Evaluation Of The Novel P Particle Vaccine Candidate Against Human Norovirus Using The Gnotobiotic Pig Challenge Model

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biomedical and Veterinary Sciences

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October 29, 2014
Blacksburg, VA

Keywords: human norovirus, T cells, P particle vaccine, gnotobiotic pigs

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ABSTRACT

Noroviruses (NoVs) are a cause of nonbacterial acute gastroenteritis affecting all ages. NoV infections result in over 200,000 pediatric deaths in developing countries annually. Vaccine development has been hindered by the lack of cell culture systems and small animal models; thus, vaccine development has relied upon recombinant VP1 capsid proteins, such as virus-like particles (VLPs) and P particles. P particles are a novel vaccine candidate derived from expression of the VP1 protruding (P) domain, while VLPs require expression of the full-length VP1. My studies utilize a gnotobiotic (Gn) pig model of human NoV infection and diarrhea to evaluate the protective efficacy and T cell responses induced by P particles and to compare them with prior NoV infection (NoVPO) and VLPs. Gn pigs received 100 µg of P particles (LoPP) or VLPs, 250 µg P particles (HiPP), or adjuvants only intranasally at post-inoculation day (PID) 0, 10, and 21. Monophosphoryl lipid A and chitosan were used as mucosal adjuvants. At PID 28, a subset of pigs were orally challenged with 10 median infectious doses (ID50) NoV. NoVPO, LoPP, HiPP, and VLPs provided partial protection from diarrhea (83%, 47%, 60%, and 60% protection rates, respectively). Only NoVPO and HiPP provided protection from shedding (49% and 60% protection rates, respectively) and also reduced the number of CD25- regulatory T cells (Tregs) in duodenum following challenge. NoV primary infection induced an overall pro-Treg and low, transient Th1 response. LoPP induced stronger overall T cell responses compared to VLPs, including activated CD4+ T cells and duodenal CD8+IFN-γ+ T cells, suggesting that P particles are more immunogenic than VLPs. I also evaluated the effects of simvastatin, a cholesterol-reducing drug that increases NoV infectivity, on P particle vaccine efficacy.

Simvastatin abolished P particle-induced protection and significantly increased diarrhea severity. Simvastatin reduced total numbers of duodenal mononuclear cells, IFN-γ+ T cells pre-challenge, and Tregs post-challenge, indicating that simvastatin impairs development of immune system and immune responses. Findings from these studies elucidate potential mechanisms behind P particle-induced immunity and reveal the negative effects of simvastatin on NoV-induced protective immunity. The knowledge will facilitate the development of effective NoV vaccines.

Dedicated to my parents, grandparents, sister, and brother for all their support and love.

Acknowledgments

First, I would like to thank my adviser, Dr. Lijuan Yuan. Even though I had no virology or immunology experience, Dr. Yuan took a leap of faith and welcomed me into her lab. To say that this work would not be possible without Dr. Yuan is a dramatic understatement; I simply could not have asked for a better adviser, teacher, and mentor. In addition to financial support and research expertise, she has always been compassionate and thoughtful of any professional and personal situations that arose and I regard her as a mother away from home. Establishing a positive Ph.D. experience is a hard task; Dr. Yuan easily accomplished this goal.

I would like to thank all current and former members of the Yuan Lab, especially Tammy Bui Castellucci, Mariah Weiss, Ernawati Giri-Rachman, Ke Wen, Guohua Li, Fangning Liu, Xingdong Yang, Christine Tin, Sarah Peloquin, and Richard Kevorkian. Not only would I have been unable to complete this work without all of you, but I also appreciate your willingness to help and answer my questions. I hope that I have made your work as easy on you as you have all made it on me. I would also like to recognize Shaohua Lei, Ashwin Ramesh, and Dr. Erica Twitchell. I am confident they will represent the Yuan Lab well.

I would like to thank our collaborators at Cincinnati Children's Hospital Medical Center, Xi Jiang, Ming Tan, Ming Xia, and Weiming Zhong, and Takeda Vaccines U.S.A., Inc., Charles Robertson, Bryan Steadman, Thomas Foubert, Sheri Klas, and Ross Taylor. Not only did they provide reagents when necessary, but provided critical technical expertise. A turning point in this project came during a phone call with Dr. Steadman that helped me make improvements to the project. I would also like to acknowledge our funding source, the National Institute of Allergy and Infectious Disease, NIH (R01 subcontract grant R01AI089634-04).

I would like to thank TRACSS, specifically Pete Jobst, Karen Hall, Kim Allen, Andrea Pulliam, Julie Holdridge, Sarah Grace Kenley, and Shannon Viers for all their hard work and

support of our pigs throughout the entire study. I would also like to thank Drs. Tiffany Borjeson, Sherrie Clark-Deener, Nicole Lindstrom, Kevin Pelzer, and Marlice Vonck for their veterinary service for our animals. I would also like to thank Melissa Makris for flow cytometry service, specifically running all of my samples and answering any questions that I had.

I would like to thank all the members of my committee, Drs. Meng, Nanthakumar, Goldstein, and Roberts. I think this was the best committee I could have had. Each member brought a different expertise to not only each committee meeting, but also each interaction that I had with them. They motivated me to be prepared for each meeting and helped train me to be the best scientist that I could be. I have learned so much from each of them.

I would like to thank the Virginia-Maryland Regional College of Veterinary Medicine and BMVS program, including Dr. Roger Avery, Dr. Ansar Ahmed, Becky Jones, and Cindy Booth. The college and program have been very supportive over the years. Becky, I especially want to thank you for being another mom away from home, always having an open door, a willing ear, and kind words.

I would like to thank the Interdepartmental Microbiology Training Program, the Fralin Life Science Institute, and Dr. David Popham. I will never forget the program that brought me to Virginia Tech, financially supported me through my first year, and afforded me the ability to explore my interests. For that, I am grateful. I am also thankful to Drs. Cory and Casey Bernhards and Saylem DePasquale for their thoughtfulness and support throughout my recruitment to the program.

I would like to thank Dr. Jennifer Koehl. I did not have an interest in microbiology until my junior year of college. After one conversation with Dr. Koehl, I was hooked. Her enthusiasm for microbiology and academia is infectious; I would have not gone to graduate school if it was

not for my time in the Koehl Staph. “Make sure you do something you love and enjoy the people you come in contact with and the lessons they teach you.”

I would like to thank all the friends from IMTP, VMRCVM, BMVS, Rivermill Drinking Club, #Kibz, Jackalopes, Team Epsom Salt, and the Unusual Suspects that I have made during my stay. I would love to name everyone, but there is simply not enough room in this section. All of you are examples of what has made Virginia Tech, the BMVS program, and Blacksburg such a positive experience. Words cannot express enough how much I appreciate your support, kindness, love, and the memories we have created.

I would also like to thank my friends from SVC. It would have been easy to fall out of touch when we moved away; it speaks to your friendships that we did not. I know that the multiple visits in either direction sustained me during my toughest moments of school. I would also like to thank former Bearcats-turned-Hokies Desiree Melonas and Drs. Bethany and Thaddeus Pajak for answering all of my questions about Virginia Tech and Blacksburg during my recruitment.

I would finally like to thank my grandparents, Edith and the late John Kocher and Helen and Frank Pugliese, my parents, Kathy and Rick Kocher, my stepmother, Julie Kocher, and my siblings, Nicole Porter and Nathan Harris. They will be the first to tell you that this degree was a long and stressful process on not only me, but on them as well. They were always supportive of me, willing to listen when I needed them, and motivating (in their own individual ways). I love you all.

Attributions

Several colleagues contributed to the projects, research, writing, and editing of each chapter of this dissertation.

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Lijuan Yuan, Ph.D. (Department of Biomedical Sciences and Pathobiology) is currently an Associate Professor at VMRCVM and is the corresponding author on these manuscripts. She aided in project development, writing, and editing of the manuscripts.

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CHAPTER 1

Human norovirus replication, epidemiology, and immunity; challenges of norovirus vaccine development and identification of norovirus-specific immunity

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1.1 Introduction

Noroviruses are non-enveloped, positive-sense single stranded RNA viruses belonging to the *Caliciviridae* family. Norovirus is one of the leading causes of nonbacterial, acute gastroenteritis worldwide, accounting for approximately 60-90% of all gastroenteritis outbreaks (1-3). Following the introduction of rotavirus vaccines reduced the occurrence of rotavirus gastroenteritis, norovirus has become the leading cause of acute pediatric gastroenteritis in the United States (4). It is responsible for approximately 21-23 million cases and 800 deaths in the US (5, 6) and over 200,000 deaths in developing nations annually (7). However, these infections may be under-reported in the young and the old (8). GII.4 NoVs account for nearly 80% of NoV gastroenteritis each year (9). A vaccine with 50% efficacy would prevent up to 2.2 million cases and reduce economic disease burden by approximately $284 million in hospitals annually (10) and up to $2.1 billion over four years (11).

Despite years of attempts, an effective cell culture system for NoV has not been established, hindering the development of vaccines, antivirals, diagnostic assays, and pathogenesis studies. Thus, NoV vaccine development has focused on recombinant capsid proteins, such as virus-like particles (VLPs) and P particles. Both particles retain similar receptor binding patterns as the native virion (12, 13) and elicit similar immune responses in mice (14). VLPs are limited by their eukaryotic expression systems, which may limit their viability as vaccines in developing countries. Conversely, P particles require an *E. coli* expression system so they may be more cost-effective vaccines.

My dissertation research involves the evaluation of the novel P particle vaccine candidate using the gnotobiotic (Gn) pig model. This is the first analysis of the protective efficacy and T cell immunogenicity of the P particle vaccine using a higher order animal model. Additionally,

we evaluated how simvastatin, a cholesterol-reducing drug that increases NoV infectivity, impacts P particle efficacy. The knowledge of this research established the P particle as a viable vaccine alternative to virus-like particles.

1.2 Norovirus

1.2.1 Norovirus overview

NoVs were first isolated in 1972 from fecal filtrates of a 1968 acute gastroenteritis outbreak in a schoolhouse in Norwalk, OH (15). NoVs are a genus of the *Caliciviridae* family of viruses, along with four other genera: *Vesivirus*, *Lagovirus*, *Nebovirus*, and *Sapovirus* (16). Sapoviruses are the only other caliciviruses that can infect humans. NoVs are further divided into five genogroups (GI-GV). Human NoVs belong to GI, GII, and GIV. Animal NoVs include porcine (GII), bovine (GIII), and murine (GV). Recently, a canine NoV has been identified and may constitute a novel genogroup, GVI, which is closely related to GII and GIV NoVs (17).

NoV causes acute, self-limiting gastroenteritis in healthy individuals across all age groups, but can cause long-term disease and fecal virus shedding in immunocompromised patients (18-20). NoV shows a distinct winter seasonality, earning the nickname “winter vomiting disease” (6, 21). In a study of children in tertiary care facilities from 2008-2009, NoV gastroenteritis had a longer duration of vomiting, but reduced duration of diarrhea or fever compared to bacterial gastroenteritis (22). NoV-induced gastroenteritis resulted in a duration of illness that lasted longer than acute gastroenteritis episodes in emergency rooms, physician’s office, and inpatient facilities (23).

NoVs have an incubation period of approximately 24 hours (24) and a disease length of approximately 24 to 72 hours (25-27). The most common symptoms are nausea, vomiting, and

diarrhea (25), but can also include abdominal cramps, fever, headache, and dehydration (28, 29). NoVs are transmitted via the fecal-oral route (28, 30), but can also be transmitted via aerosolized vomitous droplets (31, 32), contaminated food or water (27, 33), and fomites (34-36). NoVs are easily transmitted in semi-closed units, such as cruise ships and senior care facilities (25, 37). The young are more commonly infected and the elderly are more prone to severe disease outcomes (27).

1.2.2 Virion and genome structure

NoVs are non-enveloped viruses. The icosahedral capsid is 27-38 nm in diameter with cup-shaped depressions (15, 28, 38) and composed of 180 copies of the major capsid protein, VP1, organized into 90 dimers and VP2, a minor structural protein (38-41). The VP1 capsid protein is further broken down into the shell (S) domain connected to the protruding (P) domain by a hinge. The S domain consists of 225 amino acids forming an eight-stranded antiparallel β sandwich (42). The first twenty N terminal amino acids in the S domain do not affect particle formation, but the rest are critical for particle formation. Additionally, removal of the P domain yielded smooth particles (43). The P domain is further divided into two subdomains, P1 and P2. The P2 subdomain consists of 116 amino acids that fold into six β sheets inserted between the amino acids that make up the P1 subdomain (40, 43). From amino acids 226-278, P1 folds into three β sheets, while the latter half of the P1 domain forms six β strains and an α helix (40). Deletions of internal amino acids in the P domain resulted in formation of larger particles, indicating that the P domain is crucial for the size and stability of the VLPs (43). Expression of the P domain without the S domain form P dimers that retain the ability to bind to HBGA receptors (44). The addition of cysteine residues to the P domain results in formation of twelve P

dimers (24-mer), commonly referred to as the P particle (45). While S and P1 domains are conserved, P2 is highly variable and the site of antigenic diversity (40). VP2 is a minor capsid protein whose expression upregulates expression and stability of VP1 (41, 46).

The NoV genome is 7.5 – 8.0 kb long with a 5' VPg protein and a 3' polyadenylated tail and encodes three open reading frames (ORFs) (47-49). The genome has untranslated regions (UTRs) of about 110 nt at the 5' end and 48 nt at the 3' end (50, 51). The UTRs are evolutionarily conserved and form stem loops required for replication and pathogenesis (52-55). The 5' subgenomic sequences are also evolutionarily conserved among *Caliciviridae* (56). RNA secondary structures have been identified within the first 150 base pairs of the 5' end, a 3' terminal hairpin and in the non-structural/structural (NS/S) junction; there is evidence these structures are common in caliciviruses (54). These structures are predicted to be in the anti-genomic strands as well (54). GII.4 NoVs also have extra stem loops upstream of the 3' terminal hairpin (54).

ORF1 encodes a non-structural polyprotein, while ORF2 and ORF3 encode the major structural protein, VP1, and minor structural protein, VP2, respectively (47, 57). The polyprotein is cleaved by a 3C-like protease at five cleavage sites, yielding six proteins: p48, helicase, p22, VPg, protease, and RNA-dependent RNA polymerase (57-59). The 5' end of ORF2 overlaps with the 3' end of ORF1 by 17-20 bp, creating the ORF1/2 junction (57). ORF3 overlaps with ORF2 by one amino acid (57). A fourth open reading frame, ORF4, was recently found encoded in murine NoV subgenomic RNA. ORF4 encodes virulence factor 1 (VF1) (53). So far, ORF4 has not been found in the other groups of NoVs.

The *Caliciviridae* genome shares similarities to the *Picornaviridae* genome, including genome length and polarity, VPg cap, poly(A) tail, and arrangement of non-structural proteins

(60-62). The NoV non-structural polyprotein contains seven non-structural proteins: 2A/B (p48), 2C (NTPase/helicase), 3A (p22), 3B (VPg), 3C (protease), and 3D (RNA-dependent RNA polymerase) (47). However, the *Picornaviridae* genomes also have an internal ribosome entry site for translation and encode their structural proteins on the 5' end of the genome and non-structural protein on the 3' end (62). Additionally, *Picornaviridae* genomes have 1 ORF, while *Caliciviridae* have at least three (63).

1.2.3 Functions of norovirus proteins

The human NoV genome encodes six non-structural and two structural proteins: p48, 2C-like NTPase/RNA helicase, 3A-like p22, VPg, 3C-like protease, RNA-dependent RNA polymerase, VP1, and VP2. The murine NoVs (GV) encode eight homologs (NS1/2, NS3, NS4, NS5, NS6, NS7, VP1, VP2) and have a ninth protein encoded by ORF4, VF1 (53). Human sapoviruses are the only other caliciviruses that express ORF4 (48, 53, 57, 64). The lack of a cell culture system has made the function of most NoV proteins unclear, but surrogate and replicon studies have helped form at least a cursory understanding for each.

Human NoV p48 and MNV NS1/2 make up the N terminal domain of ORF1. Previous studies have indicated that p48 is related to picornavirus proteins 2A and 2B (65). These proteins are involved with polyprotein processing and viroporin formation for virion release, respectively (62). Initial cell culture studies indicated that p48 co-localized with Golgi apparatus proteins and was associated with disassembly of the apparatus and disruption of intracellular protein trafficking (65, 66). Expression of p48 has been shown to enhance the activity of the NoV RNA-dependent RNA polymerase (67). Finally, p48 has been shown to bind VAP-A, a protein associated with vesicular transport in the cell (66). Surrogate studies indicate HNV analogs, such

as MNV NS1/2 and feline calicivirus p32, localize to the endoplasmic reticulum instead of the Golgi (68, 69). Further, MNV NS1/2 does not localize with double-stranded RNA or cytoplasmic replication complexes (70). These results indicate that p48 is likely involved in the formation and localization of NoV replication complexes. However, since p48 and NS1/2 localize to different cellular organelles in cell culture, more studies are needed to fully elucidate the roles of p48.

Human NoV NTPase/helicase and MNV NS3 are the least studied proteins. The NoV NTPase was previously identified as p41 and is similar to the picornaviral 2C protein (71). The 2C protein is required for intracellular membrane rearrangement and vesicle formation, but also binds to negative-stranded RNA, suggesting potential helicase activity (62). The NoV NTPase/helicase has an affinity for binding ATP and GTP, but does not exhibit typical helicase activity, including unwinding of the nucleic acid heteroduplex (71). Transfection of MNV NS3 in Vero cells resulted in cytoplasmic vesicle formation, but this effect was not observed in infected cells. Additionally, NS3 showed co-localization with mitochondria and other unidentified vesicular structures (69). Due to the limited research conducted on this protein, its full functions have not yet been identified.

Human NoV p22 is similar to picornavirus 3A proteins, but its function has not been determined in any other caliciviruses (72). The picornaviral 3A protein plays a role in immune evasion, inhibiting expression of MHCI and intracellular membrane transport (62). Transfection of the Norwalk genome into Huh-7 cells showed that p22 localizes to the Golgi and is associated with fragmented Golgi. Though p22 could not disrupt Golgi structure as much as 3A, the proteins prevented cellular protein secretion at similar levels. Further, NoVs contain a conserved motif in p22 that mediates vesicle trafficking from ER to Golgi (72). The effects of p22 may regulate pathogenesis of NoVs (73). MNV NS4 localized around the nucleus and associated with

Golgi and endosome markers, indicating a role in Golgi membrane recruitment for replication complex formation. Damage to the Golgi was also observed (69). Golgi disruption was also observed in cells infected with poliovirus and associated with 3A, a homolog to MNV NS4 (74-76). Another homolog, feline calicivirus p30, was also responsible for Golgi disruption (77). These results indicate that NoV p22 disrupts the cellular protein secretion pathway, including disruption of the Golgi. However, the purpose of this disruption and its role in NoV pathogenesis remains unclear.

Caliciviruses do not contain a 5' cap or internal ribosomal entry site on their genome, but instead have a covalently linked VPg (viral protein genome-linked), or NS5, protein (57, 63, 78). Picornaviruses also have a VPg protein, 3B, which serves as a cap on the viral genome, but also aid in formation of replication initiation complexes and RNA synthesis (62). Unlike *Caliciviridae, Picornaviridae* do not require intact VPg for infectivity due to the presence of an internal ribosome entry site (62, 63). However, the caliciviral VPg protein is larger than its picornaviral counterpart and is more closely related to plant VPg proteins (63). Studies on human NoV VPg have remained limited as a lack of a cell culture system restricts the amount of available VPg protein. One study with human VPg indicated that the C terminal domain primarily binds cellular eIF3, but has also been associated with eIF4GI, eIF2α, and eIF4E and results in the recruitment of ribosomes for translation, but do not inhibit host protein translation (63, 79). Surrogate studies with MNV and feline calicivirus (FCV) have aided the understanding of VPg function. First, VPg is required for successful FCV infection and replication (80). Whereas human NoV VPg interacts with eIF3, MNV and FCV VPg primarily interact with eIF4E and eIF4GI (81, 82). Further, FCV mRNA translation requires interaction with eIF4E as inhibition of this factor prevented translation (83). However, MNV mRNA translation was not

prevented by inhibition of eIF4E (81). These findings indicate that VPg serves as a cap for the NoV RNA genome, permitting translation of NoV proteins following infection.

The NoV protease is similar to the picornavirus 3C protease and is commonly referred to as a "3C-like protease," which cleaves the picornaviral non-structural polyprotein (47, 62, 69). Analysis of the rabbit hemorrhagic fever disease virus, a *Lagovirus*, showed that the caliciviruses encode a cysteine protease (84). The catalytic site of the human NoV protease consists of H30, E54, and C139 amino acids (85, 86) and cleaves at five highly conserved cleavage junctions (55, 58, 87-91): Q^{330}/G^{331}, Q^{696}/G^{697}, E^{875}/G^{876}, E^{1008}/A^{1009}, and E^{1189}/G^{1190} (58). An *in vitro* study indicated that the Q-G sites are co-translationally cleaved first, releasing p48, the NTPase/helicase, and a p22/VPg/protease/polymerase complex (58, 87). This complex is further processed to p22/VPg and protease/polymerase, but likely requires host factors for catalysis (58, 88, 90). Transfected MNV NS6 co-localized extensively with mitochondria (69). MNV NS6 has also been shown to co-localize with the microtubule organizing center around the nucleus, indicating NS6 may function in reorganization of the cellular cytoskeleton for replication complex formation (92).

The NoV genome encodes an RNA-dependent RNA polymerase (RdRp) that is difficult to study due to the lack of an *in vitro* cell culture system. The NoV RdRp is similar to the MNV NS7 polymerase (93). The MNV RdRp is a homodimer with dimeric lattices (94), but is also enzymatically active in its protease-polymerase precursor (95). Both MNV and NoV RdRps link nucleotides to the VPg protein as a step preceding RNA synthesis (96-98), but only NoV RdRp can begin genome replication initiation *de novo* (99, 100) or through a VPg-dependent pathway (101). However, *de novo* synthesis decreases following cellular Akt phosphorylation of RdRp Thr33 (102). Negative-sense RNAs are believed to be synthesized *de novo* and are enhanced

through interactions with the VP1 shell (S) domain (103), while VP2 reduced polymerase activity (67). During replication, MNV NS7 diffuses around the cytoplasm along with perinuclear localization in transfected cells (69, 70).

VP1 and VP2 are the major and minor structural capsid proteins, respectively (38, 40, 41). VP1 evolution is a large determinant in host evasion, emergence of novel strains, and alteration of virulence (18, 104-108). NoV host binding has been linked to an arginine cluster at the C-terminus of the protruding (P) domain (109). VP2 increases capsid stability and interacts with the shell domain of VP1 (43, 110-112).

1.2.4 Norovirus replication and pathogenesis

The lack of a cell culture system has inhibited the elucidation of the NoV replication cycle and its pathogenesis. The knowledge of NoV replication and pathogenesis is based on *in vitro* replicon systems and MNV or Tulane virus surrogate studies (113-120). Transfection of the NoV genome results in RNA replication, viral protein production, and virion release in cell culture (114). However, this system does not result in multiple rounds of replication, indicating that NoV's fastidious nature results from complications in viral attachment, entry, uncoating, or a combination of these (114). Surrogate studies with MNV indicate replication begins approximately 6 hours post-infection and has undergone multiple cycles by 8 hours (121).

Human NoVs bind to histo-blood group antigens (HBGAs), but other unidentified cofactors may be required for viral binding, entry, and uncoating. Viral entry and uncoating may be pH-independent (122), but dependent on cholesterol and dynamin II expression (123, 124). NoVs have been shown to differentially alter cholesterol profiles in cell culture (125) and NoV infectivity increases in the presence of cholesterol biosynthesis inhibitors *in vitro* (125) and in

Gn pigs (126, 127). VLPs binding induced membrane invaginations, which may be a step in viral entry (128). Intestinal microfold (M) cells are critical for MNV replication and serve as port for the virus to cross the intestinal epithelial barrier (129, 130). However, NoVs have been detected in intestinal epithelial cells *in vitro* (131, 132) and *in vivo* (126, 127, 133, 134).

Following uncoating, the NoV VPg protein recruits cellular initiation factor eIF4E (81, 83) and eIF3 (79) then eIF4G and eIF4A proteins to form the translation pre-initiation complex (81, 115, 117). Secondary structures in the 5' end of the genomic RNA interact with host proteins La, PCBP-2, hnRNPL, and polypyrimidine tract-binding protein (PTB) (51, 54). Terminal 3' hairpins in the Norwalk genome interact with La, PTB, and poly(A) binding protein (PABP) (50, 54). The secondary structures have been found to bind other cellular proteins, including vigilin, binds cholesterol and RNA, and DDX3, a cellular RNA helicase (135). PCBP-2 and hnRNP A1 stabilize circularization of the RNA genome (136, 137).

VP1 and VP2 are translated from subgenomic RNA. HNV VP2 is produced by translation/reinitiation (138) and bovine NoV VP1 is translated in this manner as well (139). VP2 translation/reinitiation is regulated by the termination upstream ribosomal binding sites (TURBS) (140). These sites consist of three motifs with only Motif 1 (UGGGA) being conserved among caliciviruses, though Motifs 2 and 2* are found in similar positions (140-142). For HNVs, the TURBS position the ribosome at the start site, but ribosomes are capable of scanning downstream for alternate start sites (140).

The non-structural proteins are co- and post-translationally cleaved by the viral protease (58, 95) preceding formation of the viral replication complex. The replication complex forms when capsid proteins VP1 and VP2 co-localize with the polymerase and genome intermediate dsRNA (69). MNVs localize and interact with several cellular structures, including the Golgi

and endoplasmic reticulum, which are involved in vesicle formation (69, 70). MNV non-structural proteins have also been shown to interact with the microtubule organizing center surrounding the nucleus (92). HuNV proteins p48 and p22 inhibit protein secretion by Golgi disassembly or impaired trafficking from ER to Golgi (65, 66, 72, 73).

The NoV RdRp is believed to synthesize negative-sense genomic and subgenomic RNAs *de novo* (103). There is evidence that the parental or newly synthesized VP1 protein's shell domain interacts with the RdRp to stimulate *de novo* synthesis of these negative-stranded RNAs (103). Following synthesis of negative-stranded RNAs, synthesis of positive-sense RNAs in a VPg-dependent manner (81). The RdRp covalently attaches VPg via its terminal tyrosine residue to the terminal guanine on the NoV genome (67), similar to picornaviruses (62). Positive-sense subgenomic RNA is then synthesized by either premature termination during negative-sense genomic RNA synthesis or secondary stem-loop structures serving as a promoter upstream of ORF2 in negative-sense RNA (54).

NoV assembly is largely unclear. VP1 is capable of self-assembling into VLPs, indicating that cellular chaperone proteins are unnecessary (43) and RNA is likely packaged during capsid assembly (40, 42). VP2 may interact with the viral genome and localize to the interior of the VP1 protein (143). Even less is known about NoV exit from the cell. Apoptosis has been observed in intestinal biopsies following NoV infection (126, 133, 144-147). Further, MNV production is reduced by inhibition of apoptosis (147). Expression of the MNV ORF1 polyprotein *in vitro* was sufficient to induce apoptosis and down-regulate survivin (145), which was also observed in MNV-infected cells (144).

Though there are no commercially available antivirals for NoVs, antiviral development studies have been conducted. Development of NoV antiviral agents have primarily focused on

inhibition of RNA synthesis, specifically inhibition of the RNA-dependent RNA polymerase (148-151). These compounds have different inhibitory mechanisms, including blocking RNA exit from the enzyme complex (148), binding to the RdRp active site (150), or allosteric inhibition (149). Development of other NoV antiviral agents has focused on isolation of HBGA active substances to prevent NoV binding to host salivary or mucosal HBGAs (152). Norwalk virus has also been shown to be susceptible to ribavirin, a guanosine analog, *in vitro* (153). Finally, RNAi of the Tulane virus capsid proteins resulted in decreased viral titers and protein production (118).

1.2.5 Norovirus receptors

Histo-blood group antigens (HBGAs) serve as the receptors for NoVs (154, 155) and other caliciviruses (156). HBGAs are carbohydrate moieties expressed on red blood cells, gastrointestinal and respiratory epithelial cells, and in biological fluids (157) and are broken into several families: Lewis, secretor, and ABO (158, 159) that are further sub-categorized into type 1 and type 2 carbohydrates (160). Type 1 HBGAs bind NoVs more strongly than type 2 HBGAs, while GII.4 NoVs have stronger binding to all HBGAs (160). The binding patterns of NoV to HBGAs are strain-specific (152, 161-166) and is mediated by hydrogen bonds with the VP1 P2 domain (167), which contains a binding pocket with a conserved RGD/K amino acid motif surrounded by strain-specific amino acids that determine HBGA binding (168-170). Secretor status appears to be the best predictor of NoV susceptibility based on expression of the *FUT2* gene (162, 171, 172). HBGAs are also expressed in shellfish, which may contribute to bioaccumulation (173-175).

Studies have been conducted to elucidate the HBGA phenotype that confers NoV susceptibility. A study of NoV-infected children revealed all GII.4 cases involved secretors (H1+ Lewis b and/or Lewis y) or partial secretors (Lewis a and b or Lewis x and y), but not non-secretors (162). However, there were five cases of non-secretors infected with GII.3 NoV (162). Gnotobiotic (Gn) pigs also express HBGAs on their intestinal tissues (166). Norwalk- and GII.4-derived VLPs bound A+ or H+ HBGAs in Gn pigs, while GII.1- and GII.3-derived VLPs did not bind A+ tissues (166). Additionally, A+ and H+ Gn pigs had increased viral shedding than A- and H- pigs (166).

HBGAs also appear to play a role in NoV evolution. For instance, rotavirus, another enteric virus, binds to HBGAs, but does not display the same binding patterns as NoVs (176). This indicates that HBGA selection pressure may have contributed to the emergence of genogroup-specific binding patterns (177) in addition to new binding patterns to outcompete other viruses (176). The strain-specific HBGA binding properties indicate differences in binding pockets, but since all NoVs still retain similar binding properties, convergent evolution likely plays a role in NoV diversity (177, 178).

More recent studies have raised questions about HBGAs serving as the main receptor for NoV or merely acting as a cofactor. Murine NoVs have shown strain-specific binding to terminal sialic acid residues (179) and glycolipids or glycoproteins (180). Another study found NoV binding to colorectal cancer cell line Caco-2 was dependent on cellular differentiation status and not on presence of HBGAs (181). Further, NoVs have also been shown to bind to HBGA-like substances on enteric bacteria (182). The function of this binding is unknown; enteric bacteria could aid viral entry in the host or may neutralize NoVs (182). A recent study with P particles indicated that both of these hypotheses could be true; probiotics can outcompete P particles *in*

vitro, but cells pre-treated with probiotics or P particles resulted in increased P particle binding (183). However, co-infection with CagA+ *Helicobacter pylori* resulted in expression of *FUT2* by previously non-secretors, suggesting that bacteria play a role in NoV infection and disease (184). These results indicate pathogenic bacteria may increase NoV infectivity, while probiotics may inhibit NoV binding.

1.3 Norovirus transmission and economic burden

1.3.1 Transmission routes

NoV transmission occurs primarily via the fecal-oral route, but can also occur via aerosolized vomitous, contaminated fomites, contaminated food or water, or person-to-person (185). Zoonotic transmission from porcine, bovine, and canine animals remain hypothetical (185), though antibodies to canine NoVs have been detected in humans (186, 187). NoV is theoretically capable of being spread on currency, though this has yet to be proven (188). Several factors increase NoV infectivity, including prolonged shedding (189, 190), asymptomatic shedding (191-196), persistence on environmental surfaces and food (197-202), perceived ease of transfer between surfaces (36, 203, 204), and a low infectious dose (approximately 18 viral particles) (205). The median infectious dose (ID50) for GI.1 NoV is approximately 1320 and 2800 genomic equivalents in O and A positive secretor positive-humans and in all secretor-positive humans, respectively (206).

NoV is one of the most common nosocomial infections in the United States (207-209) with patient-to-patient transmission the most common method and symptomatic episodes are more likely to result in nosocomial transmission than asymptomatic infections (210, 211). Analysis of environmental samples from hospital wards indicated similarity to NoV-positive

samples from patients (208). This indicates a need for proper decontamination or closing of hospital units to prevent nosocomial infection and spread throughout the hospital (208, 212).

NoV can remain infectious following contamination of food at any point in food processing. Recent studies examining transfer of NoVs during food handling and preparation have yielded inconsistent results (36, 203, 204, 213, 214). NoV was transferred from produce to gloves more easily than vice versa (204). Norovirus can remain on mechanical slicers following slicing of contaminated food, though the transfer amount is variable (214). Simulated food handling showed that NoV-contaminated gloves used during food preparation transmitted virus particles more efficiently to food than vice versa (203). The authors estimated that contamination of gloves with 10^3 NoV copies ensures the transfer of an infectious NoV to other surfaces (203).

