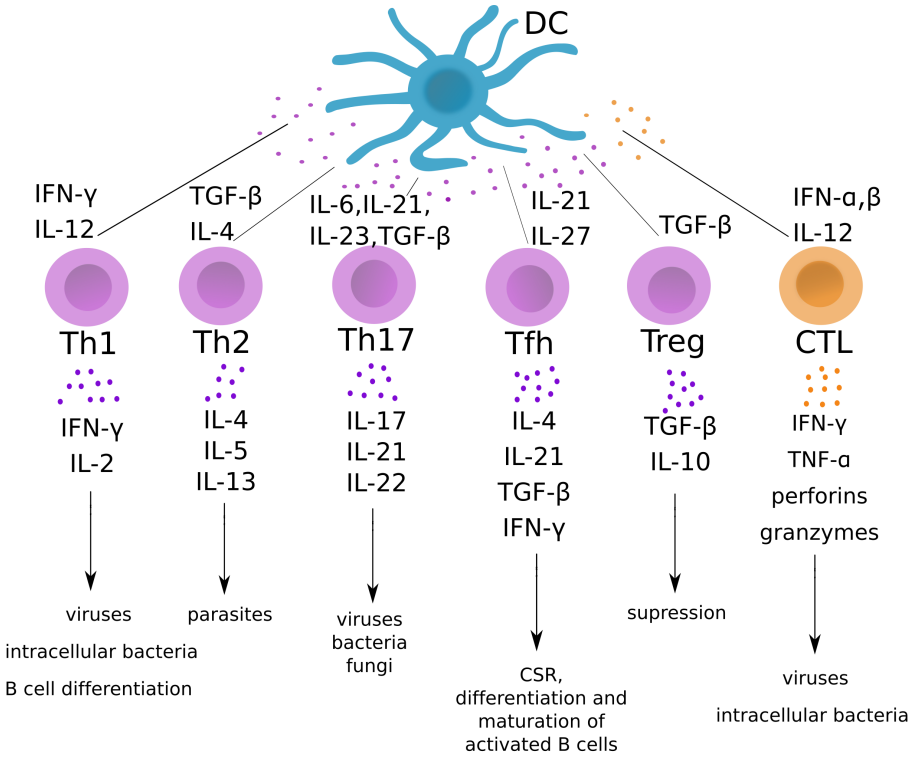


Figure 8. Activation and function of T cell subsets.

Importantly, some activated T cells develop into memory T-cells and play a protective role against future infections with the pathogen (342-344). For both CD4 and CD8 T cells there are three main subsets of memory cells: central memory (TCM), effector memory (TEM) and tissue resident memory T cells (TRM). TCMs express the C-C chemokine receptor type 7 (CCR7), which allows the cells to home to and remain in the secondary lymphoid organs where the CCR7 ligands CCL19 and CCL21 are produced. These cells rapidly respond to reactivation by the specific Ag. On the other hand, TEMs and TRMs lack CCR7, and are re-circulating (TEM) or reside in the tissues (TRM), and respond more rapidly to reinfection. They display effector functions by producing granzyme B or IFN- γ, at an early stage of infection (345, 346). The memory T cells can survive for decades with a half-life of 8-15 years providing long-lasting protection against reinfection (347).

Production of antibodies depends on the simultaneous induction of Tfh cells that support the GC reaction in the draining lymph node. Thus, the B cells

need helper factors to proliferate and differentiate into memory B cells and plasma cells for antibody production. The B cell receptor (BCR) binds specifically to the Ag, which in most cases are 3D structures that constitute the conformational epitopes. After binding, the BCR conveys signaling and the Ag can be endocytosed and degraded into peptide. These can then be presented on MHC II molecules to activated CD4 T cells that at the T-B cell border in the lymph node initiate production of helper factors, such as cytokines and CD40L expression by the CD4 T cells. This interaction leads to strong expansion of the B cell clones and their differentiation to memory B cells and long-lived plasma cells. B cell memory can be long-lived and may last for a lifetime. For example, immunity against smallpox has been shown to persist for more than 70 years in vaccinated individuals. Thus, even though smallpox was eradicated more than 30 years ago the memory B cell clones do not appear to require the presence of Ag (348).

3.2 EVOLUTION OF VACCINES

The world's first vaccine was developed by Edward Jenner against smallpox and it is an example of a live attenuated vaccine. Such vaccines consist of the living microorganism that has been made less virulent than the pathogenic strain and, hence, can provide a high level of antigen exposure, albeit not leading to severe infection. The smallpox vaccine has eradicated the disease. Also, other live-attenuated vaccines such as those against measles, mumps and rubella have been successful. These vaccines provide endogenous immunoenhancers, also called adjuvants, and, therefore, do not require the addition of such elements. Adjuvants are molecules with strong recognition by the innate immune system and these are derived from, for example, the bacterial cell wall or genomic nucleic acid, which are recognized by the innate cells by pattern recognition receptors (PRRs).

A risk with live attenuated vaccines is that they can revert to cause the emergence of more virulent strains or, which is even more important, cannot be used in immunocompromised individuals due to the risk of severe infection. For example, the live attenuated seasonal influenza virus vaccine FluMist is recommended for individuals between 2 and 49 years old only, excluding the most vulnerable populations – infants and elderly (349, 350). Live bacteria and viruses can also be used as vaccine vectors carrying recombinantly expressed Ags from other pathogens. However, a problem with this strategy is the stimulation of vector-specific immunity that may reduce the immunogenicity of the engineered Ag and in this way lower the efficacy of the vaccine (351). The adenovirus vectors are examples of this

potential risk.

Killed vaccines are safer, but less immunogenic and require an adjuvant to trigger strong immune responses. Vaccines against rabies, influenza and typhoid fever are all examples of killed vaccines. These vaccines are non-infectious, and can be used broadly among populations, including immunocompromised individuals. As they lack the endogenous activator of the innate immune response they require addition of an external adjuvant. These are most often PRRs such as lipid A, CpG or aluminum salts, but they can also be AS03, which was the adjuvant used in the Pandemrix influenza vaccine, that caused narcolepsy in some cases in Finland and Sweden (352, 353). Hence, adjuvants can be reactogenic and cause side effects and, therefore, safer and more stable vaccine adjuvants are needed.

A form of killed vaccine is the subcomponent vaccine, which hosts only fragments of the infection and not the whole microorganism, and these are attractive candidates to replace live attenuated vaccine with. They could reduce vaccine production costs, ensure safety and provide more robust and stable vaccines. Such vaccines can consist of bacterial or viral proteins or fragments of the cell wall or the virus capsid. This provides many different options and a multitude of possible approaches to the design of a new vaccine. As aforementioned, subcomponent vaccines are weakly immunogenic and require incorporation of adjuvant. In this thesis we have worked with a subcomponent approach to design a novel HuNoV vaccine. We have focused on the potential of immunogenic peptides derived from HuNoV major protein VP1 and combined these into fusion proteins that carry endogenous adjuvant activity and developed a mucosal vaccine to be given intranasally (i.n.). The mucosal adjuvant we have explored is the CTA1-DD adjuvant platform that was developed in our laboratory to circumvent the toxic side effects of cholera toxin (CT).

The Ag used in a vaccine should not only be an inducer of significant immune responses, but should also stimulate biologically relevant immune protection against the pathogen. We developed a strategy involving the selection of the best peptide candidates from the HuNoV capsid that could stimulate strong immune responses. B cell responses most often do not identify linear peptides but we identified that the MAbs we generated, indeed, could detect linear peptide epitopes. By contrast, T cells only react to linear peptide sequences, and screening for specific responses could be cumbersome and difficult, but instead *in silico* predictions using computer databases could be used to identify the affinity to MHC class I and/or II molecules that preferentially will bind the peptide. *In silico* predictions

enable faster evaluation of peptide candidates and can also predict their fitness in humans by measuring the affinity between the peptide and the HLA subsets. *In silico* tools can also be consulted to identify well conserved amino acid sequences across the family of pathogens and localize them to the whole protein. The immunogenicity of peptides should be verified *in vivo* before incorporating them into the vaccine. The approach of this thesis has been to use the DD molecule to target binding sites on the APCs. This will ensure the delivery of the Ag to its target cells. The final processing of the vaccine Ag is coordinated by the cathepsin family enzymes inside the cell. Of note, susceptibility to protease degradation must be prevented before the antigen is taken up by APCs. Finally, the vaccine candidate can be tested *in vivo,* and immune responses can be advanced by challenge studies if available for a specific pathogen.

3.3 MUCOSAL VACCINES

A majority of all pathogens gain access to the human body through mucosal surfaces. Hence, mucosal vaccination leading to protective immunity at mucosal sites is much warranted. However, at present injectable vaccines dominate the market, but these vaccines most often fail to stimulate immune protection at mucosal sites. In contrast, mucosal vaccines can stimulate antigen-specific tissue resident cell-mediated immunity as well as local secretory IgA (sIgA), which is also accompanied by strong systemic IgG responses (354, 355). Therefore, mucosal vaccines may stimulate strong and long-lasting immune protection at both mucosal and systemic sites (356, 357). Mucosal vaccines are also superior to injectable vaccines for their lower production costs and stability, as well as for being safer, hosting better compliance and for their administration via the mucosal routes, not requiring medically trained personnel. They are considered excellent for mass vaccination, as they do not carry a high risk of spreading, for example, blood-borne diseases. This can occur through the use of contaminated injection needles. Despite all these merits, few successful mucosal vaccines have been licensed. Among those are oral cholera, typhoid, polio and rotavirus, or intranasal influenza vaccines (358-360). It is crucial to consider the route of administration, the adjuvant to be used and which delivery system to employ in the next generation of mucosal vaccine (361).

3.4 DELIVERY ROUTE

The mucosal immune system displays a high degree of anatomical compartmentalization related to the migratory patterns of lymphocytes when

activated at different mucosal sites. The selective localization of mucosal lymphocytes to specific tissues is determined by "homing" or chemokine receptors. The anatomical organization of the mucosal immune response limits the choice of vaccine administration routes. This implies that whereas intranasal vaccinations provide good immunity in the respiratory tract they convey poor immunity in the GI tract. Vice versa, oral immunizations give better protection in the gut, while stimulating weaker immune protection in the lung. On the other hand, also intranasal immunization can provide gut protection as shown with HuNoV VLPs, which stimulated fecal IgA responses (362). Indeed, vaccines used intranasally with GI.1 VLPs were found to be protective against a GI.1 challenge infection (363).

