MECHANISMS OF IMMUNOMODULATION BY PROBIOTICS: INFLUENCE OF LACTOBACILLI ON INNATE AND T CELL IMMUNE RESPONSES INDUCED BY ROTAVIRUS INFECTION AND VACCINES

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MECHANISMS OF IMMUNOMODULATION BY PROBIOTICS: INFLUENCE OF LACTOBACILLI ON INNATE AND T CELL IMMUNE RESPONSES INDUCED BY ROTAVIRUS INFECTION AND VACCINES

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ABSTRACT

My dissertation research focused on studying mechanisms of immunomodulation by probiotic lactobacilli on innate and T cell immune responses induced by rotavirus infection and vaccines in a gnotobiotic pig model of human rotavirus (HRV) infection and vaccination. We first studied the effects of probiotics on antigen-presenting cells (APCs) through TLR activation. We found that a mixture of *Lactobacilli acidophilus* strain NCFM (LA) and *L. reuteri* (ATCC# 23272) induced strong TLR2-expressing APC responses and virulent HRV induced a TLR3 response. Probiotics and HRV had an additive effect on TLR2- and TLR9-expressing APC responses, consistent with the adjuvant effect of lactobacilli.

Dose effects of LA on T cell immune responses were investigated. We found that low dose LA significantly enhanced frequencies of HRV-specific IFN-γ producing CD4+ and CD8+ T cells whereas high dose LA reduced frequencies of HRV-specific IFN-γ producing CD4+ T cells. Low dose LA reduced frequencies of induced regulatory (iTreg) cells and TGF-β expression in the iTreg cells whereas high dose LA increased frequencies of iTreg cells and IL-10 expression in the iTreg cells. The dose effects of LA were independent of HRV infection/vaccination.

In addition, we demonstrated that TCR-γδ T cells play an important role in modulating immune responses to rotavirus infections. All three γδ T cell subsets showed evidence of activation after HRV infection by increasing TLR2, TLR3, TLR9 expression and IFN-γ production during the acute phase of infection. There was an additive effect between lactobacilli and HRV in inducing total γδ T cell expansion in ileum and in recruiting the cells from blood. HRV infection induced a significant expansion of the CD2+CD8+ γδ T cell subset in the ileum. This subset mainly exerts regulatory functions as evident by expressing FoxP3, secreting TGF-β and IL-10 or increasing production of the anti-inflammatory cytokines by CD4+ and/or CD8+ αβ T cells in the co-cultures. CD2+CD8- and CD2-CD8- γδ T cell subsets have mainly pro-inflammatory and anti-viral functions as evident by secreting IFN-γ or promoting CD4+ αβ T cell proliferation and IFN-γ production.

The knowledge will facilitate the development of more effective vaccination and therapeutic strategies to protect children and young animals against rotavirus gastroenteritis.

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allophycocyanin (APC)
angiogenin 4 (Ang 4)
antibody-secreting cell (ASC)
antigen-presenting cell (APC)
attenuated HRV (AttHRV)
biliary atresia (BA)
bromodeoxyuridine (BrdU)
cell adhesion molecule 1 (CAM1)
cell-culture immunofluorescent (CCIF)
colony forming unit (CFU)
conventional dendritic cell (cDC)
cytotoxic T lymphocyte (CTL)
dendritic cell (DC)
dextran sodium sulphate (DSS)
double layered particle (DLP)
double-stranded RNA (dsRNA)
Dulbecco's Modified Eagle's Medium (DMEM)
endoplasmic reticulum (ER)
enzyme-linked immunosorbent assay (ELISA)
eukaryotic initiation factor 4G (eIF4G)
fluorescein isothiocyanate (FITC)
fluorescence-activated cell sorting (FACS)
fluorescent focus-forming unit (FFU)
follicle-associated epithelium (FAE)
Foot-and-mouth disease virus (FMDV)
forkhead box protein 3 (FoxP3)
gastrointestinal (GI)
GATA binding protein 3 (GATA3)
glutamic aciddecarboxylase 65 (GAD 65)
gnotobiotic pig (Gn pig)
gut-associated lymphoid tissue (GALT)
histo-blood group antigen (HBGA)
human rotavirus (HRV)
induced Treg cell (iTreg cell)
inflammatory bowel disease (IBD)
insulinoma Ag 2 (IA 2)
interferon (IFN)
interferon regulatory factor (IRF)
intestinal epithelial cell (IEC)
intraepithelial lymphocytes (IEL)
isopentenyl pyrophosphate (IPP)

keratinocyte growth factor (KGF)
lactic acid bacteria (LAB)
Lactobacillus acidophilus (LA)
Lactobacillus reuteri (LR)
Lactobacillus rhamnosus GG (LGG)
latency-associated peptide (LAP)
lipopolysaccharide (LPS)
magnetic antibody cell sorting (MACS)
median diarrhea dose (DD_{50})
median infectious dose (ID_{50})
mesenteric lymph node (MLN)
microbe-associated molecular pattern (MAMP)
Microfold cell (M cell)
mitogen-activated protein kinase (MAPK)
mononuclear cell (MNC)
mucin (MUC)
myeloid dendritic cell (mDC)
natural killer cell (NK cell)
natural killer T cell (NKT cell)
natural Treg cell (nTreg cell)
non-structural protein (NSP)
oral poliovirus vaccine (OPV)
pattern-recognition receptor (PRR)
peridinine chlorophyll protein (PerCP)
peripheral blood mononuclear cell (PBMC)
phycoerythrin (PE)
phycoerythrin-cyanine tandem fluorochrome (PE-Cy7)
phytohaemagglutinin (PHA)
plasmacytoid dendritic cell (pDC)
polyethylene glycol (PEG)
polyinosine-polycytidylic acid (polyI:C)
porcine reproductive and respiratory syndrome (PRRS)
post-challenge day (PCD)
post-inoculation day (PID)
prostaglandin E2 (PGE2)
protein kinase B (PKB)
protein kinase C (PKC)
randomized controlled trial (RCT)
regulatory dendritic cell (rDC)
regulatory T cell (Treg cell)
retinoic acid (RA)
retinoic-acid-related Orphan Receptor C isoform 2 (RORC2)
reverse transcription polymerase chain reaction (RT-PCR)

rhesus rotavirus (RRV)
room temperature (RT)
severe combined immunodeficiency (SCID)
sialic acid (SA)
single-stranded RNA (ssRNA)
SpectralRed™ (SPRD)
subepithelial dome (SED)
T-box transcription factor (T-bet)
T cell receptor (TCR)
T helper (Th)
thymic stromal lymphopoietin (TSLP)
tissue culture infectious doses ($TCID_{50}$)
Toll-like receptor (TLR)
triple-layered particle (TLP)
ulcerative colitis (UC)
ultra-high temperature (UHT)
viral protein (VP)
virulent HRV (VirHRV)
virus-like particle (VLP)

CHAPTER 1

Human rotavirus, rotavirus vaccine, probiotics, and mucosal immunity

1.1 Introduction

Rotavirus is the most common cause of severe gastroenteritis in infants and young children and responsible for around 20 % of diarrhea-associated deaths in children under 5 years of age [1] and an estimated 500,000 deaths annually worldwide [2]. Both licensed rotavirus vaccines, RotaTeq and Rotarix, have a substantially lower protective efficacy against moderate to severe rotavirus gastroenteritis in low income countries in Southeast Asia and Africa than in the middle- and high-income countries [3-6]. There are several proposed hypotheses for explaining this disparity, including various host factors [7-15] and environmental factors [16-24] that reduce the "take" of vaccines and impair the infant's immune responses. Strategies are needed to overcome these obstacles and to improve the vaccine efficacy for children in low income countries.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [25]. Most probiotic bacteria are lactobacilli or bifidobacteria. Adjuvanticity of various *Lactobacillus* strains in enhancing cellular and/or humoral immune responses has been reported in studies of influenza, polio, rotavirus and cholera vaccines and rotavirus and *Salmonella typhi* Ty21a infections [26-33]. In a previous study, *Lactobacillus acidophilus* strain NCFM (LA) significantly increased

IFN-γ producing CD8+ T cell responses to an oral rotavirus vaccine in gnotobiotic (Gn) pigs [29]. Probiotics are increasingly used to improve human health, alleviate disease symptoms and to enhance vaccine efficacy. The strain-specific effects of probiotics are well recognized; however, the dose effects of probiotics are not clearly understood.

My dissertation research mainly focuses on improving the understanding of the influence of different doses of probiotics on the development of protective immune responses after human rotavirus (HRV) infection or vaccination since the introducing probiotics to the host influence its microbiota population and then immune responses to pathogen infection. In addition, my dissertation research wants to identify the mechanisms of the immune modulating effects and the dose effects of probiotics. The knowledge will facilitate the development of more effective vaccine strategies by using appropriate probiotic strains and doses to improve the immunogenicity and protective efficacy of rotavirus vaccines.

1.2 Rotavirus

1.2.1 Rotavirus overview

Rotavirus is the most common cause of severe gastroenteritis in infants and young children [2]. The primary site of rotavirus replication is the mature enterocytes at the tip of villi in the small intestine [34] and may also spread beyond the gastrointestinal tract resulting in an acute phase of viremia [35-38].

Rotavirus can result in both asymptomatic and symptomatic infection; symptomatic rotavirus disease presents severe watery diarrhea, fever, vomiting [39] and even death.