1.3.2 Economic burden

NoVs have become the most common cause of acute gastroenteritis in developed countries since the implementation of rotavirus vaccines (4, 23, 215-218). NoV is highly prevalent; it causes an estimated 267 million infections (219) and 200,000 deaths worldwide (7) and 5,000 quality-adjusted life years in the United States annually (220). However, its worldwide economic burden is unknown. Current estimates are based on individual countries and vary based on NoV genotype and public health response (221). For instance, the length of hospital stays for NoV gastroenteritis is associated with increased duration and severity of diarrhea, resulting in increased economic burden on hospitals (22) and earlier response to NoV outbreak management and smaller hospital wards are associated with decreased costs (222). Similarly, in Australia, outbreak duration in residential care facilities was positively associated with length of time to notify public health units (221).

Total acute gastroenteritis accounted for approximately $3.88 billion from 2006-2011; of this cost NoV and rotavirus accounted for 7% of the burden (23). In the United States, NoV gastroenteritis resulted in $284 million in healthcare charges in children from 2001-2009 (10), $180 million to $355 million in total healthcare costs from 2006-2011 (23), and $2 billion in total economic burden annually (220). A nosocomial NoV outbreak cost approximately $650,000 in 2007 (212), but these costs cannot be extrapolated. Similarly, a 2008 waterborne NoV outbreak accounted for 8.7 million Swedish krona (approximately $1.2 million) in economic costs (223). Alternatively, NoV's economic costs in the developing world are more varied and difficult to estimate. Studies focused on total acute gastroenteritis cost have estimated annual costs at a maximum of $8.25 million in Barbados (224), $1.3 million in Dominica (225), $3.9 million in Saint Lucia (226), $2.3 million in Guyana (227), and up to $20 million in Trinidad and Tobago (228).

Based on these estimates, the potential economic impact of a NoV vaccine is apparent. Computer models have shown an effective vaccine (with 50% efficacy) would prevent up to 2.2 million cases annually and reduce NoV burden by $2.1 billion over four years (11). Another study shows a NoV vaccine would reduce cost of care in deployed military personnel by approximately $1.7 million (229).

1.3.3 Complications from norovirus infection

NoV is not believed to be the causative agent of long-term intestinal disorders, such as Crohn's disease or inflammatory bowel disease (IBD) (230-232), though it may worsen IBD symptoms (233). However, asymptomatic NoV infection may disrupt gut microbiota and contribute to the development of Crohn's disease (234, 235). However, NoV has been associated

with several intestinal sequelae, such as pneumatosis intestinalis, or gas cysts in the bowel wall (236, 237), postinfectious irritable bowel syndrome (PI-IBS) (238, 239), and spontaneous bowel perforation (240). NoV also increased indigestion, constipation, and gastroesophageal reflux disease (GERD) in military recruits (241). Co-occurrence of NoV infection and ischemic colitis resulted in bloody diarrhea (242).

NoV's effects on gut microbiota may explain in whole or in part the intestinal complications, though research in this area remains limited. Mice infected with MNV-1 altered the gut microbiota profile to that of malnourished mice, including decreased *Bacteroides*/*Firmicutes* ratios (243). However, a previous study showed that MNV infection does not alter the murine microbiota following infection (244). Human NoV decreased *Bacteroides* and increased *Proteobacteria*, but did not affect *Firmicutes* in a small subset (7 of 38) of infected patients (235). The increase in *Proteobacteria* was largely comprised of non-enteropathogenic *E. coli* (235). These microbiota changes could lead to future complications from pathogenic bacteria. Further investigation into NoV's impact on gut microbiota and its effects is needed.

NoV has also been linked to various systemic complications, including febrile agranulocytosis (245), Guillain-Barré syndrome and Miller-Fisher syndrome, a variant of Guillain-Barré (246, 247), convulsions or seizures (248-252), encephalitis or encephalopathy (253-255), arthritis (256), renal failure (257, 258), increased creatinine and decreased potassium levels (258), and liver injury (259). Encephalopathy may result from NoV-induced immune responses (253), though NoV RNA has been detected in blood of a child with NoV-induced seizures (250). The prevalence of these complications remains unknown.

Chronic NoV gastroenteritis can result from a variety of conditions that compromise the host immune system, including cancer (260-264), organ transplants (265-268), and HIV (269). However, the transplant recipients responded to immunoglobulin treatment (265), while the leukemia patients did not (260). Additionally, chronic NoV infection caused the development of hemophagocytic lymphohistiocystis in a child following bone marrow transplantation (262).

1.3.4 Risk factors for norovirus infection

NoV is a highly infectious virus with as few as 18 viral particles able to cause infection (205). We are still learning of the risk factors associated with infection, disease, and transmission. The most well-recognized risk factors for NoV infection are individuals that are HBGA type O (270) and positive secretors (165, 271-273). It is also well-known that NoV has a winter seasonality (21, 274-276), but there are several other factors affecting NoV disease severity, including, among others, strain (206, 277), environment (274, 278), host age (258, 279), and immune status (260, 261, 263, 265-269, 280).

The primary risk factor for NoV community transmission involves semi-closed environments, including retirement communities, military units, and cruise ships. An association has been established showing that NoV is more easily transmitted in long-term healthcare facilities than hospitals (281). More recent studies indicated that norovirus was more common in suburban locations and is positively correlated with the number of children in a population and increased family size (282), as children <5 years of age are more infectious than older children or adults (279). Children <15 years of age are more likely to present with vomiting (283), which, combined with high titers of NoV in vomitus (206), increases the risk of transmission. Additionally, improper disinfection following outbreaks can lead to contamination of previously

clean environments or recurring infections (34, 208, 284). Notification of public health units have been inconsistently linked with reduced transmission in long-term facilities (221, 285).

Strain also plays a role in transmission as Snow Mountain virus (GII.2) resulted in more disease symptoms and more painful symptoms compared to Norwalk virus (GI.1) in humans, but Norwalk virus resulted in increased viral shedding titers and duration and increased asymptomatic infections (277). Additionally, GI NoVs are more commonly associated with waterborne outbreaks, whereas nosocomial and winter outbreaks are more commonly associated with GII NoVs (274).

A competent immune system limits NoV to an acute infection, while immunocompromised hosts are more likely to develop chronic infection or other complications (286-288). Immunocompromised hosts include the young or elderly (258, 289), HIV/AIDS patients (269), and organ transplant or graft recipients (258, 263-265, 267, 290-293). Typical clinical outcomes from NoV infection in immunocompromised hosts include longer durations of disease and shedding (258, 289), while cardiovascular disease and renal transplants increase the risk for secondary complications (258). Similarly, immunocompromised mice infected with MNV developed inflammation in the liver and lungs (294). It is also worth noting that chronic infections likely serve as a reservoir for NoV evolution (18, 295, 296) and chronic NoV shedders (297) can lead to the emergence of new NoV strains.

Malnutrition has several effects on MNV infection and disease, including more severe disease, impaired viral clearance and protective immunity, and enhanced viral evolution (243). However, pre-infection gut microbiota were not correlated with susceptibility to NoV infection (235). These results could help explain the increased fatalities in developing countries (6, 7) and may also help explain emerging strains.

Another risk factor for NoV infection is simvastatin, a cholesterol-reducing drug that inhibits HMG-CoA reductase and increases LDL receptors (298). Simvastatin has been examined as a potential treatment for chronic or autoimmune diseases, including rheumatoid arthritis (299-301), multiple sclerosis (302), and periodontitis (303, 304). Simvastatin's anti-inflammatory effects result from reducing IFN-induced MHCII expression through inhibition of the CIITA gene (305, 306), which can lead to reduced T cell proliferation (307), reduced production of IL-2 and IFN-γ (308), reduced CD4/CD8 and Th1/Th2 ratios (301), and increased Tregs (309, 310). Previous studies have shown simvastatin treatment of NoV-replicon bearing cells increases viral RNA and proteins *in vitro* (125). Studies in Gn pigs have also shown that simvastatin not only increases shedding duration and titers (127), but also increases the incidence of diarrhea (126).

1.4 Norovirus immunity

1.4.1 Innate immunity

Human norovirus immunity studies have been limited due to the lack of a small animal model. Due to the short incubation period and length of NoV disease, innate immunity is thought to be more important for clearance than adaptive responses (286). NoV surrogates have been used to examine the various mechanisms used by the innate immune system to control NoV replication *in vivo* and *in vitro*. MNV infection is detected by melanoma differentiation-associated protein 5 (MDA5), but not retinoic acid-inducible gene 1 (RIG-I) or TLR3 (114, 311). Following MDA5 recognition, interferon regulatory factors (IRF) 1, 3, 5, and 7 regulate the anti-NoV interferon (IFN) responses *in vivo* (312-314). Mice deficient in IRF-3, IRF-7, or MDA5 displayed increased MNV titers in macrophages and dendritic cells, viral dissemination to

secondary tissues, and delayed cytokine responses (311, 313). An additional knockout of IRF-5 resulted in increased lethality following challenge (312). However, macrophages still produced low levels of IFN that may be sufficient to control viral infection (313). Additionally, Murine NoVs (MNV) have a fourth opening reading frame that encodes virulence factor 1 (VF1) and is not found in other NoVs. VF1 was shown to delay apoptosis and innate immune responses, but VF1-deficient MNV were attenuated *in vivo* (53).

Innate IFNs are also important in controlling MNV infection and replication. The roles of IFNs have relied on *in vitro* and *in vivo* studies. IFN-α, IFN-β, and IFN-γ all cleared NoV replicons from cells and reduced MNV titers in permissive cells *in vitro* (116, 121, 153). These IFN effects had differential mechanisms, as IFN-γ signaling was dependent on RNA-activated protein kinase (PKR), while IFN-β was capable of inhibiting a late stage in MNV replication (121). Type I IFNs are important in controlling MNV infection (315), but do not target viral entry or genome uncoating (121). However, mice lacking receptors for IFN-α, IFN-β, and IFN-γ were more susceptible to lethal MNV infection, while mice lacking either IFN-α/β or IFN-γ receptors were not, indicating a compensatory mechanism between the IFNs in single knockout mice (286, 313).

Signal transducer and activator of transcription 1 (STAT-1) is currently thought to be a critical innate mechanism for controlling NoV infection and dissemination and is thought to be a contributor to the fastidious nature of NoV *in vitro* (116, 286, 315-318). Porcine enteric calicivirus (PEC), a NoV surrogate, is able to be cultured *in vitro*, but only in the presence of intestinal contents containing bile acids; these cultures exhibited increased cyclic AMP and decreased phosphorylation of STAT-1 (316, 319-321). Clearance of NoV replicons from cells with IFN-α was associated with a significant increase in STAT-1 (116). STAT-1 prevented

intestinal viral replication and titers, intestinal apoptosis, viral dissemination to secondary lymphoid tissues, and prevents clinical disease in mice following oral MNV infection (286, 318). MNV infection was more lethal in STAT1-deficient mice even with fully functional T and B cells and PKR (286). The increased mortality in STAT1-deficient mice is consistent with the findings in mice lacking receptors for IFNα/β and IFN-γ, as IFN signaling follows the JAK/STAT pathway to produce an antiviral state (286, 322).

MNV is the only NoV able to propagate *in vitro* and has a tropism for dendritic cells (DC) and macrophages (315). However, attempts to culture human NoVs in these cells derived from susceptible hosts were unsuccessful, though NoV proteins were present in some cells (317). Thus, the effects of MNV or NoV infection in these cell types are of interest. Stimulation of human PBMCs with Grimsby-like GII.4-derived VLPs resulted in matured DCs and production of IL-6, IFN-γ, and TNF-α (323). MNV infection reduced CD11b+ and CD103+ conventional dendritic cells (cDC) in the lamina propria of the small intestine and mesenteric lymph nodes, respectively (324). However, cDCs may also play a role in infection and persistence. Depletion of cDCs in mice not only increased intestinal and fecal viral titers and delayed antibody responses, but prevented MNV dissemination to secondary lymphoid tissues, which is required for the systemic infection seen by MNV-1 (286, 325). In contrast, plasmacytoid dendritic cells (pDC) increased in Peyer's Patches and mesenteric lymph nodes following MNV infection (324).

1.4.2 Humoral immunity

Antibodies, specifically blocking antibodies, have been shown to be important for NoV immunity. Serum HBGA blocking antibodies were identified as a correlate of protective

immunity against disease (326-328). These antibodies primarily recognize the P2 domain of the VP1 capsid protein (219, 329), but have also been detected against the NoV protease (330). There is evidence that the P domain antibody-binding sites are regulated by several factors, including viral particle confirmation, temperature, and external amino acid residues (331).

Antibodies also have an association with decreased viral shedding (286, 326), are required for clearance of MNV infection (286, 332), and prevention of viremia (286). Blocking antibodies from mature breast milk have also been shown to protect against NoV VLP binding to saliva receptors, though these effects were dependent on *FUT2* expression (333). The protective transfer of antibodies has led to the investigation of monoclonal antibodies as potential treatments (265, 334, 335).

However, little is truly known about total antibody responses to NoV infection. Surrogate studies have indicated that MNV infection of cultured macrophages results in chemokine expression profiles indicative of a weak humoral response (336), while Tulane virus resulted in B cell infiltration of the duodenum (337). Serum IgG was detected following infection of Gn calves with GIII.2 bovine NoVs (338) and in volunteers given Norwalk VLPs orally without adjuvant (339). Limited human studies have confirmed that NoV infection induces serum IgG (340).

1.4.3 Cellular immunity

The understanding of human NoV-induced cellular immunity remains limited by a reliance on human volunteer studies or viral surrogates. Infection of macrophage cells with MNV resulted in the expression of pro-Th1 chemokines (336). PBMCs isolated from felines vaccinated against feline calicivirus had increased proliferation of CD8- cells and IFN-γ production *in vitro*

against infectious FCV (341). Mice lacking CD8+ T cells developed persistent infection, implicating CD8+ T cells in clearance of viral infection (342). IFN-γ induces autophagy-independent viral clearance with the ATG12-ATG5/ATG16L1 complex through inhibition of the MNV replication complexes (ATG16L1) and viral polymerase (ATG5) (343).

There have been several studies analyzing NoV-induced T cell responses in animal models and humans. Initial studies showed Norwalk (GI.1) or Hawaii (GII.1) viruses induced lymphopenia 48 hours post-infection (344) in humans. Most importantly, CD4+ T cells, but not IFN-γ, have been shown to be a correlate of protection from MNV infection (345), though IFN-γ has been detected in serum and lymphoid tissues following NoV challenge in humans (340, 346), Gn calves (134), and Gn pigs (347). Other cytokines detected post-infection include IFN-α, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12 in Gn calves (134) and Gn pigs (347). Additionally, pro-inflammatory cytokines TNF-α and IFN-γ were detected in systemic tissues while anti-inflammatory cytokines were detected in Gn calf intestinal tissues at PID 28 (134). Perforin-secreting T cells, which have been shown to be critical for MNV clearance (287), has also been detected in human duodenal biopsies following NoV infection (146). Cross-reactive T cell epitopes have been detected, though these appear to be host- and genogroup-specific (340, 348). Taken together, these results indicate human NoV induces a predominant, yet weak, Th1 response.

1.4.4 Factors affecting norovirus immunity

1.4.4.1 Norovirus diversity

NoVs are highly diverse viruses, complicating vaccine development and surveillance. NoV genotyping is determined by capsid sequences (349), though some use the RdRp for this

purpose (350). These differing classification systems and emergence of recombinant NoV strains have caused some confusion in classification of newly isolated NoV strains (351). To overcome this problem, a new system to genotype NoVs based on both ORF1 and VP1 sequences has been proposed (349, 351, 352). Currently, NoVs are classified into genogroups, which differ by over 60% homology, and further subclassified into genotypes, which differ by 40% sequence homology (349). There are currently five genogroups, which have been subdivided into approximately 34 genotypes (353). A canine NoV was identified in 2010 and tentatively placed in two genogroups, GIV and the novel GVI (17, 155). The prototypical NoV, Norwalk virus, is GI.1, but GII.4 NoVs are the most prevalent worldwide and account for approximately 60-90% of all outbreaks (3), which may be related to the quicker evolution that occurs in GII.4 NoVs (354). GII.4 NoVs undergo epochal evolution with a new strain emerging every 2-4 years and replacing older strains (106, 355-358). The current global circulating strain of NoV is GII.4/Sydney and emerged in late 2012 (275, 357, 359-361), meaning a new pandemic strain is likely to emerge in the next 1-2 years.

There are several mechanisms that play a role in NoV evolution and diversity. Primarily, as an RNA virus, new strains result from quasispecies that survive selection pressure (362) to escape herd immunity (358, 363). Since NoV immune evasion occurs primarily in the P2 binding domain (104, 105, 355, 357, 364), these results indicate NoV strains must balance evasion of host memory responses with the ability to retain HBGA binding ability (363). NoVs are also under selection pressure by the polymorphic HBGAs (176, 178, 365). NoV quasispecies also evolve in immunocompromised hosts that cannot clear infection, creating NoVs with altered HBGA binding profiles and variation in blockade epitopes compared to the original infecting strain (18) and serving as potential reservoirs for emerging strains (18, 267, 296).

Another driving cause of NoV diversity is recombination, which has hindered epidemiology, phylogenetics, and vaccine design (366, 367). The first identified NoV recombination was identified in the GII.2 Snow Mountain virus in 1997 (368). The recombination "hot spot" is located at the ORF1/ORF2 overlap, yielding a virus containing ORF1 and ORF2/ORF3 from co-infecting viruses (366). Recombination occurs when the RdRp encounters stem-loop structures at the ORF1/ORF2 junction and processes ORF2 and ORF3 from subgenomic RNA (366). Recombinant NoVs have been detected globally, indicating their importance and prevalence (366, 367, 369-375). Though antibodies for animal NoV strains have been detected in humans, it is unclear if these can recombine with human NoV strains (186, 187, 376).

1.4.4.2 Norovirus immune evasion

Much work has been done to understand how NoVs evade the host immune response and persist in the population. NoVs confer short-term, homologous protection following infection in humans (348) and undergo epochal evolution in which new strains replace older strains (377). NoV immune evasion can occur through several mechansisms, including recombination (366), selection of quasispecies (358, 362, 363), and chronic infections in immunocompromised hosts (18, 377). Understanding NoV immune evasion is important as incidences of NoV gastroenteritis decrease as herd immunity grows following an outbreak (282, 378, 379). However, NoV immune evasion may be genotype-specific, as GII.3 NoVs have highly conserved and cross-reactive antibody-binding epitopes (380), while GII.4 NoVs evolve more rapidly than other genotypes (354).

The primary driving force for NoV immune evasion is antigenic drift caused by host immune selection pressure (358) to evade herd immunity (363). The NoV P domain controls evasion of neutralization (362, 381), specifically in Epitopes A and D of the P2 domain for GII.4 NoVs (104, 105, 355, 357, 364). Mutation in Epitope A appears to be the driving force for NoVs to escape herd immunity and undergo epochal evolution (104). A particularly promising mechanism is based on point mutations in exposed stem loops, such as the MNV E'F' loop, to evade neutralizing antibodies (329, 334, 362). However, the survival of these quasispecies and mutants are dependent on host immune strength; having too strong or weak immunity prevents proper selection pressure for NoV quasispecies survival (382).

There are several other proposed mechanisms that NoV can evade the host immune system. For instance, NoVs are capable of interrupting protein secretion pathways (69, 70) and may interfere with antigen presentation or cytokine secretion (117). Additionally, a child contracted two cases of NoV gastroenteritis within an 11 month period caused by GII.4 and GII.6 NoVs (383), indicating two NoVs within the same genogroup can be serologically distinct. This is similar to the finding previous NoV immune responses do not impair development of immune responses against future infection NoV genotypes (384).

1.5 Norovirus animal models

1.5.1 Mice

Mice are widely used animal models in microbiology, including NoVs. Mice have been used for evaluation of novel NoV vaccine candidates (356, 385-391) and elucidation of NoV replication and pathogenesis (129, 286). Mouse models also have the benefit of being natural hosts for GV NoVs, the MNVs (392), have a cell culture system (315) and a well-established

reverse genetics system (393, 394). However, studies in mice are limited as MNV does not cause gastroenteritis similar to human NoVs (294, 318) and causes a persistent infection, while human NoVs cause an acute infection (342, 395). Until recently (396), mice could not be infected with human NoVs. Mouse models are also limited by the significant differences between human and murine immune systems (397). Thus, vaccine-induced immune responses in mice are not always applicable to humans.

Recently, a mouse model was developed that could support human NoV replication (396). Wild-type BALB/c could not be infected with human NoV, while mice deficient in Rag-γc with (humanized) or without grafts (non-humanized) of CD34+ hematopoietic stem cells supported viral replication. Additionally, NoV proteins were detected in splenic and hepatic macrophages and NoV genome copies increased over the challenge dose. This includes detection of NoV capsid proteins at higher levels than the input virus. These results indicate that this mouse model may serve as a starting point for production of NoVs sufficient for attenuated or inactivated vaccine development.

However, this mouse model has several limitations. First, mice were not susceptible to oral infection with NoV; rather, they required a combined oral/intraperitoneal or intraperitoneal inoculations (396). Additionally, the mice only developed a subclinical infection. This indicates the Rag-γc- mice cannot be a suitable model for NoV transmission or disease. This limits the mouse model's potential to evaluate vaccine candidates.

1.5.2 Non-human primates

Non-human primates (NHPs) are an intriguing animal model possibility due to their similarities to humans. However, due to ethical and cost considerations, NoVs have not been

studied extensively in NHPs. NoV inoculation of NHPs has resulted in virus shedding, but not clinical gastroenteritis symptoms (337, 398, 399). Rockx and colleagues showed that common marmosets, cotton top tamarins, and rhesus macaques shed virus following oral Norwalk virus inoculation (399). Macaques inoculated with a mixture of GII.2 and GII.4 NoVs did not result in gastroenteritis or consistent virus shedding (337). In both studies, only one macaque shed virus for an extended duration (337, 399), though only one study detected virus-specific IgM and IgG (399). However, all pigtail macaques inoculated with Toronto virus via nasogastric tubes developed diarrhea, vomiting, and dehydration in addition to infectious virus shedding (400). A more comprehensive study of chimpanzees inoculated IV with Norwalk virus again did not develop gastroenteritis, but did develop shedding patterns similar to humans (398). Viral RNA was detected in intestinal and liver biopsies along with antigen detection in the lamina propria (398). Further, re-inoculated chimpanzees were resistant to infection up to 24 months in association with serum NoV-specific antibodies, while intramuscular inoculation with Norwalk-derived (but not GII) VLPs provided protection for up to 18 months (398).

Additionally, a separate genus of caliciviruses, the recoviruses, are prevalent in captive NHP populations (401, 402) and recognize HBGAs as their receptor (401). Thus, they have served as a surrogate for NoVs (403-406). The prototype strain of recoviruses is Tulane virus, which was first isolated in 2008 (403). Macaques inoculated with Tulane virus developed gastroenteritis symptoms and fecal virus shedding, along with duodenal inflammation and villous blunting (337). The macaques also developed virus-neutralizing antibodies (337). Though NHPs may not be an effective model for NoV disease, they may serve as a valuable model for zoonotic transmission. NoV antibodies have been detected in a high prevalence of NHPs previously (401, 407, 408), while recovirus antibodies (401, 409) and RNA (410) have been detected in humans.

1.5.3 Gnotobiotic calves and pigs

NoVs have been extensively studied using gnotobiotic (Gn) calves and pigs. Gn, or germ-free, animals are derived sterilely and deprived of confounding factors, such as maternal colostrum and microbiota (133). Pigs and calves are hosts for GII and GIII NoVs, respectively. Additionally, Gn pigs have similar intestinal anatomy, morphology, and immune responses as humans (411), which is crucial for the study of enteric pathogens.

The Gn pig model has been influential in the current understanding of human NoV infection and disease. The Gn pig model for NoV was developed in 2006 following inoculation with human stool filtrates containing a 2001 isolate of NoV (GII.4/HS66). Infected pigs (48/65) developed mild to severe diarrhea, though not all of these pigs shed detectable levels of virus (29/65) and the inoculum was unable to be passaged. NoV capsid and non-structural proteins were primarily detected in the duodenum and jejunum. Seroconversion and antibody titers were directly related to severity of diarrhea. Antibody-secreting cells were at higher numbers in systemic tissues than local tissues (133). Th1 cytokines were most prevalent in serum, but increased innate cytokines in intestinal tissues (347). Pigs orally or intravenously inoculated with porcine enteric calicivirus Cowden strain developed diarrhea with duodenal and jejunal villi atrophy and viremia (412).

More recent studies in the Gn pig model have verified previous findings that cholesterol plays a role in NoV replicon expression in cell culture (125). NoV had a lower ID_{50} in Gn pigs fed simvastatin, a cholesterol synthesis inhibitor, indicating that the cholesterol pathway aids in viral replication or host response to infection (126). In a similar study, simvastatin fed pigs had earlier onset and longer duration of diarrhea, increased shedding, and decreased IFN-α

expression. However, inoculation of IFN-α reduced shedding, implicating IFN-α as a potential antiviral for NoV and the effect of innate immunity on clearance of NoV infection (127).

Gn calves have also been important in the understanding of NoV infection, disease, pathogenesis, and immune responses. All Gn calves orally inoculated with human NoV (GII.4/HS66) shed virus and had diarrhea, though one calf developed lesions in the duodenum and jejunum (134), similar to what was seen in Gn pigs (133). Sixty seven percent of the calves seroconverted with higher numbers of antibody-secreting cells in the intestine than systemic tissues consistent with increased Th2 cytokine-secreting cells in the intestine (134). Overall, these lesions were less severe than duodenal and jejunal lesions witnessed following inoculation with bovine enteric calicivirus strain NB (BEC-NB) (413), which is more phylogenetically related to *Lagovirus*, which causes liver damage and systemic hemorrhage in rabbits. BEC-NB inoculated calves did not have lesions in liver or systemic tissues (413). Inoculation of Gn calves with bovine NoV CV186-OH (GIII.2) resulted in persistent diarrhea and lethargy in the absence of intestinal lesions (338). Gn calves infected with Jena virus (GIII.1) developed diarrhea that persisted for about five days and included villus atrophy in the jejunum and ileum (414). Thus, Gn pigs and calves infected with unnatural strains caused lesions in duodenum and jejunum (133, 134), while infection of Gn calves with natural strains caused lesions in distal jejunal and ileal tissues (414). Additionally, the ability of human NoVs to infect Gn pigs and calves suggest the potential for zoonotic transmission.

Gn animals are also valuable to evaluate the efficacy and immunogenicity of VLP vaccine candidates against NoV. Gn calves were inoculated with CV186-OH-derived VLPs with oil, immune stimulating complexes (ISCOM), or mLT (R192G) adjuvants via intramuscular, oral, or intranasal routes or orally inoculated with virulent CV186-OH. All vaccinated calves

shed virus following challenge with CV186-OH, while NoV infected calves did not. However, calves vaccinated IN with mLT had reduced diarrhea compared to controls and other vaccination groups. The different vaccine regimens provided different immune responses. Serum IgG and IgA antibodies were induced by VLPs co-administered with oil or mLT IM and IN, respectively. However, intranasal VLPs with mLT were the only group to induce fecal IgA like the bovine NoV inoculated group, indicating this regiment most closely protected from disease (415). A similar study in Gn pigs examined oral/intranasal inoculation of GII.4-derived VLPs with ISCOM or mLT against homologous challenge. VLPs with ISCOM and mLT provided full protection against diarrhea and 75% and 100% protection from virus shedding, respectively. However, only 57% of control pigs (adjuvants alone) shed virus, suggesting an inadequate challenge dose for the study. All vaccinated pigs seroconverted with VLP + mLT pigs having a balanced Th1/Th2 response and VLP + ISCOM pigs having a biased Th2 response with increased intestinal antibody-secreting cells (416).

1.6 Norovirus vaccine development

1.6.1 Virus-like particles (VLPs)

VLPs have been extensively studied as NoV vaccine candidates (417-419) and several formulations have gone through clinical trials (9, 339, 420-423). VLPs are derived by expression of the VP1 capsid protein in a eukaryotic expression vector, such as baculovirus, yeast, or plants (424-432) that yields a capsid similar to the native virion (Figure 1A) but lacks the NoV genome (424) and can be purified through a variety of methods (433-435). As such, VLPs are non-replicating and retain similar binding properties as wild-type NoV (158, 159, 166, 436). An alternative strategy to produce VLPs utilizes viral vectors, such as vesicular stomatitis virus

(VSV) (389, 390), Venezuelan Equine Encephalitis virus (VEEV) (437, 438), adenovirus (439, 440), or Newcastle disease virus (NDV) (391) that produce VLPs within the host.

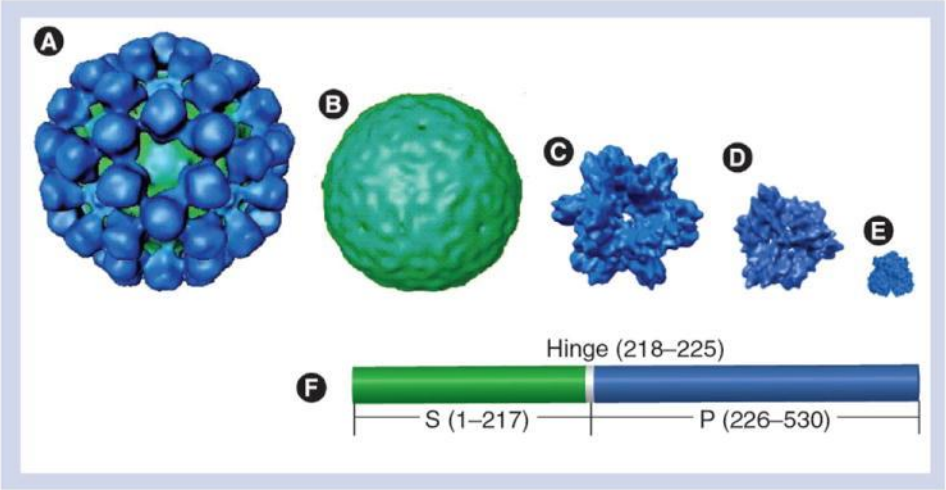

Figure 1. Structures of the five norovirus complexes that are formed by full-length or truncated norovirus VP1. (A) Virus-like particle (180-mer: ~37 nm). (B) S particle (180-mer: ~27 nm). (C) P particle (24-mer: ~20 nm). (D) Small P particle (12-mer: ~14 nm). (E) P dimer (~6 nm). (F) Linear structure of norovirus VP1 with indications of the S (green) and the P (blue) domains that are linked by a short hinge. Numbers are based on Norwalk virus VP1. P: Protruding; S: Shell. **Tan M, Jiang X.** 2012. Norovirus P particle: a subviral nanoparticle for vaccine development against norovirus, rotavirus and influenza virus. Nanomedicine **7:**889-897. [Used with permission of Future Medicine Ltd. 2014.]

VLPs have been extensively studied in animal models previously (415, 416, 442), primarily in mice (356, 443). VLPs decreased diarrhea and shedding in Gn calves (415) and provided partial protection against diarrhea and shedding in Gn pigs (416), though both findings were dependent on vaccine adjuvants. Primarily, VLPs have been shown to induce specific serum IgG and mucosal IgA (385, 387, 415, 416, 429, 443, 444) and cellular immunity (416, 444). The most promising of these studies have focused on vaccine formulations that aim to combat NoV diversity. For instance, chimeric VLPs expressing the immunodominant Epitope A from historical strains of NoV induced homotypic and heterotypic antibody responses compared to single strain VLP preparations in mice (356). However, single strain VLPs induced a stronger blockade response against their parental strain than the chimeric or multivalent vaccine preparations in mice (356). Similarly, intramuscular-administered VLPs derived from a

consensus GII.4 sequence and Norwalk virus with Alhydrogel adjuvant induced broad antibody responses against the native viruses and other variants (442). However, these responses were still confined within a genotype (442). A trivalent VLP vaccines containing GI.3 and GII.4 NoVs and rotavirus VP6 induced cross-reactive IgG antibodies in mice (445, 446).