Oral and nasal routes are the most explored administration pathways for mucosal vaccination. Other mucosal delivery routes can be identified such as, rectal, vaginal or sublingual routes. All these routes have been shown to work well as assessed in the different experimental animal models (364). While most mucosal vaccines are administered orally, this route is considered the most challenging to succeed with. The reason is the harsh gastrointestinal environment, which degrades most Ag epitopes before they are delivered to the immune inductive site. Therefore, oral vaccines nearly always require higher doses of Ag and an effective mucosal adjuvant. Another reason for poor oral immunogenicity is the fact that protein antigens induce immune suppression or tolerance instead of protective immune responses when given orally. Because of these shortcomings intranasal vaccines can be more attractive than oral vaccines due to the lower dose of Ag and adjuvant required (357, 363, 365-367). This also applies to sublingual vaccines, which are also gaining attention, because they can induce both mucosal and systemic B and T cell responses in the gastrointestinal, respiratory and genital tracts (364). This route also requires lower amount of antigen than the oral route. Finally, and in contrast to other mucosal routes, the sublingual route avoids activating cells in the central nervous system, as observed with some of the mucosal adjuvants, such as cholera toxin and *E.coli* heat-labile toxin in humans (368-370).

3.5 ADJUVANT

Immunizations with purified antigen alone are most often unable to induce a significant immune response. This is because the default pathway for mucosal immune responses to protein antigens is tolerance or immune suppression. To avoid tolerance, immunizations require co-administration of immunostimulatory agents called adjuvant (*adiuvare* translated from Latin

means to help). There are many different types of adjuvants, and these are often molecules, which act through PRRs on the innate antigen-presenting cells. Also, cytokines and bacterial toxins can exert adjuvant effects as they trigger activation and maturation of DCs resulting in efficient priming of CD4 T cells in the draining lymph nodes.

PRRs recognize molecules typical for the pathogens and are important for activation of innate immunity. For example, toll-like receptor 2 (TLR2) is activated by bacterial lipoproteins from Mycobacterium tuberculosis, which is a key component of complete Freund's adjuvant (CFA) (371). Furthermore, poly I:C mimic double stranded RNA and provides strong activation of the TLR3 pathway (372). This leads to IFN-α/β production resulting in TLR1, 2, 3 and 7 expression (373), DC activation (374) and priming of Th1 responses (375). Monophosphoryl lipid A (MPL), a detoxified derivative of lipopolysaccharide (LPS) from Salmonella minnesota R595, is an agonist of TLR4 and immunoenhances Th1 responses by triggering IL-12 and IFN-γ production (376). In fact, MPL is already used in some licensed vaccines, such as an anti-allergy vaccine (Pollinex Quattro®) (377) or a stage IV melanoma vaccine (Melacine®) (378). Moreover, TLR5 is activated by flagellin, from bacterial flagella, resulting in TNFα production, which is associated with high antibody titers and a mixed Th1/Th2 response (379). Another adjuvant formulation is imidazoquinolines, which mimic single stranded RNA, which is recognized by TLR7/8. Whereas Imiquimod is activating only TLR7, resiquimod can activate both TLR7 and TLR8. Both substances stimulate IFN-α and IL-12 production (380), and activate CD8 T cells (381). Of note, resiquimod enhances B cell responses in both humans and mice (382). CpG is oligodeoxynucleotides (ODN) composed of unmethylated CG motifs (cytosine phosphate guanidine) and is recognized by TLR9. This leads to upregulation of costimulatory molecules (CD40, CD80, CD86) and proinflammatory cytokines (IL-6, IL-12, IL-18, TNFα) (383, 384). Mannose receptors (MRs) have been described to react to α-D-mannopyranosyl residues common on glycoproteins of parasites, bacteria, yeasts and enveloped viruses (385, 386). These receptors have also been shown to trigger cytokine secretion and DC activation (385, 387). Despite a broad variety of experimental adjuvants, only few are effective or safe enough to be exploited in current vaccines under development.

Bacterial enterotoxins, such as cholera toxin (CT) or *Escherichia coli* heat labile toxin (LT) have been recognized as the golden standards for an effective mucosal vaccine adjuvant (388). They are AB protein complexes, where the A subunit is composed of the A1 and the A2 chains linked to the B pentamer subunits (CTB), i.e the CTB pentamer. The A1 portion hosts ADP-

ribosyl transferase activity that stimulates increases in intracellular cAMP. By contrast, the CTB subunit binds to GM1 ganglioside receptors present on the cell membrane of all nucleated cells, including DCs. While CT has specificity only to GM1 ganglioside, the LT toxin could also bind additional gangliosides like GM2 (389, 390). Unfortunately, despite being the most potent mucosal adjuvants CT and LT carry toxic side effects, which preclude clinical use. Therefore, different approaches have been taken to overcome this issue. Most attempts have tried mutating the A1 enzyme while hoping to have retained adjuvant function. The CTA1-DD adjuvant is derived from CT holotoxin, as it carries the enzymatically active CTA1 subunit fused to a dimer of the D-fragment from *Staphylococcus aureus* protein A (DD). The CTA1-DD exerts comparable adjuvant effects to those of CT holotoxin and has been found effective and non-inflammatory in mice, guinea pigs and monkeys (391, 392). Also, as it lacks the B subunit it fails to bind the GM1 ganglioside, which results in that it cannot accumulate in the central nervous system following intranasal administration and it remains safe after intranasal immunizations (393-395).

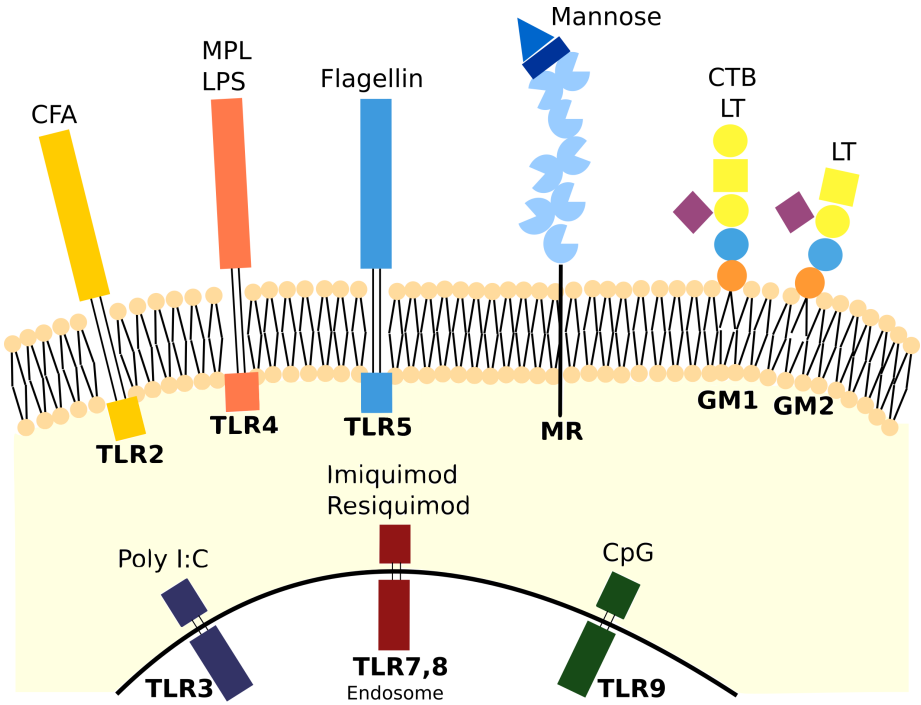


Figure 9. Experimental adjuvants recognized by PRRs and gangliosides.

3.6 VACCINE FORMULATION

The formulation of vaccines is another critical step in optimizing its' immunogenicity. An ideal mucosal vaccine delivery formulation should protect from enzymatic degradation and induce an efficient uptake and presentation of Ags by DCs. Furthermore, the physicochemical qualities of the delivery system like particle size, surface charge and hydrophobicity should be adapted to allow the Ag to cross the mucosal membrane (396). Therefore, a number of diverse delivery systems for mucosal immunization have been developed including inert systems such as microparticles, nanoparticles, liposomes, immune stimulating complexes (ISCOMs), and different live attenuated bacterial or viral vector systems (361). Mucoadhesive components could help to overcome a constantly renewing mucus layer by facilitating contact between the vaccine formulation and the mucosal membrane. Examples of such molecules are chitosan and starch, and these prolong the vaccine retention time and increase the chance of effective Ag uptake. Other strategies to target the mucosal membrane and facilitate Ag uptake are based on particulate formulations including VLPs, bacterial ghosts, microparticles, nanoparticles, liposomes and ISCOMs. These formulations can also establish close contact with the mucosal epithelium through the inclusion of adhesive (lectins, MAbs) and/or immunomodulating (TLR ligands, CTB) molecules (397, 398). It is expected that the efforts invested into new formulations of mucosal vaccines will be successful and that we will see a growing number of licensed mucosal vaccines in the future.

3.7 IMMUNITY AGAINST NOROVIRUS

It is predicted that the future success of a novel HuNoV vaccine will heavily depend on its ability to stimulate long-lived protective immunity. Modeling studies have suggested that naturally induced immunity against NoV could last 4-9 years (399). However, human challenge studies failed to find immunity lasting longer than 2 years (range between 2 months and 2 years) after NoV inoculation even with inoculums significantly higher than would be observed in naturally acquired infections (310, 400, 401). Currently no clinical data collected from challenge studies support that immunity against HuNoV of longer duration can be induced (402, 403). However, this being said it should be emphasized that antigenic diversity of norovirus strains is known to undergo great variation. In fact, already the early human challenge studies with norovirus demonstrated that naturally acquired immunity against one strain would not necessarily protect against another strain (6). Therefore, the ideal vaccine formulation should stimulate immune protection against

conserved epitopes of the HuNoV, resulting in strong cross-reactive and long-lasting immunity against both present and emerging HuNoV strains.