Rotavirus belongs to the Reoviridae family with a non-enveloped, triple-layered icosahedral capsid structure and has an 11-segmented double-stranded RNA (dsRNA) genome inside [40]. Six structural viral proteins (VP1, VP2, VP3, VP4, VP6 and VP7) of the rotavirus form the capsid of the virus and six nonstructural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) are involved in virus replication and present exclusively in the infected cells [41]. The core of the rotavirus virion is composed of VP1, VP2, VP3, and the 11 genome segments [42, 43]. VP1 acts as the viral polymerase for both transcription and replication [44]. The innermost VP2 core shell of rotavirus particle surrounds the viral genome and RNA processing enzymes (VP1 and VP3), and is also a cofactor to initiate dsRNA synthesis [45]. Viral protein VP3 interacts with the N terminus of VP2, binds GTP covalently and acts as a guanylyl and methyl transferase generating capped mRNA transcripts [46]. VP4 is a protease-sensitive protein also called the P-type antigen to be used to classify rotavirus. Because neutralization and gene sequencing assays for VP4 do not generate consistent typing results, P typing has a dual system: P serotypes by their serotype numbers (e.g., P1, P2) and P genotypes in brackets (e.g., P[8], P[4]) [47]. VP4 is involved in the virus attachment and cleaved by trypsin to form VP5* and VP8*, which enhances virus infectivity [48]. Trypsin

cleavage of VP4 is not necessary for virus binding but it is needed for virus entry into the cells possibly by exposing VP5* [49, 50] and uncoating rotavirus particles [48]. The VP8* domain is involved in binding to sialic acid (SA), whereas VP5* possibly interacts with some integrins [48, 50]. After uncoating, the resulting VP2/6 double-layered particles (DLPs) become transcriptionally competent in the cytoplasm [50]. After rotavirus infection, anti-VP4 neutralizing antibodies are induced to block virus cell entry [51, 52]. Passive immunization of mice with anti-VP4 antibodies confers protection against virulent rotavirus-induced disease [53]. Protective immunity is also induced in mice [54] or children [55] after active immunization with VP4 inoculation. The outer capsid protein VP7 is a glycoprotein, or G-type antigen. VP7 types can also be classified as serotypes by neutralization assays or as genotypes by sequencing. These two assays yield concordant results so viruses are referred to by their G serotype alone (e.g., G1, G2, G3, etc.) [47]. VP7 also has potential ligand sites for integrins such as αxβ1 and α4β1 [56, 57] and has a functional interaction with VP4 to allow rotavirus entry into the host cells [58]. After rotavirus infection, VP7 neutralizing antibodies are induced and VP7-specific CD8 T lymphocytes are generated in mice [59-61]. A 2/6/7-virus-like particle (VLP) vaccine could protect rabbits and mice from reinfection [62].

VP6 is the intermediate layer protein and is also involved in inducing immune responses. Most of the rotavirus-specific antibodies induced after natural infection and immunization are directed against VP6 [63, 64]; thus, it is

considered the most immunogenic rotavirus protein. However, antibodies against VP6 do not neutralize the infectivity of rotavirus *in vitro*. Passive immunization of severe combined immunodeficiency (SCID) mice with non-neutralizing anti-VP6 antibodies results in reduced virus shedding [65]. Furthermore, adult mice and rabbits inoculated with 2/6-VLPs are protected against rotavirus reinfection [66, 67]. The mechanism of protection by VP6-induced antibodies is probably related to inhibition of replication by binding of anti-VP6 antibodies to intracellular core DLPs during enterocyte transcytosis after surface engagement of the polymeric immunoglobulin receptor [67-69]. However, neonatal Gn pigs inoculated with 2/6-VLPs are not protected from rotavirus infection or disease [70]. Additionally, neonatal mice vaccinated with VP6 (with *E. coli* labile toxin as the adjuvant) or passively immunized with IgA antibodies against VP6 are not protected against virus challenge whereas adult mice are protected following passive immunization with anti-VP6 antibodies [53, 65, 71]. The reason for this discrepancy is not well known, but it may be related to the immaturity of the neonatal immune system or to the inability of anti-VP6 antibodies to prevent diarrhea in neonatal mice and pigs.

NSP1 can degrade interferon (IFN) regulatory transcription factor (IRF) 3, IRF5 and IRF7 in a proteasome-dependent way [72-74]. The loss of NSP1 does not seem to negatively affect rotavirus replication in cultured cells [75]. However, it plays a role in pathogenesis in some animal models by antagonizing the type I interferon responses to increase viral pathogenesis [72, 76]. A recent

study showed that systemic rotavirus strain-specific replication in the murine biliary tract is determined by viral entry mediated by VP4 and viral antagonism of the host innate immune responses mediated by NSP1 [77]. NSP1 from different strains even had different mechanisms to regulate innate immune responses. For example, human rotavirus suppressed the IFN signaling pathway mainly through the NSP1-induced degradation of IRF5 and IRF7, however, NSP1 from animal rotavirus likely target all IRF3, IRF5, and IRF7 [78].

The other nonstructural proteins are involved in rotavirus replication (NSP2, NSP3, NSP5 and NSP6) and morphogenesis (NSP4) [43]. NSP2 along with NSP5 has been implicated in viroplasm formation and genome replication. A recent study found that the proteasome activity of probable protein degradation is needed for the assembly of new viroplasms [79]. Packaging NSP3 facilitates the translation of viral mRNAs using the cellular machinery by binding viral mRNAs with its N-terminal domain, eukaryotic initiation factor 4G (elF4G) with its C-terminal domain and directing the viral mRNAs to the cellular ribosomes for protein synthesis [80, 81]. NSP6 interacts with NSP5 and might regulate the assembly of NSP5 [82, 83]. NSP4 plays a role in virus morphogenesis and enterotoxigenesis [84, 85]. Situated on the endoplasmic reticular (ER) membrane, the C-terminal cytoplasmic domain of NSP4 acts as an intracellular receptor [86] for unassembled VP6 and directs it to the ER for DLP formation [87-89]. NSP4 forms a complex with VP4 and VP7 in the ER before the formation of triple-layered particles (TLPs) [90]. NSP4 also

functions as an age-dependent enterotoxigenic agent in mice [84, 85, 91]. NSP4 induced mouse crypt cells and human cell lines to mobilize Ca^{2+} and secrete Cl^-, which is a possible mechanism for rotavirus-induced secretory diarrhea in mouse pups and human neonates [91]. A recent study reported that rotavirus disrupts calcium homeostasis by NSP4 viroporin activity [92]. In addition, NSP4 was reported to stimulate release of serotonin (5-HT) from human enterochromaffin cells and plays a key role in the emetic reflex during rotavirus infection resulting in activation of vagal afferent nerves connected to nucleus of the solitary tract and area postrema in the brain stem, structures associated with nausea and vomiting [93]. NSP4 antibodies are detected in several animal models and humans after rotavirus infection and exogenous NSP4 antibodies reduced diarrhea in neonatal mice [84, 94]. However, NSP4 antibody responses were not associated with protection against diarrhea in pigs or humans [95, 96]. NSP4 may also function as an adjuvant to enhance immune responses to other antigens [85].

1.2.2 Rotavirus pathogenesis

Rotavirus infection can result in both asymptomatic and symptomatic infection. Manifestations of rotavirus disease are severe watery diarrhea, fever and vomiting that usually lead to fluid and electrolyte disequilibrium and other secondary complications (e.g. renal failure) including death [39]. Rotavirus generally is responsible for around 20 % of diarrhea-associated deaths in

children under 5 years of age [1]. Rotavirus pathogenesis is multifactorial. Both viral and host factors affect the outcome of rotavirus infection. The most dominant host factor is age: neonates infected with rotavirus rarely have symptomatic disease; this protection is thought to be mediated primarily by transplacental transfer of maternal antibodies [97]. The peak age of rotavirus disease is between 6 months and 2 years of age [98]. Rotavirus can also infect adults normally with no severe symptomatic disease. However, an unusual virus strain or extremely high doses of rotavirus can result in severe symptoms in adults [99]. Rotavirus virulence is related to properties of the proteins encoded by a subset of the 11 viral genes: genes 3, 4, 5, 9, and 10 [99]. Gene 3 encodes the capping enzyme that affects the level of viral RNA replication [46, 100]. Genes 4 and 9 produce the outer capsid proteins required to initiate infection [51, 52]. Gene 5 codes NSP1 that functions as an interferon antagonist [72-74]. Gene 10 codes for the nonstructural protein NSP4, which functions to regulate calcium homeostasis, virus replication, and as an enterotoxin [99]. NSP2 is also involved in virulence, especially in mice [1].

Rotavirus primarily infects intestinal villus enterocytes. However, all infected individuals and animals undergo at least a short period of viremia and rotavirus can be detected in several other tissues from immunocompetent hosts in addition to the intestine [35-38]. Disease pathogenesis is multifactorial and the model is based primarily on studies in a variety of animal models.

First, rotavirus infection evokes histological changes. It is proposed that

rotavirus infection kills most of the mature enterocytes, so that crypt cells invade the villus surface to cause a decrease in the digestive and absorptive capacities of the intestine and generate a malabsorption type of diarrhea. This crypt cell invasion hypothesis, however, has never been confirmed by experiment [101]. In colostrum-deprived calves, rotavirus infection leads to a change in the villus epithelium from columnar to cuboidal, causing villi to become stunted and shortened [1]. In pigs, the macroscopic changes demonstrate the thinning of the intestinal wall and the microscopic changes include villus atrophy, villus blunting and conversion to a cuboidal epithelium [1, 102]. Studies of biopsies of rotavirus-infected infants also reveal shortening and atrophy of villi, distended endoplasmic reticulum, mononuclear cell infiltration, mitochondrial swelling and denudation of microvilli [103, 104].

Rotavirus infection influences fluid and electrolyte transport. Both the decreased lumen-to-tissue in-flux and the increased tissue-to-lumen ex-flux account for the changed sodium transport and result in rotavirus diarrhea [1]. Usually, the Ussing chamber technique is used to study electrolyte transport in virus-infected intestinal segments [105].

The cotransport of glucose and sodium in some way is impaired in intestinal segments exposed to rotavirus [106]. Disaccharidases are localized to the brush border region of enterocytes and are necessary for the monosaccharde production and then for enhancing the sodium-monosaccharide symport. In rotavirus infection, the activity of mucosal disaccharidases is markedly

attenuated [106]. The apical membrane of enterocytes is not only provided with symports for sodium/glucose but also for sodium/amino acids. The activity of the sodium-potassium ATPase pump, situated on the basolateral membrane of the enterocytes, is also attenuated in virus-infected intestines [106]. This may reflect a true decrease in ATPase activity but may also be explained by the blunting of the intestinal villi reducing the number of enterocytes.

Transepithelial electrical resistance decreases after rotavirus exposure to the apical or basolateral plasma membrane [107, 108]. It reflects increased paracellular permeability, possibly caused by a disorganization of tight junction proteins claudin, occludin and ZO-1 [107, 108]. The absorption of horseradish peroxidase and electrical tissue conductance is used to study epithelial permeability after rotavirus infection [109, 110]. Intestinal permeability is also investigated in young children with rotavirus diarrhea using polyethylene glycols (PEGs) as probes [111].

Intracellular NSP4 induces the release of Ca^{2+} from internal stores and also can disrupt tight junctions allowing paracellular flow of water and electrolytes. NSP4 also can bind to a specific receptor and trigger a signaling cascade through phospholipase C and inositol phosphatase 3 that results in release of Ca^{2+}. The increase in Ca^{2+} also disrupts the microvillar cytoskeleton. In addition, NSP4 can stimulate the enteric nervous system, in turn signaling an increase in Ca^{2+} that induces Cl^- secretion [99].