VLPs expressed from VSV have been examined in mice previously (389, 390). Insertion of VP1 in VSV attenuated viral growth *in vitro* and *in vivo* (390). However, mice inoculated with VSV-VP1 (combined IN and oral) experienced severe weight loss, suggesting this vaccine system requires further attenuation (390). Co-expression of heat shock protein 70 (HSP70) further attenuated VSV in mice, but did not prevent spread of VSV to the central nervous system (389). Mice inoculated with VSV-VP1 produced stronger serum IgG, cellular, and humoral responses than baculovirus-derived VLPs (390). VSV-HSP70-VP1 also increased vaginal IgA titers in mice, but required an increased dose to increase cellular and humoral responses (389). NDV vectored VLP vaccines have been recently examined (391). Insertion of the NoV VP1 into a modified NDV LaSota backbone resulted in formation of VLPs similar to baculovirus-derived VLPs (391). The modified NDV-VP1 vaccine induced robust immune responses, including increased levels of serum IgG than the conventional NDV-VP1 vector and baculovirus-derived VLPs and fecal IgA compared to baculovirus-derived VLPs. Further, the modified NDV-VP1 vaccine induced splenic IFN-γ, IL-2, and TNF-α secreting cells (391). Thus, recombinant viral vectors are appealing vaccines as they likely require a single dose and inoculate the host with higher amounts of VLPs than conventional VLP preparations. Although biosafety concerns and pre-existing host immunity to VSV backbones may limit the development, availability, and efficacy of the VSV vaccine candidates, pre-existing host immunity and biosafety concerns are minimal in NDV vaccines, making these more promising candidates than VSV.

VLP regimens consisting of different formulations and routes of administration have undergone or are currently going through clinical trials and have evaluated both protection and immunogenicity (9, 339, 420-423). In the first such trial, orally administered Norwalk VLPs induced serum IgG, even in humans with previous exposure (339). Similarly, intranasal Norwalk-derived VLPs provided partial protection from infection and disease (421) and elicited virus-specific intestinal homing antibodies (9) and memory B cells (422). More recently, a two-dose 50 µg intramuscular GI.1/consensus GII.4 bivalent vaccine reduced occurrence of diarrhea and vomiting (423) and increased virus-specific antibodies within 7 days after a single dose in healthy adult human volunteers (420), suggesting the vaccine boosted the anamnestic responses in previously NoV-infected hosts.

1.6.2 P particles

P particles are made by expression of from the protruding (P) domain of the VP1 capsid protein in a prokaryotic expression vector, such as *E. coli* (44, 45). The P and S domains of VP1 are linked by a short hinge (Figure 1F) (40); expression of the P domain with or without this hinge results in P dimers or 12 P dimers, respectively (Figures 1C and 1F) (44, 45). However, cysteine residues increase stability and formation of P particles (45), while other end terminal modifications yield small P particles (6 P dimers) (Figure 1D) (447). P particles contain the P2 binding domain in the outer layer and P1 domain in the inner core (Figure 2) (45). P domain complexes retain the P2 HBGA binding domain (168) and have the same binding profile as the native capsid (44, 45, 448, 449), though P particles have stronger binding capability (45).

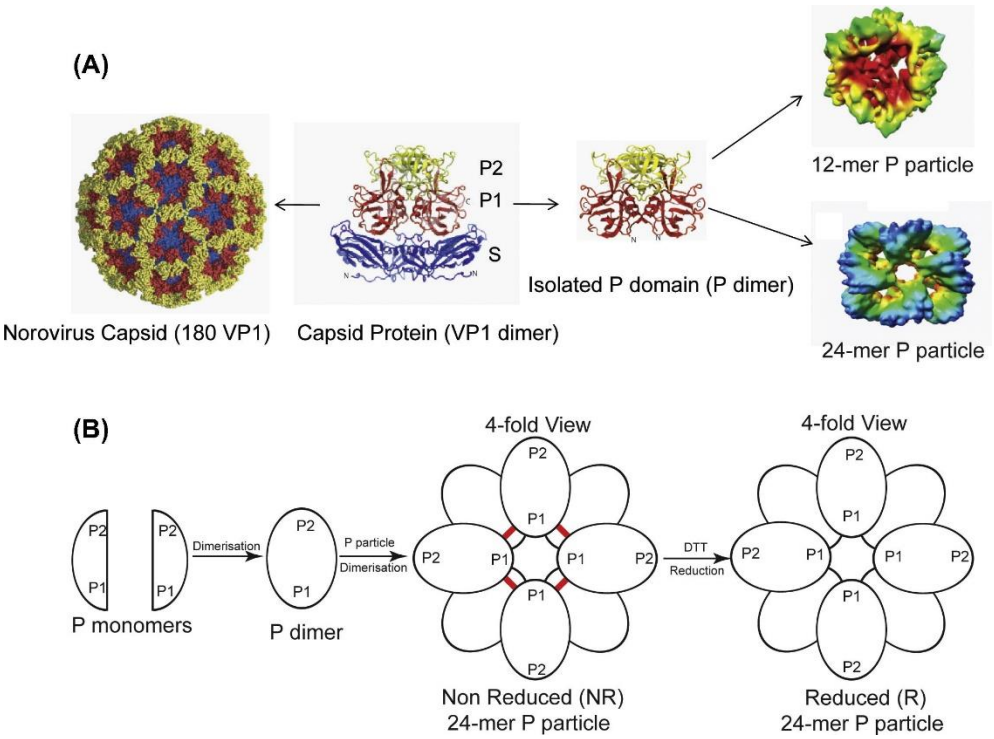

Figure 2. Formation of P particles from the norovirus P domain. (A) The dimeric norovirus capsid protein VP1 consists of an S domain and a P domain divided into two subdomains: P1 and P2. Assembly of 90 dimeric VP1 subunits forms the intact norovirus capsid. *In vitro* expression of the isolated P domain leads to the formation of the P dimer (44) that can further assemble into the 12-mer small P particle (447) and 24-mer P particle (449). (B) Schematic to show the dimerization of two P domains to form the P dimer which under non-reducing conditions form the 24-mer P particle (4-fold view is shown) that can be stabilized by interdimer disulphide bonds (indicated in red). Addition of DTT induces cleavage of disulphide bonds to form reduced P particle. **Bereszczak JZ, Barbu IM, Tan M, Xia M, Jiang X, van Duijn E, Heck AJ.** 2012. Structure, stability and dynamics of norovirus P domain derived protein complexes studied by native mass spectrometry. Journal of structural biology **177:**273-282. [Used with permission of Elsevier Limited. 2014.]

Studies on P particle immunogenicity and protective efficacy have remained limited. VA387-derived P particles induced homologous, strain-specific HBGA binding blocking antibodies after intranasal inoculation in mice (449). A study by Tamminen and colleagues (451) indicated that P particles were not as immunogenic as VLPs in mice. The study indicated VLPs induced high avidity antibodies, a balanced Th1/Th2 response including IFN-γ production, and cross-reactive B and T cells, while P particles required boosters to produce low avidity antibodies and a Th2-biased response without cross-reactivity (451). However, Tan, et al. (452) indicated that the study may have utilized the less immunogenic P dimers instead of P particles.

A later study revealed that P particles induced similar levels of central memory CD4+ T cells, polyclonal T cells, serum antibody titers, and mature DCs as VLPs in mice (14).

P particles are also capable of serving as a platform for expression of other viral antigens, including rotavirus (453), influenza (454), hepatitis E virus (HEV) (455), HIV (456), or multiple antigens (457). Immunogenicity studies expressing these antigens have produced largely positive results. NoV-rotavirus VP8 chimeric P particles reduced rotavirus shedding and increased rotavirus neutralizing antibody titers compared to free VP8 antigens and produced HBGA binding blocking antibodies in mice (453). Similar antibody and blocking responses were found in mice immunized with NoV P particles expressing influenza A M2e along with full protection against lethal influenza challenge (454). However, NoV P particles expressing HIV-1 gp41 produced weakly neutralizing antibodies in guinea pigs (456).

P particles and foreign antigens can also be expressed as polyvalent complexes following expression in *E. coli* (455, 457, 458). Fusion of P domain dimers with glutathione S-transferase (GST) dimers formed linear and network polyvalent complexes capable of also expressing influenza A M2e or rotavirus VP8 monomers (457). These polyvalent complexes increased NoV P protein binding to HBGAs, increased antibody titers and CD4+ T cells, and increased protection against challenge compared to free antigens in mice (457). Polyvalent complexes formed by fusion of HEV and NoV P domain dimers with or without GST induced higher total, HEV neutralizing, and NoV blocking antibody titers than a mixture of HEV and NoV P dimer formulations in mice (455). Larger branched-linear and agglomerate proteins were formed following expression of dimeric GST with tetrameric HEV and 24-meric NoV P proteins along with influenza A M2e or rotavirus VP8 monomers (458). These complexes increased humoral,

including neutralizing antibodies, and cellular immune responses and protective immunity compared to dimeric antigens (458).

1.7 Concluding remarks

My dissertation research involves evaluation the P particle vaccine candidate in the Gn pig model. I divided this work into three specific aims: 1) comparing P particles to VLPs and prior NoV infection, 2) evaluating the dose responses of P particles, and 3) evaluate how simvastatin affects P particles. These three specific aims involve determination of the protective efficacy and T cell immunogenicity of each group before and after NoV challenge.

The P particles are a promising vaccine candidate. Extensive work has been done to show that the immunogenicity of P particles is similar to VLPs in mice (14) and that the P particles can serve as a platform to present antigens from other viruses (441, 455). Combined with their relative ease of production compared to VLPs, the ability to present multiple viral antigens make P particles even more economically viable vaccine candidates than VLPs. Further, since NoV evolves through antigenic drift and vaccines may need reformulations during each outbreak (358), P particles may provide an easier mechanism to update vaccines. This will be the first study to analyze P particles in a large animal model.

This research project also utilizes the Gn pig model. Our lab has previously established the Gn pig model of NoV GII.4 infection and diarrhea (126), including determining the ID50 at different ages that is critical when calculating challenge dose. Gn pigs have similar intestinal morphology and immune systems to humans (411), develop diarrhea after oral NoV challenge (126, 133), and develop pathology in the intestine following challenge (126, 133). Additionally, our viral inoculum pool (GII.4/2006b variant 092895) is devoid of other enteric viruses, which

limits ambiguity about competing pathogens. These factors make our Gn pigs a more attractive and suitable model for NoV disease studies and P particle vaccine evaluation than mice. Additionally, pig litters are composed of multiple newborns, whereas calves are born individually, making pigs a more efficient model. Finally, since Gn pigs are devoid of maternal colostrum and microbiota, so the protective efficacy and immunogenicity of P particles can be evaluated without confounding factors. This eliminates previous exposure history, which may impact vaccine-induced immunity (346), or bacteria that may bind P particles or NoVs (182, 184). For these reasons, the Gn pig model is currently the ideal animal model for evaluating the P particle vaccine.

NoV immunity studies have primarily focused on humoral responses. However, cellular immunity has been shown to be important for preventing persistent infection (342), clearance of infection (287), and may serve as a correlate of protection (345). However, these conclusions were derived from MNV infection of mice, which has been established as a suitable model for NoV infection, but not disease or immune responses. NoV has been shown to induce a predominantly Th1 response with low levels of Th2 cytokines in humans (346) and Gn pigs (347). However, these studies only examined the cytokine responses and did not evaluate the T cell surface phenotypes beyond those responses. Our studies elucidated the T cell surface markers associated with protection from disease. Additionally, it provided a better model of how effector and regulatory T cells responds to NoV infection.

Finally, simvastatin is a cholesterol-reducing drug that can reduce cardiovascular events by up to 23% over 5 years in high and low-risk populations (459, 460). An estimated 50% of men and 36% of women 65-74 years of age took statins in 2010 according to the National Center for Health Statistics. The American Heart Association changed their guidelines in 2013

recommending simvastatin use for heart disease prevention in those without high LDL levels. Since simvastatin has been shown to increase susceptibility to NoV *in vitro* (125) and *in vivo* (126, 127), it may affect NoV vaccine-induced protection. Since the elderly and aging are a target population for a NoV vaccine, simvastatin's deleterious effects on the vaccine could impact an already immunocompromised population.

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CHAPTER 2

Intranasal P particle vaccine provided partial cross-variant protection against human GII.4 norovirus diarrhea in gnotobiotic pigs

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Published in *Journal of Virology* 88(17): 9728-9743 (doi: 10.1128/JVI01249-14)
PMID: 24920797 [Used with permission of American Society of Microbiology. 2014]

2.1 Abstract

Abstract

Noroviruses (NoVs) are the leading cause of nonbacterial acute gastroenteritis worldwide in people of all ages. The P particle is a novel vaccine candidate derived from the protruding (P) domain of the NoV VP1 capsid protein. This study utilized the neonatal gnotobiotic pig model to evaluate the protective efficacies of primary infection, P particles, and VLPs against NoV infection and disease and the T cell responses to these treatments. Pigs were intranasally vaccinated with GII.4/1997 NoV (VA387)-derived P particles or virus-like particles (VLPs) or orally inoculated with a GII.4/2006b NoV variant. At post-inoculation day (PID) 28, pigs were euthanized or challenged with the GII.4/2006b variant and monitored for diarrhea and virus shedding for 7 days. The T cell responses in intestinal and systemic lymphoid tissues were examined. Primary NoV infection provided 83% homologous protection against diarrhea and 49% against virus shedding, while P particle and VLP vaccines provided cross-variant protection (47% and 60%, respectively) against diarrhea. The protection rates against diarrhea are significantly inversely correlated with T cell expansion in duodenum and positively correlated with T cell expansion in ileum and spleen. The P particle vaccine primed for stronger immune responses than VLPs, including significantly higher activated CD4+ T cells in all tissues, IFN-γ+CD8+ T cells in duodenum, regulatory T cells (Tregs) in blood, and TGF-β producing CD4+CD25-FoxP3+ Tregs in the spleen postchallenge, indicating P particles are more immunogenic than VLPs at the same dose. In conclusion, the P particle vaccine is a promising vaccine candidate worthy of further development.

Keywords: norovirus, P particle, vaccine, gnotobiotic pig, protective efficacy, T cell responses

2.2 Introduction

Noroviruses (NoVs), a genus in *Caliciviridae*, are the leading cause of acute, non-bacterial gastroenteritis across all age groups, causing approximately half of all gastroenteritis outbreaks worldwide (1, 2). NoV is now the leading cause of acute pediatric gastroenteritis, accounting for approximately 1 million hospital visits for U.S. children (3), an estimated total 23 million cases in the US (4), and 1,091,000 inpatient hospitalizations and 218,000 deaths in developing nations (5) annually, though this incidence is expected to be an underestimate as NoV gastroenteritis often presents as a mild, self-limiting disease. In the United States alone, NoV gastroenteritis causes $284 million economic costs in hospitals annually (6). However, there are no vaccines or antivirals currently available. The predominant circulating NoV strains belong to GII.4 which is responsible for over 80% of NoV gastroenteritis worldwide (7).

Due to the lack of a cell culture system to isolate and propagate human NoV, vaccine development has relied upon recombinant NoV capsid proteins, such as virus-like particles (VLPs) and P particles. The capsid of NoV is composed of a single major structural protein of 55-60 kDa (VP1) that is divided into shell (S) and protruding (P) domains linked by a short hinge (8). VLPs form through expression of VP1 in a eukaryotic expression system and retain the antigenic structure and histo-blood group antigen (HBGA) receptor binding function (9, 10). P particles form when the P domain is expressed in *E. coli* (11). Each P particle contains 24 copies of the P domain with a total molecular weight of ~840 kDa and a diameter of ~20 nm, which is an ideal size for an immunogen (11). P particles display similar HBGA binding patterns as VLPs and elicit similar innate, humoral and cellular immune responses as VLPs in mice (12). A previous study by Tamminen et al. (13) comparing the immunogenicity of VLPs and P particles in mice suggested that VLPs induce a superior immune response than P particles. However, mice

are resistant to human NoV infection so the protective efficacy cannot be evaluated to address the disparities in these studies. In addition, Tan et al. (14) raised concerns that this study utilized P dimers instead of P particles. A future study indicated that P dimers induce weaker immune responses than P particles (12), which may have impacted Tamminen et al.'s results. From a vaccine production point of view, VLPs require a eukaryotic system whereas P particles can be easily produced by *E. coli* at a higher yield than VLPs (15, 16). P particles have also been shown to be a useful vaccine platform for dual vaccine development (17, 18). Thus, P particles may be more economically viable vaccine candidates than VLPs.

The gnotobiotic (Gn) pig model has been used for the study of NoV pathogenesis and vaccines (19-22). Gn pigs have similar intestinal physiology and immune systems to humans and are well-suited for studies of vaccine-induced immune responses due to the lack of interference from maternal antibodies and extraneous pathogens (19, 20). We recently reported studies of NoV infectivity in the presence or absence of a cholesterol-lowering drug, simvastatin, in a Gn pig challenge model using a large inoculum pool of a human GII.4/2006b NoV variant (22). The median infectious dose (ID_{50}) of the NoV inoculum in Gn pigs at 33-34 days of age was determined. The present study utilizes this well-established Gn pig challenge model to evaluate the immunogenicity and protective efficacy of a GII.4/1997 NoV P particle vaccine candidate and to compare the P particle vaccine with the corresponding VLPs and primary NoV infection.

For non-replicating vaccines, effective adjuvants and delivery systems are important to the immunogenicity of the vaccine antigens. We used monophosphoryl lipid A (MPL) and chitosan in the P particle vaccine formulation. MPL is a natural substance derived from *Salmonella* Minnesota and a potent TLR4 agonist recently approved by the FDA and other regulation agencies globally. Intranasal administration of hepatitis B antigen, tetanus toxoid, or

influenza antigens with MPL resulted in increased mucosal and cellular immunity (23). Chitosan is a polysaccharide derived from the partial deacetylation of chitin. The mucoadhesive properties of chitosan increase antigen uptake by mucosal surfaces and reduce clearance by cilia (24). Additionally, chitosan has been shown to shift a biased Th1 response to a balanced Th1/Th2 response (24) and had adjuvanticity with intranasal HIV (25) and anthrax vaccines (26). Chitosan and MPL have been used in previous NoV VLP studies (7, 27, 28)

In this study, we evaluated the protective efficacies conferred by the P particles, VLPs or primary NoV infection in Gn pigs challenged with homotypic NoV GII.4. We also examined the total T helper (Th) cell, cytotoxic T cell (CTL), virus-specific effector/memory T cell, and regulatory T cell (Treg) responses in the intestinal and systemic lymphoid tissues of Gn pigs at challenge and/or postchallenge. Protective immunity, especially among T cells, against NoV infection and diarrhea has not been fully understood. Previous infection studies have indicated that NoV infection provides short-term, homologous protection (29, 30) in humans. Immunity to NoV has been linked to serum HBGA blocking antibodies and CD4+ T cells (28, 31, 32). Effector T cells play an important role in clearance of NoV infection in humans (33, 34) and mice (35, 36). To our knowledge, this is the first study to compare the protective efficacies induced by P particles, VLPs and primary NoV infection and to comprehensively examine the T cell responses induced in Gn pigs.

2.3 Materials and Methods

2.3.1 Virus

A pool of human stool containing GII.4/2006b variant 092895 (GenBank accession number KC990829) was collected by Dr. Xi Jiang's laboratory at Cincinnati Children's Hospital Medical Center from a child with NoV gastroenteritis in 2008. It was processed as we previously described and used for the virus primary infection and challenge studies (22). The ID_{50} of the inoculum in Gn pigs at 4-5 days of age is 2.74 x 10^3 viral RNA copies and at 33-34 days of age is 6.43 x 10^4 viral RNA copies (22). Ten times the ID_{50} was used for primary infection and challenge. This inoculum dose is consistent with the challenge dose in a previous study in humans (28).

2.3.2 Amino acid sequencing of NoV 092895 VP1

RNA from the stool was extracted with QIAmp Viral Isolation Kit (Qiagen) and DNA contamination was eliminated by Turbo DNAse (Life Technologies). DNAse was subsequently cleaned by using RNAeasy Kit (Qiagen). RT-PCR was performed using oligodT primer and Maxima H Minus First Strand cDNA synthesis kit (Thermo Scientific). The 1.6 kb of VP1 capsid gene was amplified by PCR using MyTaq HS DNA Polymerase (Bioline) and re-PCR using PrimeStar HS DNA Polymerase (TaKaRa) with the following primers: cog2F(5_CARGARBCNATGTTYAGRTGGATGAG-3_) (37) and JV24 reverse (5_-TTATAATGCACGTCTACGCCC-3_) (38). The amplified fragment was sequenced, ligated into a pBlueScriptSKII(+) and cloned into NEB10-beta Chemically Competent *E.coli* cells (New England Biolabs). The VP1 sequence (GenBank Accession no: KC990829) was derived from sequencing of resultant recombinants performed by Virginia Bioinformatics Institute (Virginia Tech, Blacksburg, VA).

2.3.3 Vaccine preparation

P particles and VLPs were both derived from GII.4 VA387 (1997 Farmington Hills variant) as described previously (11, 39) and sterilized with short-wave UV light for 30 minutes. Synthetic MPL (Avanti Polar Lipids, Inc.) was dissolved in 0.5% triethanolamine and heated at 65° C for 5 minutes. MPL-TeOH was sonicated in a bath sonicator and pH adjusted to 7.0. Chitosan (Novamatrix) was dissolved in Water for Inoculation (Life Technologies) and 0.2 μm filter sterilized. Vaccines contained 100 μg of P particles or VLPs, 5 mg chitosan, 50 μg MPL, and TNC buffer (40) to a final volume of 1 ml. Sterility of all solutions was monitored by culture on blood agar plates and fluid thioglycollate medium. Endotoxin levels of the P particles was determined with the ToxinSensor^{TM} Chromogenic LAL Endotoxin Assay (GenScript) to be 0.8 EU/ml, which is below the recommended level for a recombinant subunit vaccine (41).

2.3.4 Treatment and inoculation of Gn pigs

Near-term Large White cross pigs were derived via hysterectomy and maintained in germ-free isolator units as described (42). Pigs were confirmed to be A+ or H+ prior to inoculation and sterility was monitored as previously described (22). Pigs (both male and female) were randomly divided into four groups. Each group is composed of at least 6 pigs that were derived from at least 3 different litters - 3 experimental replicates. Pigs in P particle or VLP groups were intranasally inoculated with 3 doses of the vaccines using mucosal atomization devices (MADs, LMA North America) at post-partum day (PPD) 5 (post-inoculation day [PID] 0), PID 10, and PID 21. Pigs in the NoV infection group (NoVPO) were orally inoculated with 10 ID_{50} NoV (2.74 x 10^4 viral RNA copies) at PPD 5. Control pigs received diluent or adjuvants only. Pigs were given 4 ml of 200 mM sodium bicarbonate 10 minutes prior to oral inoculation

to reduce gastric acidity. A subset of pigs in each group was orally challenged with 10 ID_{50} NoV (6.43 x 10^5 viral RNA copies) at PID 28 (postchallenge day [PCD] 0) and monitored daily for clinical signs and virus shedding until PCD 7. All pigs were euthanized at PID 28 or PID 35 (PCD 7) to isolate mononuclear cells (MNCs) from duodenum (20 cm), ileum (20 cm), spleen (whole organ), and blood (70 ml) as previously described (43). All animal experimental procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Virginia Tech.

2.3.5 Detection of NoV shedding and assessment of diarrhea

Rectal swabs were collected daily following NoV primary infection and challenge for assessment of diarrhea and virus shedding. Diarrhea was scored based on our previously used scaling system (22). Virus shedding was monitored with conventional RT-PCR and TaqMan® real-time RT-PCR as previously described (22).

2.3.6 Flow cytometry analysis of total CD3+CD4+ (Th) cells, total CD3+CD8+ (CTLs), and IFN-γ-producing CD4+ and CD8+ T cells

Flow cytometry was used to determine the numbers of total CD4+ and CD8+ T cells and NoV-specific IFN-γ producing CD4+ and CD8+ T cells in intestinal (duodenum, ileum) and systemic (spleen) tissues and blood of Gn pigs. Cells were stimulated *in vitro* for 17 h and stained as previously described (44, 45). The P particles from VA387 (6 µg/ml for duodenum, ileum and peripheral blood lymphocytes [PBL] and 12 µg/ml for spleen; the concentrations were optimized in pilot studies) were added in the MNC cultures as stimulating antigen. Total numbers of each T cell subset was calculated by multiplication of frequencies of the specific

subset among lymphocytes, frequencies of lymphocytes among MNCs, and total MNCs isolated per tissue. The data are presented as mean numbers per tissue. Total numbers of IFN-γ+ producing T cells are presented as adjusted mean numbers. Adjusted numbers were derived by subtraction of total numbers of the mock-stimulated MNCs from P particle-stimulated MNCs. Isotype-matched irrelevant antibodies were used to establish positive and negative gates of Th, CTL, and IFN-γ producing T cells. At least 100,000 cells were collected on a BD FACSAria flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.4 software (Tree Star, Inc).

2.3.7 Flow cytometry analysis of activated nonregulatory (FoxP3-) and IL-10 and TGF-β producing Treg (FoxP3+) cells

MNCs were stained on the day of isolation for activated nonregulatory T cell and Treg analysis as previously described (44). The activated nonregulatory T cells were identified as CD25+FoxP3- T cells. Tregs were identified as CD4+CD25-FoxP3+ and CD4+CD25+FoxP3+. Numbers of IL-10 and TGF-β producing Tregs were calculated by multiplication of frequencies of IL-10+ and TGF-β+ Tregs among lymphocytes, frequencies of lymphocytes among MNCs, and total MNCs isolated per tissue and are presented as mean numbers. All numbers are presented after subtraction of background numbers. Isotype-matched irrelevant antibodies were used to establish positive and negative gates for Tregs and cytokine-producing Tregs.

2.3.8 Statistical analysis

One-way analysis of variance (ANOVA-general linear model [GLM]) followed by Duncan's multiple range test was used to compare mean durations of diarrhea and shedding. Fisher's exact test was performed to compare percentages of pigs with diarrhea and virus

shedding. Kruskal-Wallis rank-sum test was used to compare the area under the viral diarrhea and shedding curves and numbers of T cell subsets. All statistical significance for these tests was assessed at $p<0.05$. Spearman's rank correlation coefficient was used to evaluate correlations between T cell subsets and protection rates. All correlations were evaluated at $p<0.0001$. All statistical analyses were performed using SAS Program 9.3 (SAS Institute, NC, USA).

2.3.9 Nucleotide sequence accession number

The complete VP1 sequence of NoV GII.4/092895 was deposited in the GenBank database under the accession number KC990829 in August 2013.

2.4 Results

2.4.1 The VP1 sequences of VA387 and 092895 have 93.5% sequence homology

The VP1 sequences was determined for 092895. Sequence alignment indicates that 092895 is a GII.4/2006b variant (data not shown). The amino acid sequences for VA387 VP1 (GenBank Accession no: AY038600 and AAK84679) and 092895 VP1 (GenBank Accession no: KC990829) have 93.5% sequence homology. Amino acid alignment showed that 33 substitutions were present, including 21 substitutions in the P2 domain (Table 1). There are 4 substitutions in epitope A, 1 substitution in epitope B, 1 substitution in epitope C, 3 substitutions in epitope D (including 1 deletion), and 3 substitutions in epitope E (Table 2).

Table 1. Amino acid differences in the S, P1-1, P2, and P1-2 domains between NoVs VA387 and 092895

Domain	Amino acid substitution (total)	Amino acid substitution within one group	Amino acid substitution between different group	Amino acid substitution which potentially influence the protein folding	Insertion/ deletion
S	3	2	1	-	-
P1-1	2	1	1	-	-
P2	21	9	10	1	1
P1-2	7	4	2	1	-

Table 2. Amino acid differences in epitopes A-E of NoVs VA387 and 092895

	Epitopes																			
	A							B			C			D				E		
VLP	294	296	297	298	368	372		333	382		340	376		393	394	395		407	412	413
092895	A	S	R	N	A	E		V	K		G	Q		S	T	T		S	N	V
VA387	A	S	**H**	**D**	**T**	**N**		**M**	K		**E**	Q		**N**	**N**	**-**		**N**	**T**	**G**

2.4.2 The P particle and VLP vaccines provided similar protection rates, which were lower than that of primary NoV infection, against a homotypic NoV challenge

The protective efficacy for each group was evaluated following GII.4/2006b NoV challenge. Clinical signs and NoV shedding were monitored daily postchallenge (Table 3). Primary NoV infection (NoVPO) significantly reduced the occurrence of diarrhea (protection rate 82.9%). The P particle and VLP vaccines reduced diarrhea at a similar rate (protection rate 46.7% and 60.0%, respectively). Compared to control pigs, NoVPO and VLPs slightly shortened the mean duration of diarrhea (by 1.5 and 1.1 days, respectively), while P particles reduced mean area under the curve (AUC) of diarrhea slightly, but these differences were not statistically significant. Though only one NoVPO pig developed diarrhea, several NoVPO pigs had a diarrhea score of 1.5 for 4-6 days following challenge.

Primary NoV infection not only provided substantial protection against diarrhea, but also from homologous virus reinfection, as evidenced by reduced percent of shedding (protection rate 48.6%). NoV primary infection and P particles also decreased the mean AUC of virus shedding (by 5.6- and 3-fold, respectively), though these differences were not statistically significant. VLPs did not have an effect on virus shedding.

Table 3. Clinical signs and protective efficacy in previously infected or vaccinated Gn pigs after challenge with GII.4 2006b NoV[a]

		Diarrhea[b]					Virus shedding				
Group	n	Mean % of pigs with diarrhea (no. of pigs with diarrhea/total no.)[c]	Mean no. of days with diarrhea[d] (SEM)	Mean AUC from PCD 1 to PCD 7 (SEM)	Fold reduction in AUC	Rate of protection against diarrhea (%)[e]	Mean % of pigs shedding virus (no. of pigs shedding/total no.)	Mean no. of days with shedding[d] (SEM)	Mean AUC from PCD 1 to PCD 7 (SEM)	Fold reduction in AUC	Rate of protection against diarrhea (%)[e]
NoVPO	7	14% (1/7) [B]	0.3 (0.3)	6.7 (0.6)	1.0	82.9	43% (3/7)	2.9 (1.4)	20775 (57893)	-5.6	48.6
100 µg P particle + MPL/chitosan (IN)	9	44% (4/9) [AB]	1.3 (0.6)	5.4 (0.8)	-1.2	46.7	89% (8/9)	1.8 (0.4)	38216 (10568)	-3.1	0.0
100 µg VLP + MPL/chitosan (IN)	6	33% (2/6) [AB]	0.7 (0.4)	6.2 (1.0)	-1.1	60.0	100% (6/6)	2.0 (0.4)	96428 (25627)	-1.2	0.0
Diluent #5 (oral) or MPL/chitosan (IN)	6	83% (5/6) [A]	1.8 (0.6)	6.7 (1.0)	1.0	NA	83% (5/6)	2.0 (0.7)	116960 (78189)	1.0	NA

[a] Gn pigs were challenged with a human NoV GII.4 2006b variant 092895 at 33-34 days of age. Rectal swabs were collected daily after challenge to determine diarrhea and virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents.

[b] Fecal scoring system: 0, solid; 1, pasty; 2, semiliquid; 3, liquid. Pigs with scores of 2 or higher were considered diarrheic.

[c] Values in the same column followed by different letters differ significantly (P, <0.05 by Fisher's exact test); there is no significant difference between values followed by the same letter.

[d] From PCD 0 to PCD 7.

[e] Calculated as [1 - (percentage of immunized pigs in each group with diarrhea or shedding/percentage of control pigs with diarrhea or shedding)] x 100.

2.4.3 NoV challenge increased the numbers of Th cells and CTLs in the duodena of naïve (control) pigs

To comprehensively evaluate T cell responses induced by oral NoV inoculation (mimic primary natural infection) versus intranasal vaccination, we examined total Th cells, CTLs, activated nonregulatory CD25+FoxP3- T cells, virus-specific IFN-γ producing effector/memory T cells, CD4+CD25-/+FoxP3+ Tregs, and IL-10/TGF-β producing Tregs in the intestinal and systemic lymphoid tissues. The total Th and CTLs were identified using flow cytometry by gating lymphocytes and co-expression of CD3 with CD4 (Th) or CD8 (CTL) (Figure 1A) and presented as total numbers of cells isolated from each tissue (Figure 1B). Since the P particles and VLPs produced similar protection rates in Gn pigs, similar dendritic cell and T cell responses and cytokine production patterns in mice (12), and the P particle vaccine is targeted for further development in our future studies, we focused on comparing T cell responses induced by the P particle vaccine to NoV infection and controls in Gn pigs.

Total Th and CTLs were compared among NoVPO, P particle, and control pigs pre- and postchallenge. P particle vaccinated pigs had significantly higher numbers of Th cells in PBL compared to the NoVPO and control pigs (Figure 1B) pre-challenge. Following challenge, control pigs displayed significant increases of Th and CTLs in duodenum.