3.7.1 HUMORAL IMMUNITY

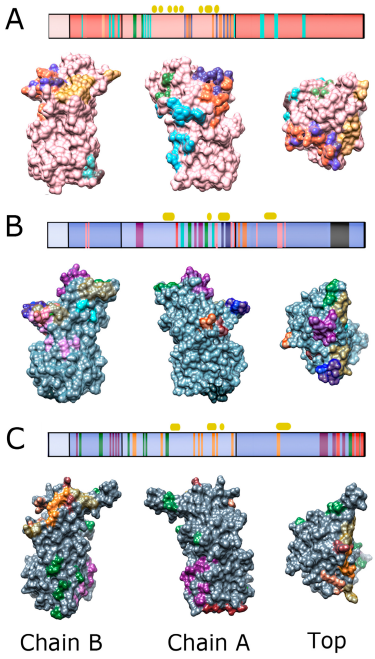
Many studies have been conducted to analyze humoral immunity against HuNoV. Compared to T cell mediated immunity, antibodies against HuNoV are thought to last longer, but there may still be a lack of memory B cell development following a HuNoV infection (310, 404-406). Nevertheless, the importance of protective antibodies in serum has been correlated to a reduced infection rate and less severe gastroenteritis (86, 400, 407, 408). Also, immunocompromised individuals can develop chronic HuNoV infections clearly emphasizing the need of antibodies to resolve infection (67, 409, 410). It is believed that protection against HuNoV is primarily mediated by neutralizing antibodies, but other mechanisms could be operational in protection. To this category we should count in antibody-dependent cellular cytotoxicity (ADCC), which has been shown to play a protective role against influenza infection (411, 412). A large number of studies have been published evaluating MAbs against HuNoV in different *in vitro* or *in vivo* assays. These have reported on a protective capacity when recognizing epitopes in the VP1 capsid protein (413). Further efforts are needed to understand the relationship between HuNoV diversity and host protective immunity, as well as the mechanisms used by the virus to evade the immune response.

3.7.2 B CELL EPITOPES

Genogroup specificity

Blocking antibodies have been found to convey protection against clinical norovirus gastroenteritis (414). Hence, MAbs and nanobodies have been generated against different norovirus VLPs and used to define blocking epitopes on the capsid of norovirus. The blocking capacity of the different MAbs and nanobodies was evaluated using two different assays, the hemagglutination inhibition (HAI) and HBGA binding assays. Despite this, only very few antibody-blocking epitopes have been identified for the GI.1 and GII.10 genotypes. Interestingly, most anti-GI.1 MAbs and nanobodies uniquely bind to the GI.1 genotype, which suggests sequence diversity across the genogroups reflecting differences in the P domain. This would also be important when considering antibody treatment in combined therapeutic interventions (415, 416). By contrast, the GII.4 genotype has 8 predicted antibody-blocked epitopes, A through H. Epitopes A through E were predicted based on sequence alignments and comparison of variable residues

across pandemic GII.4 sequences (417). Epitope A has shown immunodominance over the other GII.4 epitopes. Around 40% of serum antibodies have been found to block the epitope A (272, 417). Interestingly, changes in epitope A have been found to correlate with the emergence of new virus strains of the GII.4 genotype. The A epitope is exposed in the P2 domain and independent of the VLP conformation (272, 418). On the other hand no blocking MAbs have been mapped to the B epitope, which is hidden in the dimer between two VP1 monomers and could be important for residues located on the surface of the VLP. Epitope C is also located on the surface, directly proximal to the HBGA binding pocket, and has been associated with the strain-specific HBGA blockade (419). Epitope D faces the outer surface of the VLP in the P2 domain and does not change due to the NERK domain effect (420). This domain hosts the binding site for MAbs and is also close to the HBGA binding domain. Importantly, polymorphisms in the D epitope have been implicated in escape mutants from herd immunity and HBGA ligand switch (272, 421, 422). It has been shown that changes in the D epitope can modify affinity for different HBGAs by stabilizing bonds with non-H (such as Le^x and sialyl-Le^x) antigen HBGAs (277, 423). Such information may give insights into virus adaptation to higher virulence and larger populations of susceptible people. Epitope E is lateral to A and D, but is less exposed (421). It has a high variability among major GII.4 epidemic strains indicating that it is a critical site for the emergence of antigenically novel GII.4 strains (273, 421). Furthermore, Epitope E has been shown to be temperature sensitive (424). A similar temperature-dependent epitope is the F epitope, which is conserved among GII.4 strains. Interestingly, it has been observed that changes in Epitope F have affected binding of MAb to Epitope E (424), despite that these epitopes do not share amino acid residues. Epitopes G and H have not been characterized yet, but it is thought that Epitope G might be located close to Epitope F, and Epitope H might be influenced by Epitope A (420, 425). Of note, also less well described epitopes exist and are predicted to be important for virus pathogenicity and immunogenicity. Studies using nanobodies have identified that some nanobodies might be cross-reacting with 7 different GII genotypes and block GII.4 VLP binding to HBGAs (419). Most importantly, a nanobody has also been mapped to a linear epitope located on the P1 domain and another nanobody reacted only with GII.4 and GII.10 epitopes when the P particles were dimeric and not monomeric (426). Blocking epitopes mapped to GI.1, GII.4 and GII.10 P domains are depicted in Figure 10.



GI.1 blockade epitopes
(HBGA binding residues – aa 327, 329, 338, 342, 344, 375, 377, 378, 380)

Epitope	Residues	Conformation	Blocks	Mechanism	Color
mAb 54.6*	280, 291-293, 302	Monomeric VP1	GI.1	Steric hindrance	Green
mAb 5I2	346, 348, 350-352, 380, 381, 383, 394, 396, 398	Monomeric VP1	GI.1	Steric hindrance	Dark blue
Nano-7	Chain A: 275, 276, 280, 282, 303, 305, 310, 312, 313, 318, 404, 446, 449 Chain B: 237, 463, 464, 465, 498, 500	Dimeric VP1	GI.1	Conformational changes	Light blue
Nano-94	264, 348-350, 352, 379, 381, 382, 391, 392, 394, 397, 400, 401	Monomeric VP1	GI.1	VLP aggregation	Orange

GII.4 blockade epitopes
(HBGA binding residues – aa 342-347, 374, 389-393, 440, 441)

Epitope	Residues	Conformation	Blocks	Mechanism	Color
Epitope A*	294-298, 368, 372, 373	Monomeric VP1	GII.4	Steric hindrance	Purple
Epitope B*	333, 382 (*put.*)	Monomeric VP1	GII.4	Conformational changes	Light blue
Epitope C*	340, 376	Monomeric VP1	GII.4	Conformational changes	Green
Epitope D*	391, 393-396	Monomeric VP1	GII.4	Steric hindrance	Dark blue
Epitope E*	407, 412, 413	Monomeric VP1	GII.4	Conformational changes	Orange
Epitope F*	327, 404	Monomeric VP1	GII.4	Conformational changes	Red
M6	517-528	Monomeric VP1	GII.4	(*unknown*)	Black
2C3G3*	245, 247, 389, 390, 397, 435, 443-446, 448	Monomeric VP1	GII.4	Direct competition / Steric hindrance	Pink

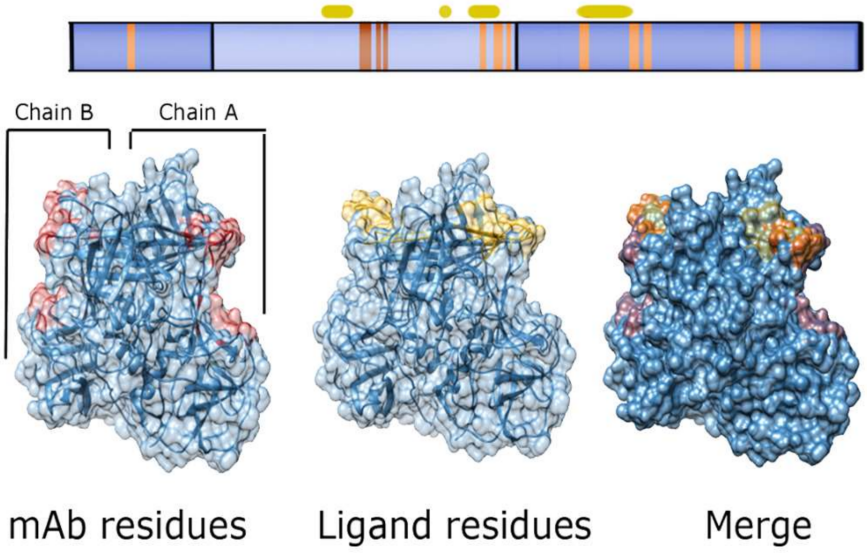
GII.10 blockade epitopes
(HBGA binding residues – aa 355, 356, 381, 382, 385, 449, 451, 452)

Epitope	Residues	Conformation	Blocks	Mechanism	Color
Nano-14	Chain A: 298, 299, 333, 361, 363, 381, 382, 403, 449 Chain B: 358, 384	Dimeric VP1	GII.10	Direct competition	Orange
Nano-26	Chain A: 231, 488 Chain B: 269, 271, 272, 274, 276, 316, 470-472, 475	Dimeric VP1	GII.4 GII.10	VLP disassembly	Purple
Nano-32	Chain A: 236, 287, 314, 316, 343, 344 Chain B: 247, 248, 492, 518, 519	Dimeric VP1	GII.10	VLP aggregation	Green
Nano-85	477, 525, 528-530, 532, 533, 534	Monomeric VP1	GII.4 GII.10	VLP disassembly	Red

Figure 10. HBGA blockade epitopes mapped to (A) GI.1, (B) GII.4 and (C) GII.10 P domains. Golden color above the linear P domain presentation – HBGA binding residues; asterisk (*) – do not have an antibody/virus structure associated with their epitope definition. (413)

Neutralizing epitopes

Do MAbs against HuNoV host neutralizing properties? This was investigated in recent studies, but relatively few of these studies have been published (121). To date only one blocking and neutralizing anti-GII.4 MAb, 10E9, has been mapped to GII.4 P domain. The epitope of 10E9 overlapped with the HBGA binding pocket (Fig. 11)(427, 428). Interestingly, though, 10E9 MAb could neutralize GII.4 virus from patient stool samples, but it failed to block GII.4 NSW 2012 VLP binding to HBGAs (428).