The other symptom cuased by rotavirus infeciton is biliary atresia (BA), which

is a neonatal obstructive cholangiopathy (biliary epithelial cell pathogenesis) that results in obstruction of the biliary tree [112, 113]. Recent study using a murine model found that gene VP4 of rhesus rotavirus (RRV) strain plays an important role in inducing murine BA and VP3 regulates this effect [114]. However, Feng et al. found that RRV NSP4, instead of VP7 and VP4, is important to induce BA because mouse pups infected with a RRV strain that was subjected to NSP4 silencing had lower incidence of BA than after VP7 or VP4 silencing. Another study found that an anti-enolase antibody cross-reacts with RRV proteins and indicated that molecular mimicry might activate humoral autoimmunity to promote BA [115]. This cross-reactivity between rotavirus and host antigens is also found in type 1 diabetes caused by rotavirus infection, in which rotavirus VP7 have high sequence similarity to T cell epitope peptides in the islet autoantigens: tyrosine phosphatase-like insulinoma Ag 2 (IA2) and glutamic acid decarboxylase 65 (GAD65) [116]. The inflammatory responses in cholangiocyte triggered by rotavirus infection are also important to inducing BA. In the in vivo study with RRV infected Balb/c pups and in vitro study with an immortalized cholangiocyte cell line (mCl), the chemokine expression (such as chemokines macrophage inflammatory protein 2, monocyte chemotactic protein 1) by RRV-infected cholangiocytes may trigger a host inflammatory process to cause bile duct obstruction [117]. NK cells also are suggested as important initiators for cholangiocyte injury via Nkg2d [118].

1.2.3 Rotavirus epidemiology

Group A rotavirus is the leading cause of gastroenteritis in infants and young children worldwide. Within Group A, at least 19 G and 28 [P] sero/genotypes of rotavirus have been identified [47]. Serotypes G1, G2, G3, G4, and G9 are responsible for 90 % of all rotavirus infections in North America and Europe; however, these serotypes account for less than 70 % of cases in Africa [119]. P[8] and P[4] account for over 90 % of circulating P types worldwide; however, the relative frequency of these two serotypes is lower in Africa where P[6] accounts for around a third of all detected P types [119]. There are 2^{11} different combinations of G and P proteins that can be generated. However, the actual number of G and P combinations is less than the possible number because most combinations are not fit and do not survive subsequent rounds of replication in host.

To date, rotaviruses circulating in humans are characterized as common genotypes (G1P[8], G2P[4], G3P[8], G4P[8]), reassortants among human genotypes (G1P[4], G2P[8], G4P[4]), reassortants between animal and human genotypes (G1P[9], G4P[6], G9P[8], G12P[8]), and likely zoonotic introductions (G9P[6], G9P[11], G10P[11], G12P[6]) [47, 120]. Among these, G1, G2, G3 and G4 in combination with P[4] and P[8] represented over 88 % of strains worldwide based on studies published between 1998 and 2004 [119]. The data from the surveillance networks of the WHO also indicate that G1P[8], G9P[8] and G2P[4] account for 75 % of samples genotyped in the WHO regions

of North America, Europe, southeast Asia and western Pacific regions. However, greater diversity of strains was seen in the Africa and Mediterranean regions [121]. This indicates that the surveillance of rotavirus epidemiology is very important and critical for each specific region.

Currently, two rotavirus vaccines are commercially available globally: Rotarix (GlaxoSmithKline Biologicals) and RotaTeq (Merck) [122]. The G or P serotypes of more than 85 % strains circulating in US from 1996 through 2005 are covered in both the licensed vaccines [123]. G1-G4, G9, and P[8] types are still found to be the most prevalent overall. However, in Indonesia [124], only 56 % of strains contained a G or P antigen common to both vaccines and 23 % of strains comprised types not represented in either vaccine. A strong predominance of G12 strains in Nepal is now recognized as a global phenomenon [125, 126]. The G12 genotype continues to be described in several parts of the world [127-131] and raises the question of whether current vaccines will provide protection against this strain. The G12 genotype has been reported in combination with different P genotypes and exhibits varying electropherotypes, indicating reassortant with other strains [128]. Infections by other unusual strains of regional importance as well as unusual reassortants of commonly circulating G and P types are also described. These include G8 in Malawi, G3P[10] and G3P[19] in Thailand, G1P[19] in western India, and G2P[11] and G3P[11] in north India [128, 131-134]. It is obvious that rotavirus surveillances generate valuable data on circulating rotavirus strains in each

specific region and are also vital to inform vaccine development, to track emergent types, and to help assess vaccines introduced. Also, the rotavirus surveillances can track interspecies transmission of rotavirus strains [135, 136].

Most reassortants involve genes encoding VP7 and VP4, suggesting that immune selection pressure may influence virus evolution. As a result, porcine serotype G5 and bovine serotype G8 emerge as important regional human pathogens in South America and Africa, respectively [119, 123, 137]. For example, in Rio de Janeiro the incidence of G5 strains peaked at almost 60 % in the mid-1990s, although they seem to be decreasing lately, whereas, in Malawi, G8 strains comprised 42 % of rotavirus samples in the late 1990s and have been reported elsewhere in Africa, albeit in smaller numbers [119, 123, 137, 138]. Meanwhile G9 and more recently G12 strains have most likely emerged from porcine origins in Asia and spread globally, whereas P[6] bearing strains are found throughout Africa [119, 123, 125, 131, 138, 139]. Infections by bovine-human reassortants and the presence of several unusual strains in cases of infant diarrhea suggest that animal rotaviruses could have a significant zoonotic impact [140]. One recent study showed that a G6P[7] reassortant strain KJ9-1 (containing six bovine-like gene segments: VP7 (G6), VP6 (I2), VP1 (R2), VP3 (M2), NSP2 (N2), and NSP4 (E2), four porcine-like gene segments:VP4 (P[7]), NSP1 (A1), NSP3 (T1), and NSP5 (H1), and one human-like gene segment: VP2 (C2)) induced severe diarrhea in colostrum-deprived calves with dramatically intestinal villous atrophy and viral RNA was detected in serum,

mesenteric lymph node, lungs, liver, choroid plexus, and cerebrospinal fluid. This reassortant strain also replicated in colostrum-deprived piglets but without clinical symptoms present [141].

The genomic diversity of rotavirus strains has important implications for vaccine development as strains that fail to share serotype antigens with vaccines may evade vaccine-induced immune protection.

1.2.4 Rotavirus vaccine development

The studies of rotavirus vaccines were initiated in the early 1980s on the basis of the classical "Jennerian" approach: using cowpox as a surrogate vaccine to induce immunity to smallpox [142]. This approach is based on normal attenuation of animal virus strains in human and/or cell culture attenuation. Even though rotavirus can reassort among different species, rotavirus exhibits species restriction in inducing infection [143] and so both animal strains and cell culture adapted human rotavirus can be applied for human rotavirus vaccine development. Rotavirus animal strains RIT4237 (bovine strain), Wistar Calf 3 (bovine strain, WC3) and MMU18006 (rhesus strain) displayed good tolerance and did not cause illness in young children [142].

Bovine rotavirus vaccine RIT4237 was the first one for clinical trial [144], which displayed nonreactogenicity even with a maximum 1×10^8 50 % tissue culture infectious doses ($TCID_{50}$) [144], but were capable of inducing high protection in Finland [145, 146] and Peru [147]. However, neutralizing

antibody responses in serum was only homotypic (G6) in the first infected infants, despite showing booster responses to infants previously exposed to G1 rotaviruses [146]. In the following trials, RIT4237 vaccine showed little protection rates in Rwanda, Gambia and the Navajo reservation [148-150]. The reason behind this low efficacy likely is the high levels of preexisting antibodies against rotavirus which neutralize the vaccine strains and decrease vaccine titers [148, 149]. RIT4237 was ultimately abandoned as a candidate for further application to human infants.

Bovine rotavirus vaccine WC3 [151, 152] offered 100 % and 76 % protection rate against severe and all rotavirus gastroenteritis, respectively, in a G1 serotype dominant season in Philadelphia [153]. However, WC3 did not demonstrate a similar protection rate in Cincinnati [154] and in the Central African Republic [155] and no longer was developed as a single candidate vaccine, but it was used for the development of a more efficacious vaccine, RotaTeq.

MMU18006 is an attenuated rhesus strain vaccine and had variable protection rates in clinical trials [150, 156-159]. Furthermore, MMU18006 vaccine could induce fever in more than 1/5 of vaccinees even at a relative low vaccination doses: 10^4 or 10^5 colony forming unit (CFU). The MMU18006 vaccine is no longer being developed as a single candidate vaccine, but it was one component of a candidate tetravalent vaccine, RotaShield.

The failures of those single animal rotavirus strain vaccines to induce

consistent protection in young infants caused researchers to explore alternative avenues. Because of the reassortant characteristics of rotavirus, it is possible to include human rotavirus VP4 and VP7 serotypes to broaden antigenic coverage and provide protection against the epidemiologically important rotavirus serotypes [160].

The first multivalent live-attenuated oral rotavirus vaccine produced was the rhesus-human reassortant tetravalent vaccine, RotaShield, which consisted of RRV and three RRV-based reassorted strains. G3 is from RRV and shows strong cross-reactivity with human G3 serotype, while G1, G2 and G4 are provided by human strains [160]. All reassorted rotavirus strains retained the VP4 serotype-specificity of RRV, P5B[3]. The vaccine demonstrated 49-83 % efficacy against all rotavirus gastroenteritis and 70-95 % efficacy against severe disease [156, 158, 159, 161-164]. RotaShield were licensed in the US in 1998, but was removed from the market in October 1999 due to possibly causing intussusception [160].

Currently, there are two human single strain vaccines are under development, the 116E in India and the RV3 in Australia. Both strains are isolated from asymptomatic rotavirus infected neonates [165].

1.2.5 Currently licensed rotavirus vaccines

Considering the higher efficacy and higher neutralizing antibody titers against human rotavirus by RotaShield, a pentavalent rotavirus vaccine (RotaTeq) was

further developed and it contains 5 human-bovine reassortant rotavirus strains. The G1-G4 and P1 serotypes are derived from the human rotavirus strain [122, 166].

In February 2006, the pentavalent rotavirus vaccine (RotaTeq) was approved by the FDA in the US, and RotaTeq is currently licensed in more than 90 countries. The monovalent attenuated human rotavirus vaccine (Rotarix) is also approved by the FDA in the US in 2008 and licensed in over 100 countries worldwide. Rotarix is based on the attenuated human strain, 89-12, a G1P1A[8] strain [122, 167]. As a result of the possible association of RotaShield with intussusception, very large Phase III randomized and controlled clinical trials were required to show that RotaTeq and Rotarix were well tolerated and not associated with intussusception [18, 19].