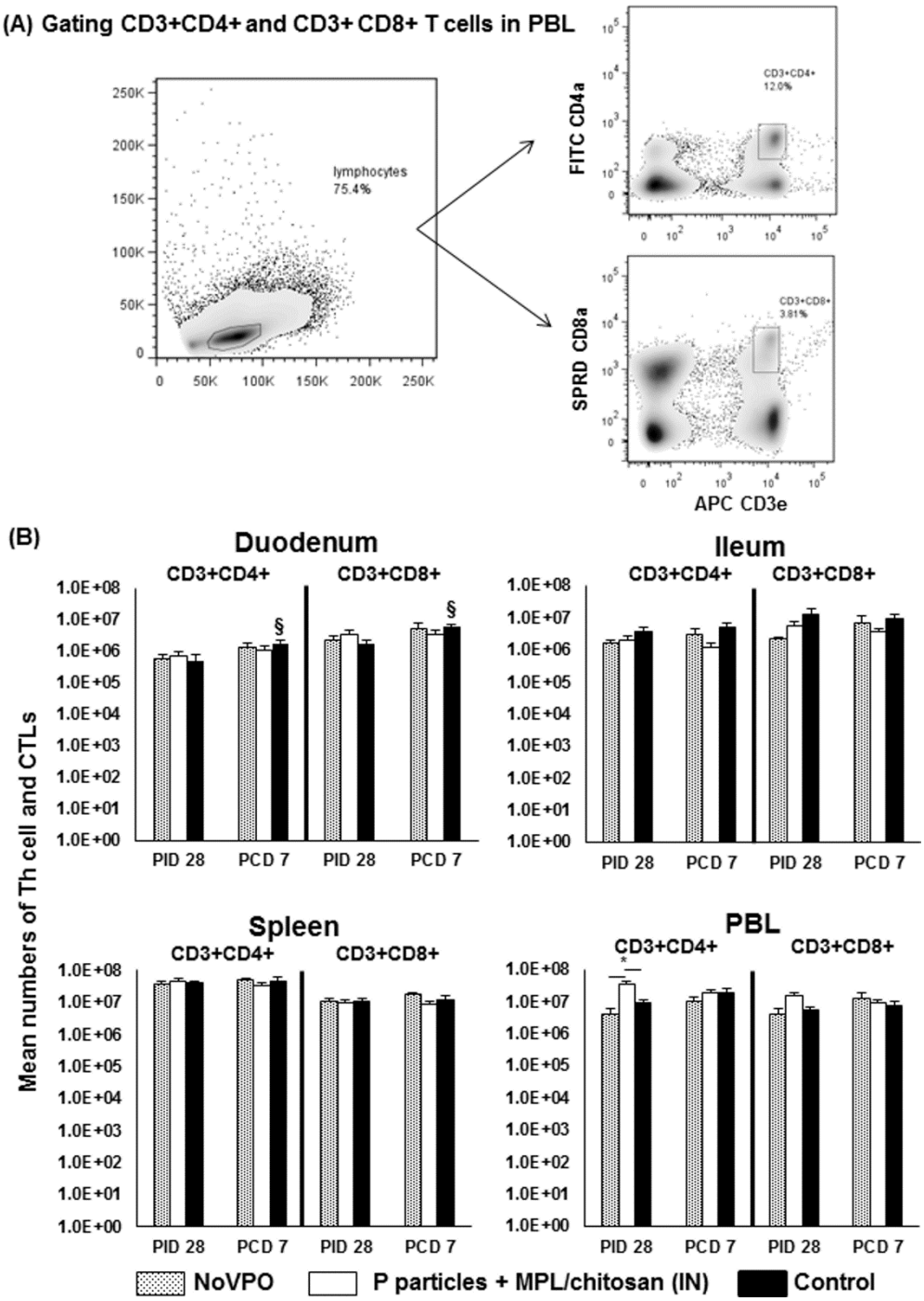

Figure 1. Th cells and CTLs induced by NoV infection or vaccination pre- and postchallenge. (A) Representative dot plots of frequencies of CD3+CD4+ and CD3+CD8+ T cells among lymphocytes from PBL of mock-vaccinated pigs postchallenge. FITC, fluorescein isothiocyanate; SPRD, Spectral Red; APC, allophycocyanin. (B) Mean total numbers plus standard errors of the means (n, 6 to 10) of CD3+CD4+ and CD3+CD8+ T cells in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues pre- and postchallenge. An asterisk above the error bars indicates a significant difference among groups for the same cell type and tissue at the same time point (P, <0.06 by Kruskal-Wallis one-way ANOVA). A section sign indicates that the numbers increased significantly following challenge in the same group. A number sign indicates that the numbers decreased significantly following challenge in the same group. IN, intranasal.

2.4.4 P particles increased the number of activated nonregulatory CD4+ T cells in the circulation prechallenge

Activated nonregulatory CD4+ and CD8+ T cells were identified by gating CD4+ or CD8+ lymphocytes expressing CD25, but not FoxP3 (Figure 2A). The data are presented as the mean number of cells per tissue (Figure 2B). Pre-challenge, P particle-vaccinated pigs had significantly higher numbers of activated CD4+ T cells in PBL compared to both NoVPO and control pigs (Figure 2B). This coincides with significantly higher numbers of total Th cells in P particle-vaccinated pigs in PBL (Figure 1B). Additionally, P particle pigs had significantly higher numbers of activated CD8+ T cells in PBL compared to NoVPO pigs at PID 28. These data suggest that intranasal administration of P particles with MPL and chitosan adjuvants effectively induced expansion of circulating activated T cells.

2.4.5 The duodenum and spleen are the major effector and memory sites, respectively, for activated T cells postchallenge

Following challenge, P particle-vaccinated pigs had significant increases in duodenal activated CD4+ T cells, which coincided with a significant decrease in splenic activated CD4+ T cells (Figure 2B). NoV challenge also significantly increased activated CD4+ T cells in duodenum of control pigs, which reflects the primary T cell response at the site of viral replication. This increase was confirmed by significant increases in total Th in duodenum of control pigs (Figure 1B). NoVPO pigs displayed significant increases in activated CD8+ T cells in spleen, indicating activation of memory CD8+ T cells following challenge at PCD 7. NoVPO pigs had significantly higher numbers of activated CD4+ and CD8+ T cells in spleen compared to P particle-vaccinated pigs. P particle-vaccinated pigs also had significantly lower numbers of activated CD4+ T cells in spleen following challenge (Figure 2B).

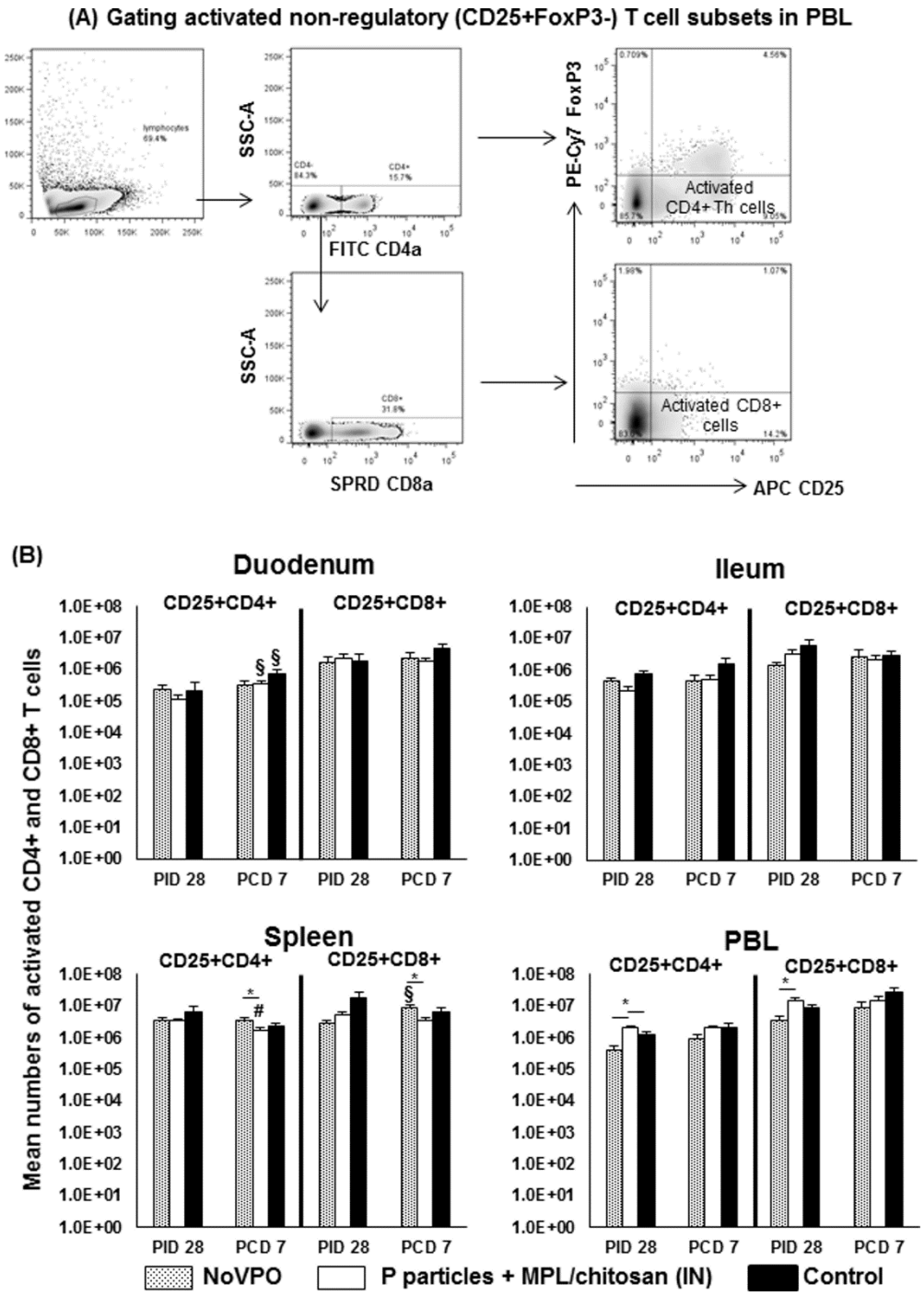

Figure 2. Activated nonregulatory CD4+ and CD8+ T cells pre- and postchallenge. (A) Representative dot plots of frequencies of CD4+CD25+FoxP3- and CD8+CD25+FoxP3- activated T cells in PBL from P-particle-vaccinated pigs prechallenge. PE, phycoerythrin. (B) Mean total numbers plus standard errors of the means (n, 6 to 10) of CD4+CD25+FoxP3- and CD8+CD25+FoxP3- activated T cells prechallenge and postchallenge in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues. See the legend to Figure 1 for an explanation of the symbols indicating statistical significance.

2.4.6 NoV-specific IFN-γ-producing T cell responses were low and transient in all groups

Virus-specific IFN-γ producing T cells act as effector cells to eliminate virus-infected cells and their magnitudes in the small intestine are significantly correlated with protective immunity against rotavirus, another enteric virus (45). NoV-specific effector/memory CD4+ and CD8+ T cells from the NoVPO, P particle and control groups at PID 28 and at PCD 7 were detected in intestinal and systemic lymphoid tissues by flow cytometry (Figure 3A). MNCs were stimulated with P particles or with PHA and medium-only as positive and background controls, respectively. The low numbers of IFN-γ expressing mock-stimulated MNCs were subtracted from the numbers of the P particle-stimulated MNCs to derive the adjusted virus-specific numbers. The mean numbers of virus-specific IFN-γ+ T cells pre- and postchallenge are shown in Figure 3B. Neither primary infection nor P particle vaccination significantly altered T cell IFN-γ production at PID 28 compared to control pigs. These data indicate that primary IFN-γ producing T cell responses after NoV infection or P particle vaccination are short-term.

Postchallenge, P particle-vaccinated pigs had significantly lower numbers of IFN-γ+CD4+ T cells in duodenum compared to control pigs (Figure 3B). Control pigs displayed significantly increased numbers of IFN-γ+CD8+ T cells in the duodenum compared to pre-challenge, reflecting the development of primary effector T cells at the site of NoV replication. This CD8+ effector T cell expansion is consistent with the expansion of total CTLs in duodenum (Figure 1B). The numbers of IFN-γ+CD4+ and IFN-γ+CD8+ T cells in ileum of the challenged NoVPO and P particle pigs were about 5-fold higher (not statistically significant) compared to the challenged control pigs at PCD 7 (Figure 3B).

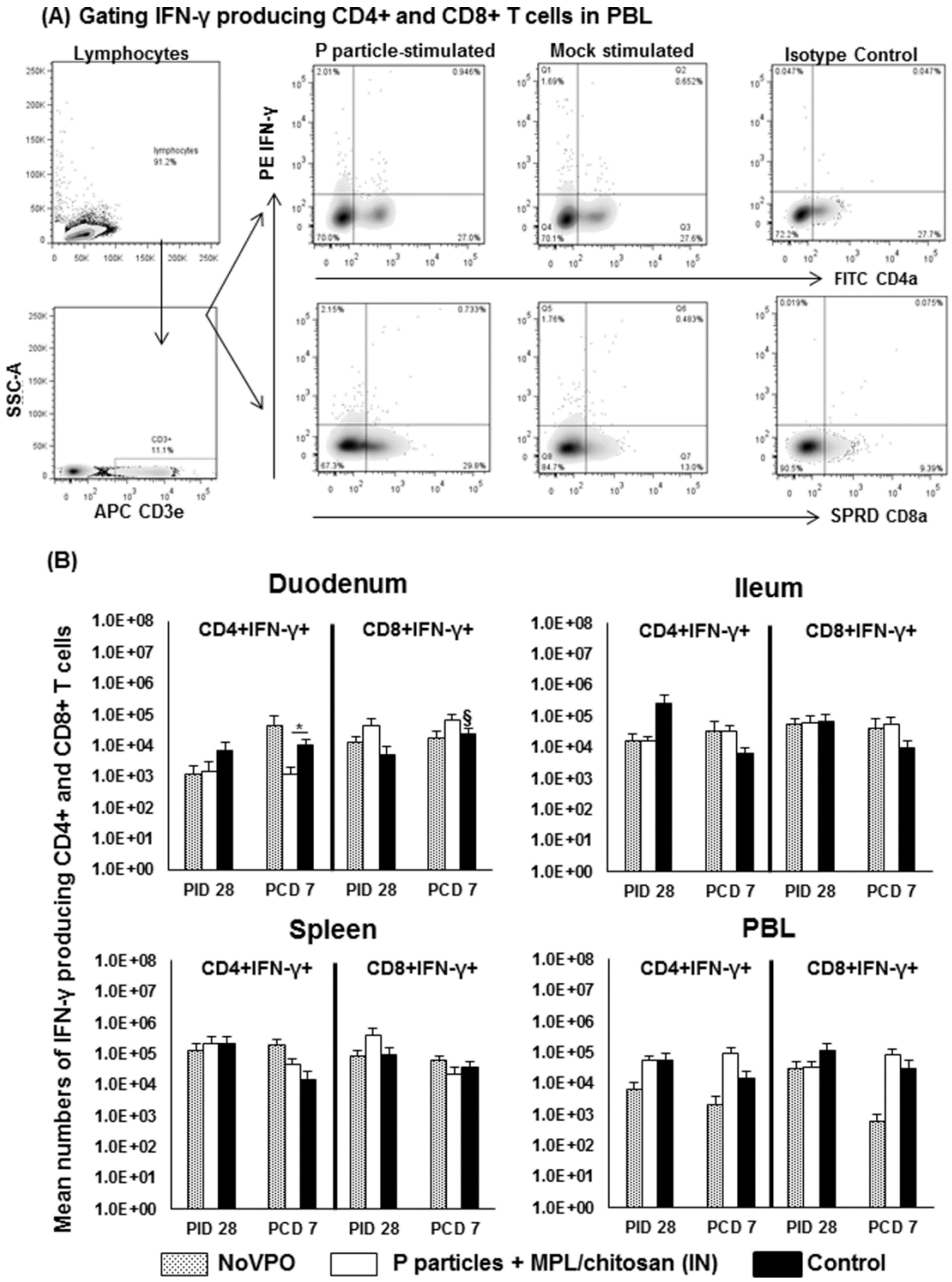

Figure 3. NoV-specific IFN-γ-producing CD4+ and CD8+ T cell responses pre- and postchallenge. (A) Representatitve dot plots of frequencies of NoV-specific CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ effector T cells in PBMCs isolated from the NoVPO group postchallenge and stimulated with P particles (17 h at 37ºC). IFN-γ was detected using intracellular staining and flow cytometry. (B) Numbers of IFN-γ-producing CD4+ and CD8+ T cells following subtraction of isotype control and mock-stimulated background numbers. Data presented are mean total numbers plus standard errors of the means (n, 6 to 10) prechallenge and postchallenge in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues. See the legend to Figure 1 for an explanation of the symbols indicating statistical significance.

2.4.7 Lower numbers of CD4+CD25-FoxP3+ Tregs in the duodenum are associated with increased protective efficacy against NoV challenge

Previous studies suggest that higher frequencies of functional CD4+CD25- Tregs could be an indicator for poorer protective immunity against rotavirus (46). In this study, we assessed the relationship between Treg numbers and protective immunity against NoV. Intestinal and systemic Tregs were evaluated using intracellular staining and flow cytometry on the days of MNC isolation at PID 28 and PCD 7 (Figure 4A). Figure 4B shows the total numbers of Tregs in each tissue.

Pre-challenge, there were no significant differences in the intestinal lymphoid tissues among the three groups. NoVPO pigs had significantly reduced numbers of CD4+CD25+FoxP3+ Tregs in PBL compared to P particle-vaccinated and control pigs. However, P particle pigs had overall higher numbers of CD4+CD25-FoxP3+ Tregs in spleen and significantly higher numbers of CD4+CD25+FoxP3+ Tregs in PBL than control pigs at PID 28. The increases in CD4+CD25+FoxP3+ Tregs of P particle-vaccinated pigs are consistent with increases in total Th cells (Figure 1B). Additionally, P particle-vaccinated pigs had significantly higher numbers of CD4+CD25-FoxP3+ Tregs in spleen and PBL compared to NoVPO pigs.

After NoV challenge, NoVPO pigs had significantly lower numbers of CD4+CD25-FoxP3+ Tregs in duodenum compared to controls, which corresponded to the highest protection rate conferred by primary NoV infection. P particle and control pigs had significant increases of both Treg subsets in duodenum compared to pre-challenge levels, while NoVPO pigs did not. Similar to the behaviors of activated nonregulatory CD4+ T cells (Figure 2B), the expansion of both Treg subsets in duodenum of P particle pigs coincided with their significant reductions in

spleen and the significantly lower numbers of CD4+CD25+FoxP3+ Tregs in spleen compared to NoVPO pigs postchallenge at PCD 7 (Figure 4B).

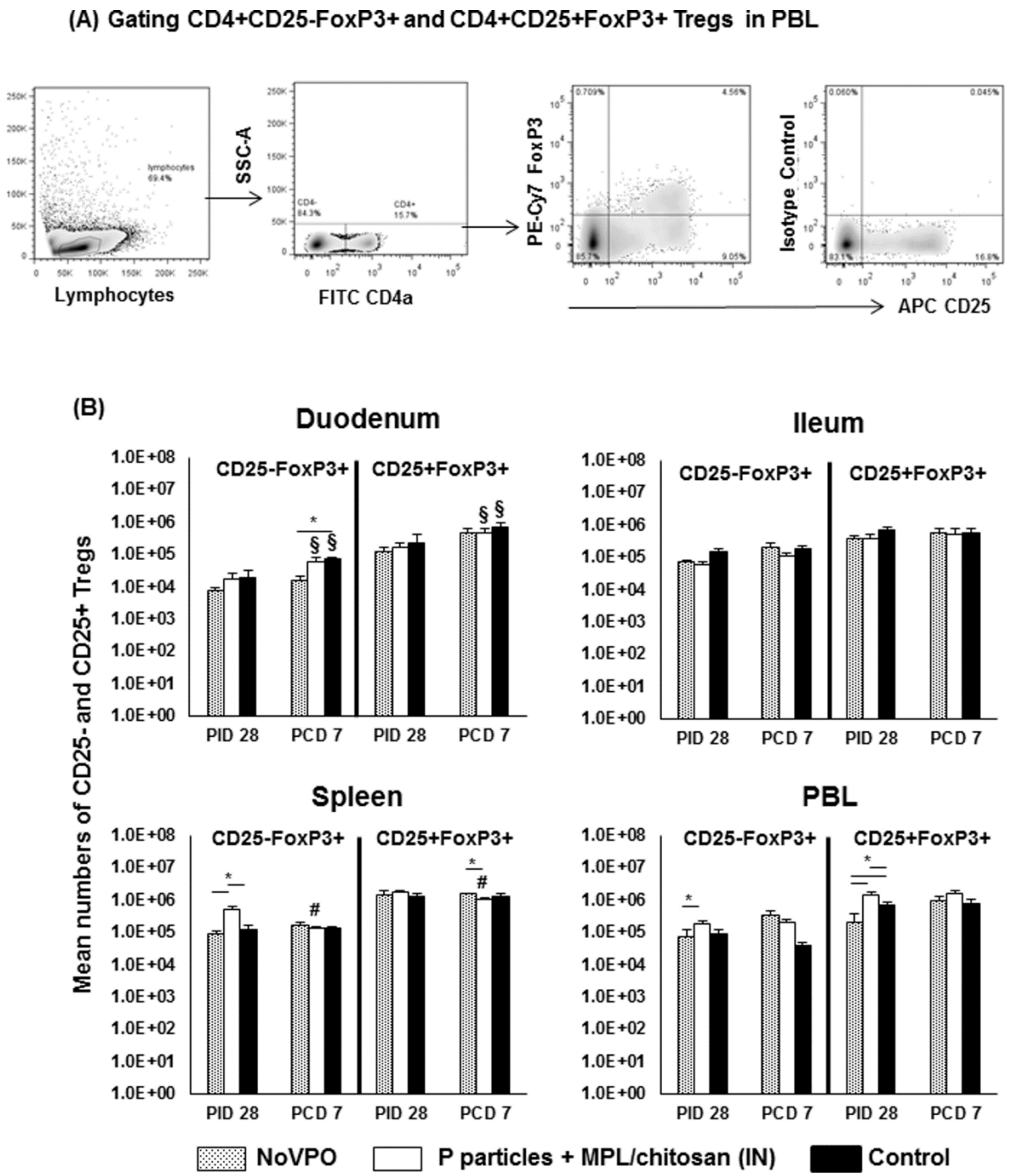

Figure 4. Treg responses induced by NoV infection or vaccination pre- and postchallenge. (A) Representative dot plots of frequencies of CD4+CD25-FoxP3+ and CD4+CD25-FoxP3+ Tregs from PBL of P-particle-vaccinated pigs prechallenge. (B) Mean total numbers of CD25- and CD25+ Tregs plus standard errors of the means (n, 6 to 10) among total MNCs prechallenge and postchallenge in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues. See the legend to Figure 1 for an explanation of the symbols indicating statistical significance.

2.4.8 NoVPO and P-particle-vaccinated pigs had reduced levels of TGF-β-producing Tregs in the ileum prechallenge

The cytokine-producing Treg responses were evaluated by intracellular staining of TGF-β and IL-10 producing CD25- and CD25+ Tregs (Figures 5 and 6). Pre-challenge, control pigs had significantly higher numbers of TGF-β producing CD25- Tregs in ileum compared to NoVPO and vaccinated pigs (Figure 5B). P particle-vaccinated pigs had significantly higher numbers of TGF-β producing CD25+ Tregs in spleen compared to NoVPO and control pigs. This pattern was similar to the IL-10 producing CD25+ Tregs in spleen (Figure 6B) pre-challenge.

After challenge, NoVPO pigs had significantly lower numbers of TGF-β producing CD25- Tregs in duodenum compared to vaccinated pigs and control pigs (Figure 5B). P particle-vaccinated pigs had significantly higher TGF-β producing CD25- Tregs in spleen compared to NoVPO and control pigs. Control pigs had significant reductions in numbers of TGF-β producing CD25- Tregs in ileum and in both Treg subsets in PBL. NoVPO pigs had significant reductions in TGF-β producing CD25- Tregs in spleen after challenge.

Control pigs, however, had significantly higher numbers of IL-10 producing CD25+ Tregs in duodenum compared to NoVPO and vaccinated pigs (Figure 6B). NoVPO pigs had significantly lower numbers of IL-10 producing CD25- Tregs in spleen compared to vaccinated and control pigs. P particle-vaccinated pigs had significantly higher numbers of IL-10 producing CD25+ Tregs in spleen compared to NoVPO and control pigs. Vaccinated pigs also had significantly higher numbers of IL-10 producing CD25- Tregs in PBL compared to NoVPO pigs. Challenge significantly decreased IL-10 producing CD25- Tregs in duodenum of NoVPO pigs.

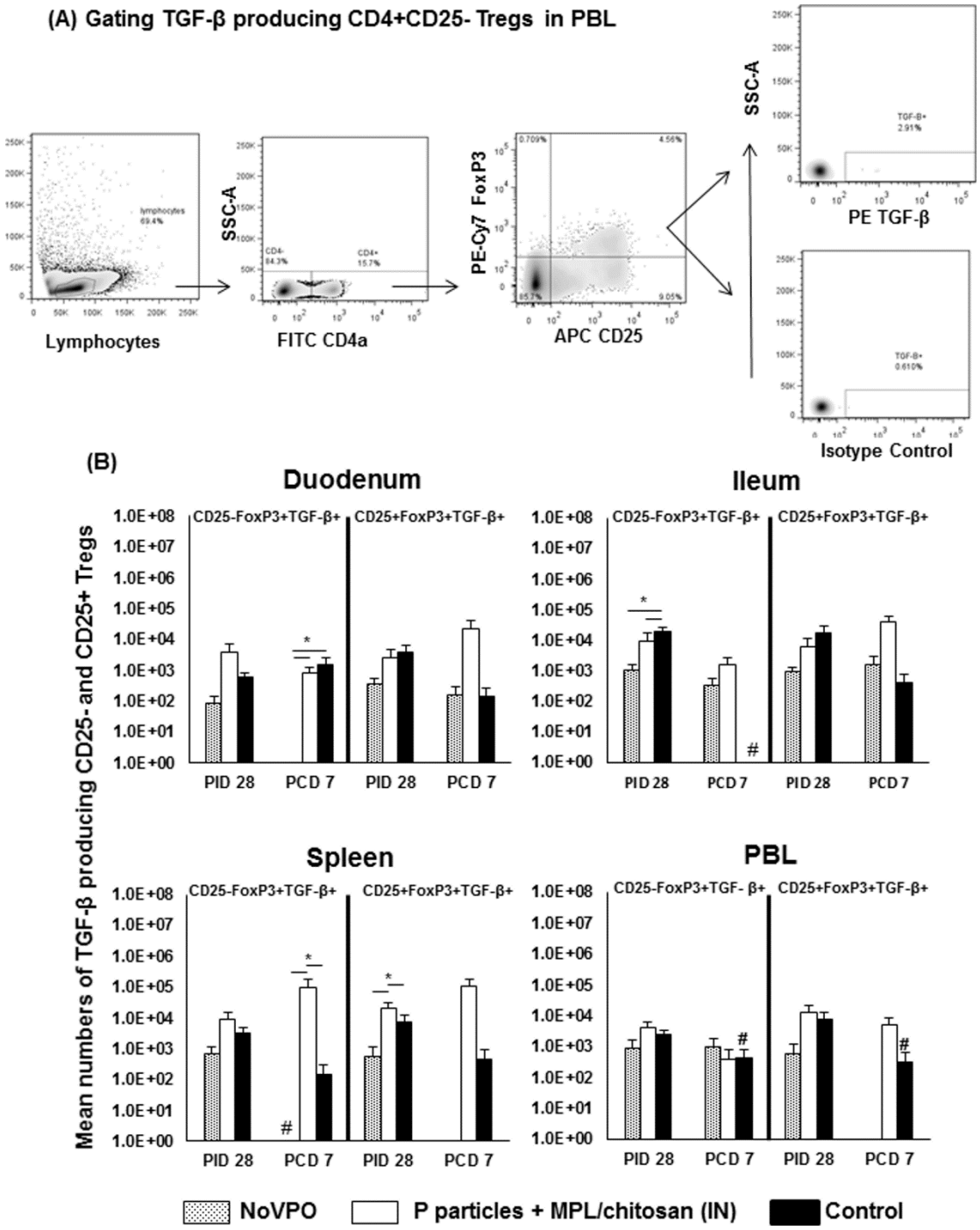

Figure 5. TGF-β-producing Treg responses induced by NoV infection or vaccination pre- and postchallenge. (A) Representative dot plots of frequencies of cytokine-secreting Tregs from unstimulated MNCs from PBL of P-particle-vaccinated pigs prechallenge. TGF-β production was detected using intracellular staining and flow cytometry. (B) Mean numbers plus standard errors of the means (n, 6 to 10) of cytokine-producing Tregs among Tregs prechallenge and postchallenge. See the legend to Figure 1 for an explanation of the symbols indicating statistical significance.

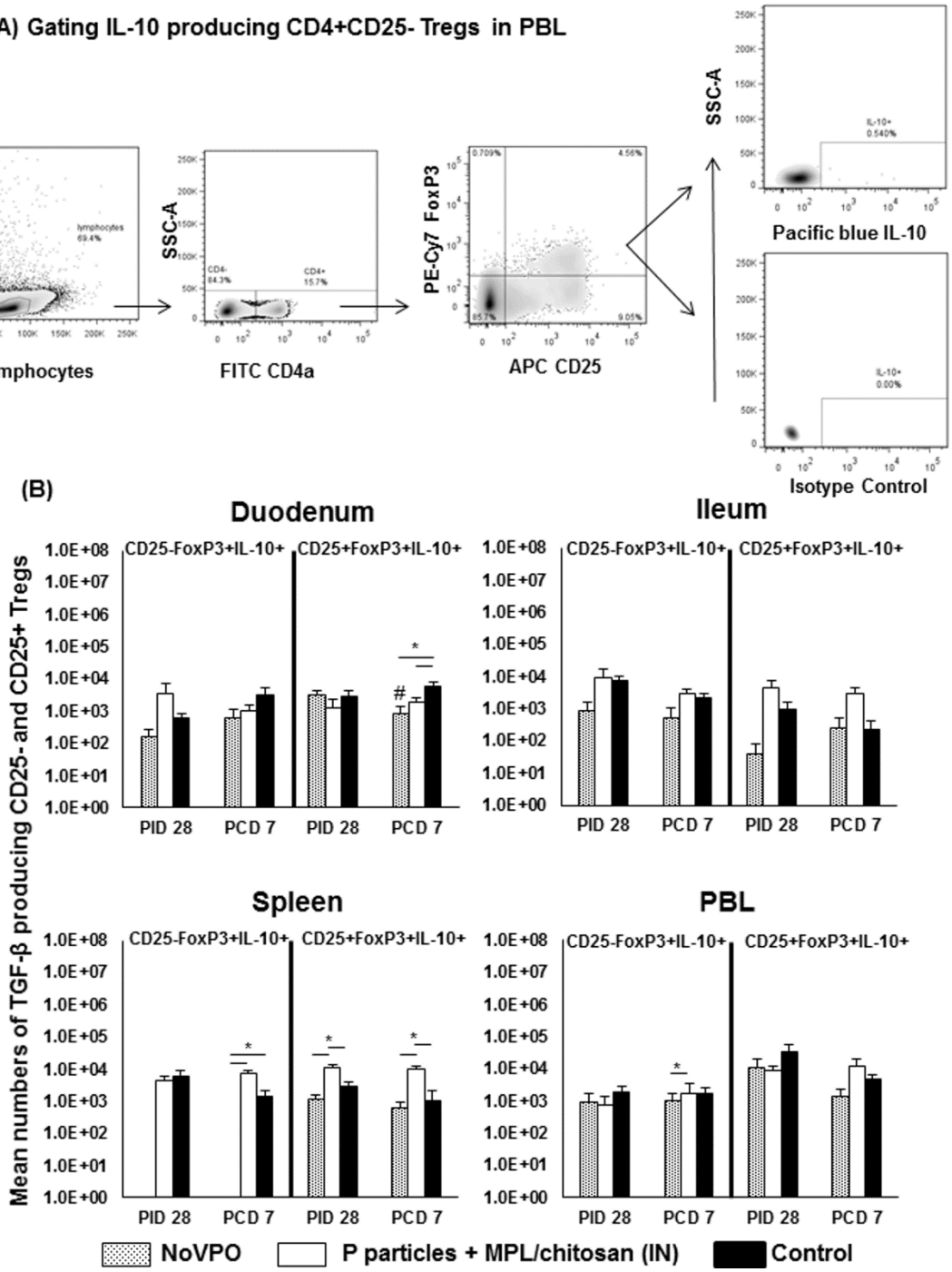

Figure 6. IL-10-producing Treg responses induced by NoV infection or vaccination pre- and postchallenge. (A) Representative dot plots of frequencies of cytokine-secreting Tregs from unstimulated MNCs from PBL of P particle-vaccinated pigs prechallenge. IL-10 production was detected using intracellular staining and flow cytometry. (B) Mean numbers plus standard errors of the means (n, 6 to 10) of cytokine-producing Tregs among Tregs prechallenge and postchallenge. See the legend to Figure 1 for an explanation of the symbols indicating statistical significance.