GII.4 blockade epitope (HBGA binding residues – aa 342-347, 374, 389-393, 440, 441)					
Epitope	**Residues**	**Conformation**	**Blocks**	**Mechanism**	**Color**
10E9	Chain A: 391, 394-395, 397, 435, 444, 446, 448, 504, 506 Chain B: 250, 340-341, 343, 345	Dimeric VP1	GII.4	Direct competition	Red

Figure 11. MAb 10E9 relation to HBGA binding site in GII.4 P domain. Golden color – HBGA binding pocket (413).

Linear B cell epitopes in norovirus

Though a majority of B cell epitopes identified by MAbs has been conformational, some MAbs have recognized linear epitopes mapping to S and P1 domains of the GI and GII genogroups (413). Amino acid sequences spanning the S domain are more conserved than those in the P1 domain (420, 424). These MAbs, mapping to linear epitopes, were found to be cross-reactive and have been used in diagnostic assays. However, their protective capacity remains to be evaluated (429-431).

3.7.3 VIRAL ESCAPE FROM BLOCKING ANTIBODIES

There are two factors determining the blocking capacity of the specific MAbs. The first is the amino acid sequence of the MAb epitope and the second is whether antibodies can get access to the epitope, which partly may be influenced by the flexibility of the P domain. A majority of HBGA

blocking antibodies identify epitopes that are within or proximal to the HBGA binding site. Accordingly, the direct binding of MAbs to the HBGA site should prevent viral infection. However, this may not be the case should an antigenic drift affect the P domain, which would eliminate the blocking effect of the MAbs. But, interestingly, reports have indicated that amino acid changes close to, but not in, the binding pocket are the ones that most affect the blocking ability of the MAbs (272). This could result in the complete loss of MAb binding without any critical change in the actual HBGA binding pocket. Furthermore, amino acid changes can lead to conformational changes of the HBGA pocket. This could be yet another mechanism to prevent MAbs from binding to the epitope (424). During evolution the virus could adjust changes to the amino acid sequence around the HBGA binding pocket and in this way expand the pool of potentially susceptible people to infect (423). Moreover, the norovirus capsid has been shown to be flexible depending on the temperature, and conserved epitopes, buried within the VLP at room temperature, can become exposed at 37°C (420). This feature might be particularly important for virus transmission (420). The "viral breathing" dependent on temperature is determined by the motif of amino acid residues called NERK (amino acids 310, 316, 484 and 493). Together these amino acids might comprise a proposed viral "breathing core" and regulate epitope presentation and ligand binding to the P domain (424). This notion suggests that exposure to changes in body temperatures might influence the binding efficiency of specific antibodies and, therefore, it should be considered when interpreting results from *in vitro* experiments. Interestingly, these temperature camouflaged epitopes could be particularly important targets for interventions based on MAb recognition.

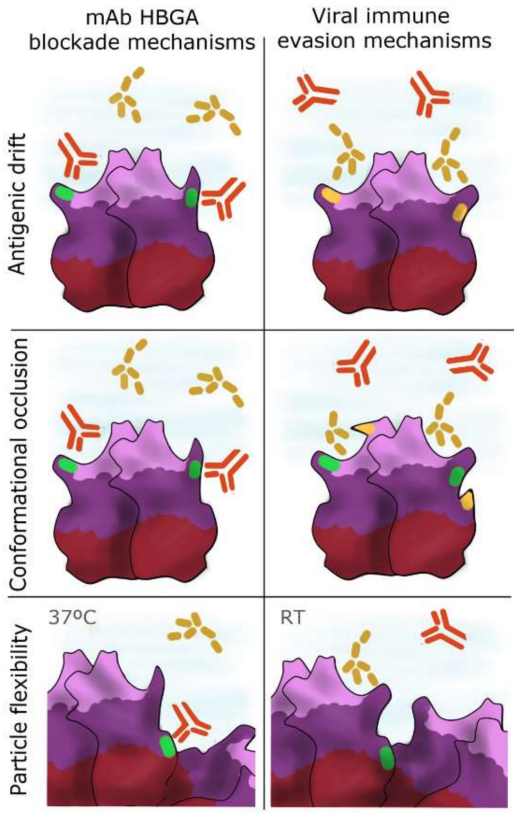


Figure 12. Mechanisms of MAb mediated blockade to HBGA binding pocket and viral immune escape strategies (413).

3.7.4 CELLULAR IMMUNITY

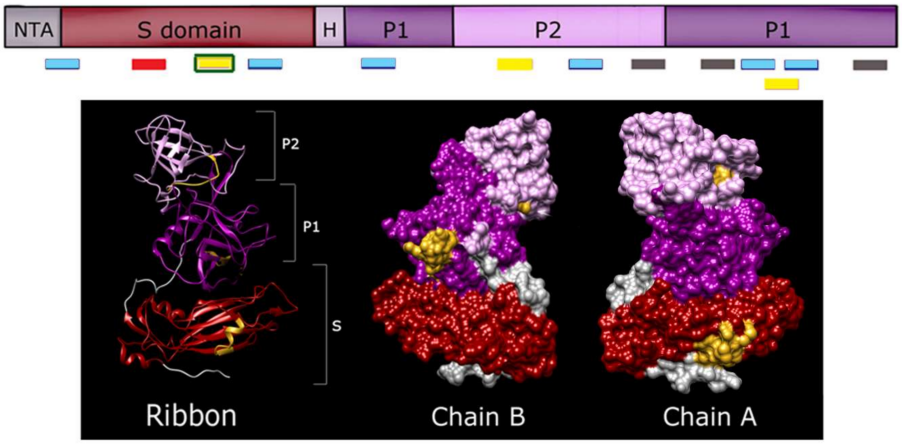
Human norovirus infection leads to recruitment of monocytes to the infected tissue and the production of cytokines, in particular IFN-γ, IL-2 and TNF-α (81, 432-435). Elevated levels of these cytokines were detected in human serum and stools within a few days in the course of infection. Concomitantly it was observed that Th1 and cytotoxic CD8+ T cell immunity were upregulated (415, 432, 436, 437). The detection of high levels of IL-10 at 3 days into the infection might reflect a down-modulation of the immune response, but this must be more thoroughly investigated (432, 436). Also IL-4, IL-5, IL-6 and IL-8 could be detected already after a few days into the infection (435, 436, 438) and these might represent Th2 and Tfh cell activity, which is critical for the B cell response. Whereas a majority of these findings have been made with peripheral blood cells further studies are needed to investigate local immune responses in the intestine, at the site of infection.

While no animal model exists for studies of HuNoV there are excellent models of murine NoV (MuNoV), which allow for detailed studies of protective immunity and viral pathogenesis. For example, MuNoV has been found to be endocytosed through the M cells that overlay the Peyer's patches (PPs) in the small intestine. The MuNoV is then transported into the PPs and can get access to cells also in the mesenteric lymph nodes (MLN). Thus, the MuNoV may infect the host cells differently and with a different tropism than that of HuNoV (439, 440). The MuNoV is also known to infect macrophages, DCs, B and T cells in the PPs (99, 102, 122, 439). Infected cells release type I IFNs, which initiate innate immune responses and play a key role in limiting MuNoV replication (441-443). Adaptive immunity against MuNoV is dominated by activated CD4 and CD8 T cells, as well as antibody producing plasma cells (335, 444). Indeed, specific CD4 T cells correlated with protection after re-exposure to MuNoV in mice (445). Nevertheless, it remains difficult to extrapolate findings in the mouse model to humans directly.

3.7.5 T CELL EPITOPE MAPPING

The diversity of HuNoV strains makes the identification of cross-reactive T cell epitopes difficult. Few challenge studies with the GI.1 or GII.2 norovirus genotypes have found cross-reactive T cells or IFN-γ responses to heterologous VLPs (415, 435). Another study identified varying T cell activation levels over the course of infection (436). Interestingly, gnotobiotic pigs immunized with human P particles intranasally or orally demonstrated activation of CD4 T cells in the intestinal and systemic secondary lymphoid tissues, and CD8 T cells in the duodenum (90). On the other hand, the same study found P particles to elicit weaker heterogeneous protection than VLPs, suggesting that the S domain is of importance for cross-reactivity. To date there have been only a few T cell epitopes identified for HuNoV (Fig. 13). Most of them were identified by stimulating peripheral blood T cells from VLP-immunized individuals, which were reported conserved epitopes shared by GI and GII strains (446, 447). Another example is the study that was conducted in mice and which identified T cell epitopes spanning the VP1 region of human GII.4-1999 strain, GII.4 genotype, or even genogroup GII (448). The peptides were restricted to either CD4 or CD8 T cell recognition. It should be emphasized that the results of the latter study disagree with earlier findings reported by Dr. R. Baric group (447). Whereas, both groups identified the same peptide in recall experiments it was claimed that one was reactive to CD4 T cells while the other one was reported as a CD8 T cell epitope. This discrepancy might be due to different mouse models, immunization routes or even reagents used for the studies. Another study

reported a human CD8 T cell epitope, which was cross-reactive in four patients of the same HLA-A(*)0201 (449). The 10 amino acid long peptide belongs to the S domain and is highly conserved across GI and GII genotypes, but not found in the GII.4 Sydney strain. Additional studies are needed to get a better picture of how specific CD4 and CD8 T cells can convey protective immunity and their location and dynamics in the affected tissues. Also, studies of the earlier history of exposure to HuNoV infection and individual HLA patterns in different age groups could contribute to a better understanding of how protection against HuNoV infections works. Overall, it remains of high importance to identify dominant T cell epitopes that are conserved among different strains of HuNoV in human.