Currently, these two rotavirus vaccines are commercially available globally [122, 166, 167]. However, only 17 countries have introduced routine rotavirus vaccination. Rotarix is orally administered in 2 doses at 2 and 4 months of age [122]. RotaTeq is administered as a 3-dose course at 2, 4, and 6 months of age [122].

These two licensed vaccines have a protective efficacy of more than 85 % against moderate to severe rotavirus gastroenteritis in middle and high-income countries [6]. However, the efficacy of RotaTeq is 39.3 % against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa [3] and 48.3 % in developing countries in Asia [4]. Rotarix showed an overall

efficacy of 61.2 % in South Africa and Malawi [5]. Since the introduction of rotavirus vaccines into the US from 2006, rotavirus seasons have been delayed and the magnitude diminished. Rotavirus caused hospitalizations, emergency department visits, and outpatient visits have declined dramatically in children < 5 years of age [168]. Specifically, rotavirus-related hospitalizations decreased by 50 % in 2007-2008 and by 29 % in 2008-2009 [169]. The dominant serotype was also changed. G1 was the dominant G-type during 2005-2006 and 2006-2007 seasons, whereas G3 was the most frequently detected strain in the 2007-2008 seasons [170]. Lanzieri et al. also found that the introduction of rotavirus vaccines to Brazil dramatically decreased rates of gastroenteritis-related deaths in children, especially with < 1 year of age [171]. The children rotavirus vaccination program even provided indirect protection to older children and adults in the US [172] and Australia [173, 174]. This suggests that rotavirus vaccination has a substantial public health impact on rotavirus disease and overall diarrhea events and the sustained declines reaffirm the health benefits of the rotavirus vaccination program [175].

1.2.6 Factors affecting rotavirus vaccine efficacy

There are fundamental differences in the behavior of live oral vaccines in the gut of infants in low-income countries from developed countries that may significantly affect received vaccine titers. This problem was initially seen for oral poliovirus vaccine (OPV) tested in India [176-178] and for live oral cholera

vaccine trials conducted in Thailand and Indonesia [179].

Because Rotarix is derived from human rotavirus strain, it can grow very well in the gut and two doses of each 1×10^6 CFU could induce a sufficient immune response and provide a high protection rate to children in European and Latin America. However, RotaTeq grows less well and 2×10^6 to 116×10^6 CFU of each reassortant have to be reached to offer good protection rate in 3 dose series. This makes it reasonable to explain the lower efficacy of those vaccines in developing countries by those host or environmental factors which may reduce the "take" of vaccines and also might reduce the immunogenicity and efficacy of live oral vaccines in infants.

There are three factors that can decrease vaccine titers delivered to the gut. The transplacental antibodies in infants from mother could neutralize vaccine rotavirus and inhibit the immune response. The second factor is the immune and nonimmune components of breast milk [15-21] which includes three main aspects: neutralizing antibody present in breast milk [15], practices around the time of breastfeeding [22], and the effect of breastfeeding. IgA antibodies in breast milk can neutralize vaccine virus and some receptor analogues in breast milk can attach to virus and prevent its attachment [23]. A recent study found that breast milk from Indian women had higher IgA and neutralizing titers against rotavirus RV1, 116E and RV5 G1 strains than those from Korean, Vietnamese and American women, with the lowest in American's [15]. Another study showed that some human rotavirus strains may use histo-blood group

antigen (HBGA) as receptors; thus, the HBGA in breast milk may reduce the susceptibility of rotavirus infection and also reduce the take of rotavirus vaccines [180]. The third factor is the amount of gastric acid in the infant's digestive tract. Low pH can destroy rotavirus and decrease rotavirus titers [24]. Thus, it is unclear whether the difference in levels of gastric acidity between developed and developing countries might influence vaccine uptake because it is hard to get these data from humans.

There are also three factors that affect the host responses to the oral vaccines. The first one is malnutrition (e.g. zinc and vitamin A). Vitamin A is used in many developing countries and demonstrates a positive impact on gastrointestinal function [9]. Zinc was also tested in several studies and demonstrated effectiveness in prevention of childhood diarrhea and respiratory illnesses [7, 181]. However, the roles of vitamin A and zinc on the efficacy of rotavirus vaccines need further study.

The components of gut flora [182] and oral administration of probiotics definitely influence the development of host immune system and thus regulate the immune responses to vaccines. Commensal bacteria in the gut are known to be involved in oral tolerance [183-187] and the balance change of commensal bacteria caused by either external introduced probiotics or others may alter the gut hemostasis and affect the immune responses to vaccines, including rotavirus oral vaccines. The health status of the host, such as malaria, diarrhea or HIV infection, could also interfere with their immune responses to rotavirus vaccines

and influence the protection rate of vaccines [188]. Finally, inconsistency between circulating rotavirus serotypes and rotavirus vaccine serotypes might also cause low protection rate [189]. As discussed previously, there are dramatically different circulating serotypes of rotaviruses between the developing and developed countries, even among special regions in the same country [12, 190].

1.2.7 Improvement of rotavirus vaccines

An infant's immune responses to a live oral vaccine may be influenced by the factors that decrease the effective titer of the vaccine virus reaching the intestine and the factors that might impair the infant's immune response. The high titers of transferred maternal antibodies must be overcome to increase the titer of vaccine virus. One study showed that the immune response to Rotarix was enhanced when the vaccination schedule was changed from 6 and 10 weeks of age to 10 and 14 weeks of age, which may be caused by the decreasing maternal antibodies among the vaccinees giving the delayed vaccination schedule as the half-life of transplacental antibody is 3-4 weeks in the infant. Breastfeeding could be delayed for a period after the vaccine is administered to examine whether breastfeeding at the time of vaccination might affect the infant's immune response to vaccines. Zinc, vitamin A, or probiotics may be used to enhance immune responses to vaccines.

1.3 Probiotics

1.3.1 Probiotics overview

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [25]. Most probiotic bacteria are lactobacilli or bifidobacteria. Different species and strains of probiotics induce different immune responses and may differentially regulate immune responses induced by pathogen infection. For example, germfree mice colonized with different bifidobacteria strains reveal strain-specific immune responses [191]. *B. longum* or *B. lactis* Bb12 induce a T helper (Th) 2 polarization with high IL-4 and IL-10 expression and leads to B cell-mediated humoral immune responses [192]; however, *B. bifidum* and *B. dentium* induce a Th1 polarization with increasing IFN-γ and TNF-α secretion by splenocytes and polarize to T cell-driven cellular responses. Our study also found that different lactobacilli strains had different immune regulatory functions. Probiotic *L. acidophilus* strain NCFM treatment of the non-transformed porcine jejunum epithelial cells (IPEC-J2) prior to rotavirus infection significantly increased the IL-6 immune responses to rotavirus infection, whereas *L. rhamnosus* strain GG (LGG, ATCC# 53103) strain treatment post-rotavirus infection down-regulated the IL-6 response suggesting LGG has antiinflammatory effects [193]. Even different isolates of *L. acidophilus* have different immune regulatory functions [194]. For example, *L. acidophilus* strain NCFM prolonged survival of adult and neonatal bg/bg-nu/nu mice, however, *L. acidophilus* strain LA-1 did not improve the survival of bg/bg-nu/nu mice. The same strains of probiotics in

different studies reportedly to induce different immune modulatory responses possibly due to the different doses used. For example, administration of *L. casei* at higher doses suppressed proinflammatory cytokine expression by CD4+ T cells and up-regulated immunoregulatory cytokine IL-10 and TGF-β levels [195, 196], whereas another study using lower doses found that *L. casei* were pure Th1 inducers [197].

Probiotics are widely accepted to have strain-specific health benefits as discussed above; however, dose effects of probiotics has not been studied in detail. Different research groups recommend different doses to be used to obtain a beneficial effect [198]. Fang et al. [199] found that at least 6×10^8 CFU of *L. rhamnosus* 35 were needed to have a positive effect on reducing fecal excretion of rotavirus. However, Guandalini [200] recommended at least 5×10^9 CFU of *L. rhamnosus* should be used. In addition, one study [201] showed that *B. lactis* HN019 was efficient at enhancing the immune responses (polymorphonuclear cell phagocytosis and NK cell tumor killing activity) at a comparatively low dose (5×10^9 organisms/d) relative to a 10-fold higher dose [202]. In addition, Konstantinov et al. showed that the cytokine profiles of dendritic cells (DCs) induced by *in vitro* interacting with *L. acidophilus* strain NCFM were concentration-dependent: the higher ratios (1,000:1 and 100:1) of *L. acidophilus* strain NCFM:DCs enhanced IL-10 expression; whereas the relatively lower *L. acidophilus* NCFM strain:DCs ratios (100:1 and/or 10:1) induced proinflammatory cytokines (IL-12p70, TNF-α, and IL-1β) [203].

1.3.2 Mechanisms of probiotic effects in health and in diseases

Different species of probiotics differentially regulate immune responses. There are many mechanisms behind probiotics' functions [204]. Probiotics mainly function by promoting intestinal development [205-207], by regulating intestinal epithelial cell function [193, 208-220], and by regulating inflammatory cytokine responses [187, 216, 221-226]. In addition, probiotic bacteria contribute to host health by promoting polysaccharide digestion [205, 227-230]. Probiotics also function to inhibit pathogens by lowering intestinal pH [231-233] or penetrate some pathogen cells, secreting bactericidal materials to reduce the population of other microorganisms [234-239] and modulating gut microbiota composition by competition [240-253].

The most beneficial effect of probiotic bacteria to the host is to promote intestinal development. Germfree animals show several developmental abnormalities, including immature intestinal mucosal surfaces. However, monocolonization with *Bacteroides thetaiotaomicron* in germfree adult mice can shift this phenomenon [205]. Germfree mice have an immature pattern of high sialyltransferase and low fucosyltransferase activities in the intestinal brush border. Colonization with conventional bacteria shifts these patterns to maturational development [206]. Colonization of mice with conventional bacterial flora or monocolonization with *B. thetaiotaomicron* can restore the mature villus capillary network [207]. In addition, some probiotics, such as lactobacilli, could stimulate up-regulation of mucous genes in intestinal goblet

cells to promote their maturation and also regulate intestine immune responses [210].