2.4.9 Expansions of T cells in the duodenum were inversely correlated with protection rates, while T cell expansion in ileum was positively correlated with protection rates

We calculated the expansion of each type of T cells in each tissue after NoV challenge by dividing postchallenge numbers with pre-challenge numbers for the NoVPO, P particle, and control pigs (Figure 7). Values >1 indicate numbers of T cell subsets increased following challenge, while values <1 indicate a decrease. We found that the expansion of T cells (activated CD4+CD25+ T cells, CD3+CD8+IFN-γ+ T cells, CD4+CD25-FoxP3+ Tregs, and TGF-β+CD4+CD25-FoxP3+ Tregs) in duodenum, which is the site of NoV replication, were all significantly inversely correlated with protection rates against diarrhea ($R = -1$, $p<0.0001$). It is also important to note that duodenal CD3+CD4+IFN-γ+ T cells expanded 37-fold in NoVPO pigs postchallenge. However, the expansion of T cells in ileum (including activated CD8+CD25+ T cells, CD3+CD4+/CD8+IFN-γ+ T cells, CD4+CD25+/-FoxP3+ Tregs, TGF-β+CD4+CD25-FoxP3+ Tregs, and IL-10+CD4+CD25+/-FoxP3+ Tregs) were all significantly positively correlated with protection rates ($R = 1$, $p<0.0001$). Ileum is the induction site of gut associated lymphoid tissues. In addition, expansion of T cells (activated CD4+CD25+ and CD8+CD25+ T cells, and CD3+CD4+IFN-γ+ T cells) in spleen were also significantly positively correlated with protection rates ($R = 1$, $p<0.0001$).

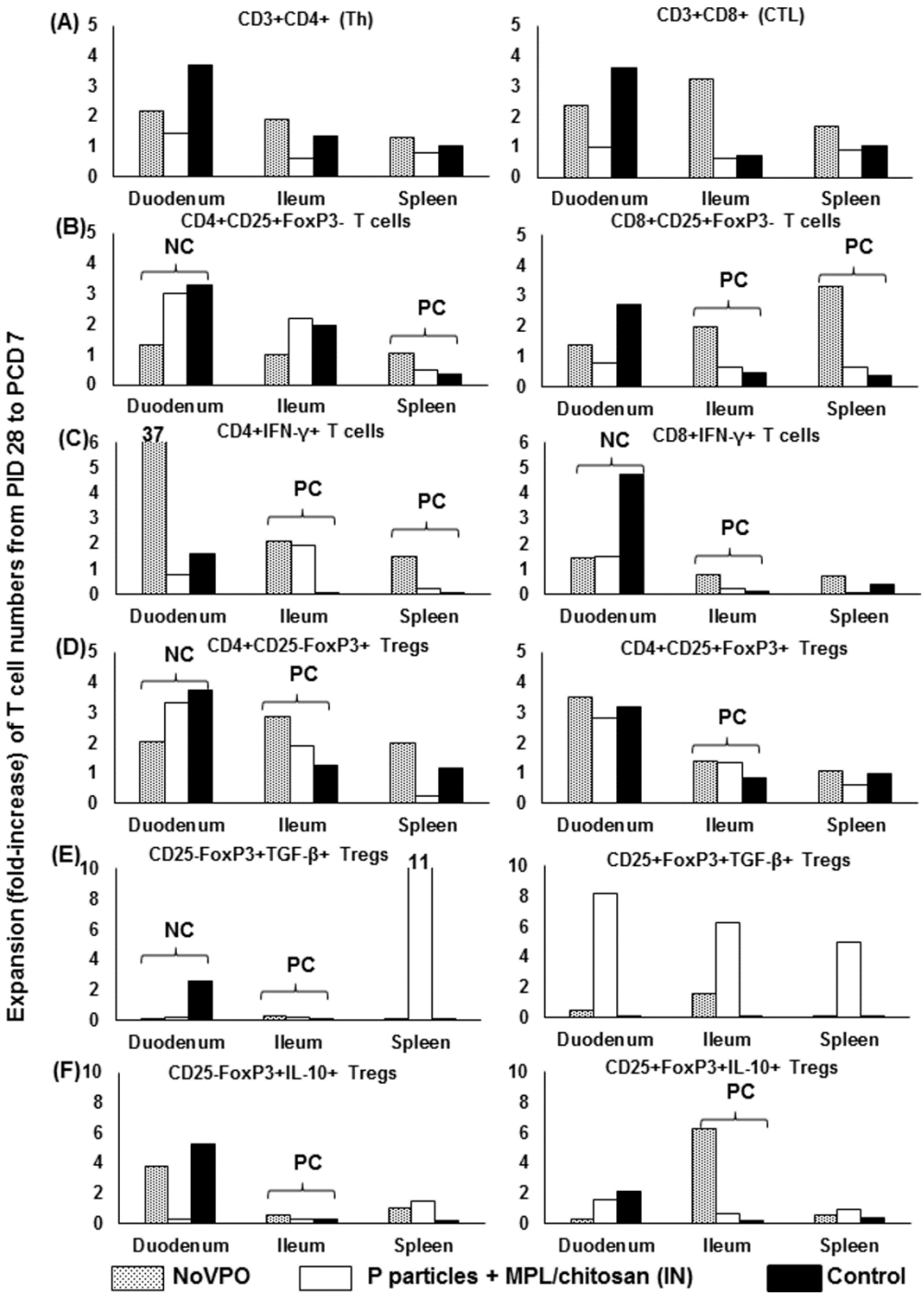

Figure 7. Expansion of T cell subsets following NoV challenge. Expansion was determined by dividing the postchallenge numbers of each T cell subset by the prechallenge numbers. Shown is the expansion of Th cells and CTLs (A), activated nonregulatory CD25+FoxP3- T cells (B), IFN-γ-producing T cells (C), CD25- and CD25+ Tregs (D), and TGF-β (E)- and IL-10 (F)-producing Tregs among NoVPO, P-particle-vaccinated, and control pigs. Correlations between T cell expansion and the protection rate against diarrhea were determined with Spearman's rank correlation coefficient (R, +1 or -1; P, <0.0001) in SAS. NC indicates a significant negative correlation with the protection rate against diarrhea (R, -1; P, <0.0001); PC indicates a significant positive correlation with the protection rate against diarrhea (R, 1; P, <0.0001). See Figures 1 to 6 for the analysis of cell types.

2.4.10 P-particle-vaccinated pigs had stronger effector and regulatory T cell responses than VLP-vaccinated pigs postchallenge

T cell responses in P particle- versus VLP-vaccinated pigs postchallenge were compared. The total numbers of activated nonregulatory T cells, IFN-γ producing T cells, Tregs, and TGF-β producing Tregs are shown in Figure 8. P particle pigs had significantly higher numbers of activated CD4+CD25+ T cells in all tissues examined (Figure 8A). These pigs also had significantly higher numbers of CD8+IFN-γ+ T cells in duodenum (Figure 8B). Additionally, P particle pigs had significantly higher numbers of CD4+CD25-FoxP3+ and CD4+CD25+FoxP3+ Tregs in PBL (Figure 8C) and TGF-β producing CD4+CD25-FoxP3+ Tregs in spleen compared to VLP pigs (Figure 8D).

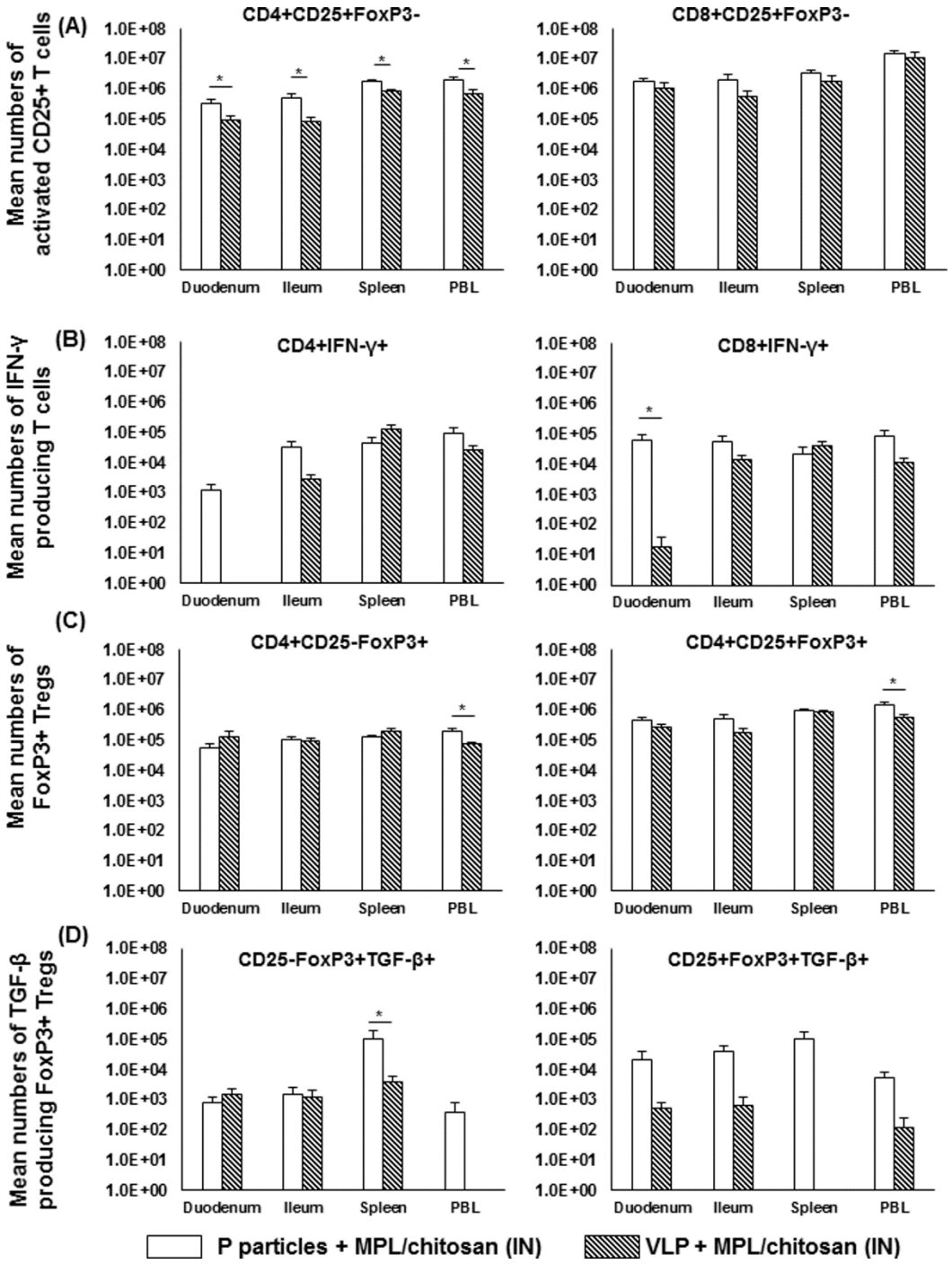

Figure 8. Comparisons of P-particle- and VLP-induced T cell responses following NoV challenge. Shown are total numbers of activated nonregulatory CD25+FoxP3- T cells (A), IFN-γ-producing T cells (B), FoxP3+ Tregs (C), and TGF-β-producing Tregs (D) among P-particle-vaccinated or VLP-vaccinated pigs postchallenge. Error bars indicate the standard errors of the means. See Figures 2 to 5 for the analysis of cell types. See the legend to Figure 1 for an explanation of the symbols indicating statistical significance.

2.5 Discussion

Studies of NoV infection and vaccines in humans and mice have mainly focused on evaluating humoral immune responses (13, 27, 28, 34, 47). In this study, we compared the protective efficacies of NoV primary infection, P particles, and VLPs to homotypic GII.4 challenge in Gn pigs. We also evaluated the T cell responses induced by NoV infection and vaccination pre- and/or postchallenge.

Prior NoV GII.4/2006b infection conferred the best protection by significantly reducing the number of pigs with diarrhea (82.9% protection rate) and reducing the number of pigs that shed virus (48.6% protection rate) following challenge with the same NoV variant (homologous protection). The difference in protection rates between diarrhea and infection are likely the result of asymptomatic infection in 2 out of 7 challenged pigs postchallenge. Asymptomatic NoV infection is common in humans (48). Three intranasal doses of GII.4/1997 VA387 (Farmington Hills) variant-derived P particle or VLP vaccines provided protection against diarrhea but not virus shedding in GII.4/2006b challenged Gn pigs. Previous studies comparing VLPs and P particles in mice (12, 13) have offered conflicting results and have been limited by the inability to infect mice with human NoV. The present study illustrates that P particles are not only capable of partial protection against human NoV-induced diarrhea in a higher order animal model, but can provide similar protection to VLPs. Further, the protection rates (46.7% and 60.0%, respectively) closely resemble the relative efficacy (47%) of a 2-dose intranasal VLP vaccine in human volunteers challenged with 10 ID_{50} homologous Norwalk virus (28), which was the same challenge dose used in this study.

A previous study of VLP vaccine in Gn pigs reported that oral/intranasal administration of the GII.4 HS66 variant-derived VLPs adjuvanted with ISCOM or mLT provided 75% or 100% protection against diarrhea, respectively, and 100% protection against infection following

homologous challenge (21). However, only 57% of the challenged control pigs shed virus in the study, indicating that almost half of the reported protection was due to insufficient viral challenge dose. Thus, the relative protective efficacy of the HS66 VLP-mLT vaccine was similar to the protection rates of the P particles and VLPs that we reported in this study. The Norwalk VLP vaccine conferred a 26% protection rate against virus infection in humans (28), whereas the VA387 VLP and P particles in our study did not reduce the incidence of virus shedding in Gn pigs, suggesting that higher vaccine doses may be needed. However, these vaccines reduced the diarrhea AUC 1.2- and 3.1-fold, respectively (not statistically significant). The duration of protection conferred by the P particle or VLP vaccines remains to be determined. Previous studies in humans showed that a NoV infection may confer protection from a range of 2 months to 2 years (30) or up to 9 years (29) against homologous challenge.

NoV primary infection conferred only partial protection (48.6%) against reinfection upon challenge with 10 ID_{50} of the same virus inoculum. In contrast, human rotavirus primary infection conferred 100% protection against reinfection when challenged with 10^5 ID_{50} of the virulent Wa strain human rotavirus in Gn pigs (45). Comprehensive T cell immune responses to NoV infection or P particle and VLP vaccinations have not been reported previously in humans or Gn pigs. Limited evidence has suggested that NoV infection in humans induces a primarily Th1 type immune response. NoV-specific IFN-γ was predominantly produced by CD4+ cells in antigen-stimulated PBMCs of humans infected with Snow Mountain virus (33). Norwalk VLPs induced IFN-γ production in the absence of IL-4 in PBMCs of volunteers (34). Both CD4+ and CD8+ T cells were shown to be crucial for clearance of NoV infection in mice (35, 36).

Consistent with the disparity in protection rates conferred by rotavirus versus norovirus primary infection, virus-specific IFN-γ producing T cell responses at challenge and

postchallenge in NoVPO pigs were overall weaker compared to that of rotavirus-infected pigs (45, 49). Thus, virus-specific IFN-γ producing T cells are not a correlate of protection against NoV challenge, confirming the findings in mice (32). However, NoVPO pigs had a 37-fold increase in IFN-γ producing CD4+ T cells in duodenum following challenge while control pigs experienced significantly increased IFN-γ producing CD8+ T cells following challenge (Figures 3B and 7C). P particle-vaccinated pigs had a more balanced expansion in CD4+ and CD8+ IFN-γ producing T cells. These data suggest that CD4+IFN-γ+ T cells in duodenum may be important for protection from re-infection. Additionally, these results may indicate the importance of effector CD8+ T cells in duodenum for clearance of viral infection. Future studies using CD8-knockout pigs will further elucidate the role of these cell types in NoV immunity and infection.

We reported previously that rotavirus infection reduced frequencies and numbers of tissue-residing Tregs and decreased the frequencies of IL-10 and TGF-β producing CD4+CD25-FoxP3+ Tregs in ileum, spleen and blood at PID 28 (46). The frequencies of IL-10 and TGF-β producing CD4+CD25-FoxP3+ Tregs in all sites at PID 28 were significantly inversely correlated with the protection rate against rotavirus diarrhea upon virulent rotavirus challenge. In the present study, we identified that the numbers of Tregs in duodenum at PID 28 are inversely associated with protection rates.

Zhu et al. (32) reported that CD4+ T cells are correlates of immunity in mice following murine norovirus infection, while IFN-γ+ and CD8+ T cells are not, though these effects may be strain-specific (32). It is worth noting that the study only examined immune responses from spleen-derived cells. Our study supports their conclusion that IFN-γ and CD8 T cells are not correlates of protection. We were only able to establish an inverse association between duodenal

Tregs at PID 28 and protection rate against diarrhea, but not CD8 or CD4 T cells as a correlate of protection.

The present study identified correlations between postchallenge expansions of T cell subsets and protection. Interestingly, the correlations are tissue-dependent, regardless of T cell subset. Responses in the effector site, duodenum, which is also the site of NoV replication, were significantly inversely correlated with protection. However, NoVPO pigs had a 37-fold increase in CD4+IFN-γ+ T cells in the duodenum, which is consistent with the Th1 responses seen in human volunteers (33). Conversely, significant responses in the memory sites (ileum and spleen) were positively correlated with protection. These data have multiple implications. First, they illustrate the importance in selection of tissues for the study of immune responses to NoV infection and vaccines. Second, they suggest that primary NoV infection drives T cell proliferation in the primary infection site, while vaccination or previous infection drives T cell expansion in spleen or homing to the ileum and these T cells are recruited to the effector site upon challenge.

Previous studies comparing the immunogenicities of VLPs and P particles in mice have provided conflicting results (12, 13). Tamminen et al. (13) showed that VLPs induced a balanced Th1/Th2 response and P particles induced a Th2-biased response. Tan et al. (14) raised concerns that P dimers were used in the study instead of P particles. Fang et al. (12) found that P particles induced similar immune responses to VLPs. The present study shows that the P particle vaccine primed for stronger T cell responses overall than VLP postchallenge in Gn pigs. P particles induced significantly higher numbers of activated CD4+ T cells in all tissues, IFN-γ+CD8+ T cells in duodenum, Tregs in PBL, and TGF-β producing CD4+CD25-FoxP3+ Tregs in spleen. These results suggest that at the same dose, the P particle vaccine is more immunogenic than the

VLP vaccine in a higher order animal model. Given their similar protection rates and the T cell profiles, P particles are a viable alternative NoV vaccine candidate to VLPs.

Another major finding of this study is that the P particle vaccine can provide partial cross protection between two distinct GII.4 variants separated by over nine years. Amino acid sequence analysis suggests antigenic drift in the capsid proteins and in immunogenic epitopes of the VA387-derived P particles/VLPs and 092895 challenge strain (Tables 1 and 2). The NoV 092895 inoculum in this study is a 2006b variant, while the P particles and VLPs were derived from VA387, a 1997 Farmington Hills variant. These two strains have 93.5% VP1 sequence identity. However, despite these suggested antigenic drifts, the major binding pockets remain highly conserved between these two GII.4 variants (50).

NoVs undergo epochal evolution with the emergence of a new circulating pandemic strain replacing previous pandemic strains every 3-7 years (51) through mutations in VP1 or replication structures (52) and evasion of herd immunity (53). Previous studies have indicated a lack of protection among different pandemic strains and genogroups (33), including weak heterologous responses (54-56). Vaccine-induced heterologous responses to distinct antigenic NoVs are inconsistent and usually require cocktail vaccines (47, 57, 58). An effective NoV vaccine must induce cross-reactivity with different variants in the same genogroups and any emerging strains without impacting costs of vaccine production.

In this study, the NoV challenge inoculum was derived from a 2008 isolate of the GII.4/2006b strain, while the P particles and VLPs were derived from a GII.4 Farmington Hills variant isolated in 1997. Accordingly, there have been three antigenic shifts between these two strains (59). Blockade Epitope A in the P2 domain is considered to be the immunodominant GII.4 epitope that mutates over time to escape herd immunity and contributes to the epochal

evolution witnessed by NoV (60). The strains in this study have four substitutions among the six Epitope A amino acids (Table 2), suggesting that these strains are antigenically distinct in this epitope. This study is the first indication that a P particle vaccine candidate can provide protection from disease against an antigenically different challenge strain. Further, stimulation of MNCs isolated from pigs pre- and postchallenge with P particles induced production of IFN-γ, including from the NoVPO and control pigs that have only been inoculated with the challenge NoV GII.4/2006b inoculum (Figure 3B). These results indicate that VA387-derived P particles can stimulate the effector/memory T cell response against antigenically distinct strains. There is promise that a GII.4 vaccine candidate derived from current vaccine technologies may not only provide protection against parent strains, but potentially emerging or re-emerging strains.

To summarize, this study has several implications for NoV immunity and vaccine development. First, we have shown that previous infection provides substantial protection from NoV-induced gastroenteritis and partial protection from NoV re-infection. Additionally, we have shown that P particles are capable of providing partial protection against NoV diarrhea at a similar rate as VLPs. Second, we showed that VA387-derived P particles and VLPs provided cross-protection against a different variant of GII.4 NoV, indicating that current NoV vaccine technologies are capable of providing protection against antigenically distinct NoVs. Third, we showed an inverse association between pre-challenge duodenal Tregs and protection rates against diarrhea. Finally, we have shown that P particles induced stronger T cell responses than VLPs in an animal model that closely resembles the NoV disease in humans. These results indicate that P particles are a viable alternative to VLPs for NoV vaccine development.

In conclusion, primary NoV infection and both vaccine candidates reduced occurrence of diarrhea, while only primary NoV infection provided partial protection from re-infection. To our

knowledge, this is the first study to compare the protective efficacies of VLPs and P particles to NoV infection in a higher order animal model and also to indicate cross protection induced by antigenically distinct NoV vaccines. Additionally, this is the first study to comprehensively evaluate T cell profiles elicited by NoV primary infection, VLPs, and P particles prior to and/or following homotypic challenge. A study using a computer simulation model has suggested that a NoV vaccine conferring 50% protective efficacy lasting for 12 months could prevent 1.0-2.2 million cases of NoV gastroenteritis and save up to $2.1 billion in total economic burden in the US (61), including the $284 million economic cost to hospitals (6). Based on our findings and these economic considerations, we believe that the P particle vaccine merits further investigation.

2.6 References

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CHAPTER 3

Intranasal P particle norovirus vaccine is immunogenic and reduces diarrhea and infection following viral challenge in gnotobiotic pigs

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Submitted to *Human Vaccines & Immunotherapeutics*

3.1 Abstract

Abstract

Norovirus became the leading cause of viral gastroenteritis worldwide since the introduction of rotavirus vaccines in 2006. We previously showed that three intranasal doses of 100 µg of norovirus P particles provided similar protection against diarrhea compared to virus-like particles (VLPs) but superior T cell responses in gnotobiotic (Gn) pigs. This study examined the dose effects of P particles on protective efficacy and T cell immunogenicity using the Gn pig model. Pigs were intranasally inoculated with three doses (post-inoculation days [PIDs] 0, 10, and 21) of GII.4/VA387-derived P particles (100 or 250 µg/dose) with MPL and chitosan adjuvants. A subset of pigs were orally challenged with 10 median infectious doses (ID50) of a human norovirus GII.4/2006b variant. Protection was monitored for 7 days post-challenge. Intestinal and systemic T cell responses were determined pre- and post-challenge. High dose (250 µg) P particles conferred protection from viral shedding (60%) and slightly increased protection rate against diarrhea compared to the low dose (60% vs. 47%). High dose P particles primed for increased activated T cells, IFN-γ producing T cells, and decreased regulatory T cells (Tregs) post-challenge. Protection rate against diarrhea was positively correlated with activated T cells and IFN-γ producing T cell expansion in ileum and spleen and was negatively correlated with CD4+CD25-FoxP3+ Treg expansion in duodenum. A higher dose of NoV P particles is required for protection against NoV infection and diarrhea and for induction of robust T cell responses. Promoting activation of T cells while down-regulating Tregs are important for P particle-induced protective immunity.

Keywords: Human norovirus, P particle, vaccine, gnotobiotic pig, protective efficacy, T cells

3.2 Introduction

Human noroviruses (NoVs) have become the leading cause of acute gastroenteritis since the introduction of rotavirus vaccines (1-6). In the United States, NoV-derived gastroenteritis accounts for approximately 19-21 million cases, 2 million hospital visits and an estimated $284-$493 million in healthcare costs (7-9). On a worldwide basis, NoVs claim over 200,000 lives in children <5 years of age in developing countries annually (10). Overall, NoV accounts for approximately 20% of all acute gastroenteritis cases (11). GII.4 NoVs, the predominant genotype, account for 60-90% of outbreaks (12) and are associated with more severe disease (13, 14). It is estimated that a vaccine with a 50% protective efficacy for 12 months would avert 1.0-2.2 million cases of NoV gastroenteritis annually in the US (15). However, there are currently no vaccines or antivirals available.

The lack of a cell culture system has greatly hindered vaccine development through the traditional strategies of attenuated or inactivated vaccines. Thus, recombinant NoV capsid proteins, such as virus-like particles (VLPs) and P particles, have been examined as vaccine candidates. VLPs are produced via expression of the full-length capsid protein (VP1) in a eukaryotic expression system, while the P particles form after expression of the protruding (P) domain of VP1 in *E. coli* (16-18). Both particles retain similar histo-blood group antigen (HBGA) binding profiles as NoV (18). Several NoV VLP vaccines are currently going through clinical trials (19-21). Our previous study showed that VLPs and P particles provided similar protection rates against GII.4 NoV diarrhea in gnotobiotic (Gn) pigs, though the P particles induced stronger T cell responses (22). P particles had also been shown to elicit similar immune responses as VLPs in mice (23).

Dose responses are an important factor that must be considered in vaccine development. Previous studies showed that dose affected VLP-induced immune responses in adult humans (20, 21, 24, 25). Increasing the doses (up to 100 µg) of two dose intranasally-administered VLPs promoted development of memory B cells (24) and intestinal homing virus-specific IgG and IgA antibodies (20). Additionally, two doses of 50 µg intramuscular bivalent NoV vaccines increased serum antibody and HBGA blocking antibody titers compared to 5 µg and 15 µg doses (21). Two doses of oral VLPs at 250 µg/dose increased serum IgG production similar to higher doses (500 µg or 2000 µg) (25). However, both regimens of 250 µg or 500 µg induced transient IFN-γ production, while the 2000 µg regimen did not (25). These results indicate that NoV vaccines operate within ideal range of 50-250 µg/dose or 100-1000 µg over the entire vaccine course.

This current study builds upon our recently published P particle study (22) and further evaluates the P particles as vaccine candidates. We evaluated the dose effects of the P particles in protection and immunogenicity against GII.4 NoV challenge and disease utilizing our established Gn pig challenge model (22, 26). We also compared the dose effects on P particle vaccine-induced T cell responses. Since we previously showed that P particles provided similar protection against NoV diarrhea and increased T cell responses as VLPs at the dose of 100 µg (22) and VLP immunogenicity is dose-dependent (20, 21, 24, 25), we hypothesize that P particles' protective efficacy and immunogenicity are dose-dependent as well. To our knowledge, this is the first study to evaluate the dose responses of the P particle vaccine on protective efficacy and T cell immunity.

3.3 Materials and Methods

3.3.1 Virus

Human stool containing human norovirus GII.4/2006b variant 092895 (GenBank accession number KC990829) was collected and processed as previously described (26). Briefly, confirmed NoV-positive stool was diluted to 10% and subjected to repeated high speed centrifugation for removal of bacterial and other fecal contaminants. We previously established an ID_{50} (6.43 x 10^4 RNA copies) for this variant at 33-34 days of age (26). Ten times the ID_{50} (6.43 x 10^5 RNA copies) was used for challenge as in our previous study (22) and is consistent with the challenge dose used in human volunteer studies (19).

3.3.2 Vaccine preparation

P particles were derived from GII.4 VA387 (1997 Farmington Hills variant) as previously described (18, 22). P particles were sterilized with UV light as previously described (22). Synthetic monophosphoryl lipid A (MPL) (Avanti Polar Lipids, Inc. 699800P) and chitosan (NovaMatrix UP-G-213) adjuvants were prepared as previously described (22). Vaccines were prepared as previously described (22). Briefly, each vaccine dose consisted of 100 µg (LoPP) or 250 µg (HiPP) P particles with 5 mg chitosan and 50 µg MPL diluted in TNC buffer (36) to a final volume of 1 ml. Control pigs received adjuvants alone. Each vaccine dose was administered intransally using mucosal atomization devices (MADs) (LMA North America MAD300). Sterility was monitored with blood agar plates and thioglycollate media as previously described (22).

3.3.3 Treatment and inoculation of Gn pigs

Near-term Large White cross pigs were derived via hysterectomy and maintained in germ-free isolator units (37). Pigs were confirmed to be A+ or H+ prior to inoculation and sterility was monitored as previously described (26). Pigs (male and female) were randomly divided into three groups consisting of ≥6 pigs/group from at least 2 different litters. Vaccinated pigs were inoculated with three intranasal doses of 100 µg (LoPP) or 250 µg (HiPP) P particles. Control pigs were inoculated with adjuvants only. Intranasal doses were given at post-inoculation day 0 (PID 0), PID 10, and PID 21 using MADs. A subset of pigs in each group was orally challenged with 10 ID_{50} NoV (6.43 x 10^5 RNA copies) as previously described.(22) Challenged pigs were orally treated with 200 mM sodium bicarbonate 20 minutes prior to challenge to neutralize gastric acidity. All pigs were euthanized at PID 28 (pre-challenge) or PCD 7 (post-challenge) and mononuclear cells (MNCs) were isolated from duodenum, ileum, spleen, and blood as previously described (22, 38). Each group consists of: 9 pigs (LoPP PID 28), 6 pigs (HiPP PID 28), 10 pigs (Control PID 28), 9 pigs (LoPP PCD 7), 6 pigs (HiPP PCD 7), and 6 pigs (Control PCD 7). All animal experimental procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Virginia Tech.

3.3.4 Detection of NoV shedding and assessment of diarrhea

Pigs were rectal swabbed daily for clinical signs and shedding from PCD 0 to PCD 7. Diarrhea was scored using rectal swabs as previously described (26). Briefly, diarrhea was assessed on the following scale: 0, normal; 1, pasty; 2, semi-liquid; 3, liquid. Scores of ≥2 were

considered diarrheic. Pigs were considered positive for diarrhea if they presented with diarrhea for at least day from PCD 1 to PCD 7.

Virus shedding was monitored with TaqMan® real-time RT-PCR as previously described (26). Pigs were considered positive for shedding if NoV was detected in a stool sample for at least one day from PCD 1 to PCD 7.

Diarrhea severity and shedding titers are presented as AUCs to include duration and magnitude of disease and shedding and were used in our previous study (22). AUCs were calculated for disease and shedding from PCD 1 through PCD 7 using GraphPad Prism 6.

3.3.5 Flow cytometry

The frequencies of T cell subsets in intestinal and systemic tissues were determined by using intracellular staining and multicolor flow cytometry. The evaluated subsets were defined as T helper cells (Th, CD3+CD4+), cytotoxic T lymphocytes (CTL, CD3+CD8+), NoV-specific IFN-γ producing CD3+CD4+ and CD3+CD8+ T cells, activated non-regulatory (CD25+FoxP3-) CD4+ and CD8+ T cells, and TGF-β producing CD4+CD25-FoxP3+ and CD4+CD25+FoxP3+ regulatory T cells (Treg) as previously described (22, 39, 40). Briefly, IFN-γ producing CD4+ and CD8+ T cells and total Th and CTLs were *in vitro* stimulated with P particles for 17 h (the final 5 h with Brefeldin A) following MNC isolation, while activated T cells and Tregs were stained freshly on the day of cell isolation. Total numbers and expansion of T cell subsets were calculated following subtraction of background and mock-stimulated numbers as previously described (22). Data presented are total numbers of each subset.