MHC Source (Allele)	T cell restriction	Reactive region	Amino acids	Stimulatory activity confirmed against
Mouse	CD4+	VP1 – S domain	83-94	GI.1 (NV, WC-02)
Mouse	**CD4+**	**VP1 – P1 domain**	**461-473**	**GII.1*, GII.4 (FH-02, LV-87, LV-97, Hu-04, Sak-05*), GII.14***
Mouse	CD4+	VP1 – P1 domain	471-485	GII.4 (FH-02, LV-97*, Hu-04*)
Mouse	CD4+	VP1 – P1 domain	235-248	GII.4 (VA387)
Mouse	CD8+	VP1 – P2 domain	388-405	GV (MNV-CR6*)
Mouse	CD8+	VP1 – P1 domain	424-441	GV (MNV-CR6*)
Mouse (H-2kb)	CD8+	VP1 – P1 domain	519-527	GV (MNV-CR6, MNV-CW3)
Mouse	CD4+	VP1 – S domain	22-39	GII.4 (1999, NO, Sydney2012)
Mouse	CD4+	VP1 – S domain	176-193	GII.4 (1999, NO, Sydney2012)
Mouse	**CD8+**	**VP1 – P2 domain**	**318-326**	**GII.4 (1999, NO, Sydney2012), GII.12**
Mouse	CD4+	VP1 – P2 domain	344-354	GII.4 (1999)
Mouse	CD8+	VP1 – P1 domain	449-466	GII.4 (1999)
Human (HLA-A*02:01)	**CD8+**	**VP1 – S domain**	**139-148**	**GI.1, GI.3, GII.1, GII.2, GII.3*, GII.4 (1999, FH-02, Apeldoorn 2003, Hu-04, NO-09, Sydney2012), GII.10, GII.12, GII.17**

Figure 13. Identified T cell epitopes and their mapping to the VP1. Grey – MuNoV specific epitopes; red – GI – specific epitopes; blue – GII – specific epitopes; bolded peptides and yellow in 3D model – cross-genotype reactive epitopes. The 3D structure is of GI.1 VP1 (413).

3.8 COMPETING VACCINE STRATEGIES

While several norovirus vaccine candidates are under development or are being evaluated in clinical trials there is still a need to concentrate on preclinical investigations of HuNoV vaccine candidates. Two groups are developing VLP-based vaccines, where one vaccine contains GI.1 and GII.4 VLPs and is presently in Phase 2b clinical trials (Takeda), and another vaccine contains GI.3 and GII.4 VLPs and remains in preclinical studies. Two other groups have developed vaccines based on recombinant adenovirus serotype 5 vectors expressing norovirus VP1. One is based on a GI.1 norovirus sequence and is in a Phase 1 clinical trial (Vaxart), while the other is based on a GII.4 sequence and is undergoing preclinical studies. A fifth group has published extensive preclinical data on a GII.4 P particle-based vaccine (450). The Takeda bivalent vaccine is administered intramuscularly and contains adjuvants aluminum hydroxide and MPL. Though this vaccine induces specific serum antibody responses, increases HBGA-blockade antibody levels, stimulates plasmablast responses and memory B cells against norovirus vaccine strains, it has failed to demonstrated long-lasting protective immunity (145, 146, 451, 452). This is an essential aim for a mucosal vaccine against HuNoV. While intramuscular administration of the vaccine has poor chances to induce effective mucosal immune responses, the Vaxart vaccine was developed as a tablet for oral delivery. Lately its' safety and immunogenicity were confirmed in a Phase 1 clinical trial. This study showed norovirus-specific IgA antibodies in feces for up to 6 months following immunization (144). Further studies are awaited to confirm the efficacy of this vaccine. Noteworthy, the use of an adenovirus vector as a delivery system could potentially lower the vaccine efficacy as pre-existing adenovirus-specific antibodies could reduce the amount of vaccine Ag available following vaccination (351).

We have chosen to develop a novel mucosal subcomponent vaccine based on the inherent adjuvanticity of the CTA1-DD platform and selected peptides from the virus capsid. We have then explored the potential of our mucosal vaccine candidates by assessing the immunogenicity of the T and B cell epitopes and the ability to recognize the selected peptides by specific MAbs or serum antibodies from infected people. To this end we choose 15-mer peptides for determining binding sites for antibodies against epitopes of the Dijon strain. The identified B and T cell epitopes were selected on the basis of to providing strong cross-protective immunity resulting in better viral clearance, enhanced memory responses and increased protection against reinfection.

4 AIMS

To advance our current knowledge on the pathogenesis and prevention of gastrointestinal NoV infections this thesis has focused on two crucial issues. The first was to identify membrane elements that are required for HuNoV infection, specifically in epithelial cells of human intestinal enteroid cultures. The second was to identify highly immunogenic peptides from the human norovirus capsid for generating a subcomponent vaccine that stimulates strong virus-specific immune responses. Therefore, we focused on two challenges that are discussed along the thesis:

1. To characterize the membrane components of human gastrointestinal cells allowing for NoV infection.
2. To test the viability of a novel way to design a mucosal NoV subcomponent vaccine by employing information on VLP-specific monoclonal antibodies and their binding patterns to the capsid of the virus.

5 METHODOLOGICAL ASPECTS

This is an overview of the main methods used in this thesis work. A detailed description of each method can be found in the individual papers. Since, the work plan addressed two different fields of research, namely glycobiology and immunology, this is also reflected in the methods used.

Virus-like particles (VLPs)

It has been shown that VLPs preserve binding and antigenicity features of the native virus (312). Thus, we employed VLPs of different strains to study virus-glycan binding and virus immunogenicity. Paper I used norovirus GII.4 004/95M VLP (GenBank access number: AF080551.1), which was His-tagged to enable direct measurements of the VLP-glycan interactions. The polyhistidine tag was added at the C terminal of the VP1 and is described elsewhere (453). Paper II has used GII.4 Sydney VLP (GenBank access number: AFV08795.1), which represents the latest strain of known globally spread GII.4 genotypes. In Paper IV we used GII.4 Dijon VLPs (GenBank access number: AAL79839.1) to immunize mice and develop MAbs against this strain. We used the sequence of the GII.4 Dijon capsid to generate a peptide array to identify distinct peptides that the MAbs reacted with. All VLPs were produced in insect cells using the well-established baculovirus expression system (454). Since each VLP was made in a different laboratory minor differences exist in their protocols. For example, GII.4 004/95M and GII.4 Dijon VLPs are assembled of the VP1 capsid part only (455, 456), while the GII.4 Sydney VLP is a result of the VP1 and VP2 expression (457). Though the VP2 is important for the stability of the virion, but how it influences the whole VLP is less well understood. Nevertheless, we used GII.4 VLPs due to their higher prevalence in norovirus caused outbreaks.

Human intestinal enteroids (HIEs)

Human norovirus pathogenicity studies have been delayed for a long time due to the lack of study models. Just recently it became possible to replicate the virus in laboratory settings in stem cell derived HIEs. The stem cells are isolated from intestinal crypts obtained from human intestinal surgery or biopsy samples. These cultures retain the genetic and biologic properties of the donors, which can lead to the discovery of host-specific factors that affect susceptibility to infection and result in personalized approaches to treat individuals. HIEs recapitulate the natural intestinal epithelium and are non-transformed, physiologically active cultures that can be maintained endlessly.

They produce self-organizing structures that contain the multiple differentiated intestinal epithelial cell types including enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Since HIEs recapitulate the HBGA phenotype of the donor genotype, they are an excellent model to extend our understanding of how host HBGA and HuNoV interact and which specific requirements are needed to allow infection. In this thesis we analyzed the glycoconjugate and lipid composition of HIEs recapitulating the small intestine of 6 individuals of different HBGA status.

Glycosphingolipids

The reference GSLs used in Paper I, II and IV were purified and characterized as previously described (458-462). The type 1 chain GSLs were isolated from human meconium samples of either a single individual or individuals pooled according to their ABO blood group phenotype. The type 2 chain structures were purified from canine small intestine. The glycolipid extracts from an adult human intestinal epithelium were a gift from Prof. Michael Breimer and the GalCer and GlcCer references were a gift from Assoc. Prof. Niclas Karlsson.

Folch partitioning

The Folch partitioning method was introduced in 1957 (463). It describes a now classical procedure, which allows extraction of lipids from a tissue and separation of lipids based on their polarity into two phases, the lower phase and the upper phase, respectively. The lower phase containing non-polar lipids collects all the lipids, including sphingolipids with shorter glycan chains from a tissue, while the upper more polar phase contains glycosphingolipids with longer glycan chains, gangliosides and salts. Importantly, the highly polar glycolipids can be separated from other lipids by Folchs' partition and the denaturated proteins are found mainly in the disc in-between the two phases. Neutral glycolipids with 5-7 monosaccharides are found in both the lower and the upper phases, and the clear-cut between them is dependent on each glycolipid structure but also on the coexisting phospholipids, other lipids and the concentration of ions present in the lipid extract. It has been observed that if the method is applied to human erythrocyte glycolipids, the upper phase contains 90% of the GSL with more than 5 glycan residues and higher gangliosides, while the lower phase contains 90% of GSL with less than four carbohydrates residues (464). Furthermore, both phases can be used for further purification of sphingolipids and glycosphingolipids. For example, alkaline hydrolysis of the lower phase degrades all the phospholipids leaving the sphingolipid part of the sample. In

turn, the upper phase can, after a desalting step, also be used for further purification and characterization of glycosphingolipids and gangliosides. Overall, Folch partitioning is a very simple and efficient method to extract lipids from a tissue or cell line of interest. We used Folch partitioning in Paper II to characterize lipids and sphingolipids from HIEs.

Thin-layer chromatography (TLC)

TLC involves a stationary phase, which is a layer of particles of relatively uniform size (usually silica, alumina or cellulose) stuck onto a backing plate made of glass or metal. A complex mixture is applied on the plate as a spot or a band, is dried and the chromatogram is then developed in a suitable solvent, allowing the sample constituents to travel with different speed across the plate relating to the chemical and physical properties of the constituents present in the sample. Components of the sample have different preferences for the stationary and mobile phase. For instance, the sample mixture would separate according to the polarity of the solvents used over a silica coating. Subsequently the analytes are detected and identified based on their relative migration distance and based on their color reaction to chromogenic spray reagents ability to absorb light. For example, anisaldehyde stain is excellent for carbohydrate detection, while resorcinol is specific to visualize sialic acid containing structures. TLC may be used for both qualitative measurements as well as for semi/quantitative purposes. Since it is a fast, easy to handle and inexpensive procedure, it is one of the most widely used chromatography techniques. TLC was used to visualize extracted lipids from lower and upper phases from HIEs in Paper II.