Probiotics regulate intestinal epithelial cell function, including enhancing epithelial barrier function, proliferation, differentiation, and cell survival. LA (ATCC# 4356) and *Streptococcus thermophiles (*ATCC# 19258) can prevent the disruption of intestinal epithelial barrier function in Caco-2 cells caused by enteroinvasive *E. coli* infection [214]. A mixture of probiotic bacteria enhanced tight junctions and prevented *Salmonella enterica* seropyte dublin-induced tight junction dissolution and ZO-1 redistribution in T84 cells which is a transplantable human carcinoma cell line [212]. The gram negative probiotic *E. coli* Nissle 1917 strain (EcN1917) also protected mice from dextran sodium sulphate (DSS)-induced colitis by increasing ZO-2 and ZO-1 expression to restore intestinal barrier function [216] and preventing disruption of the mucosal barrier by enteropathogenic *E. coli*. EcN1917 restored mucosal integrity in T84 epithelial cells through the enhanced expression and relocation of ZO-2 and protein kinase C (PKC) [220]. Maintaining the intestinal barrier function is also found with *L. casei* DN-114 001 [213] and VSL#3 (a mixture of four *Lactobacillus spp.,* three *Bifidobacterium spp.* and *S. thermophiles*) by the increasing expression of mucin 2 (MUC2) and MUC3 [212]. Lactobacilli also enhanced MUC3 synthesis and secretion in HT29 cells and inhibited enteropathogenic *E. coli* binding to these cells [209]. Our study found that LGG increased the production of membrane-associated MUC3 in IPEC-J2 cells

[193]. Probiotics reduced DSS-induced colitis in mice [208, 211, 217] possibly because of their ability to improve barrier function and regulate pro- and antiinflammatory cytokine responses [216, 221, 222].

Another mechanism of probiotics in maintaining the intestinal barrier function is through prevention of epithelial cell apoptosis. For example, some components secreted by LGG can prevent cytokine-induced cell apoptosis by activating the anti-apoptotic protein kinase B (PKB) and by inhibiting the pro-apoptotic p38/mitogen-activated protein kinase (MAPK). Furthermore, probiotics can function to restore intestinal barrier function by regulating inflammatory cytokine responses in some inflammatory symptoms, such as DSS-induced colitis in mice [216, 221, 222]. LGG inhibited lipopolysaccharide (LPS)- or *Helicobacter pylori*-stimulated macrophage cytokine production [223] and also was found to trigger the synthesis of IL-10 and decreased the release of IFN-γ, IL-6 and TNF-α [224]. *L. bulgaricus* strain LB10 and *L. casei* strain DN-114001 can reduce the frequencies of TNF-α secreting CD4+ T cells and the TNF-α expression by intraepithelial lymphocytes (IEL) [225].

Probiotic or commensal bacteria play a key role in maintaining intestinal immune homeostasis by regulating inflammatory cytokine responses. Both pathogenic and probiotic/commensal bacteria possess molecular recognition patterns detected by pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) [254]; however, probiotics/commensals normally do not initiate the pathogenic inflammatory response. The γ-irradiated VSL#3 was

capable of decreasing the severity of inflammation by interaction with TLR2 or TLR4 but not with TLR9 in DSS-induced colitis in mice [255]. EcN1917 also exerted its effects on DSS-induced colitis in mice through TLR2 or TLR4 [256] in addition to directly suppressing the IL-8 function on a human colonic epithelial cell line, HCT15 [257]. Tobita et al. also found that heat-treated *L. crispatus* strain KT could induce TLR2 and NOD2 activation to reduce allergic symptoms in mice [258]. Our study found that lactic acid bacteria (LAB) (a mixture of LA strain NCFM and *L. reuteri* (ATCC# 23272)) enhanced TLR2 and TLR9 expressing antigen-presenting cell (APC) responses to rotavirus, but had a suppressive effect on the TLR3 and TLR9 expressing CD14- APC responses in spleen [259]. TLR2 expression was up-regulated by LGG in IPEC-J2 cells [193]. Even DNA from some probiotics showed a systemic antiinflammatory effect compared to DNA from pathogenic bacteria which induced an inflammatory immune responses by TLR9 [255]. For regulating inflammatory cytokine responses, LGG (ATCC# 53103) inhibits macrophage cytokine production stimulated by LPS or *H. pylori* [223]. Probiotic *E. coli* or lactobacilli also suppress monocyte populations, increase IL-10 production, and down-regulate proinflammatory cytokine expression when incubated with intestinal epithelial cells [226]. Our study found that LGG (ATCC# 53103) treatment of the IPEC-J2 cells post-rotavirus infection down-regulated the IL-6 response, suggesting the antiinflammatory effect of LGG [193].

DCs are responsible for sensing and collecting antigens from the gut by

several receptors, such as the aforementioned TLRs, and present them to naive T cells. Different probiotics can regulate different DC phenotype and cytokine production profile by either enhancing or down-regulating immune responses [260-265]. For example, *Klebsiella pneumonia* (AZU R574) modulates DC function and induces a Th1 immune response [262] whereas *L. rhamnosus* modulates DC function to produce relative lower TNF-α, IL-6, and IL-8 production by immature DC and lower IL-12 and IL-18 production by mature DC and induces T cell hyporesponsiveness [187, 262]. One recent study [265] found that a probiotic mixture (LA, *L. casei*, *L. reuteri*, *B. bifidium*, and *Streptococcus thermophilus*) up-regulates CD4+Foxp3+ regulatory T cells by the regulatory dendritic cells (rDCs) that express high levels of IL-10, TGF-β, COX-2, and indoleamine 2, 3-dioxygenase. *L. reuteri, L. casei* and VSL#3 are capable of stimulating IL-10 production by human DCs [266, 267]. Our previous study found that LAB (a mixture of LA strain NCFM and *L. reuteri* (ATCC# 23272)) enhanced antibody responses to rotavirus infection in Gn pigs [268], suggesting that LAB are involved in enhancing Th2 cell responses.

Additionally, probiotic bacteria can contribute to host health by promoting polysaccharide digestion [229]. A number of bacteria-regulated genes promoting polysaccharide digestion in gut epithelial cells are identified [205]. Germfree mice show altered patterns of expression of oligosaccharides [230]. The regulation of glycoproteins and glycolipids by commensal bacteria likely contributes to the overall host health [227, 228] because they are key

components of a number of functional processes, such as metabolism, receptor signaling and coordination of the immune response. *B. thetaiotaomicron* colonization was shown to increase ileum Na^+/glucose cotransporter, colipase, and apolipoprotein expression [205].

In addition, most probiotics are lactic acid producers and can lower the local pH environment, in turn inhibiting the growth of lactic acid-sensitive bacteria and permeating the outer membrane of Gram-negative bacteria [231-233]. Probiotics can also secret bactericidal materials. Commensal bacteria in human milk inhibit the growth of *Staphylococcus aureus* [238]. The flagella of EcN1917 induce β-defensin production in Caco-2 cells [234, 235]. Some probiotics strains enhance the defensin/cryptidin expression by paneth cells to reinforce the mucosal barrier function [269]. *B. thetaiotaomicron* strain VPI-5482 stimulates Paneth cells to produce Angiogenin 4 (Ang4), which exhibits bacteriocidal activity against several pathogens [239]. Some lactobacilli strains produce low-molecular-weight and high-molecular-weight bacteriocins [236] to decrease the numbers of pathogenic pathogens. In addition, probiotics *L. reuteri* (ATCC# 55730) produce certain antibiotics such as reuterin [237].

Probiotics directly modulate gut microbiota composition [243] and balance the composition of host commensal bacteria to prevent pathogenic bacteria colonization. For example, ingestion of *L. casei* strain Shirota or *L. johnsonii* strain La1 increased the numbers of bifidobacteria and lactobacilli but decreased those of pathogenic bacteria, including enterobacteria or clostridia [244, 245].

Ingestion of bifidobacteria-containing milk reduced the numbers of *Bifidobacterium vulgatus* in the gut of ulcerative colitis (UC) patients [246]. VSL#3 increased the numbers of total gut bacteria and restored the intestinal microbiota diversity in pouchitis patients [247]. Additionally, VSL#3 increased caecal bifidobacteria numbers and modified the metabolic activity of caecal bacteria in mice with DSS-induced colitis [248]. EcN1917 was demonstrated *in vitro* to protect epithelial cells from invasion by *S. enterica*, *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila*, *Listeria monocytogenes* and *E. coli* [249, 250]. Competitive exclusion of receptor sites between probiotics and pathogenic bacteria is the main reason for these observations [240-242, 251, 252]. In addition, probiotics prevent pathogen adhesion by increasing mucin production. Mack et al. [209] showed that *L. plantarum* strain 299v or LGG induced MUC3 production in HT20-MTX cells. *B. lactis* strain Bb12 and/or LGG reduced the adhesion of *Salmonella*, *Clostridium* and *E. coli* to pig intestinal mucus [270]. However, some probiotic strains increased the adhesion of *E. coli*, *L. monocytogenes* and *S. typhimurium* to human mucus to prevent their invasion [241]. Other modes of prevention of pathogens by probiotics possibly include carbohydrate receptor degradation by secreted proteins, biofilm establishment, receptor analogue production and biosurfactant induction. For example, some lactobacilli (*Lactobacillus casei* strain DN-114 001 and *Lactobacillus plantarum* strain 423*)* and *B. bifidum* strains secreted factors to prevent *S. typhimurium* invasion of host epithelial

cells [253, 271]. *L. kefir* strains shed S-layer protein to mediate the antiinvasive effect [272]. Antitoxin components secreted by some probiotics mainly function in preventing diarrhea [273, 274]. Probiotics also compete for some resources against other microorganisms and inhibit the population of these potential disease induced microorganisms. For example, lactobacilli do not need iron for survival [275]. *L. acidophilus* and *L. delbrueckii* can efficiently bind ferric hydroxide and make it unavailable to pathogenic microorganisms [276]. EcN1917 relies on iron but competes effectively against pathogenic microorganisms for this limited resource [277, 278].

1.3.3 Probiotics in rotavirus diarrhea

There are a large number of randomized controlled trials (RCTs) in using probiotics for the prevention of nosocomial or community-acquired acute diarrhea, including rotavirus gastroenteritis, in infants and young children [279-288]. The effect of probiotics for the prevention of nosocomial diarrhea (mainly rotavirus-induced) appears to be inconsistent [279-282, 289] and the effects of probiotics on rotavirus-induced diarrhea are only descriptive and the mechanisms behind are not fully understood.