3.3.6 Statistical analysis

One-way ANOVA-general linear model (ANOVA-GLM) followed by Duncan's multiple range test was used to compare mean durations of diarrhea and shedding. Fisher's exact test was performed to compare percentages of pigs with diarrhea and virus shedding. Kruskal-Wallis rank-sum test was used to compare the diarrhea and virus shedding AUCs and numbers of T cell subsets. Correlations between T cell expansion and protection rates were assessed with Spearman's rank correlation coefficient. All statistical significance was assessed at $p<0.05$. All statistical analyses were performed using SAS Program 9.3 (SAS Institute, NC, USA).

3.4 Results

3.4.1 HiPP provide partial protection against infection upon NoV challenge

The protective efficacies for both P particle doses were monitored following GII.4/2006b NoV challenge. The clinical signs and shedding are shown in Table 1. Data for both low dose P particles (LoPP) and control pigs were partially presented previously (22). Both P particle doses provided partial protection from diarrhea (46.7% and 60.0% protection rates), while high dose P particles (HiPP) slightly reduced the mean number of days with diarrhea compared to LoPP and control (0.8 vs. 1.3 and 1.8 days, respectively). Both doses had similar reductions in areas under the curve (AUCs) of diarrhea (1.2 and 1.3-fold) compared to controls. AUCs of diarrhea were calculated from the line graphs made using the daily diarrhea score and the days of diarrhea. Only 33% of HiPP pigs shed virus resulting in a 60% protection rate against shedding, while LoPP did not reduce shedding. Further, HiPP reduced the mean number days of shedding by 1.5 days and reduced AUC by 53-fold compared to control pigs. AUCs of shedding were calculated from the line graphs made using the daily virus shedding titer and the days of virus shedding. Although neither dose reached statistical significance in reduction of diarrhea or shedding, these trends are evident. It is worth noting that NoV shedding titers were not higher than the inoculum titer on any day in any group post-challenge, but as we had previously showed that total NoVs in the whole intestine of mock-vaccinated, challenged control pigs was higher than that in the inoculum (22). This result confirms that the Gn pig model supports NoV viral replication.

Table 1. Clinical signs and protective efficacy in P particle-vaccinated Gn pigs after challenge with GII.4/2006b NoV[a]

		Diarrhea[b]					Virus shedding				
Group*	n	Mean % of pigs with diarrhea (no. of pigs with diarrhea/total no.)	Mean no. of days with diarrhea[c] (SEM)	Mean AUC from PCD 1 to PCD 7 (SEM)	Fold reduction in AUC	Rate of protection against diarrhea (%)[d]	Mean % of pigs shedding virus (no. of pigs with shedding/total no.)	Mean no. of days with shedding[c] (SEM)	Mean AUC from PCD 1 to PCD 7 (SEM)	Fold reduction in AUC	Rate of protection against shedding (%)[d]
LoPP**	9	44% (4/9)	1.3 (0.6)	5.4 (0.8)	-1.2	46.7	89% (8/9)	1.8 (0.4)	38216 (10568)	-3.1	0.0
HiPP	6	33% (2/6)	0.8 (0.7)	5.1 (1.1)	-1.3	60.0	33% (2/6)	0.5 (0.3)	2191 (1395)	-53.4	60.0
Control**	6	83% (5/6)	1.8 (0.6)	6.7 (1.0)	1.0	NA	83% (5/6)	2.0 (0.7)	116960 (78189)	1.0	NA

[a] Gn pigs were challenged with a human NoV GII.4 2006b variant 092895 at 33-34 days of age. Rectal swabs were collected daily after challenge to determine diarrhea and virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents.

[b] Fecal scoring system: 0, solid; 1, pasty; 2, semi-liquid; 3, liquid. Pigs with scores of 2 or higher were considered diarrheic.

[c] From PCD 0 to PCD 7.

[d] Calculated as [1 – (percentage of immunized pigs in each group with diarrhea or shedding/percentage of control pigs with diarrhea or shedding)] x 100. NA, no applicable

* Abbreviated group names: LoPP (100 µg P particles + MPL/chitosan); HiPP (250 µg P particles + MPL/chitosan); Control (MPL/chitosan alone).

** Data were presented in Chapter 2.

3.4.2 HiPP prime for increased Th and CTL in intestine and blood

The pre- and post-challenge total Th (CD3+CD4+) and CTL (CD3+CD8+) are depicted in Figure 1A. Interestingly, LoPP pigs had significantly higher numbers of Th in PBL compared to HiPP and control pre-challenge. HiPP pigs had significantly higher numbers of Th and CTL in duodenum and ileum, respectively, compared to LoPP pigs post-challenge. There were significant increases in numbers of Th and CTL in duodenum, ileum and PBL of HiPP pigs following challenge. The fold expansion of Th and CTL following challenge are show in Figure 1B. HiPP induced the most expansion of Th and CTL in each tissue examined, while LoPP induced the lowest expansion, though these differences were not statistically significant.

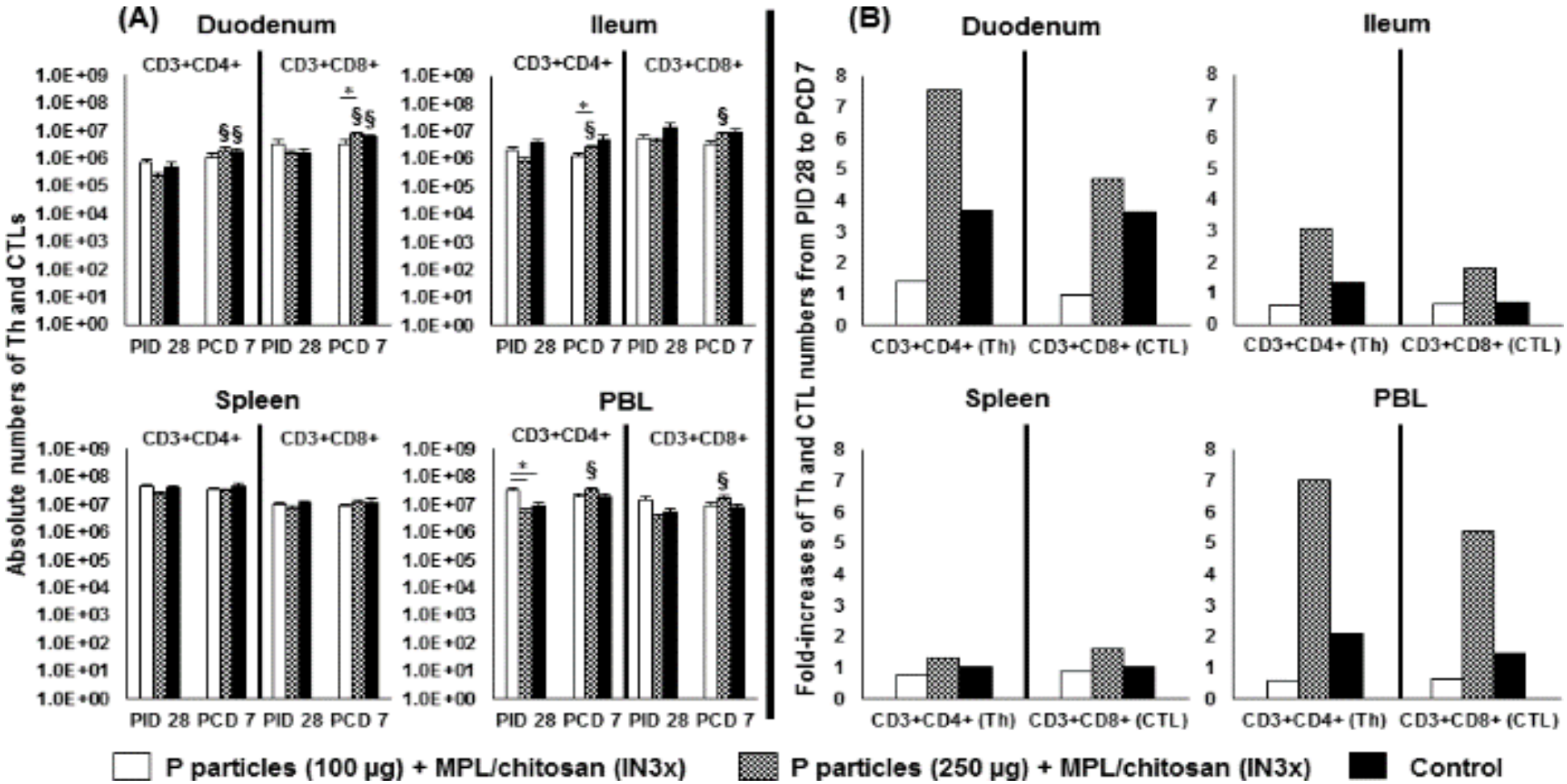

Figure 1. Th and CTL responses induced by low dose and high dose P particle vaccination pre- and post-challenge. Gn pigs were intranasally inoculation with VA387-derived P particles at PIDs 0, 10, and 21. A subset of pigs were orally challenged with human NoV at PID 28/PCD 0. Mean total numbers + standard errors of the means (n, 6 to 10) of CD3+CD4+ (Th) and CD3+CD8+ (CTL) T cells in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues at PID 28 (pre-challenge) and PCD 7 (post-challenge) (A). Frequencies of Th and CTL were determined using intracellular staining and multicolor flow cytometry. Total numbers were calculated using volume and concentration of isolated MNCs. An asterisk indicates a significant difference among groups for the same cell type and tissue at the same time point (Kruskal-Wallis one-way ANOVA, P<0.05). A section sign indicates that numbers increased significantly following challenge among the same group and a number sign indicates that numbers decreased significantly following challenge among the same group. Total expansion of Th or CTL following NoV challenge (B). Expansion of Th and CTL were calculated by dividing post-challenge numbers by pre-challenge numbers. Values >1 indicate Th or CTL increased following challenge, while values <1 indicate Th1 or CTL decreased following challenge. Correlations between protection rate against diarrhea and T cell subset expansion were determined using Spearman's rank correlation coefficient. PC indicates a positive correlation between protection rate against diarrhea and T cell subset expansion (R=1, P<0.0001), while NC indicates a negative correlation (R=-1, P<0.0001).

3.4.3 HiPP prime for increased activated T cells post-challenge

The activated non-regulatory CD4+ and CD8+ T cells are shown in Figure 2A. At PID 28, HiPP pigs had significantly lower activated CD4+ T cells in spleen than both LoPP and control pigs and in PBL compared to LoPP pigs. HiPP pigs also had significantly lower activated CD8+ T cells in spleen and PBL compared to control and LoPP pigs, respectively. Following challenge, HiPP pigs had significantly higher numbers of activated CD8+ T cells in ileum compared to LoPP pigs. HiPP pigs had significant increases in both activated T cell subsets in all tissues with the exception of CD4+ T cells in spleen following challenge. The NoV challenge-induced fold expansions of activated T cells are shown in Figure 2B. HiPP pigs had the biggest fold expansion of activated T cell in all tissues examined. Activated CD4+ and CD8+ T cells in ileum and spleen were positively correlated with protection rate against diarrhe

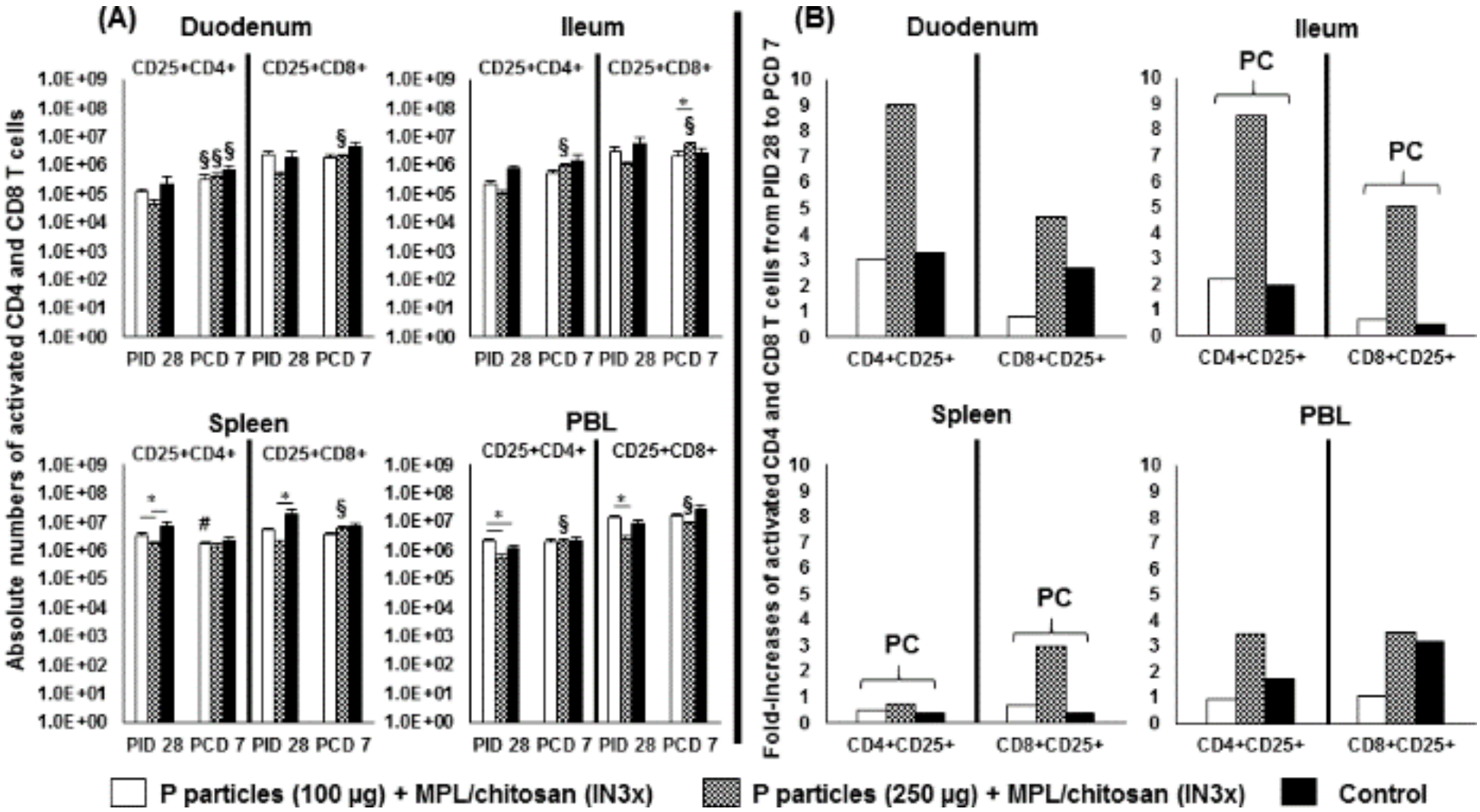

Figure 2. Activated non-regulatory CD4+ and CD8+ T cell responses induced by low dose and high dose P particle vaccination pre- and post-challenge. Gn pigs were intranasally inoculated with VA387-derived P particles at PIDs 0, 10, and 21. A subset of pigs were orally challenged with human NoV at PID 28/PCD 0. Mean total numbers + standard errors of the means (n, 6 to 10) of CD4+CD25+FoxP3- and CD8+CD25+FoxP3- T cells in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues at PID 28 (pre-challenge) and PCD 7 (post-challenge) (A). Frequencies of activated non-regulatory T cells were determined using intracellular staining and multicolor flow cytometry. Total numbers were calculated using volume and concentration of isolated MNCs. Total expansion of activated CD4+ and CD8+ T cells following NoV challenge (B). See Figure 1 legend for description of statistical analysis.

3.4.4 HiPP prime for increased IFN-γ producing effector T cells

The numbers of NoV-specific IFN-γ producing CD4+ and CD8+ T cells are shown in Figure 3A. There were no significant differences between treatment groups at either time point. HiPP pigs did show a trend for increased IFN-γ+ T cells compared to LoPP and control pigs post-challenge. The fold expansions of IFN-γ+ T cells following challenge are shown in Figure 3B. HiPP pigs had high levels of expansion, ranging from 20-fold to 975-fold, following NoV challenge. Additionally, there were positive correlations between protection rates from diarrhea and expansion of ileal CD4+IFN-γ+ and CD8+IFN-γ+ T cells, splenic CD4+IFN-γ+ T cells, and PBL CD4+IFN-γ+ and CD8+IFN-γ+ T cells. Importantly, the only two HiPP pigs to shed virus post-challenge lacked virus-specific CD8+IFN-γ+ T cells in duodenum (data not shown).

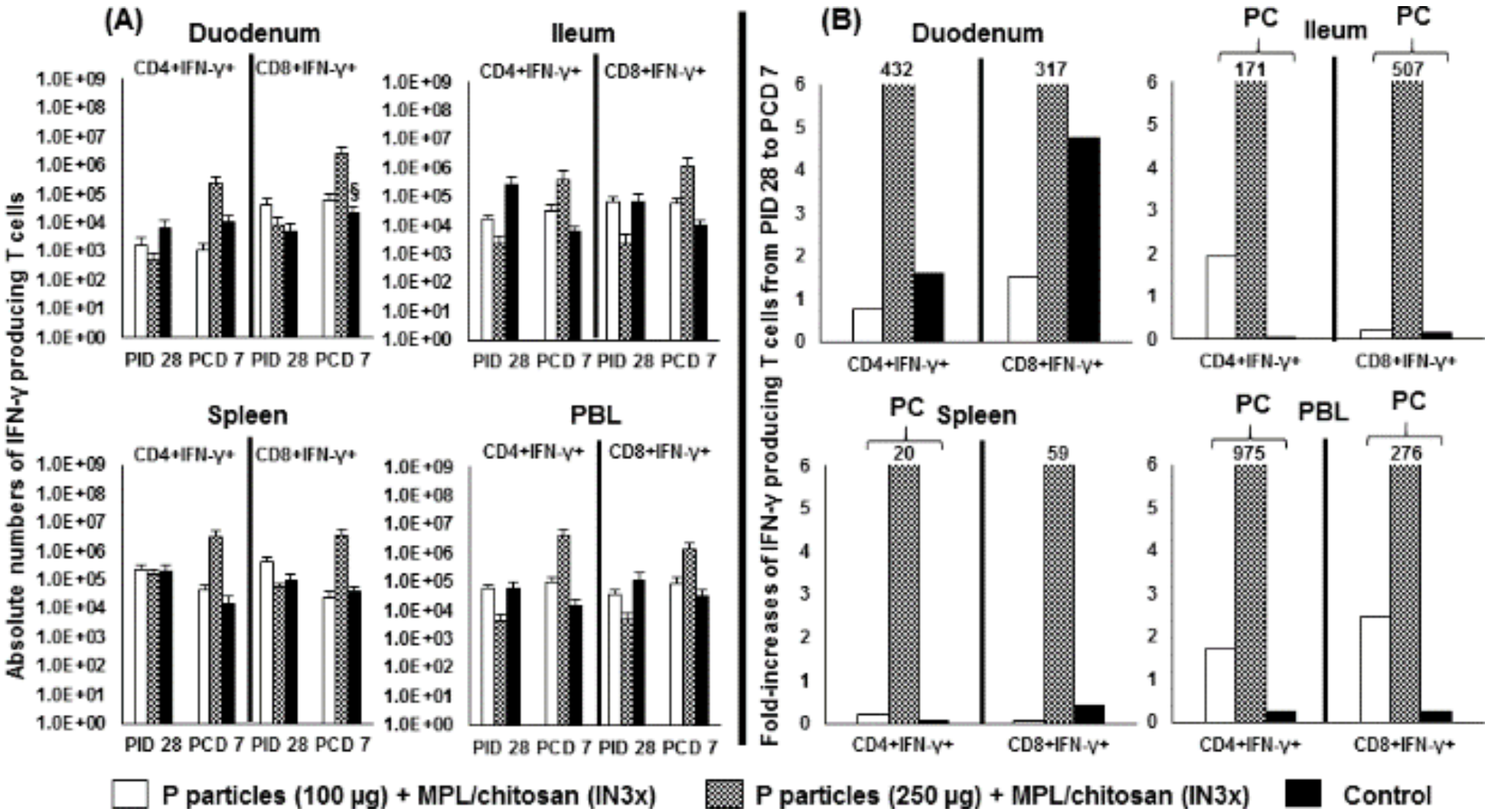

Figure 3. IFN-γ producing CD4+ and CD8+ T cell responses induced by low dose and high dose P particle vaccination pre- and post-challenge. Gn pigs were intranasally inoculated with VA387-derived P particles at PIDs 0, 10, and 21. A subset of pigs were orally challenged with human NoV at PID 28/PCD 0. Mean total numbers + standard errors of the means (n, 6 to 10) of CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T cells in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues at PID 28 (pre-challenge) and PCD 7 (post-challenge) (A). Frequencies of IFN-γ-producing T cells were determined using *in vitro* stimulation, intracellular staining, and multicolor flow cytometry. Total numbers were calculated using volume and concentration of isolated MNCs. Total expansion of IFN-γ-producing CD4+ or CD8+ T cells following NoV challenge (B). Expansion of IFN-γ-producing CD4+ and CD8+ T cells was calculated by dividing post-challenge numbers by pre-challenge numbers. See Figure 1 legend for description of statistical analysis.

3.4.5 HiPP down-regulate systemic Treg responses

The pre- and post-challenge Treg responses of vaccinated and control pigs are shown in Figure 4A. At PID 28, there were no significant differences between groups in intestinal tissues, though HiPP pigs had slightly reduced numbers of CD25+FoxP3+ Tregs in duodenum and ileum and CD25-FoxP3+ Tregs in duodenum compared to both LoPP and control groups. Additionally, HiPP pigs had significantly lower numbers of both Treg subsets in spleen and PBL compared to LoPP and control pigs. Following challenge, HiPP pigs had significantly lower numbers of CD25-FoxP3+ Tregs in duodenum and spleen and CD25+FoxP3+ Tregs in spleen compared to both LoPP and control pigs. Challenge significantly increased numbers of both Treg subsets in the duodena of all groups compared to pre-challenge numbers. HiPP pigs also had significantly increased CD25+FoxP3+ Tregs in ileum and both subsets in PBL post-challenge. The fold expansions of Tregs in each group following challenge are shown in Figure 4B. The fold expansions of CD25+FoxP3+ Tregs in ileum and CD25-FoxP3+ Tregs in PBL were positively correlated with protection rate from diarrhea, while expansion of CD25-FoxP3+ Tregs in duodenum was negatively correlated.

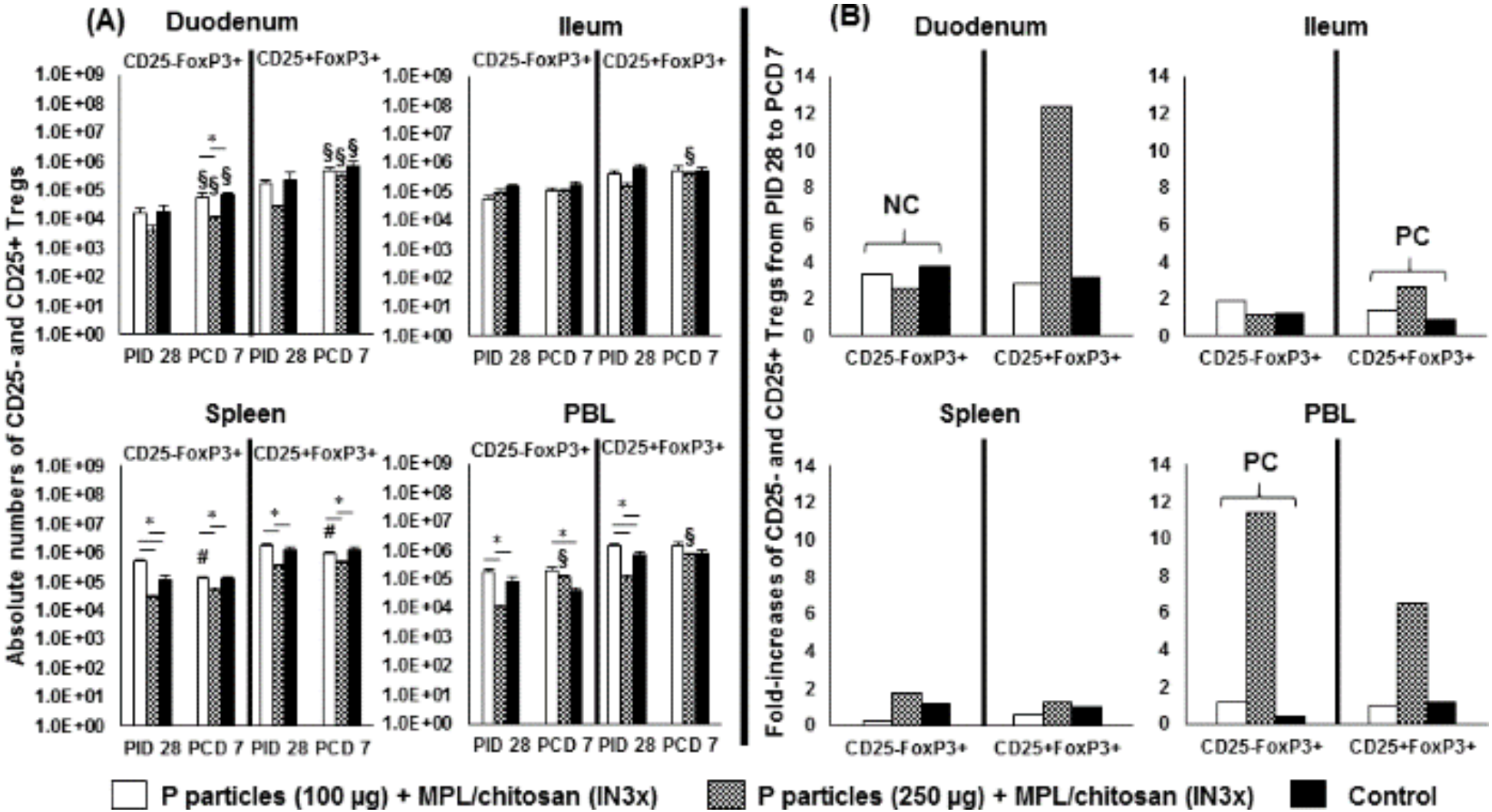

Figure 4. Treg responses induced by low dose and high dose P particle vaccination pre- and post-challenge. Gn pigs were intranasally inoculated with VA387-derived P particles at PIDs 0, 10, and 21. A subset of pigs were orally challenged with human NoV at PID 28/PCD 0. Mean total numbers + standard errors of the means (n, 6 to 10) of CD4+CD25-FoxP3+ and CD4+CD25+FoxP3+ Tregs in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues at PID 28 (pre-challenge) and PCD 7 (post-challenge) (A). Frequencies of Tregs were determined using intracellular staining and multicolor flow cytometry. Total numbers were calculated using volume and concentration of isolated MNCs. Fold expansion of Tregs following challenge (B). See Figure 1 legend for description of statistical analysis.

3.4.6 Correlations between protection rate against diarrhea and Treg expansion post-challenge

The TGF-β producing Treg responses in intestinal and systemic tissues are shown in Figure 5A. HiPP pigs had significantly lower numbers of TGF-β+CD25+ Tregs in spleen compared to LoPP pigs pre-challenge. It is worth noting that HiPP pigs had no detectable TGF-β+CD25- Tregs in PBL at PID 28. Following challenge, HiPP pigs had significantly higher numbers of TGF-β+CD25- Tregs in ileum and PBL and TGF-β+CD25+ Tregs in spleen compared to control pigs. HiPP pigs had significantly lower numbers of TGF-β+CD25- Tregs in spleen compared to LoPP pigs. HiPP pigs had significantly increased TGF-β+CD25- Tregs in PBL and TGF-β+CD25+ Tregs in spleen compared to pre-challenge. The fold expansions of TGF-β+ Tregs following challenge are shown in Figure 5B. We identified a positive correlation between protection rate against diarrhea and expansion of TGF-β+CD25+ Tregs in spleen and PBL and TGF-β+CD25- Tregs in ileum.

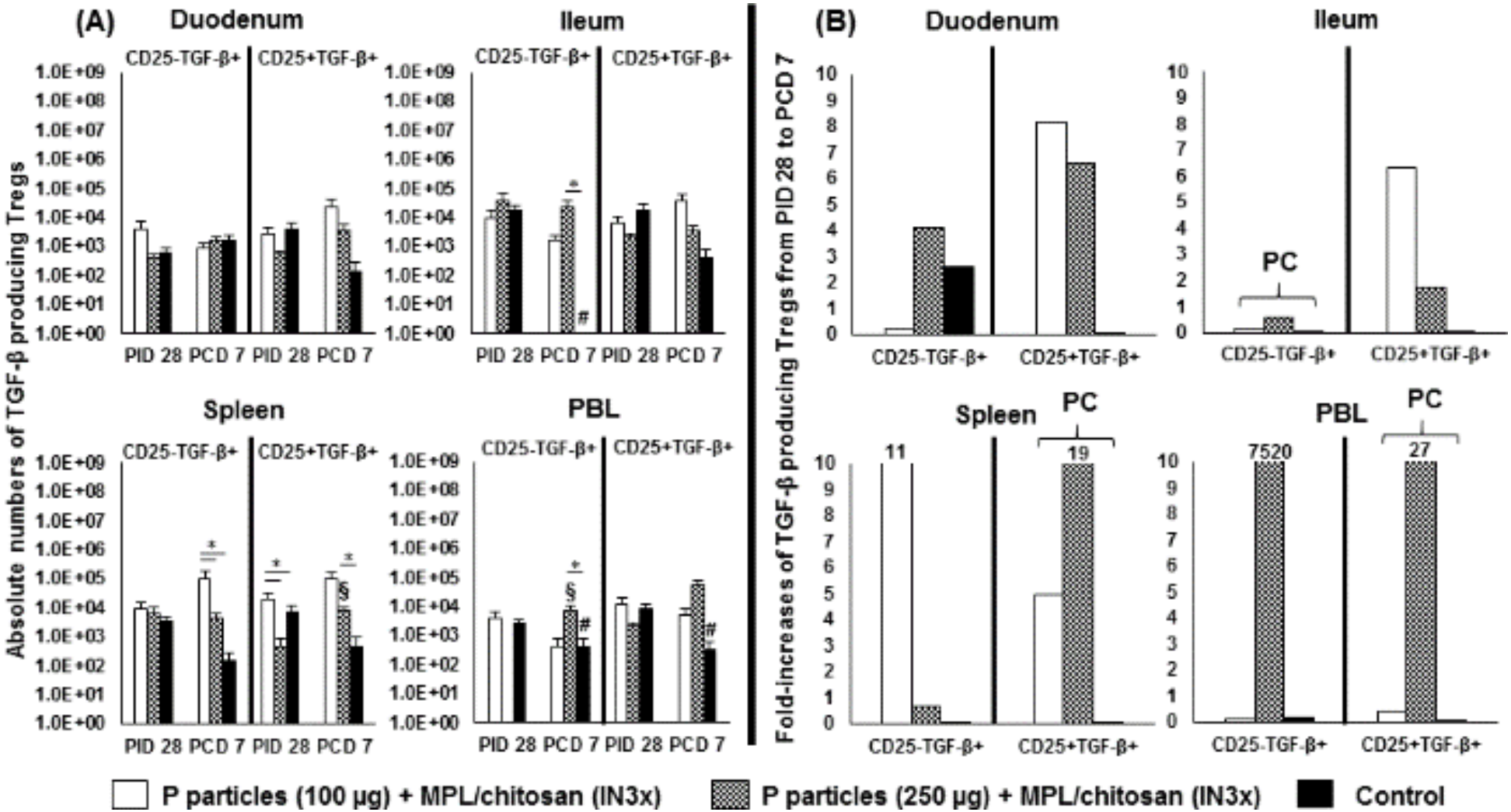

Figure 5. TGF-β producing Treg responses induced by low dose and high dose P particle vaccination pre- and post-challenge. Gn pigs were intranasally inoculated with VA387-derived P particles at PIDs 0, 10, and 21. A subset of pigs were orally challenged with human NoV at PID 28/PCD 0. Mean total numbers + standard errors of the means (n, 6 to 10) of CD4+CD25-FoxP3+TGF-β+ and CD4+CD25+FoxP3+TGF-β+ Tregs in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues at PID 28 (pre-challenge) and PCD 7 (post-challenge) (A). Frequencies of TGF-β-producing Tregs were determined using intracellular staining and multicolor flow cytometry. Total numbers were calculated using volume and concentration of isolated MNCs. Total expansion of TGF-β-producing Tregs following NoV challenge (B). See Figure 1 legend for description of statistical analysis.