Chromatogram binding assay (CBA)

CBA is a now classical method to study protein-GSL interactions. After developing a TLC of GSLs, the plate is coated with plastic and overlayed with the glycan binding protein of interest. The plastic coating protects the silica gel from detachment and covers the surface with a hydrophobic film, which improves the presentation of hydrophilic oligosaccharides (465). Though the method was originally established to study interactions between cholera toxin and gangliosides (466), it soon became extensively applicable for studies of other glycans binding proteins, including mono- and polyclonal antibodies, lectins, bacterial adhesins and viruses such as norovirus (292, 306), parvovirus B19 (467, 468) and other (465). The CBA method was thoroughly used in Paper II to phenotype the lipid extracts from HIEs using HBGA recognizing antibodies and to study the binding of GII.4 Sydney VLP to pure glycolipids.

Total internal reflection fluorescent microscopy (TIRFM)

Many studies have raised discussions about the nature of glycan-protein interactions and claimed that a static picture of glycan recognition might ignore differences in the presentation of the glycan epitope when compared to membrane bound glycans. The classical binding assays fail to encounter the complexity of glycan-protein interactions, including the fluidity of the membrane together with the potential lateral diffusion of membrane proteins and GSLs in the context of lipid rafts. Thus, studies of the role of specific glycoproteins or GSLs in viral entry require membrane-based assays. Indeed, such assays can unravel multiple questions regarding the very early steps of viral binding to cellular membranes, and also be applied as a screening method to identify viral receptors or attachment factors. TIRFM serves as an excellent platform to resolve both aspects (469).

TIRFM was first introduced in 1956 (470) and since then it has been extensively used to study the properties of events at cellular membranes (471) and of transmembrane biomolecules (472, 473). The specific feature of TIRFM is that it uses evanescent waves to selectively illuminate the region just above the glass surface, with zero background from fluorescence in the solution above. When an incident beam of light is projected at the glass-aqueous interface of the sample at an incident angle larger than the critical angle, it will be totally internally reflected. This leaves the electromagnetic evanescent waves on the surface of the glass, which exponentially decays as a function of perpendicular distance from the interface. Evanescent field penetration depths of <100nm can be readily achieved from total internal reflection at glass-aqueous interfaces, providing an opportunity to selectively excite fluorophores close to the interface. This allows a 5-10 fold advantage over the optical resolution achievable with confocal microscopy ($\approx$ 500 nm).

The technique consumes low amounts of material and is highly sensitive. It has the potential to detect association and dissociation events of vesicles binding to a single VLP on a lipid membrane. Furthermore, the setup is rather simple and the readout is rich in quantitative kinetics data, including analysis of different VLP-gycan interactions based on their binding strength and the real-time kinetics behavior of the GSL population over different durations of residence times. Most importantly, TIRFM may be of great use for optimization in drug design where the effect of inhibitors on either the VLP-vesicle or the VLP-glycan kinetics data might be important for choosing the most interesting candidates.

In this thesis, the TIRFM technique was exploited through different strategies in Papers I, II and IV, where protocols are described in greater detail.

Lipidomics

Lipidomics is a newly emerged analysis studying cellular lipids on a global scale based on analytical chemistry principles and novel technological tools, particularly mass spectrometry (MS). The discipline emerged in 2003 and has largely advanced due to the development of MS. A typical workflow for lipidomic analysis of biological samples starts with sample preparation, is followed by chromatographic separation and mass spectrometry-based analysis and is completed with data processing. Biological lipid extracts are typically complex, including the diversity of lipid classes/subclasses/molecular species and the vast dynamic range in the endogenous contents and concentrations of individual species. In order to reduce the complexity of lipid extracts and to improve the identification and quantification of individual species in the complex extracts, it is recommended to use liquid chromatography (LC)-based lipidomics. In addition, it allows the enrichment of the low abundance species. Overall, the LC-MS lipidomics identify lipid species from both chromatogram and mass spectral data, which adds valuable features to the species identification (474). The final output is qualitative and quantitative lipidomics data, which allow to characterize and to explore underlying mechanisms of lipid content and metabolism in health and disease, leading to applications in biomedical sciences, including the discovery of biomarkers, drug targets and guidance in precision medicine. The lipidomics work in Paper II was led by Assoc. Prof. Marcus Ståhlman at the Lipidomics Core Facility, University of Gothenburg.

Glycoproteomics

Glycoproteomics is used to identify and characterize proteins containing glycans and includes the identification of glycoproteins, elucidation of glycosylation sites and the structural analysis of glycans at each site of the glycoproteins. Since neither the proteome nor the transcriptome can accurately predict such a complex target, the glycoproteome must be analyzed unbiased, whether on a single glycoprotein or in a complex mixture of glycoproteins. Analysis of the complete set of glycoproteins from a biological sample, including their glycan structures and sites of attachment, is primarily dependent on the analysis of (often tryptic) glycopeptides, which combine physical and chemical characteristics of both peptides and glycans. Thus, in a reversed phase LC-MS/MS set up the glycopeptides often migrate in close proximity to their corresponding un-glycosylated peptides and also

fragment in MS2 at peptide bonds giving typical B- and Y- ions identifying the glycoprotein sequence carrying the glycan attachment site. Similarly, by using different fragmentation techniques and energies the glycopeptide parent ions will fragment their glycan parts at glycosidic bonds giving rise to diagnostic oxonium ions of released saccharides and sequence ions of the partially fragmented glycopeptides.

Glycomics

Different approaches and techniques are used to characterize released N-linked and O-linked glycans of soluble or membrane bound glycoproteins or released sulfated GAGs of proteoglycans.

In the case of the soluble glycoproteins, the N-glycans can be released enzymatically or chemically, separated by high performance liquid chromatography (HPLC) and sequenced by MS with or without further glycosidase treatments. The analysis of O-linked glycans would follow the same procedure, but has one important drawback that there is no single enzyme, which can release the O-linked glycans in general, which complicates the analysis of often large and complex O-glycoproteins. Thus chemical means are used for hydrolysis of O-linked glycans being bound to proteins or to ceramides. Moreover, MS of glycans has challenges to determine the isomeric nature of the constituent monosaccharide units, linkage configurations and positions. Since different structures can have the same mass, often co-elute in separation systems and fragment similarly (although not identically) in MS/MS analysis, it is also challenging to assign a full structural analysis of glycans with only one method. It is therefore recommended to deliver glycan structures as possible assignments from one technology and to confirm them by at least one additional technology. There is an increasing amount of bioinformatics tools and databases becoming available to handle all structural data obtained from proteomic, glycoproteomics and glycomic analyses but mass spectral analysis is still heavily dependent on the expert manual interpretation. Paper III includes an extensive analysis of glycoproteins found in HIEs, the interpretation of which was conducted mainly by Assoc. Prof Jonas Nilsson.

Monoclonal antibodies (MAbs)

While the immune response to an antigen is usually polyclonal in nature, in 1975 Köhler and Milstein were the first to describe the *in vitro* production of murine MAbs from hybridomas (475). Later technologies allowed to develop engineered human MAb on the basis of information gained from studies with

murine hybridomas. Today many functions of MAb technology are used. These include Fc region engineering, conjugation of drug to the MAbs, tissue targeting, and bispecific MAbs that can bind two independent epitopes. The *in vitro* phage display technology enables the generation of fully human compatible MAbs. While there is lack of information on whether the phage display generated MAbs behave differently in the clinical setting, this possibility has to be considered. Therefore, as a complementary technology to the classical hybridoma method phage display approached are highly attractive. MAb technology has had major impact on clinical treatments and they can serve as essential tools for diagnosis or therapeutic interventions. Also, MAbs are used heavily in pre-clinical research to identify immunogenic sites, epitope mapping, antigen detection, and in various neutralization assays. Moreover, MAbs can be used for gene cloning, protein purification, identification of cell surface markers, and analysis of different cell functions. They are also widely used in a number of immunoassays, for example for particle agglutination, radioimmunoassays, enzyme-linked immunosorbent assays, immunofluorescent antibody assays and immunohistology. Clearly, without MAbs-technology modern medicine would not be what is today. In current work we developed specific MAbs against HuNoV VLPs as presented in Paper IV.

Enzyme-linked immunosorbent assay (ELISA)

Since its' invention in 1971 by two Swedish scientists Eva Engvall and Peter Perlman (476), ELISA has offered a wide palate of possibilities used primarily for detection of specific proteins or antibodies against the specific proteins. Indeed, ELISA is considered to be a routine method in both clinical diagnostic practice and in experimental systems. The ELISA method provides quick and accurate determinations, is highly sensitive, and relatively simple to perform. The technology can easily be automated and it is convenient for a large number of applications. In our study presented in Paper IV, ELISA was used to identify MAb binding to peptides relevant to HuNoV infections.

Peptide array

Peptide arrays are powerful tools for investigation of protein-protein interactions. In contrast to classical epitope characterization methods like X-ray crystallography or multidimensional NMR, peptide arrays allow a large-scale epitope identification, and evaluation of possible cross-reactivities in defined MAbs. This technology provides detailed epitope information on virus binding MAbs. Peptide arrays can be synthesized on solid phase

surfaces, which simplifies handling of large numbers of peptides or MAbs, and can eliminate the need for identification of peptides by de-novo sequencing. Epitope mapping is used for screening of biologically active sites in any given protein. The peptide sequences are generated by shifting a frame with a distinct peptide length over a protein sequence. The smaller the shift of the frame, the more precise will be the localization of the binding region. Thus, in Paper IV we screened for MAb-reactivity to epitopes on the HuNoV capsid, and the anti-norovirus MAbs were tested for binding to 526 norovirus peptides of 15-mers and 14 amino acids overlap.

Immunizations

To stimulate antibody production *in vivo,* we immunized Balb/c mice through the classical peritoneal route. Next we isolated splenic lymphocytes for the *in vitro* production of MAbs. We extracted activated B cells from the spleen, and fused these with hybridoma cells in hypoxanthine-aminopterin-thymidine (HAT)-discriminating medium.