In a RCT with 40 children who were hospitalized with acute diarrhea (75 % positive for rotavirus) [289], Shornikova et al. demonstrated that *L. reuteri* strain SD 2112 (10^{10}-10^{11} CFU/day for the duration of hospitalization or up to 5 days) reduced the duration of watery diarrhea by an average of 1.2 days

compared to the placebo group. Two RCTs evaluated the use of LGG in preventing diarrhea [279, 280]. One of the RCTs with 81 children showed that 6×10^9 CFU of LGG (given orally twice per day during a hospital stay) reduced the risk for diarrhea by 80 % and the risk for the rotavirus-induced diarrhea by 87 % compared to the placebo group [279]. Another RCT with 220 children did not show a significant effect of 10^{10} CFU of LGG given orally once per day on the incidence of rotavirus-induced gastroenteritis compared to the placebo group [280].

There were two RCTs to test the efficacy of *B. bifidum* and *S. thermophilus* in the prevention of rotavirus-induced diarrhea [281, 282]. The first trial involved 55 infants and it showed that *B. bifidum* and *S. thermophilus* reduced the incidence of diarrhea by 80 % and the incidence of rotavirus gastroenteritis by 70 % compared to the placebo group [281]. The second RCT involved 90 healthy infants; however it showed that *B. lactis* did not reduce the incidence of diarrhea compared with the placebo group [282].

1.3.4 Probiotics as mucosal adjuvant

Probiotics are known to influence both mucosal and systemic immune responses and function as adjuvants by promoting proinflammatory cytokine production, enhancing both humoral and cellular immune responses. As discussed before, probiotics can induce antigen-specific and non-specific IgA antibody responses at mucosal surfaces [290, 291] to prevent invasion by

pathogenic microorganisms. Oral administration of LA strain L-92 in mice led to a significant increase of IgA production in Peyer's patches [292]. *L. casei* strain CRL 431 increased induction of IgA secreting cells in the gut of mice [293]. In human clinical trials, probiotic administration was also associated with higher levels of fecal IgA [294] and increased levels of total serum IgA [295]. LGG enhanced rotavirus-specific IgA antibody-secreting cell responses in humans and promoted recovery from rotavirus diarrhea [26]. Another study also found that LGG enhanced rotavirus-specific IgM secreting cells and rotavirus IgA seroconversion and had an immunostimulating effect on oral rotavirus vaccination in neonates [32]. In a double-blind RCT, LGG or *L. acidophilus* strain CRL 431 increased serum poliovirus neutralizing antibody titers and poliovirus-specific IgA and IgG titers for 2- to 4-fold in adult human volunteers vaccinated with oral live polio vaccine [296]. The daily consumption of a fermented dairy drink (*L. casei* strain DN-114 001 and yoghurt ferments) was shown to increase relevant specific antibody responses to influenza vaccination in individuals of over 70 years of age [33]. In addition, *L. acidophilus* strain La1 and bifidobacteria enhanced specific serum IgA titer to *S. typhi* strain Ty21a and also total serum IgA in humans [297]. Specific strains of probiotics may also act as adjuvants to enhance humoral immune response (especially IgG) following oral cholera vaccination [28].

In addition to enhancing humoral immune responses, probiotics also exert adjuvant properties by inducing proinflammatory cytokine and promote Th1 cell

responses. For example, *L. fermentum* strain CECT5716 enhanced the Th1 responses induced by an influenza vaccine in addition to enhancing virus-neutralizing antibody responses and may provide enhanced systemic protection from infection [27]. LA strain NCFM enhanced HRV-specific IFN-γ producing CD8+ T cell responses in ileum and spleen, ileal IgA and IgG antibody-secreting cell responses, and serum IgM, IgA and IgG antibody and virus neutralizing antibody titers induced by attenuated HRV (AttHRV) vaccine in pigs [29]. Taking probiotic bacteria plus vitamins and minerals for at least three months can increase the population of lymphocytes and monocytes and reduce the incidence and the severity of symptoms of common cold infections in humans [30]. Feeding with *L. acidophilus* enhanced the antigen-specific immune responses to *Bacillus anthracis* and also increased the bioavailability of this antigen by dendritic cells [31], indicating the direct adjuvanticity of *L. acidophilus*. *L. lactis* and *L. plantarum* induced the production of IL-12 and IFN-γ by mice splenocytes in a dose-dependent manner [298]. Sashihara et al. [299] also demonstrated that some lactobacilli strains induced high levels of IL-12 by mice splenocytes. Kekkonen et al. [300] tested 11 different probiotic strains for cytokine production in human peripheral blood mononuclear cells and showed that each tested bacterium induced the production of TNF-α and strain-specifically induced the production of IL-12 and IFN-γ.

In summary, probiotics can function as adjuvants by promoting proinflammatory cytokine production, enhancing both humoral and cellular

immune responses.

1.4 Immune cells mainly involved in rotavirus infection and immunity

1.4.1 Macrophages and dendritic cells

Monocytes, derived from myeloid progenitor cells, are released from the bone marrow into the peripheral blood. These monocytes migrate to tissues under either the steady state or in response to occurring inflammation and develop into the major tissue macrophages [301, 302]. Inflammatory insults can result in the rapid recruitment of blood-borne precursors to the tissues and develop into macrophages [301, 303-306]. Tissue macrophages are also maintained through local proliferation, such as alveolar macrophages [307-309], splenic white-pulp and metallophilic macrophages [310], liver Kupffer cells [303], and brain microglia [311]. Tissue macrophages have various functions in the maintenance of tissue homeostasis, including clearance of senescent cells, tissue remodeling, wound healing and repair, as well as the genesis and resolution of the inflammatory responses.

Macrophages have been classified as M1 macrophages (high IL-12 and low IL-10 production) and M2 macrophages (low IL-12 and high IL-10 production). M1 macrophages develop during cell-mediated immune responses and require both IFN-γ and TNF for their development [312]. M1 macrophages are essential for eradication of intracellular microorganisms and enhance the host defense through production of inflammatory cytokines, such as IL-1, IL-6, IL-

12, and IL-23 [313]. However, M1 macrophages are also involved in chronic inflammatory disorders, such as inflammatory bowel disease (IBD). M2 macrophages include those with either wound healing or regulatory properties [313]. The initial increase of IL-4 and/or IL-13 drives tissue macrophages to function into wound healing through production of extracellular matrix [314]. However, the subsequent increase of IL-10 and TGF-β promotes regulatory macrophages to inhibit the immune responses and limit inflammation [313]. The production of IL-10 and TGF-β and the ability to suppress IL-12 production are the signatures of regulatory macrophages [315]. LAB colonization and HRV infection influenced the distribution and frequencies of monocytes/macrophages and DCs in neonatal Gn pigs [316] and also influenced the systemic TLR2, TLR3, and TLR9 expressing monocyte/macrophage and conventional dendritic cell (cDC) responses [259].

Rhesus rotavirus (RRV), but not other animal or human rotaviruses, can infect a hepatic macrophage cell line, Raw 264.7 cells. These infected macrophages stimulated the chemotaxis of neutrophils by releasing Mip2/Cxcl2 [317]. Rotavirus also replicated in dendritic cells, B cells and macrophages in mice (both murine rotavirus EC and RRV strains) [318] and macrophages in rats (both human rotavirus HAL1166and RRV strains) [319].

There are two major subsets of DCs, plasmacytoid DCs (pDCs) and myeloid DCs or conventional DCs (mDCs or cDCs). The pDCs act as the front line to prevent virus infection because of their ability to rapidly produce high quantities

of type I interferon [320, 321]. The pDCs have a functional plasticity. When exposed to some viruses, such as live influenza virus, pDCs can induce the expansion and differentiation of antigen-specific memory B and T lymphocytes into plasma cells [322] and cytotoxic T lymphocytes (CTLs), respectively [323, 324]. The cDCs are mainly involved in presenting antigens to T cells. In the steady state, nonactivated cDCs present self-antigens to T cells, leading to tolerance [325, 326]. The mechanisms include (i) T cell deletion [327-329], (ii) T cell unresponsiveness induction [330], and (iii) Treg cell activation [331-334]. However, activated antigen-presenting cDCs polarize T cells into antigen-specific T cells [335, 336], leading to T cell proliferation and differentiation into helper and effector T cells. The cDCs are also important in initiating humoral immunity because of their capacity to directly interact with B cells [337, 338] and to present unprocessed antigens to B cells [339-342].

Gonzalez et al. [343] found that the frequencies of intestinal pDCs and cDCs increased after HRV infection in Gn pigs. These DCs showed significantly lower VLP uptake/binding compared to mock-infected pigs suggesting higher activation of pDCs and cDCs in infected piglets. The pDCs showed both anti- and proinflammatory functions. A 100-fold higher inoculation dose of HRV enhanced the anti-inflammatory effects of pDCs. The study suggested that stimulation of the anti-inflammatory effects of pDCs after the high dose, without increasing their proinflammatory impacts, may be critical to reduce further immunopathology during HRV infection [343]. Our study found that

LAB colonization influenced the distribution and frequencies of DCs in HRV infected neonatal Gn pigs [316] and influenced the systemic TLR2, TLR3, and TLR9 expressing cDC immune responses [259]. Live or inactivated RRV stimulated human pDCs to produce IFN-α, but these pDCs did not support rotavirus replication. Conversely, RRV was able to replicate in a small subset of human pDCs with diminished IFN-α production [344]. In mice, wild-type murine rotavirus (EDIM strain) infected DCs increased the expression of mRNA for IL-12/23p40, TNF-α, IFN-β, and IL-10, suggesting that DCs function in controlling rotavirus infection and simultaneously avoiding excessive inflammatory immune responses after rotavirus clearance [345].

1.4.2 αβ T cells (CD4+, CD8+)

T cells are defined by their T cell receptor (TCR) expression into αβ T cells or γδ T cells. The αβ T cells differentiate into several different subsets: CD4+, CD8+ and CD4+CD8+ T cells. CD8+ T cells act primarily to kill intracellular pathogen infected cells but also contain regulatory cells that down-regulate immune responses. CD4+ T cells are named as helper cells and act primarily to facilitate the cellular and humoral immune responses. Some CD4+ T cells also play an important regulatory role and act to down-regulate immune responses. A small subset of αβ T cells expresses the NK1.1 natural killer (NK) cell antigen and is called natural killer T (NKT) cells. NKT cells are usually CD4-CD8-, recognize CD1d presenting glycolipid antigens, and produce several

cytokines: IFN-γ, IL-4, GM-CSF, TNF, and others [346].