3.5 Discussion

In this study, we demonstrated the dose-dependence of the protective efficacy and immunogenicity of P particle vaccines. Studies with NoV VLPs have indicated the proper dose for a NoV vaccine likely range from 100 µg to 1000 µg per vaccine course (2 doses at 50 – 500 µg/dose) based on protective and immunological parameters (19-21, 24). The present study utilizes two vaccine regimens (3 doses at 100 and 250 µg/dose) that fall within this ideal range (total 300 µg and 750 µg per vaccine course). HiPP increased protective efficacy against diarrhea and shedding and primed for increased activated T cells and IFN-γ producing T cells post-challenge.

We previously showed that P particles and VLPs provided similar protection against NoV diarrhea, though neither provided protection from shedding (22). However, prior NoV infection (NoVPO) provided the best protection against diarrhea as well as partial protection against NoV shedding (22). The present study shows that while both doses of P particles provided similar protection rates and slightly reduced severity of diarrhea, only HiPP provided partial protection against virus shedding. In fact, HiPP slightly increased protection against shedding compared to NoVPO from our previous study.(22) The protection rates against diarrhea conferred by the three-dose high dose P particles are slightly higher than the protection rate of a two-dose intranasal Norwalk-derived VLP vaccine in humans (47%) (19). However, the VLP vaccine only provided 26% protection against infection (19), while HiPP provided 60% protection. Since VLPs provided similar protection against diarrhea in our previous study (22) as HiPP in the present study, but did not provide any protection against infection, these results suggest that HiPP can be more protective and immunogenic than VLPs and are similar to prior NoV infection.

Previous studies have indicated that CD4+ helper T cells are required for protection (27), while CD8+ effector T cells play a role in clearance of primary infection (25, 28, 29). The dose effects of NoV vaccines on T cell responses are understudied. The most effective dose will need to be sufficient for immune priming but avoid tolerance development. The dose effects on VLP-induced immune responses have been reported previously (20, 21, 24, 25). However, this is the first study to evaluate the dose effects of P particles. In addition to the better protection, we observed that HiPP produce superior T cell responses to LoPP. Our results indicate that HiPP prime for increased intestinal and peripheral Th, CTLs, and activated T cells, increased IFN-γ producing T cells, but decreased systemic and peripheral Tregs. It is also worth noting ileal CD25- Tregs produced TGF-β in vaccinated pigs, but not control pigs. This suggests an inflammatory state in control pigs as a response to primary infection, while vaccinated pigs are returning to immune homeostasis. Taken together, our data show that the HiPP vaccine appears to strike the balance between immune priming and tolerance.

It has been reported that serum HBGA blocking antibodies and CD4+ T cells are correlates of protection for norovirus (27, 30, 31), though T cells are necessary for clearance of viral infection (25, 28, 29). Indeed, the two HiPP pigs that shed virus lacked detectable duodenal CD8+IFN-γ+ T cells. Additionally, our previous study showed an inverse association with duodenal Tregs and protection as well as the importance of T cell expansions in protection against NoV diarrhea (22). The present study identified several positive correlations in ileum (activated CD4+ and CD8+ T cells, CD4+IFN-γ+ and CD8+IFN-γ+ T cells, CD25+ Tregs, and CD25-TGF-β+ Tregs), spleen (activated CD4+ and CD8+ T cells, CD4+IFN-γ+ T cells, and CD25+TGF-β+ Tregs), and PBL (CD4+IFN-γ+ and CD8+IFN-γ+ T cells, CD25- Tregs, and CD25+TGF-β+ Tregs). We also identified a negative correlation between expansion of CD25-

Tregs in duodenum and protection rate. Collated with our previous findings (22), we have found an overall positive correlation between activated CD4+ and CD8+ T cells in spleen and protection rate against diarrhea, as well as a negative correlation with CD25- Treg expansion in duodenum and protection rate against diarrhea. These findings illustrate the importance of P particle-induced expansion in systemic lymphoid tissues in NoV expansion. Additionally, they show the importance of vaccines down-regulating CD25- Tregs in the duodenum following expansion and could have implications in evaluating host susceptibility to NoV disease.

We also noted previously that NoVPO and controls pigs had CD4+IFN-γ+ biased and CD8+IFN-γ+ biased expansions in duodenum post-challenge, respectively, while LoPP had a more balanced expansion (22). The present study shows that HiPP also have a balanced expansion between these cell types following NoV challenge. These results indicate that P particle vaccination induces balanced effector expansion, while the CD4+ biased expansion following re-infection may limit clearance of virus from infected cells by CD8+ cells.

Overall, our data on T cell responses indicate that HiPP vaccination promotes development of activated T cells in secondary lymphoid tissues and down-regulates expansion of Tregs in the primary effector site following challenge. However, it is worth noting that all groups in the present study displayed significant increases in total numbers of duodenal Tregs from pre-challenge to post-challenge, indicating that NoV infection primarily induces a Treg response. NoV vaccines should aim to down-regulate this expansion.

Though higher doses of P particles were required to provide similar protection against NoV disease as VLPs, P particles were able to protect against viral shedding while low dose P particles and VLPs were not (22). The duration of these protective effects needs to be determined in human clinical trials. Since P particle vaccines can be produced easily at low cost via a

prokaryotic expression system, an increased dose should not be an issue for developing countries. This three-dose 250 µg VA387 (GII.4)-derived intranasal P particle vaccine increased both protection rates against diarrhea and infection (60% and 60%, respectively) compared to the protection rates (47% and 26%, respectively) witnessed following the two-dose 100 µg Norwalk (GI.1)-derived VLP intranasal vaccine in humans (19). This high dose P particle vaccine regimen should be tested in human clinical trials. Additionally, P particles can serve as a platform to display antigens from other NoV strains or viruses (32-35), which has the potential to maximize their economic impact. These results further demonstrate that P particles are viable vaccine alternatives to VLPs and that the vaccine dose response needs to be evaluated.

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CHAPTER 4

Simvastatin reduces P particle vaccine efficacy against GII.4 human norovirus diarrhea and suppresses intestinal T cell immune responses in gnotobiotic pigs

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Manuscript in preparation

4.1 Abstract

Abstract

Noroviruses (NoVs) are a leading cause of acute gastroenteritis worldwide. P particles are derived from the NoV capsid protruding (P) domain and are a promising vaccine candidate against NoV. Simvastatin is a cholesterol-reducing drug that has been shown to increase NoV infectivity. This study examines simvastatin's effects on P particle-induced protective efficacy and T cell immunogenicity using the gnotobiotic (Gn) pig model. Pigs were intranasally inoculated with three doses (100 µg/dose) of GII.4/VA387-derived P particles with MPL and chitosan adjuvants at post-inoculation days (PID) 0, 10, and 21. Simvastatin-fed pigs received 8 mg/day orally for 11 days prior to challenge. A subset of pigs were orally challenged with 10 ID_{50} of a NoV GII.4/2006b variant. Protection was monitored for 7 days post-challenge. Intestinal and systemic T cell responses were determined pre- and post-challenge. Simvastatin abolished the P particle's protection and significantly increased NoV diarrhea severity, but only. Simvastatin-fed control pigs shed for significantly more days than vaccinated pigs. Simvastatin significantly decreased duodenal CD4+IFN-γ+, CD8+IFN-γ+, and regulatory T cells (Tregs) pre-challenge. Simvastatin also significantly reduced total duodenal activated CD4+ and CD8+ T cells in vaccinated pigs at PID 28. Following challenge, simvastatin eliminated splenic IFN-γ+ T cells in vaccinated pigs. Vaccinated pigs had significant decreases in splenic Tregs and simvastatin-fed pigs had significant decreases in CD25+ Tregs in PBL compared to pre-challenge. These results indicate simvastatin impairs P particle-induced protection and immunity and have implications on the elderly, a target population for a NoV vaccine, and primary consumers of simvastatin.

Keywords: human norovirus, P particle, simvastatin, gnotobiotic pig, T cells

4.2 Introduction

Noroviruses (NoVs) are a member of the *Caliciviridae* family and a leading cause of acute, non-bacterial gastroenteritis. Since the introduction of rotavirus vaccines, NoVs have become the leading cause of gastroenteritis in developed countries (1, 2). NoVs account for approximately 20% of all cases of gastroenteritis worldwide (3) with GII.4 NoVs believed to cause 60-90% of all outbreaks (4). NoVs cause an estimated $284 million in economic cost to hospitals across all groups in the United States each year (5). NoVs are easily transmitted in semi-closed communities, such as senior care homes and cruise ships (6, 7).

NoV vaccine development has been hindered by the lack of a small animal model and cell culture system. Thus, vaccine development has relied on recombinant NoV capsid proteins, including virus-like particles (VLPs) and P particles. P particles are a novel vaccine candidate derived from expression of the VP1 protruding (P) domain in a prokaryotic expression vector (8-10). Previously, we reported that an intranasal three-dose 100 µg P particle regimen provided a similar protection rate as VLPs against diarrhea following cross-variant, homotypic NoV challenge in gnotobiotic (Gn) pigs (11). Both provided similar protection to intranasal two-dose 100 µg Norwalk-derived VLPs in humans (12). Additionally, P particles primed for increased activated CD4+ T cells, duodenal CD8+IFN-γ+ T cells, CD25-FoxP3+ regulatory T cells (Tregs) in PBL, and CD25-FoxP3+TGF-β+ Tregs in spleen compared to VLPs following NoV challenge in Gn pigs (11). Additionally, we showed that an intranasal three-dose 250 µg P particle vaccine regimen increased protection against NoV infection and primed for increased activated T cells, IFN-γ producing T cells, and decreased Tregs following challenge (manuscript under review). These data indicate the importance in examining the P particles as a vaccine candidate against NoV.

Simvastatin is a cholesterol-reducing drug commonly marketed under the name Zocor. Simvastatin inhibits HMG-CoA reductase, an enzyme in the cholesterol biosynthesis pathway, resulting in reduction of low density lipoprotein (LDL) cholesterol levels (13). Forty mg of simvastatin decreases LDL cholesterol and the risk of cardiovascular events by 23% over 5 years (14) with similar effects witnessed in low-risk populations (15). Based on the report by the National Center for Health Statistics, 50% of men and 36% of women who are 65-74 years old take statin type drugs in 2010. In 2013, the American Heart Association and American College of Cardiology released new guidelines which expand the recommendation for the use of simvastatin in the prevention of heart diseases even to people without high LDL levels. This new guideline will likely result in a large increase of the percentage of the elderly adult human population who take statin-type drugs. Simvastatin has also been shown to reduce the severity of several other diseases, including rheumatoid arthritis (16-18), multiple sclerosis (19), and periodontitis (20, 21). These pleiotropic effects can be explained by simvastatin's role in the down-regulation of IFN-induced MHCII expression by inhibition of the CIITA gene (22, 23). This down-regulation of induced MHCII has many downstream effects, including reduced NK cell cytotoxicity (24, 25), reduced *in vitro* T cell proliferation (25), reduced production of IL-2 and IFN-γ (26), reduced CD4/CD8 and Th1/Th2 ratios (17), increased Tregs (27, 28), and impaired lymphocyte homing to secondary lymphoid organs (29).

Cholesterol pathways have been shown to play a role in murine NoV and bovine NoV VLP cellular entry (30-32) and in Norwalk replication (33). Norwalk replication was directly associated with increased expression of LDL receptor (LDLR) mRNA in cells bearing the Norwalk replicon (33). Simvastatin has been shown to increase the expression of LDLR (13) and has been shown to increase the production of Norwalk proteins and RNA in replicon-bearing

cells (33). The roles of simvastatin on NoV infection and disease have also been investigated *in vivo*. Gn pigs treated with simvastatin had earlier onset and longer duration of fecal NoV shedding, including increased viral titers compared to pigs not fed simvastatin (34). Oral inoculation of IFN-α reduced the effects of simvastatin on NoV infectivity, indicating simvastatin down-regulates innate immunity (34). Previously, our lab showed that simvastatin feeding increased the susceptibility of Gn pigs to infection by a NoV GII.4 variant, incidence of diarrhea compared to non-simvastatin fed Gn pigs, and reduced the ID_{50} of the GII.4 variant in older Gn pigs to a similar ID_{50} observed in neonatal Gn pigs (35). We have also shown that simvastatin reduces *in vitro* T cell proliferation, NoV-specific duodenal IFN-γ producing T cells, and increases Tregs (unpublished data).

In this study, we used our well-established Gn pig model to evaluate the effects of simvastatin on the protective efficacy of the P particles vaccine following homotypic GII.4 NoV challenge. We also examined simvastatin's immunomodulatory effects on the T cell profile induced by the P particles before and after challenge in the intestinal and systemic lymphoid tissues. We previously implicated the importance of splenic activated T cells and duodenal Tregs in NoV immunity (11). Simvastatin is primarily consumed by the elderly and aging, a target population for a NoV vaccine, but our understanding of its role in affecting NoV vaccine-induced immunity remains limited. To our knowledge, this is the first study to evaluate simvastatin's effects on P particle-vaccine induced protection and immunity.

4.3 Materials and Methods

4.3.1 Virus

A pool of human stool containing GII.4/2006b variant 092895 (GenBank accession number KC990829) was collected at Cincinnati Children's Hospital Medical Center by Dr. Xi Jiang's laboratory. The stool pool was collected from a family with confirmed NoV gastroenteritis in 2008. The inoculum was processed by high speed centrifugation as we previously described (35). The ID_{50} of the inoculum for pigs at 33-34 days of age was determined to be 6.43 x 10^5 viral RNA copies in a previous study (35) and 10 ID_{50} was used for challenging pigs with or without simvastatin treatment.

4.3.2 Vaccine preparation

P particles were both derived from NoV GII.4 VA387 (a 1997 Farmington Hills variant) as previously described (36) and UV sterilized as previously described (11). Synthetic MPL (Avanti Polar Lipids, Inc.) and chitosan (Novamatrix) were prepared and filter sterilized as previously described (11). Vaccines consisted of 100 µg P particles with 5 mg chitosan and 50 µg MPL adjuvants in TNC buffer (37) at a final volume of 1 ml. Sterility of all solutions were monitored as previously described (11).

4.3.3 Simvastatin preparation

Simvastatin (Dr. Reddy's Laboratories, Ltd) was prepared as previously described (35). Tablets (80 mg) of simvastatin were dissolved in 100% ethanol for a final concentration of 8 mg/ml and filter sterilized. Serum cholesterol was monitored pre-feeding and post-feeding to verify simvastatin's effects in Gn pigs. Data presented as the mean serum cholesterol levels in mg/dL.

4.3.4 Treatment and inoculation of Gn pigs

Near-term Large White cross pigs were derived via hysterectomy and maintained in germ-free isolator units as previously described (38). Pigs (both male and female) were randomly divided into four groups: (1) P particles without simvastatin (P+S-), (2) P particles with simvastatin (P+S+), (3) Control without simvastatin (CS-), and (4) Control with simvastatin (CS+). Each group was composed of at least 6 pigs. All pigs in the P+ groups were intranasally inoculated with 3 doses of the vaccine using mucosal atomization devices (MADs, LMA North America) at post-partum day (PPD) 5 (post-inoculation day [PID] 0), PID 10, and PID 21. Control pigs received adjuvants alone at the same time points. Simvastatin-treated pigs were orally inoculated with 8 mg/day of simvastatin in diluent #5 for 11 days prior to challenge (PID 17-27). Serum cholesterol levels were evaluated pre-simvastatin and post-simvastatin feeding by the Virginia-Maryland Regional College of Veterinary Medicine Hospital laboratory to confirm reduction in cholesterol. At PID 28, a subset of pigs from each group were challenged with 10 ID_{50} (6.43 x 10^5 viral RNA copies) at PID 28 (post-challenge day [PCD] 0). Challenged pigs were given 4 ml of 200 mM sodium bicarbonate 10 minutes prior to oral inoculation to reduce gastric acidity. Challenged pigs were monitored daily for diarrhea and virus shedding until PCD 7. All pigs were euthanized at PID 28 (pre-challenge) or PCD 7 (post-challenge) to isolate mononuclear cells (MNCs) from duodenum (20 cm), ileum (20 cm), spleen (whole organ), and blood (70 ml) as previously described (39). The total numbers of MNCs were calculated by multiplying the volume and concentration of cells isolated from each tissue. All animal experimental procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Virginia Tech.

4.3.5 Detection of NoV shedding and assessment of diarrhea

Rectal swabs were collected daily following NoV challenge for assessment of diarrhea and virus shedding. Diarrhea was scored on our previously used scaling system (35). Briefly, diarrhea was assessed on the following scale: 0, normal; 1, pasty; 2, semi-liquid; 3, liquid. Scores of ≥2 were considered diarrheic. Pigs that had a score of 2 or greater for at least one day from PCD 1 to PCD 7 were considered positive for diarrhea. Virus shedding was detected using TaqMan® real-time RT-PCR as previously described (35). Pigs were considered positive for shedding if NoV was detected in a stool sample for at least one day from PCD 1 to PCD 7.

Diarrhea severity and shedding are presented as areas under the curve (AUCs). AUCs were calculated from the line graphs using the daily diarrhea score or daily shedding titer (Y-axis) and the days of diarrhea or virus shedding (X-axis). AUCs were calculated for disease and shedding from PCD 1 through PCD 7 using GraphPad Prism 6.

4.3.6 Flow cytometry analysis of total Th, CTLs, and IFN-γ producing CD4+ and CD8+ T cells

Flow cytometry was used to determine the total numbers of CD4+ and CD8+ T cells and IFN-γ producing CD4+ and CD8+ T cells in intestinal (duodenum and ileum) and systemic (spleen) tissues and peripheral blood lymphocytes (PBL) of Gn pigs as previously described (11). T helper (Th) and cytotoxic T lymphocytes (CTLs) were defined as CD3+CD4+ and CD3+CD8+, respectively. Cells were stimulated with P particles *in vitro* for 17 h and stained as previously described (11, 40, 41). Total numbers of each T cell subset per tissue were calculated as previously described (11). Data are presented as mean numbers of cells per tissue. Numbers of IFN-γ producing T cells are presented as mean adjusted numbers following removal of mock-

stimulated MNCs and isotype-matched irrelevant controls. At least 100,000 events were collected using a BD FACSAria flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.4 software (Tree Star, Inc.).

4.3.7 Flow cytometry analysis of activated non-regulatory (FoxP3-) and IL-10 and TGF-β producing Tregs (FoxP3+) cells

MNCs were stained freshly on the day of isolation for activated non-regulatory T cells and Tregs as previously described (40). Activated non-regulatory T cells were defined as CD25+FoxP3- T cells and Tregs were defined as CD25-FoxP3+ and CD25+FoxP3+ and were identified as previously described (11). Total numbers of Tregs and IL-10 and TGF-β producing Tregs per tissue were calculated as previously described (11). Isotype-matched irrelevant antibodies were used to establish positive and negative gates. At least 100,000 events were collected using a BD FACSAria flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.4 software (Tree Star, Inc.).

4.3.8 Statistical analysis

One-way analysis of variance (ANOVA)-general linear model (GLM) followed by Duncan's multiple range test was used to compare mean durations of diarrhea and shedding. Fisher's exact test was used to compare percentages of pigs with diarrhea and virus shedding. Kruskal-Wallis rank-sum test was used to compare diarrhea and viral shedding AUCs and numbers of T cell subsets. A two-tailed paired Student's *t*-test was used to compare mean serum cholesterol levels following simvastatin feeding. Spearman's rank correlation coefficient was used to evaluate correlations between T cell subsets and protection rates. All statistical

significance was assessed at $p<0.05$. All statistical analyses were performed using SAS Program 9.3 (SAS Institute, NC, USA).

4.4 Results

4.4.1 Simvastatin significantly reduced serum cholesterol in Gn pigs

Serum cholesterol was monitored before and after simvastatin feeding to verify its effects in Gn pigs. Simvastatin feeding significantly reduced serum cholesterol in Gn pigs. After 11 days of simvastatin feeding, mean serum cholesterol levels had decreased from 133 mg/dL at PID 17 to 72 mg/dL at PID 27 for a 44% reduction ($p<0.0001$) (data not shown). Mean serum cholesterol levels in age-matched non-simvastatin fed pigs were 110 mg/dL at PID 17 and 99 mg/dL at PID 27 for an 8% reduction (data not shown).

4.4.2 Simvastatin reduced the protective efficacy conferred by the P particle vaccine

Simvastatin's effects on the protective efficacy of P particle vaccines against NoV diarrhea and shedding following GII.4/2006b NoV challenge was evaluated. Diarrhea and fecal NoV shedding were monitored up to PCD 7 (Table 1). We partially published the results for the non-simvastatin fed pigs previously (11) and the data (protection and immune responses) are compared with simvastatin-fed pigs in this study to reduce the numbers of animals used. All P+S+ pigs had diarrhea (0% protection rate) whereas P+S- pigs had a 47% protection rate. CS+ and CS- had the same occurrence of diarrhea (83%), regardless of simvastatin feeding. P+S+ and CS+ pigs had significantly higher diarrhea AUCs compared to P+S- pigs (8.8 vs. 5.4). Simvastatin did not have an effect on the occurrence of shedding, however it reduced the fold-reduction of AUC in vaccinated pigs from the corresponding controls (P+S- 3.1 fold vs. P+S+ 2.3 fold). It is important to note, though, in simvastatin-fed pigs, the P particle vaccine significantly shortened the duration of virus shedding compared to control pigs (P+S+ 4.2 days vs. CS+ 1.7 days) following NoV challenge.

Table 1. Clinical signs and protective efficacy in P particle-vaccinated Gn pigs after challenge with GII.4/2006b NoV[a]

		Diarrhea[b]					Virus shedding				
Group*	n	Mean % of pigs with diarrhea (no. of pigs with diarrhea/total no.)	Mean no. of days with diarrhea[c] (SEM)	Mean AUC from PCD 1 to PCD 7 (SEM)**	Fold reduction in AUC	Rate of protection against diarrhea (%)[d]	Mean % of pigs shedding virus (no. of pigs with shedding/total no.)	Mean no. of days with shedding[c] (SEM)**	Mean AUC from PCD 1 to PCD 7 (SEM)	Fold reduction in AUC	Rate of protection against shedding (%)[d]
P+S-***	9	44% (4/9)	1.3 (0.6)	5.4 (0.8)[B]	-1.6	46.7	89% (8/9)	1.8 (0.4)[B]	38216 (25627)	-3.1	0.0
P+S+	6	100% (6/6)	3.0 (0.7)	8.8 (1.1)[A]	0.0	0.0	83% (5/6)	1.7 (0.4)[B]	18697 (17496)	-2.3	0.0
CS-***	6	83% (5/6)	1.8 (0.6)	6.7 (1.0)[AB]	NA	NA	83% (5/6)	2.0 (0.7)[AB]	116960 (78190)	NA	NA
CS+	6	83% (5/6)	2.5 (0.6)	8.8 (0.7)[A]	NA	NA	83% (5/6)	4.2 (1.3)[A]	42212 (26330)	NA	NA

[a] Gn pigs were challenged with a human NoV GII.4 2006b variant 092895 at 33-34 days of age. Rectal swabs were collected daily after challenge to determine diarrhea and virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents.

[b] Fecal scoring system: 0, solid; 1, pasty; 2, semi-liquid; 3, liquid. Pigs with scores of 2 or higher were considered diarrheic.

[c] From PCD 0 to PCD 7.

[e] Calculated as [1 – (percentage of immunized pigs in each group with diarrhea or shedding/percentage of control pigs with diarrhea or shedding)] x 100. NA, no applicable

* Abbreviated group names: P+S-, non-simvastatin fed P particle-vaccinated (+MPL/chitosan) pigs; P+S+, simvastatin-fed P particle-vaccinated (+MPL/chitosan) pigs; CS-, non-simvastatin fed control (MPL/chitosan alone) pigs; CS+, simvastatin-fed control (MPL/chitosan alone) pigs.

** Means in the same column followed by different letters (A, B) differ significantly (One way ANOVA, $p<0.05$); while shared letters indicate no significant difference.

*** Data were presented in Chapter 2.

4.4.3 Simvastatin decreased total Th and CTLs in duodena of vaccinated pigs

To evaluate the effects of simvastatin on P particle vaccine-induced T cell responses, we evaluated total Th, CTLs, activated non-regulatory CD25+FoxP3- T cells, IFN-γ producing effector/memory T cells, and CD4+CD25-FoxP3+ and CD4+CD25+FoxP3+ regulatory T cells (Tregs) in intestinal and systemic lymphoid tissues. The total numbers of Th and CTLs were compared among P particle-vaccinated and control pigs with or without simvastatin pre- and post-challenge. To assess the immunomodulatory effects of simvastatin, we compared the vaccinated pigs with and without simvastatin (P+S+ and P+S-), as well as control pigs with and without simvastatin (CS+ and CS-). To assess the immunogenicity of P particles in the presence of simvastatin, we compared the simvastatin-fed vaccinated pigs to simvastatin-fed non-vaccinated control pigs (P+S+ and CS+). Pre-challenge, P+S+ pigs had significantly lower numbers of Th and CTLs in duodenum compared to P+S- pigs. Following NoV challenge, duodenal Th and CTLs increased significantly from PID 28 to PCD 7 in all groups except P+S- pigs. In duodenum, P+S+ pigs had significantly higher numbers of CTLs compared to P+S- and CS+ pigs. CS- pigs also had significantly higher numbers of CTLs in duodenum compared to CS+ pigs. In ileum, CS+ pigs had significant increases in Th from pre-challenge including significantly higher Th compared to P+S+ pigs. In PBL, P+S+ pigs had significantly higher Th compared to P+S- pigs.

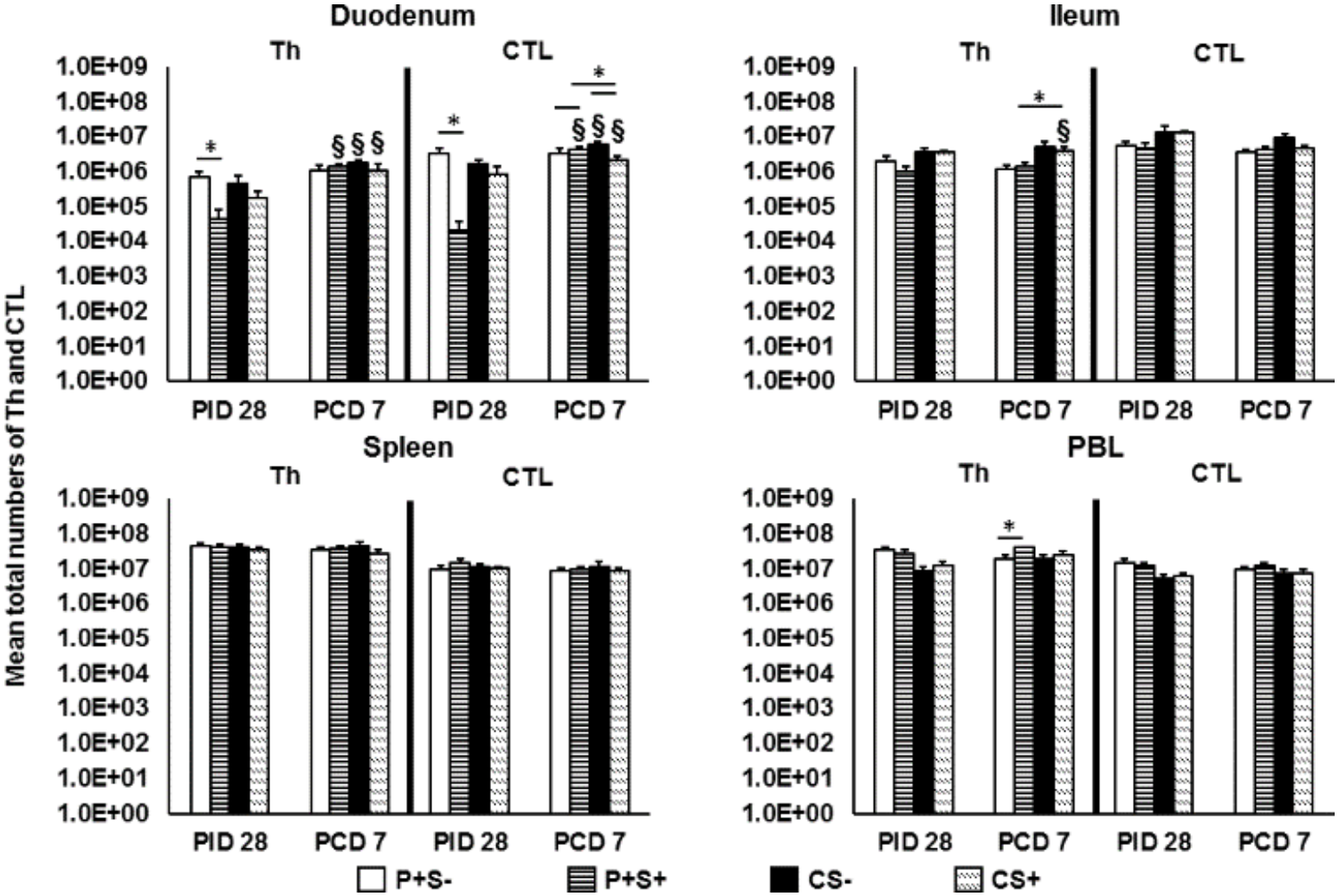

Figure 1. Th and CTLs induced by P particle vaccination with or without simvastatin feeding pre- and post-challenge. MNCs were gated as previously described (11) following *in vitro* stimulation. Mean total numbers plus standard errors of the mean (*n*, 6 to 10) of CD3+CD4+ and CD3+CD8+ T cells in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues pre- and post-challenge. An asterisk above error bars indicates a significant difference among groups for the same cell type and tissue at the same time point (*P*, <0.05 by Kruskal-Wallis one-way ANOVA). A section sign indicates that the numbers increased significantly following challenge in the same group. A number sign indicates that the numbers decreased significantly following challenge in the same group. Sim, simvastatin.

4.4.4 Simvastatin reduced activated CD4+ and CD8+ T cells in duodena of vaccinated pigs pre-challenge

To evaluate the activated non-regulatory T cells pre- and post-challenge, MNCs were stained freshly on the day of isolation and gated by CD4+ or CD8+ T cells for CD25+ and FoxP3- cells as previously described (11). The mean numbers of activated CD4+ and CD8+ T cells are shown (Figure 2). Pre-challenge, P+S- pigs had significantly higher number numbers of activated CD4+ and CD8+ T cells in duodenum compared to P+S+ pigs. Following challenge, the activated CD4+ T cells in duodenum increased significantly from pre-challenge in all groups. P+S+ pigs had significant increases in duodenal activated CD8+ T cells but significant decreases in splenic activated CD8+ T cells. In ileum, CS+ pigs had significant increases in activated CD4+ T cells compared to pre-challenge; CS+ pigs had significantly higher numbers of activated CD4+ T cells compared to P+S+ pigs. In spleen and PBL, P+S- pigs had significantly higher numbers of activated CD8+ T cells compared to P+S+ pigs.