For mucosal immune responses we employed intranasal immunizations and evaluated the success by assessing antigen-specific T cell responses to recall antigen *in vitro* after isolating lymphocytes from the draining mediastinal lymph nodes (mLN). Additional analysis of antibody responses was carried out by ELISA, while thymidine incorporation was used for determining T cell proliferation and enzyme-linked immunospot assay (ELISPOT) was used for assessing cytokine production from antigen-specific T cells in the immunized mice.

Assays to measure T cell immune responses

We used H^3-labeled thymidine incorporation and IFN-γ ELISPOT to measure T cell responses. Thymidine is incorporated into the replicating DNA of cultured cells, which results in a quantitative measure of the rate of DNA synthesis. This rate is proportional to the rate of cell division and, hence, reflects T cell responses to a certain antigen. The ELISPOT method is quantitative for assessing cytokine secreting effector T cells. It is a sensitive method for detection of single T cell responses. Both methods are conducted *in vitro* and are technically simple, easy to perform, require small numbers of cells and reagents. The combination of data from these assays is used to more precisely validate the findings that we obtain.

In silico studies

Lately there has been a growing availability and refinement of computational resources and access to algorithms that can be applied for gaining better insights into the complex field of structure-and-function relationships between proteins. For instance, *in silico* methods can be used alone or in combination with experimental techniques for molecular-level understanding and protein engineering in drug design (477). The evolution of machine learning techniques has resulted in the development of large databases that can be exploited for different predictions of protein conformation, binding affinities and modes of ligands (dominant placement, conformation, orientation) bound to large protein complexes. Combinations of prediction tools can be applied to create solutions for problems in such areas as the design of vaccine compounds, drug-like molecules, or the discovery of novel materials. In Paper IV of this thesis *in silico* databases were consulted to optimize the design of the novel mucosal HuNoV vaccine formulation that we have developed.

6 KEY FINDINGS AND DISCUSSION

In late 2015, at the time when this PhD project was started, there was no efficient cell culture system available to study HuNoV in laboratory settings. The majority of experimental norovirus studies relied on HuNoV VLP and MuNoV models. The accumulated evidence from challenge studies and binding experiments was pointing to the importance of the HBGAs in single individual's susceptibility to the infection and norovirus VLP binding/attachment. Guided by the idea to explore the possibilities to design a novel mucosal vaccine against norovirus, we raised monoclonal antibodies against one of the HuNoV GII.4 genotype strains and conducted HBGA blocking experiments in a TIRF microscopy setting allowing for antigen presentation on a membrane model (Paper IV). However, testing of immunogenic compounds against norovirus was limited to only immunizations due to lack of an animal model to study HuNoV infection. Finally, the human norovirus field attained a revolutionary discovery from the group led by Dr. Mary K. Estes when they published that HuNoV can indeed be replicated in human intestinal enteroid cultures (121). This finding was a breakthrough and spurred us to initiate Paper II and Paper III, which could contribute to the receptor/attachment factor research and future model studies in the field. Furthermore, we introduced an advanced study of VLP-HBGA binding on the membrane model, where VLP binding could be traced directly (Paper I) and could have crucial implications for the studies of virus-host interactions.

6.1 PAPER I. NOROVIRUS VLPS FORM CLUSTERS UPON BINDING TO GLYCOSPHINGOLIPIDS

The main concept of this paper using current biomembrane physical chemistry is studies of the formation of lipid rafts and their interaction with viruses or biologically relevant nanoparticles. This paper specifically explores the interactions employing his-tagged VLPs of a HuNoV GII.4 strain and reference GSLs, H type 1 chain pentaglycosylceramide (H-1) and B type 1 chain hexaglycosylceramide (B-1), presented in supported lipid bilayers. Importantly, the paper is the first attempt to measure norovirus VLP binding dynamics directly on the membrane surface with post-binding labeled VLPs, thus, indicating that the binding of viruses or nanoparticles may induce deformations of the lipid bilayer. This advancement introduces measurements of events during and after VLP binding to the

receptor/attachment factors on the supported lipid bilayer, also providing a niche for future inhibition studies.

The binding affinity of the VLP to supported lipid bilayer hosting a range of GSL concentrations was compared using QCM-D to measure the corresponding binding kinetics. It was evident that the binding rate of VLP to GSLs was dependent on the concentration of the attachment factor on the surface. However, to achieve a firm attachment the VLP required at least 3 times lower concentrations of the B-1 antigen than of the H-1. This finding points out a difference in the VLP binding affinity towards different GSLs in the supported lipid bilayer, where the affinity to B-1 was higher than that to H-1. The affinity of the receptor/attachment factors might play an important role in the virus uptake mechanism.

The major difference observed upon the post-binding labeled VLP in binding to B-1 or H-1 antigens using TIRFM, was the heterogeneous versus the homogenous distribution of the fluorescent emission, respectively. The heterogeneous distribution appeared to originate from discrete objects of heterogeneous signal intensity and spatial extension, and was reproduced with both increasing concentrations of either the B-1 antigen or of the VLP. Importantly, there was no VLP aggregation in solution. In fact, if the aggregation in solution was the reason for the appearance of aggregates/clusters, it would have been observed irrespectively of the GSL type on the supported lipid bilayer. In addition, we could conclude that the size of aggregates/clusters was at least 3 times larger when the VLP was bound to the B-1 antigen-presenting surface. Furthermore, employment of pre-labeled VLPs revealed an increasing number of discrete objects as well as increasing intensity of the signal from these clusters over the time period studied. This could be explained either by the newly arriving VLP particles preferentially binding to the already bound particles or by the clustering of the laterally bound particles. Indeed, the latter explanation could be applied based on TIRFM time movies, but was difficult to analyze due to weak signals of single particles and the dye photobleaching effect on the signal. However, we could exclude the theoretical GSL-cluster formation by using a fucose-binding lectin, a competitor to the VLP for binding to the GSLs. After lectin addition, the VLP clusters became mobile. Taken together these observations lead to the speculation that the observed clusters are related to the interactions between the GSL in the supported lipid bilayer and the VLPs, which is supported by a more pronounced formation of clusters in the B-1 antigen presence where the ligand-receptor/attachment factor interaction is stronger. Thus, the formation of a greater number of bonds between the VLP and the B-1 antigen appears more favorable. Indeed, despite the weaker

energy of the individual interactions, the multivalent interactions could compensate and be strong enough to induce the deformation of the lipid bilayer locally at the binding sites. As a result, the VLP is partially wrapped or enveloped by the supported lipid bilayer. Importantly, these findings might reflect membrane-deformation-induced virus clustering on cell membranes and might function as signaling intermediates or low-energy transition states between the bound and internalized states *in vivo*. Similar membrane invaginations were previously reported on giant unilamellar vesicles with GII.4 Dijon VLP (296). Overall, Paper I emphasizes that the affinity of norovirus VLP/virus to the receptor/attachment factors most likely plays a critical role in viral endocytosis. Hence, this should be exploited in more detail in future studies of GSLs-interactions with the virus. Especially, studies of virus strains with significantly different binding affinities, coupled with labeling strategies capable of resolving structural changes at the molecular scale are highly warranted.

6.2 PAPERS II AND III. GLOBAL ANALYSIS OF LIPIDS, SPHINGOLIPIDS AND GLYCOPROTEINS FROM HUMAN INTESTINAL ENTEROIDS SUSCEPTIBLE OR RESISTANT TO HUMAN NOROVIRUS

The introduction of an infection model of HuNoV in HIEs has allowed for detailed studies aimed at revealing the virus-host interactions at the molecular level. This is an essential step towards the development of a human norovirus vaccine. HIEs provide a simplified model of human intestinal epithelium to study host-pathogen relationships *in vitro* and to mimic the pathobiology in the human gut. The importance of HBGAs in norovirus infection and binding has been extensively discussed earlier. The conceptual idea behind Papers II and III was to structurally characterize membrane components and HBGA epitopes on glycosphingolipids (Paper II) and glycoproteins (Paper III) from 7 different HIEs, relating the HBGA glycans to the host geno- and phenotypes, and to correlate these observations with the known infectious susceptibility of these cell cultures.

The major lipid components from all the HIEs were essentially identical and in the same quantitative ranges (Paper II). This finding is important not only as a support to the biological relevance of the HIEs model system, but also for the knowledge of complex lipid composition of differentiated adult intestinal cells, which can be applied in future studies of host-pathogen

interactions, drug delivery, or membrane models with a long-term goal to develop preventive and therapeutic treatments. However, important to note is that the general composition presented in Paper II does not address compositional asymmetries that apply over basolateral or apical parts of the membranes, nor does it differentiate between lipids from the plasma membranes or from endosomes and other subcellular organelles.

Sphingolipids, as a subset of cellular membrane components, have been shown to influence lipid raft formation and have been proposed to play a role in cell signaling, trafficking, sorting, polarization and apoptosis. We found that 10% of all lipids of HIEs were comprised of sphingolipids (Paper II). The most dominant sphingolipids were sphingomyelin, ceramide, sulfatide and non-acidic glycosphingolipids. Galactosylceramides (GalCers) made up about 0.2% of all the lipids, but the amount of complex glycosphingolipids in HIEs was not assessed. Considering the lipid distribution within biological membranes, it is important to understand that lipids, based on their chemical properties and enzymatic transporters, are distributed heterogeneously. If we estimate that the majority of glycosphingolipids are concentrated in the outer layer of the apical surface of the plasma membrane, this should lead to a high density of glycosphingolipids there compared to other lipid classes, which emphasizes the risk of underrating the importance to study glycosphingolipids. Based on TLC technique we could identify HBGA epitopes on sphingolipids of cells, which comprise amounts similar to that of GalCers. Importantly, we could pinpoint major ABH and Lewis antigens present on sphingolipids using monoclonal antibody overlays. The distribution of these antigens among different HIEs correlated to the known ABO, Lewis and secretor geno- and phenotype of the cell cultures. We could not detect HBGA epitopes on type 2 chains GSLs, but they were all dominated by type 1 chain (lactoseries) structures nor could we detect any sialic acids in the lipid extracts from HIEs. Recent studies have shown that the replication of HuNoV GII.4 strains in HIEs is dependent on the secretor gene. In fact, secretor negative HIEs are resistant to the GII.4 infection, while overexpression of *FUT2* gene in such cells leads to a secretor positive phenotype and allows for infection. Indeed, secretor-dependent GII.4 replication is not an unexpected finding, based on earlier literature data, but the lipid characterization of HIEs provides background data for a new experimental setting to further study different steps of this infection. Our TIRF microscopy binding experiments could, for the first time, assay lipid extracts from different HIEs as to their binding of HuNoV VLPs. Our data confirm that GII.4 VLPs, similar to the GII.4 virus in inocula, preferentially bind to secretor gene dependent structures in HIE lipid extracts containing HBGA epitopes on glycosphingolipids. This finding supports

glycosphingolipids to be one of the important attachment factors for human norovirus.