Both CD4+ and CD8+ T cells differentiate into functionally distinct subsets after exposure to antigens. Resting naive CD4+ Th cells release very low levels of cytokines. After stimulation, naïve Th cells begin to produce IL-2 and are designated as Th0 cells. As the Th0 cells continuously respond to the activating signals, they differentially polarize into Th1, Th2, Th17 and Treg cells depending on the cytokine environment [347]. IL-12 produced by macrophages or NK cells induces Th0 cell differentiation toward Th1 cells; IL-4 produced by NKT cells, basophils, or mast cells induces Th0 cell differentiation toward Th2 cells; TGF-β and IL-6 induce Th0 differentiation toward Th17 cells, and TGF-β and all-trans retinoic acid induce Th0 differentiation toward Treg cells. Th1 cells are characterized by the expression of T-box transcription factor (T-bet), and by IL-2, IFN-γ and lymphotoxin production. Th2 cells are characterized by the expression of transcription factor GATA binding protein 3 (GATA3) and produce IL-4, IL-5, IL-9, IL-13, and GM-CSF. Th17 cells express the transcription factor Retinoic-acid-related Orphan Receptor C isoform 2 (RORC2) and produce the cytokines IL-17a and IL-17f [348, 349]. Th17 cells are induced early in the adaptive immune responses to extracellular bacteria and help to recruit neutrophils to eliminate these bacteria. Th1 and Th2 cells often function together in inducing immune responses. However, Th1 cells generally support cell-mediated immune responses whereas Th2 cells support humoral and allergic responses. There is a reciprocal relationship between Th17 and

Treg cell development [350, 351]. Treg cells will be discussed in detail in a later section.

Similar to T cells in other species, porcine T cells are defined by CD3 expression [352]. Porcine αβ T cells consist of four subsets: CD4+CD8−, CD4+CD8lo, CD4−CD8lo, and CD4−CD8hi, while porcine γδ T cells consist of three subsets: CD2+CD4−CD8−, CD2+CD4−CD8+, and CD2−CD4−CD8− [353] in peripheral sites. Porcine CD4+CD8− T cells are referred to as naive Th0 cells. These cells recognize foreign antigens in a MHC II-restricted manner and respond to the antigens by cell proliferation and expression of CD8 and MHC II molecules [354]. Porcine CTLs bear the CD3+CD5+CD6+CD8hi phenotype, whereas porcine NK cells bear the CD3−CD5−CD6−CD8lo phenotype [353]. The CD8 molecules of porcine CTLs are αβ heterodimers, whereas the CD8 molecules of porcine NK cells are αα homodimers [355]. CD4+CD8lo T cells (double positive T cells) can be found in extrathymic sites in normal pigs, while this extrathymic population is found only in humans and mice with physiological disorders [356]. Porcine CD4+CD8lo T cells express MHC II and act as memory T helper cells of pigs and recognize recall antigens in a MHC II-restricted fashion and respond to the antigens by up-regulation of CD25, cell proliferation, production of the cytokines IFN-α, IL-2, and IFN-γ, and stimulation of Ig production by porcine B cells [356]. Human and mice do not have peripheral CD4+CD8lo T cells with similar functions as those in pigs [357]. Markers to differentiate CD4+CD8lo from CD4+CD8- Th0 cells include

β1 integrins (CD29), CD45RC, MHC II, and 2E3. Naive porcine Th0 cells are CD29loCD45RC+MHC II−2E3+, whereas memory Th cells are CD29hiCD45RC−MHC II+2E3− [358]. There are also several differentiation markers of CD4+CD8lo from CD4+CD8+ αβ thymocytes [355]. CD4+CD8lo T cells have αα homodimers of CD8 molecules instead of αβ heterodimers in CD4+CD8+ thymocytes and express MHC II molecules, but do not express thymocyte-specific CD1 molecules.

The primary importance of CD8 T cells in rotavirus infection may lay in the resolution of infection while CD4 T cell function may lay in their helper function for antibody production and cytokine production [359]. For example, CD8 T cells can mediate almost complete short-term and partial long-term immune responses to rotavirus infection in mice [360]. In virulent HRV (VirHRV)-infected or AttHRV-vaccinated pigs, HRV-specific IFN-γ producing CD4 or CD8 T cells reside primarily in ileum and provided protection against rotavirus-induced diarrhea upon VirHRV rechallenge [361].

1.4.3 γδ T cells

γδ T cells and αβ T cells have different antigen recognition requirements and recognize different sets of antigens [362]. γδ T cells do not recognize antigens in the context of MHC class I or II. Some of these cells recognize antigens in the MHC I-related protein CD1 [363]. Also, a subset of double-negative γδ T cells recognizes the MHC I chain-related proteins designated as MIC [364].

Despite these differences, most γδ T cells and αβ T cells produce similar cytokines, mount cytotoxic responses, and require the development in the thymus prior to entering the periphery sites [365].

Antigen-naive γδ T cells mainly produce IL-17, whereas antigen-experienced γδ T cells mainly produce IFN-γ [366], suggesting that γδ T cells have different effector functions depending on γδ TCR signaling strengths and functional specifications. This is in dramatic contrast to the activation requirements of αβ T cells, which require an initial antigen-specific priming event by professional APCs before developing into effector cells. IL-17 is a T cell cytokine which regulates the expansion and recruitment of neutrophils and monocytes to initiate the inflammatory responses [367, 368]. The initial IL-17 responses must be elicited without prior antigen exposure in acute inflammation responses to prevent against pathogen infection. Given the ability of γδ T cells to produce cytokine immediately upon TCR stimulation, they may be the first one to produce IL-17 at the onset of the inflammatory responses [366]. For example, γδ T cells are the major early producers of IL-17 in several infections in mice [369]. Although most γδ T cells produce IL-17 or IFN-γ, some γδ T cells also produce IL-4. These IL-4-producing γδ T cells largely are present in mice with defective TCR signaling [370, 371]. In our studies of Gn pigs, we found that all three porcine γδ T cell subset produced IFN-γ after rotavirus infection; CD2+CD8+ γδ T cells produced IL-10 constitutively and produced TGF-β after phosphoantigen isopentenyl pyrophosphate (IPP) stimulation (see Chapter 5).

Due to the lack of an appropriate antibody against porcine IL-17, we did not detect IL-17 production by γδ T cells in our study.

Peripheral porcine γδ T cells consist of three subsets: CD2+CD4−CD8−, CD2+CD4−CD8+, and CD2−CD4−CD8− [353]. The percentages and distribution of total γδ T cells and three subsets (CD2+CD8+, CD2+CD8- and CD2-CD8-) in blood and secondary lymphoid tissues (spleen, popliteal lymph nodes, mesenteric lymph nodes [MLN] and tonsil) of pigs (28 days of age) was reported previously [353, 372]. Our study reported the distribution of the three γδ T cell subsets in ileum, spleen and blood of neonatal (5 days of age) and older (33 days of age) Gn pigs. In pigs, γδ T cells form a major T cell subpopulation in peripheral blood of the young [373]. It was shown that CD2+CD8+ and CD2+CD8- γδ T cells preferentially reside in lymphoid tissues, while CD2-CD8- γδ T cells are the predominant subset in blood [374]. Our study also found that in naïve piglets, the predominant γδ T cell subset in blood is the CD2-CD8- (85 %) and in ileum is the CD2-CD8- (48 %) and the CD2+CD8+ (39 %) followed by the CD2+CD8- (8 %). In spleen, all the three subsets have similar frequencies (27-39 %). The highest mean frequencies of CD2-CD8- subset was found in blood, CD2+CD8+ in ileum, and CD2+CD8- in spleen (see details in Chapter 4). Porcine CD2+CD4−CD8− and CD2+CD4−CD8lo γδ T cells recognize foreign antigens in a non-MHC-restricted manner and respond to antigenic stimulation primarily by cell proliferation and the expression of cytokines including IL-1, IL-6, IL-8, and

IFN-α [375], IFN-γ and TGF-β (see details in Chapter 5). Both CD2+CD4−CD8lo γδ T cells and NK cells are CD8αα homo-dimers. However, γδ T cells are CD3+ and CD5lo, whereas porcine NK cells are CD3− and CD5− [355]. Porcine CD2+ γδ T cells express MHC II and CD80/86 molecules. They are reported to have antigen-presenting activity to stimulate porcine αβ T cells via their MHC II molecules [376].

Porcine γδ T cells respond to several viral infections, such as porcine reproductive and respiratory syndrome virus (PRRSV) and foot-and-mouth disease virus (FMDV) [375, 377]. However, there is no report on γδ T cell immune responses to rotavirus until our studies (will be discussed in Chapters 4 and 5). Studies of human, murine, bovine, and porcine γδ T cells have suggested that different γδ T cell subsets have opposite functions and can be proinflammatory (Vδ2 in humans; Vγ1 in mice, WC1+CD2-CD8- in cattle and CD8- subsets in pigs), regulatory (Vδ1 in humans and mice; WC1-CD2+CD8+ in cattle, and CD8+ subset in pigs) [378], or promote epithelial healing [379]. γδ T cells have demonstrated both pro- and anti-inflammatory roles in response to infections [380, 381].

1.4.4 Role of γδ T cells in rotavirus infection

The γδ T cell response to rotavirus infection has not been studied in detail in any species, including mice, and the role of this T cell subpopulation in rotavirus infection and immunity is much less understood compared to αβ T

cells. After calves were infected with rotavirus, WC1+ γδ T cells increased significantly (1.7-3.8-fold) in the distal small intestinal epithelium at post-inoculation days (PID) 4-8 [382], suggesting that a specific immune response to rotavirus infection involving γδ T cells. In another study of the role of lymphocyte subpopulations in controlling virus excretion and mucosal antibody responses in Gn calves infected with rotavirus, monoclonal antibodies were used to deplete CD4+, CD8+ or WC1+ lymphocytes in Gn calves prior to rotavirus inoculation. Rotavirus excretion was significantly increased in the calves depleted of CD8+ cells, but not in the calves depleted of WC1+ lymphocytes. Fecal and serum antibody responses to rotavirus were reduced in CD4+, but not WC1+ or CD8+, lymphocyte-depleted calves. The authors concluded that a CD8+ cell population is involved in limiting primary rotavirus infection, while CD4+ or WC1+ lymphocytes are not [383].