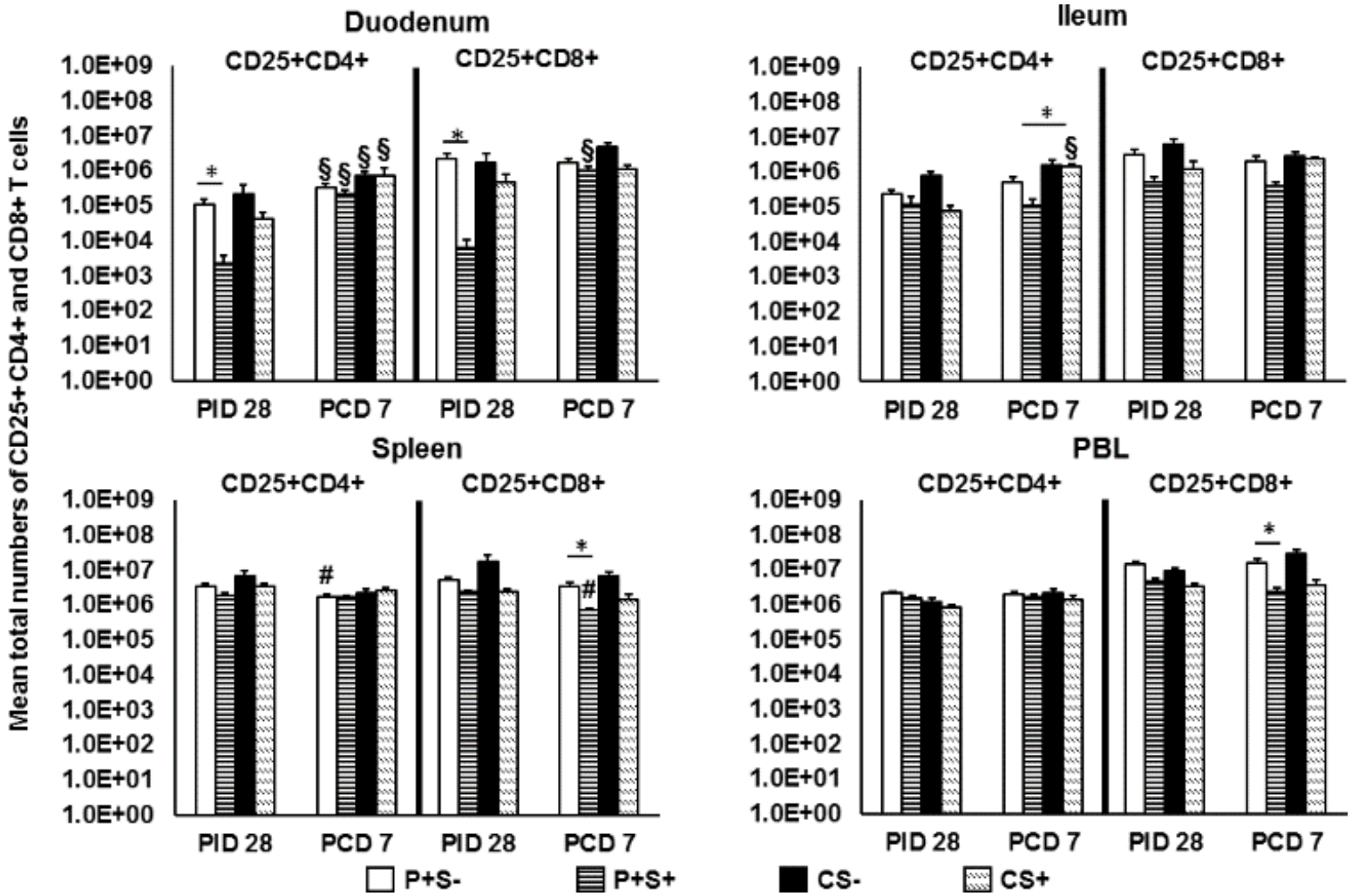

Figure 2. Activated non-regulatory CD4+ and CD8+ T cells pre- and post-challenge. MNCs were gated as previously described (11) freshly on the day of cell isolation. Mean total numbers plus standard errors of the means (*n*, 6 to 10) of CD4+CD25+FoxP3- and CD8+CD25+FoxP3- activated T cells pre-challenge and post-challenge in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues. See the legend to Fig. 1 for an explanation of the symbols indicating statistical significance.

4.4.5 Simvastatin feeding reduced CD8+IFN-γ+ T cells in duodenum and PBL

We previously showed that NoV challenge induced a 37-fold expansion in duodenal CD4+IFN-γ+ T cells in previously NoV-infected pigs (11). The results indicate the importance of an intestinal Th1 response in NoV immunity. In the present study, we identified IFN-γ+ producing CD4+ and CD8+ T cells in intestinal and systemic lymphoid tissues at PID 28 and PCD 7 following *in vitro* stimulation and flow cytometry (11). IFN-γ+ T cells were identified following subtraction of isotype controls and mock-stimulated MNCs and are presented as mean total numbers (Figure 3). Pre-challenge, simvastatin significantly reduced IFN-γ producing CD4+ and CD8+ T cells in duodenum and CD8+IFN-γ+ T cells in PBL compared to non-simvastatin pigs. In vaccinated pigs, simvastatin significantly reduced IFN-γ producing CD4+ and CD8+ T cells in spleen.

Following NoV challenge, P+S+ pigs had significantly lower numbers of CD4+IFN-γ+ and CD8+IFN-γ+ T cells in spleen compared to P+S- pigs. In fact, P+S+ pigs had no detectable CD4+IFN-γ+ and CD8+IFN-γ+ T cells in spleen post-challenge. Similarly, CS+ pigs had a complete lack of detectable CD8+IFN-γ+ T cells in PBL, which were significantly lower than CS- pigs. Interestingly, CS+ pigs had significantly higher CD4+IFN-γ+ T cells in spleen compared to CS- pigs.

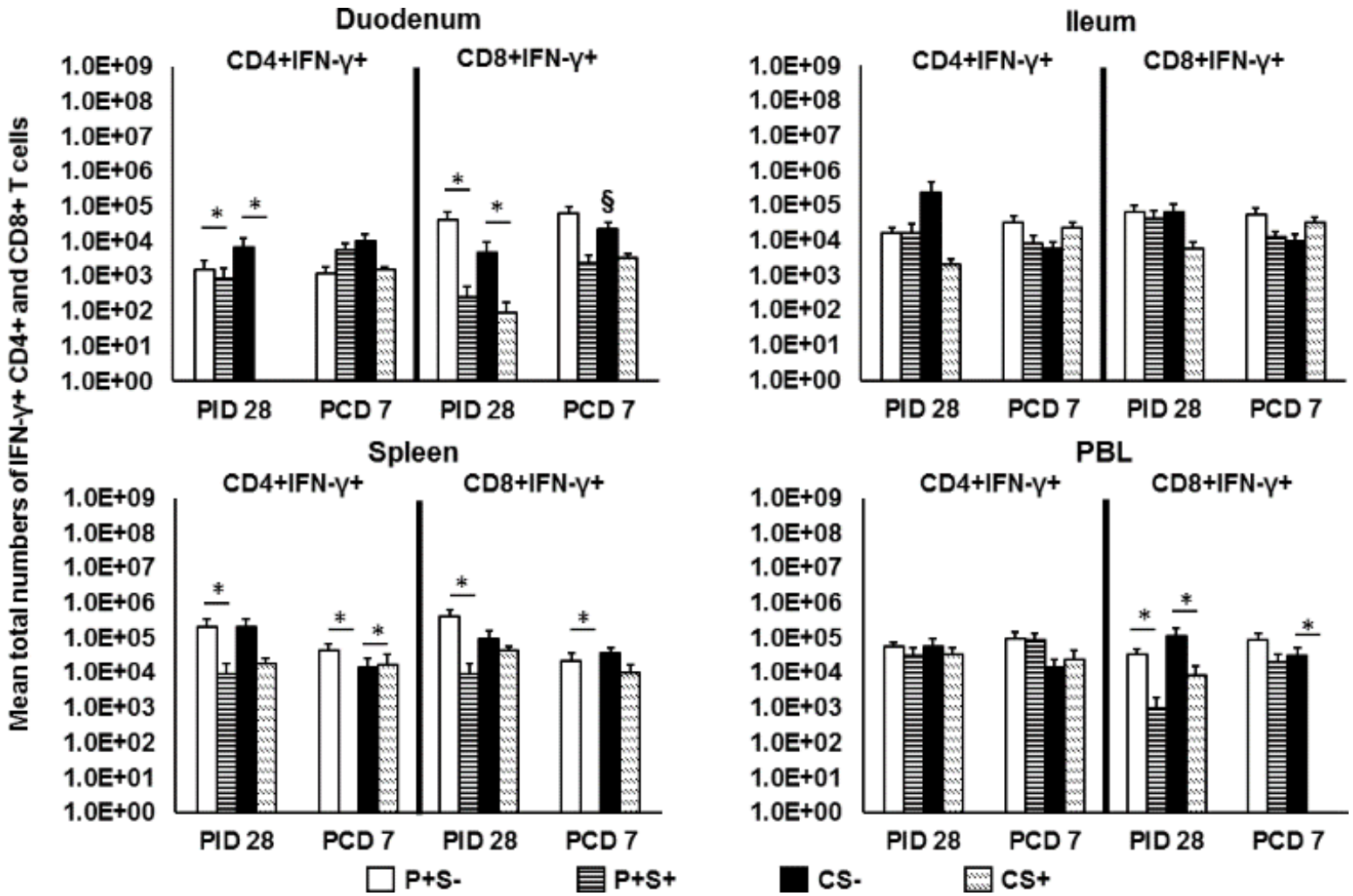

Figure 3. IFN-γ producing CD4+ and CD8+ T cell responses pre- and post-challenge. MNCs were gated as previously described (11) following *in vitro* stimulation. Mean total numbers of IFN-γ producing CD4+ and CD8+ T cells following subtraction of isotype control and mock-stimulated background numbers. Data presented are mean total numbers plus standard errors of the means (*n*, 6 to 10) pre-challenge and post-challenge in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues. See the legend to Fig. 1 for an explanation of the symbols indicating statistical significance.

4.4.6 Simvastatin-fed pigs had reduced CD25-FoxP3+ Tregs in duodena at PID 28

Previously, we showed the importance of reducing Tregs in the duodenum in NoV protective immunity (11), though simvastatin has been shown to increase Tregs *in vivo* (42, 43). In the present study, we evaluated simvastatin's effects on P particle-vaccine induced Tregs pre- and post-challenge. To identify Tregs, MNCs were intracellularly stained on the day of isolation and flow cytometry on PID 28 and PCD 7. The mean total numbers of CD25-FoxP3+ and CD25+FoxP3+ Tregs are shown in Figure 4.

At PID 28, simvastatin feeding significantly reduced the numbers of CD25-FoxP3+ Tregs in duodenum and spleen. P+S+ pigs had significantly lower numbers of CD25-FoxP3+ Tregs in ileum and PBL and CD25+FoxP3+ Tregs in duodenum and PBL compared to P+S- pigs. Following challenge, simvastatin feeding significantly reduced the numbers of Tregs in PBL compared to non-simvastatin fed pigs. P+S+ pigs had significantly lower numbers of both Treg subsets in duodenum and spleen compared to P+S- pigs; P+S+ pigs also had significantly lower numbers of CD25+FoxP3+ Tregs in ileum compared to P+S- pigs.. However, P+S+ pigs still had significantly reduced numbers of CD25-FoxP3+ Tregs in duodenum and spleen and CD25+FoxP3+ Tregs in ileum and spleen compared to CS+ pigs. CS+ pigs had significant increases in both Treg subsets in ileum compared to pre-challenge. Interestingly, all groups had increases or significant increases in CD25-FoxP3+ and CD25+FoxP3+ Tregs in duodenum following challenge. In spleen, both P+S+ and P+S- groups had significant decreases in both Treg subsets following challenge compared to pre-challenge numbers. In PBL, simvastatin-fed pigs (P+S+ and CS+) had significant decreases in CD25+FoxP3+ Tregs compared to pre-challenge.

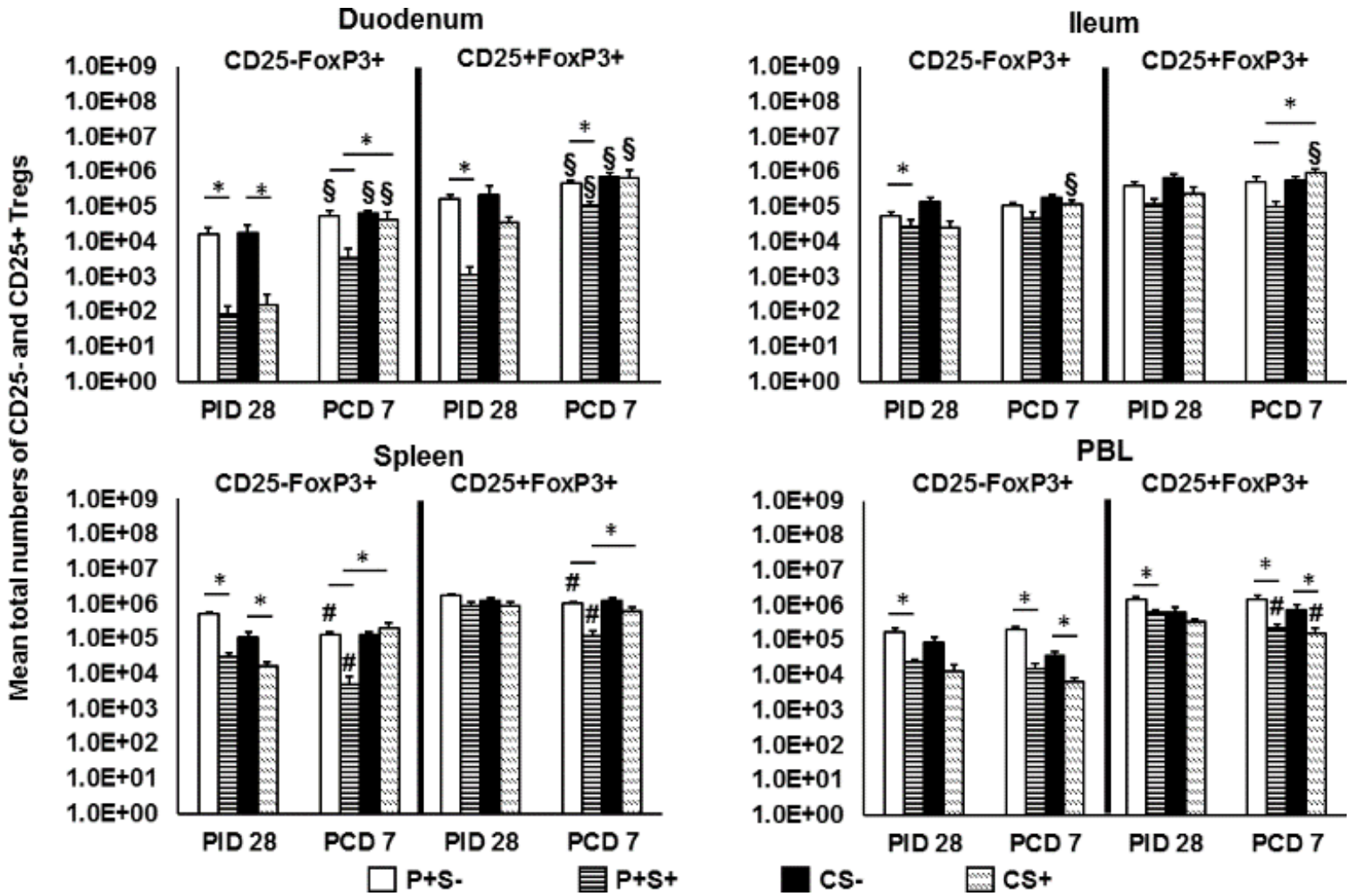

Figure 4. Treg responses induced by P particle vaccination with or without simvastatin feeding pre- and post-challenge. Tregs were gated as previously described (11) following staining freshly on the day of cell isolation. Mean total numbers of CD25- and CD25+ Tregs plus standard errors of the means (*n*, 6 to 10) among total MNCs pre-challenge and post-challenge in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues. See the legend to Fig. 1 for an explanation of the symbols indicating statistical significance.

4.4.7 Simvastatin feeding decreased total MNCs isolated from PBL post-challenge

To evaluate how simvastatin affects total numbers of MNCs, we calculated the total numbers of MNCs isolated from each tissue pre- and post-challenge (Figure 5). At PID 28, simvastatin feeding significantly reduced the total number of MNCs in duodenum of vaccinated pigs (P+S- vs. P+S+). Interestingly CS+ pigs had significantly higher numbers of MNCs in duodenum compared to P+S+ pigs. Post-challenge, simvastatin-fed pigs had significantly lower numbers of MNCs in PBL compared to non-simvastatin fed pigs with or without vaccination. Total MNCs significantly increased in duodenum of all groups except P+S- pigs following challenge. MNC also increased significantly in ileum of CS+ pigs post-challenge.

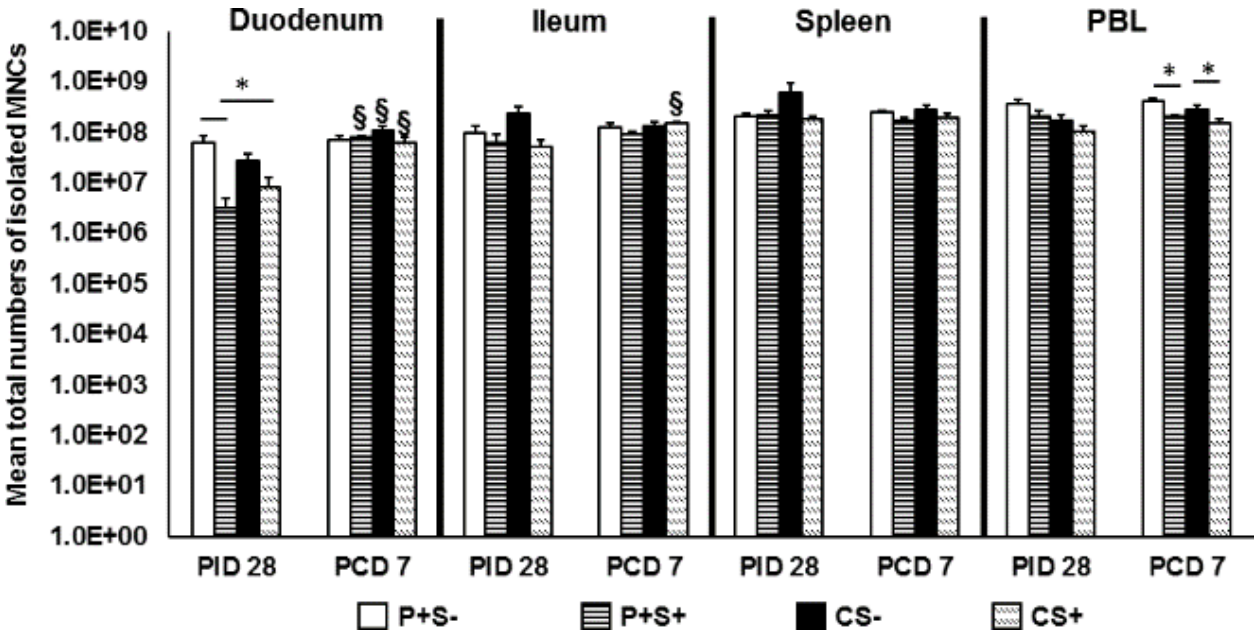

Figure 5. Total MNCs isolated following P particle vaccination with or without simvastatin feeding pre- and post-challenge. Total MNCs were determined based on the concentration and total volume of MNCs on the day of cell isolation. Mean total numbers of total MNCs plus standard errors of the means (*n*, 6 to 10) pre-challenge and post-challenge in intestinal (duodenum, ileum) and systemic (spleen, PBL) tissues. See the legend to Fig. 1 for an explanation of the symbols indicating statistical significance.

4.5 Discussion

Simvastatin is a cholesterol-reducing drug. In addition to being used to reduce the risk of heart diseases and stroke, it has been shown to have pleiotropic effects, including reducing severity in rheumatoid arthritis (16, 17), multiple sclerosis (19), and periodontitis (20, 21). However, simvastatin has been shown to increase host susceptibility to NoV infection *in vitro* (33) and *in vivo* (34, 35). Simvastatin's immunomodulatory effects include down-regulating IFN-inducible MHCII expression (44) and Th1 responses (45), up-regulating Treg proliferation and cytokine production (42, 43), and reducing Th1/Th2 and CD4/CD8 ratios (17). We previously showed that P particles provide partial protection against GII.4 NoV diarrhea and induce stronger T cell responses than virus-like particles (VLPs) in Gn pigs (11). In this study, we evaluated how simvastatin affects the protective efficacy of P particles against cross-variant, homotypic NoV challenge. We also evaluated simvastatin's effects on the T cell immunogenicity induced by P particles pre- and post-challenge as simvastatin is primarily consumed by the elderly and aging, a target population for a NoV vaccine.

Three intranasal doses of 100 µg (total: 300 µg) of P particles provide partial, cross-variant protection (47% protection rate) against homotypic infection in non-simvastatin fed pigs. However, simvastatin feeding eliminated this observed protection as all P+S+ pigs had diarrhea. Interestingly, simvastatin did not result in 100% occurrence of diarrhea in the control pigs. The increased occurrence of diarrhea has been reported in Gn pigs previously (34, 35). Among vaccinated pigs, simvastatin resulted in significantly higher AUC compared to non-simvastatin fed pigs, abolishing the reduction of AUC (1.6 fold vs 0) induced by the vaccination. Since simvastatin only slightly increased the mean number of days with diarrhea, the significant increase in AUC mainly resulted from increased daily diarrhea scores.

Since P particles previously did not reduce viral shedding (11), it is not surprising that simvastatin-fed vaccinated pigs also did not reduce the occurrence of viral shedding. Vaccination still resulted in slightly decreased shedding AUC compared to control pigs. However, among simvastatin-fed pigs, vaccination significantly lowered numbers of days with virus shedding compared to control pigs. Hence even with the presence of simvastatin, P particle vaccine can still exert some effect to reduce the duration of virus shedding. Curiously, one pig in each group did not shed virus. Additionally, we did not observe an increase in viral titers shed following simvastatin feeding, which was reported in a previous study (34). The simvastatin feeding schedule in the present study ended on the day before challenge, while the previous study's feeding schedule included simvastatin feeding 5 days pre-challenge and 5 days post-challenge. We noted a slight association between diarrhea severity and decreased viral shedding titers. Increased watery diarrhea may have prevented the collection of enough fecal material on rectal swabs, affecting the detection of NoV.

Despite recent efforts, NoV T cell immunity remains understudied. We previously reported that GII.4/VA387-derived P particles induced stronger T cell immune responses than VA387-derived VLPs following homotypic NoV challenge (11). Studies of mice have indicated that both CD4+ and CD8+ T cells are required for protection from murine norovirus (MNV) infection (46). Specifically, CD8+ T cells are required for viral clearance (47), while CD4+ T cells have been shown to be a correlate of protection from MNV (48). We also showed that NoV induces strong expansion of CD4+IFN-γ T cells in duodenum following challenge in Gn pigs (11), which is consistent with the predominant Th1 response found in PBMCs of humans following challenge (49).

The present study builds upon our previous findings in the role of T cells in NoV immunity. Importantly, P+S- pigs were the only group to provide partial protection against NoV diarrhea and these pigs had higher numbers of Th and CTLs and significantly higher numbers of activated CD8+ T cells in duodenum compared to all other groups at PID 28 (Figure 2). P+S- pigs also did not have significant increases in duodenal Th and CTLs following challenge (Figure 1). These findings indicate the importance of Th and CTLs in protection from NoV diarrhea and the result is similar to the findings in mice (47, 48). We previously reported on the correlations between T cell expansion in tissues and protection rate from diarrhea (11). Specifically, there were positive correlations between T cell expansions in ileum and spleen and protection rate, while T cell expansions in duodenum and protection rate are negatively correlated. Taken together this study with our initial study (11) and our dose response study (manuscript under review), we found several significant correlations. Expansion of activated CD8+ T cells in spleen were significantly positively correlated with protection rate from diarrhea ($R = 0.94$, $p = 0.0051$), while expansion of CD25-FoxP3+ Tregs in duodenum were negatively correlated with protection rate from diarrhea ($R = -0.94$, $p = 0.0051$) (data not shown). The exact phenotype of the splenic activated CD8+ T cells are unknown and require further investigation.

The focus of this study was to identify the mechanisms underlying simvastatin's deleterious effects on P particle-induced protection and immunity. First, simvastatin did not have an effect on circulating Th cells in PID 28 (Figure 1), indicating that P particles can adequately stimulate the immune system in the presence of simvastatin. However, simvastatin significantly reduced numbers of all analyzed cell types in duodenum of vaccinated pigs at PID 28. Interestingly, P+S+ pigs had significant decreases in splenic activated CD8+ T cells following challenge, while P+S- pigs had significant decreases in splenic activated CD4+ T cells following

challenge (Figure 2). The decrease in this cell type is in stark contrast to the aforementioned correlations with expansions of activated CD8+ T cells and protection rate from diarrhea in the P+S- pigs and at least partially explains the lack of protection in P+S+ pigs.

Simvastatin also down-regulated effector/memory CD4+IFN-γ+ and CD8+IFN-γ+ T cells in duodenum and effector/memory CD8+IFN-γ+ T cells in PBL at PID 28 compared to non-simvastatin fed pigs. This was expected, as simvastatin down-regulates IFN-inducible MHCII expression (44). Since simvastatin does not affect constitutive expression of MHCII (22) and P+S+ pigs had high levels of Th in PBL, P particles were initially able to stimulate the innate and adaptive immune responses, but the pigs were unable to develop memory immune responses. This is evident in the complete lack of splenic IFN-γ+ producing T cells following challenge in P+S+ pigs (Figure 3), which indicates a lack of memory response to NoV. Future studies should be conducted to determine the duration of IFN-γ+ T cell survival in spleen following simvastatin feeding. These results also indicate that approaches to overcoming simvastatin's negative effects on HuNoV vaccines should focus on IFN-inducible genes.

Our results also illustrate how simvastatin affects the primary T cell immune response to NoV infection. NoV immunity studies in humans are confounded by prior infections (49) and existence of cross-reactive CD4+ T cell epitopes (50), but NoV epochal evolution and immune escape (51-54) make improving our understanding of NoV primary immune responses imperative. Previously, we showed that primary NoV induced significant increases in total Th and CTLs, CD8+IFN-γ+ T cells, and Tregs in duodenum at PCD 7 in Gn pigs (11). IFN-γ was detected in humans following Snow Mountain virus (GII NoV), including IFN-γ secretion from antigen-pulsed PBMCs (49). Following NoV challenge, CS+ pigs had significantly reduced duodenal CTLs, PBL CD8+IFN-γ+ T cells, and PBL Tregs compared to CS- pigs. CS+ pigs had

significant increases in ileal Th, ileal activated CD4+ T cells, and ileal Tregs, but significant decreases in PBL CD25+ Tregs following challenge. Finally, CS+ pigs had significantly higher numbers of ileal Th, and ileal activated CD4+ T cells compared to P+S+ pigs. These results indicate that simvastatin feeding shifts the primary NoV immune response from duodenum, the primary site of viral replication (35), to the ileum in Gn pigs.

The most important effects of simvastatin were determined by the total MNCs isolated. Gn pigs fed simvastatin had significantly lower numbers of MNCs isolated from PBL at PCD 7, while the total MNCs in P+S+ pigs were significantly lower than P+S- pigs at PID 28 in duodenum (Figure 5). These significant decreases likely contribute to the significantly lower numbers of all T cell types analyzed in duodenum at PID 28. Similarly, simvastatin-fed pigs had significantly lower numbers of Tregs in PBL at PCD 7. These results provide a broader view to simvastatin's overall immunomodulatory effects. Not only does simvastatin impair T cell activation and memory development, but impairs the development of all MNCs, presumably including impaired antigen-presenting cells and B cells. Serum HBGA blocking antibodies and CD4+ T cells are believed to be correlates of protection (48, 55), but innate immunity has also been shown to be critical for control of MNV infection (56). NoV immunity requires intact innate and adaptive immune responses; simvastatin's impairment of total MNC development and immune activation is likely responsible for the increased diarrhea severity and duration of shedding that we witnessed. Simvastatin's effects on B cells and innate immunity are currently under investigation.

In summary, this study has several implications for simvastatin's effects on NoV infectivity and vaccine-induced immunity. First, we have shown that simvastatin feeding increases the severity of NoV-induced diarrhea. Second, we have reported that expansion of

splenic activated CD4+ T cells and duodenal CD25- Tregs are positive and negative correlates of protection, respectively. Third, we have shown that P particles still primed CD4+ and CD8+ T cells in the presence of simvastatin, which are important for protective immunity and viral clearance. Fourth, we have shown how simvastatin impairs the P particle vaccine's protective efficacy and immunogenicity. Finally, we have shown how simvastatin impairs total MNC development in hosts.

In conclusion, simvastatin decreased the protective efficacy of the P particle vaccine and inhibited MNC development in the duodenum of vaccinated pigs pre-challenge and in PBL post-challenge. To our knowledge, this is the first study to evaluate how simvastatin affects P particle vaccine protective efficacy and its underlying mechanisms. A robust immune response including B cells and CD4+ and CD8+ T cells are required for viral clearance (46, 47). Since the elderly and aging are one of the target populations for a NoV vaccine and the primary consumers of simvastatin, the implications that simvastatin can increase NoV-induced diarrhea and decrease the development of overall T cell responses are important for not only NoV protective immunity, but other pathogens (57). Simvastatin has also been shown to affect other viral infections, including influenza (58), hepatitis C virus (59), and HIV (60). As immunocompromised patients and chronic shedders serve as potential reservoirs for new NoVs (61-63) and simvastatin increases NoV shedding (34) and disease, simvastatin's down-regulation of IFN-inducible MHCII expression could lead to the emergence of potentially pandemic new strains of NoVs.

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CHAPTER 5

Future Directions

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5.1 Proposed experiments

This dissertation research has shown the P particle vaccine is a promising vaccine candidate against human noroviruses using the gnotobiotic (Gn) pig model. We have also shown that simvastatin negatively impairs P particle-induced immunity and elucidated the T cell responses to P particle vaccination and NoV infection, including several T cell subsets that are correlated with protection from diarrhea. Future experiments will continue to explore the P particles as vaccine candidates and further elucidate the roles of T cells in NoV immunity. These experiments include: 1) evaluating the GII.4/VA387 P particles against other genotypes of NoVs, 2) evaluating P particles as platforms for other viral antigens, 3) evaluating probiotics and rice bran as P particle vaccine adjuvants, 4) evaluating ways to overcome simvastatin's deleterious effects on P particles, and 5) further identifying the important T cell subsets in NoV immunity.

Our current studies have shown that P particles provide a similar protection rate as virus-like particles (VLPs) and prior NoV infection (1). However, these protective effects have only been determined between two GII.4 variants, though we considered these two variants antigenically distinct. Other strains have been implicated in recent outbreaks, including GI, GII.2, GII.3, GII.5 and GII.6 NoVs (2, 3). Additionally, NoVs undergo epochal evolution with a new strain emerging every 2-4 years (4-6), indicating a new strain of NoV will emerge in the next 1-2 years to replace the current pandemic strain GII.4/2012 Sydney. NoV vaccines will have to provide broad protection against other genotypes and emerging strains. The GII.4/VA387-derived P particle's protective effects will need to be determined against another common genotype, such as GII.3 or a recombinant strain, and the future pandemic strain.

The P particle vaccine can also serve as a platform to present other viral antigens. Previous studies have indicated P particles expressing other viral antigens, including rotavirus, influenza, and hepatitis E virus (7-11), are immunogenic in mice. The potential economic

benefits of a vaccine that can protect against several pathogens are vast, especially in developing countries. While NoV has become the leading cause of nonbacterial acute gastroenteritis in the developing world since the advent of rotavirus vaccines (12, 13), rotavirus remains the most common cause of pediatric gastroenteritis in the developing world. However, it remains unknown how addition of foreign antigen impacts the effectiveness of P particles and the vaccine efficacy needs to be evaluated in our animal models. We have a well-established Gn pig rotavirus challenge model (14) in addition to the norovirus model. These models can be used to evaluate the protective efficacy and immunogenicity of a P particle platform virus expressing rotavirus VP8 (7) against NoV infection, rotavirus infection, or a co-infection.

The influence of pathogenic, commensal, and probiotic bacteria on NoV infectivity and NoV vaccines has yet to be studied. However, studies have shown that GI.1 and GII.6 VLPs bind to enteric bacteria (15) and GII.4 infectivity is increased in the presence of *Helicobacter pylori* (16). Further, *in vitro* studies have indicated probiotics can outcompete P particles for binding, but cells already bound with P particles or probiotics had an increased retention of P particles (17). These results directly indicate that NoVs and P particles are capable of interacting with pathogenic and probiotic bacteria. The implications of these interactions are unknown; pathogenic bacteria appear to increase NoV infectivity while probiotics may enhance immunogenicity of P particle vaccines. We have a well-established Gn pig model of probiotics (14, 18) and Gn pig model transplanted with human gut microbiota (19) that can be used to evaluate how probiotic bacteria and gut microbiota impact NoV infectivity and P particle-induced protective immune responses.

Our studies have shown simvastatin increases occurrence and severity of NoV diarrhea in P particle-vaccinated pigs. This confirms previous studies that show simvastatin increases NoV

infectivity *in vitro* (20) and *in vivo* (21, 22). These findings are related to simvastatin's down-regulation of IFN-inducible MHCII (23), which likely inhibits the formation of memory responses against NoV. However, simvastatin has beneficial effects on reducing heart disease and stroke, in addition to other chronic conditions (24-29). Strategies must be developed to overcome simvastatin's negative effects on P particle vaccine-induced protective efficacy, but not impact simvastatin's beneficial effects. Studies in Gn pigs have indicated oral IFN-α treatment reduced simvastatin's negative effects (22). It would be beneficial to determine how oral IFN-α treatment could improve P particle vaccine efficacy.

We also plan to elucidate further the T cell subsets that play a role in NoV immunity. Previous studies have indicated both CD4+ and CD8+ T cells are important for protection and clearance (30), with CD8+ T cells responsible for viral clearance (31) and CD4+ T cells serving as a correlate of protection (32). Our studies have shown activated CD8+ T cells in spleen and CD25-FoxP3+ Tregs in duodenum are positively and negatively correlated with protection rate against diarrhea, respectively. However, we do not know the full phenotype of these cells. Further studies will be conducted to determine the surface markers and cytokines expressed by these cell types in NoV immunity.

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