Our glycoproteomics approach to characterize glycoproteins from HIEs is the first global characterization of such cell cultures (Paper III). There was only one attempt to describe glycoproteins from human intestine before referring to findings from two individuals (478). Paper III represents findings from enteroids derived from stem cells from the small intestine of six individuals, and emphasizes the feasibility of the HIE experimental setting. Moreover, it allows us not only to look into the content of membrane proteins, but also to compare findings among different HIEs, thus among different individuals. We describe the diversity and complexity of tryptic glycopeptides not only from one protein, but from more than 100 glycoproteins. The majority of glycoproteins analyzed contained high-mannose N-linked glycans, while others were of the hybrid or complex types. Some identified glycosites contained only high-mannose glycoforms or only complex type glycans whereas others contained high-mannose, hybrid and complex types reflecting the glycosylation process from early synthesis in the ER to Golgi and final presentation on the plasma membrane. Though we looked into membrane proteins from HIEs, the employed extraction method does not allow the separation of plasma membrane from cellular organelles. Nevertheless, conclusions from the 40 most common glycoproteins among all 7 tested HIEs narrows the candidate list down to a possible viral receptor of proteinaceous nature for HuNoV. In fact, defining and comparing HBGA epitopes from just a few selected glycoproteins of 4 individual HIEs, suggested essentially the same glycan patterns irrespectively of the glycoprotein analyzed. Most importantly, this study found a strong correlation of HBGA epitopes on glycoproteins to the known geno- and phenotype of the cells, and has given support for a dependence of HBGA epitopes on glycoproteins for effective GII.4 HuNoV infection. Though it is difficult to resolve the chain type of HBGA epitopes using MS analyses, we argue that the glycans studied were dominated by, but not exclusively, the type 1 chain due to the correlation to an active secretor gene (*FUT2*) expression in human intestine. Finally, the results from both Paper II and Paper III are in agreement with each other's detailed characterization of glycosphingolipid and glycoprotein HBGA epitopes. Future studies should resolve if any of the described HBGA epitope carriers serve as a functional receptor to facilitate adhesion to target cells for HuNoV.

6.3 PAPER IV. TOWARDS A NOVEL MUCOSAL SUBCOMPONENT VACCINE AGAINST HUMAN NOROVIRUS INFECTION

We do not have a vaccine against HuNoV, but clinical trials are ongoing. However, the development of a vaccine against norovirus has been facing several challenges over the years. More specifically, the lack of experimental models has slowed the progress in vaccine development and left complete reliance on testing in clinical trials. Hence, the outcome of clinical testing is not preceded by pre-clinical testing of the vaccine and, thus, little information exists as to what to expect from a fully protective NoV vaccine. Therefore, the unavailability of a good animal model or the lack of a cell culturing system has greatly hampered vaccine development. A majority of experimental studies has been relying on VLPs or P particles as vaccine components, which are thought to retain most of the immunogenicity of the whole virus. Human challenge studies using different genogroups of the human virus have revealed the lack of cross-reactive antibodies against virus strains. Indeed, it is thought that antigenic drift among new strains of HuNoV is one of the important reasons for the lack of protection against the virus in humans. Furthermore, neither challenge studies nor the ongoing clinical phase trials of vaccine candidates could find immune responses lasting for more than 2 years or more than 12 months, respectively. Clearly, it appears that the VLP-vaccine poorly stimulates immunological memory, which is required from an effective NoV vaccine. Therefore, in Paper IV we have explored a novel strategy for generating a mucosal subcomponent vaccine and have designed the vaccine based on an adjuvant active fusion protein, CTA1-DD.

We developed MAbs against GII.4 norovirus VLP and tested their reactivities towards peptides, derived from the same VLP using a peptide array with overlapping 15-mers. One of the tested MAbs demonstrated reactivity to several fragments from the capsid, as well as cross-reactivity against antigenically distant norovirus VLPs. Furthermore, selected peptides were exposed on the capsid suggesting a good proximity to the MAb binding site. In addition, these peptides contained preserved amino acid sequences shared by a high proportion of norovirus strains. Interestingly, using *in silico* affinity prediction method, we could identify that one of the selected peptides was also found to host a high affinity to MHC class II molecules. Another CD4 T cell restricted peptide was identified after screening all the peptides from the same capsid by consulting the *in silico* algorithms. Furthermore, these MHC class II restricted peptides were predicted to be of high affinity in both mice

and humans. Finally, we could confirm the superior immunogenicity of soluble peptides in the mouse model, which supports the *in silico* predictions and the validity for our choice of strategy.

Next step was to generate different fusion proteins to test whether a peptide-specific response could be stimulated. The 5 peptides were inserted into the fusion protein, CTA1-DD, which has self-adjuvanting properties. To our surprise, none of the immunizations with the designed fusion proteins could stimulate an immune response, albeit the peptides had earlier proven to stimulate acceptable responses when given combined with an adjuvant. Because of this result we speculated that maybe it was possible that the critical epitopes were degraded and could not be presented by DCs. We investigated whether cathepsin D, could digest the fusion proteins into fragments that had lost their immunogenicity. The most abundant protease, found in endosomes of antigen presenting cells, hydrolyzed our fusion proteins *in vitro*, including the inserted peptides, into non-immunogenic fragments. This outcome indicated that the peptides were susceptible to cathepsin D cleavage and could possibly be exposed and hydrolyzed by the protease also *in vivo*. Therefore, we have considered redesigning of our fusion proteins to better resist degradation.

7 FUTURE PERSPECTIVES

Glycosphingolipids and glycoproteins from HIEs

HIEs open up tremendous new opportunities to investigate the complexity of glycosylation in individual intestinal epithelia at the molecular and transcriptional levels. It is now possible to analyze binding kinetics of vesicles prepared from lipid extracts of target cells to VLPs of different strains using TIRFM technology. Furthermore, single binding glycosphingolipid components can be examined with specific addition of cholesterol or sphingomyelin on a relevant glycerophospholipid background. Since VLPs can also be labeled, it will be of interest to trace binding dynamics on the surface prepared from lipid extracts of HIEs. Just by studying HIEs from six individuals led us into unexpected genotype vs. phenotype results expressed on glycosphingolipids in a single individual. This particular HIE line (J10) has been genotyped as secretor negative (*FUT2*-/-), Lewis negative (*FUT3*-/-) and A positive (*AO*), but the lipid extracts from J10 were repeatedly reactive to an anti-A antibody, indicating the presence of small amounts of an A-like antigen on glycosphingolipids. This unexpected and divergent finding will be addressed in greater detail in future studies, in which we will also investigate the presence of HBGA structures of different types on glycosphingolipids and glycoproteins. We will also include an extended glycome analysis of glycosphingolipids/glycoproteins in J10 cells, including genetically engineered J10 clones with overexpression of *FUT2* and *FUT3* genes. The role of glycoproteins in HuNoV infection of HIEs will require additional data from the infection model. This is possible to address through engineered HIEs losing their susceptibility to the infection, or other cell lines gaining sensitivity to the infection. These studies should then be complemented by enrichment and LC-MS/MS analysis of specific glycoproteins from susceptible HIE lines. In this way, HIE studies will identify receptors and attachment factors essential for HuNoV infection caused by specific virus strains/genotypes/genogroups. Indeed, HIEs allow studies of single individuals in a simplified experimental model, where numerous questions can be applied to study the physiology and pathology of intestinal cells from different segments of the gut. HIEs will allow the setup of different infectious and cancer models, therapeutic interventions, and even co-cultures with additional types of cells forming small functional organisms *ex vivo* in a single well. Certainly, extended panels of individual HIEs will open doors into precision medicine of advanced prevention and treatment strategies in humans, including strategies against HuNoV infections.

Redesign of fusion proteins

To optimize the design of our vaccine, we will improve the presentation of immunogenic peptides in the fusion protein. After identifying linear CD4 T-cell specific peptides, we now know these could be completely degraded by intracellular processing. The studies with cathepsin D have clearly identified that we can engineer the fusion protein to host less susceptible peptides for protease degradation. This could be improved by modifying the sensitive regions of the fusion protein to achieve a better protected set of peptides less easily digested by the antigen processing cell. This will, hopefully, preserve their immunogenicity. Using our strategy to identify immunogenic peptides it is also possible to identify peptides that are less sensitive to proteases. Subsequently, insertion of selected peptides into the CTA1-DD adjuvant platform adding novel cleavage sites to attract proteases away from the peptides could be a way forward. Similarly, the B cell epitopes would require a better presentation so that they are more effective in cross-binding the B cell receptor so that B cells become activated. We identified several peptides reactive with our MAb, including possible linear B cell epitopes. Presentation of these linear epitopes in the fusion protein construct failed due to the digestion by cathepsin D. An alternative method is to replace sensitive regions of the fusion protein by substitution of amino acids in the fusion protein. Indeed, the linear epitope may transform and undergo conformational changes when inserted into the fusion protein. Therefore, another strategy is to employ tagging of the peptides to scaffolds, which might be a better approach to preserve the presentation of linear B cell epitopes. Nevertheless, we will challenge the classical VLP-based vaccine approach and develop a mucosal subcomponent vaccine against HuNoV. We will use carefully selected peptides and the adjuvant CTA1-DD formulation in further refining the vaccine candidate. Future pre-clinical studies of vaccine candidates against norovirus would benefit from an engineered animal model expressing receptors and attachment factors allowing the HuNoV infection *in vivo*.

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