In a similar study using mice [384], γδ TCR knockout (γδ-/-) mice were infected with rotavirus and the virus clearance and immune responses were compared to those of normal mice. The γδ-/- mice cleared infection at the same rate as control mice and rotavirus-specific intestinal IgA responses were similar in the γδ-/- mice as in the control mice, suggesting that γδ T cells were not necessary for efficient clearance of primary rotavirus infection in mice. Both studies concluded that the absence of γδ T cells in animals infected with rotavirus did not prevent the clearance of rotavirus infection, but these studies did not investigate the γδ T cell responses or functions per se after rotavirus

infection. Our studies of porcine γδ T cell subset distribution and kinetics in response to rotavirus infection showed that rotavirus infection significantly increased frequencies of total γδ T cells and the CD2+CD8+ subset in ileum of Gn pigs at PID 3 and PID 5. In our studies of the immune modulating functions of porcine γδ T cell subsets, we found evidence of increasing TLR2, TLR3, TLR9 expression and IFN-γ production in each of the γδ T cell subsets during the acute phase of infection. These dynamic γδ T cell responses indicate that γδ T cells are activated by rotavirus infection and they are important components of the immune response to rotavirus infection.

1.4.5 Regulatory T (Treg) cells

Regulatory T cells are classified into natural (nTreg) and induced Treg (iTreg) cells. The nTreg cells develop in the thymus and are characterized by the expression of CD4, CD25 and the forkhead box protein 3 (FoxP3) transcription factor. TGF-β and IL-10 secreted by nTreg cells function to regulate immune responses [385] with TGF-β acting in a membrane-associated form [386]. The iTreg cells are thought to differentiate in the periphery sites from naive CD4+ T cells. They are called adaptive or iTreg cells because they develop in response to specific antigen stimulation. IL-10 seemingly is required for their initial differentiation. FoxP3 expression is variable and IL-10 is a main secreted product from these iTreg cells [387].

There are only a few studies investigating the immune responses of Treg cells

to rotavirus infection [388-390]. RRV-infected mice increased hepatic Treg cells (Foxp3+CD4+CD25+) by 10-fold within 3 days [390]. These Treg cells suppressed NK cell activation by hepatic dendritic cells and decreased the production of proinflammatory cytokines, including TNF-α and IL-15 *in vitro* [390]. Murine rotavirus infection induced an expansion of Treg cells in MLN and spleen and the magnitude of the T cell-mediated immune responses to rotavirus was down-regulated by Treg cells [388]. Depleting Treg cells with anti-CD25 antibodies led to an increase in IFN-γ producing T cells [388, 389].

1.4.6 B cells

B cells and antibodies are critical elements of the humoral immune responses against a variety of pathogens. B cell immunity functions in two ways: T cell-dependent B cell responses and T cell-independent B cell responses.

In T cell-dependent B cell responses, T cells secret cytokines to induce B cell maturation, including both induction of isotype switching and activation of somatic mutation. B cells are professional APCs. They capture antigens through membrane attached Ig, and internalize the antigens and process them for surface presentation on MHC II. The costimulatory molecules CD80/86 are up-regulated in the B cell antigen presenting procedure. The costimulatory molecules and antigen-MHC II complex activate T cells which then signal back to B cells through the interaction of CD40 ligand (CD40L on T cells) with CD40 (on B cells). CD40L-CD40 interaction is essential for the induction of

isotype switching [391]. These reciprocal signals between T cells and B cells are also critical for somatic mutations. Isotype switching and somatic mutations are tightly associated with the development of B cell memory responses [392], which are characterized by the production of IgG, IgA, or IgE and by somatic mutations of the antigen-binding domains on these antibodies.

In T cell-independent B cell responses, B cells are activated without help from T cells. Because of no costimulations from T cells, monomeric antigens cannot activate B cells alone, whereas polymeric antigens with a repeating structure can sufficiently activate B cells most likely due to the cross-linking and clustered Ig molecules on the surface of B cells. These polymeric antigens include bacterial LPS, other polysaccharides, and some polymeric proteins. Isotype switching and somatic mutations do not occur in most T cell-independent antibody responses. Consequently, immune memory responses are normally weak in these T cell-independent antibody responses [393, 394]. For this reason, it is difficult to produce fully protective vaccines against bacterial polysaccharide components.

All B cells express CD79a and only immature B cells express SWC3a in pigs. CD2+CD21+ B cells represent naive mature B cells that down-regulate CD2 following activation, but they can reexpress CD2 again to become CD2+CD21+ [395]. This suggests that CD21, but not CD2, can be considered a maturation marker [395]. Porcine B cells can be divided into 7 subpopulations based on expression of CD79a, SWC3a, CD21, CD2, and IgM [395]. Further maturation

of activated B cells includes down-regulation of CD21. The resulting CD2+CD21− B cells can become μHC- large proliferating plasma blasts or small nondividing plasma cells that eventually become CD2−CD21− [395].

Swine do not transfer maternal antibodies to their fetuses *in utero*, but do so after birth through the colostrum and milk. Porcine colostrum is characterized by very high levels of IgG, which can be up to four times higher than the level of serum IgA and IgG. This is quite different from mammals that use placental transport to provide IgG to the offspring pre-partum [396, 397]. Piglets can absorb all proteins from the colostrum during the first 24 h to 36 h post-parturition [398]. After that, piglets cannot absorb any Ig. The concentration of IgA in the colostrum/milk is the highest, which is 5-fold higher than IgG. Sows continue to provide these IgA antibodies throughout lactation although absorption of IgA into blood is no longer possible [399, 400]. From this point on, the composition of Ig in sow's milk resembles that of human colostrum and milk. The milk IgA is not continuously absorbed into plasma, but milk IgA antibodies provide their function against pathogens within the gastrointestinal (GI) tract. The B cell and antibody responses to rotavirus infection will be discussed in later sections.

1.4.7 Epithelial cells

The intestinal epithelium is the first line of defense against enteric pathogens. It exhibits numerous physical adaptations to separate the host tissues from the

harsh external gut environment. Intracellular tight junctions between epithelial cells prevent paracellular traffic and leaking. Extended microvilli form a brush border on the apical surface of the epithelium, which impedes microbial attachment and prevents their invasion [401]. In addition, the physical barrier is strengthened by chemical junctions, such as glycocalyx formed by secretion and apical attachment of a mucin-rich layer by goblet cells. Altogether, these form a viscous and relatively impermeable sheet on the apical surface of the epithelium to prevent uncontrolled invasion of pathogens into the host tissues [402, 403]. The beneficial effect of probiotics on keeping the epithelial barrier has been discussed extensively in section 1.3. This brings up one new question: how can intestinal epithelial cells (IECs) discriminate between probiotic/commensal and pathogenic bacteria upon IEC-bacteria interaction? Several mechanisms have been proposed to explain their ability, including the selective expression and distribution of PRRs, such as TLRs, and the active modulation of IEC function by commensal bacteria. For example, IECs express little or no TLR2 and TLR4 and CD14 under steady-state conditions and minimize the recognition of bacterial LPS [404-406]. Furthermore, intracellular localization of TLRs, such as 3, 7, 8, and 9, in the endosomes of IECs may assist the discrimination between commensal versus pathogenic bacteria [407]. These intracellular TLRs only recognize invading pathogenic bacteria/viruses. In addition, the contact time between IECs and commensal bacteria seems to be critical. Short-term stimulation with LPS led to the activation of proinflammatory signaling

cascades in IECs, whereas prolonged exposure resulted in a state of hyporesponsiveness in IECs [408].

IECs also play a critical role in mucosal immunity against enteric bacteria [409]. IECs express MHC I and II and MHC I-like molecules to present antigens to T cells and lead to secretion of cytokines [410-412]. IECs also express a variety of potential immunomodulatory factors to regulate the function of intestinal DCs. First, the migration of DCs is influenced by chemokines produced by IECs. For example, IECs can release CCL9 and CCL20 to attract DCs to the subepithelial dome of Peyer's patches to induce immune responses [413]. In addition, Butler et al. [414] demonstrated that IECs create a tolerogenic environment to modulate the phenotype and function of DCs towards tolerogenic DCs that release increasing levels of TGF-β. IECs also constitutively secrete thymic stromal lymphopoietin (TSLP), and consequently induce noninflammatory DCs that produce IL-10 but no IL-12 [415].

Our previous study used IPEC-J2 cells as an *in vitro* model of rotavirus infection and probiotic treatment [193]. We found that the TLR2 expression was up-regulated by LGG and peptidoglycan, corresponding to the increased IL-8 and decreased IL-6 responses, which suggests a mechanism of regulating TLR2 expression on intestinal epithelial cells by probiotics to achieve protective effects. The total DNA of LA or LGG also significantly decreased the TLR9 expression; thus probiotic bacterial DNA may confer protection to intestinal

epithelial cells by TLR9 down-regulation.

1.5 Innate and adaptive immune responses to rotavirus infection

1.5.1 Toll-like receptor responses

The observed immune responses to rotavirus infection are mainly based on several animal models, especially the murine and neonatal Gn pig models. Each model offers certain advantages and disadvantages. For example, mice develop lifelong sterilizing immunity and there are several gene knockout immunodeficient mice available for study, but the mouse model is only an infection one [416]. The pig model can be used to evaluate protection against diarrheal disease and is more susceptible to illness following heterologous HRV infection [417]. However, the window to evaluate vaccination of piglets is relatively short [418] and there are some critical issues that need to be addressed before a knockout pig model is widely available [419].

TLRs, a type of PRR, play an important role in viral antigen recognition, innate immunity and in bridging innate and adaptive immune responses [420]. TLRs are expressed on many cell types, such as monocytes/macrophages [259, 421], DCs [259, 422], B cells [423], T cells [424], Treg cells [424] and epithelial cells [193]. TLR4 is the most thoroughly studied TLR because of its outstanding role in antibacterial defense and its peculiar modes of TLR signal transduction [425]. Together with TLR1 or TLR6, TLR2 recognizes a wide variety of microbial lipoproteins and plays a crucial role in the immune

response to fungi and Gram-positive bacteria [426-428]. Other TLRs with antibacterial activity are TLR5 and TLR9, which recognize flagellin and bacterial DNA, respectively. TLR11 also has antibacterial activity but its ligand is currently unknown [426-428]. TLR3, TLR7, and TLR8 are important regulators of the antiviral responses through the recognition of single- or double-stranded RNA species (ssRNA and dsRNA, respectively) [426-428]. The TLR family members can also be classified into two groups according to their subcellular localization: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are expressed on the plasma membrane, whereas TLR3 TLR7, TLR8, and TLR9 are found in the endolysosomal compartment [426-428]. TLR activation induces type I interferon production through several signaling pathways that lead to antiviral and proinflammatory cytokine responses and induction of adaptive immune responses [429, 430]. The regulation of immune responses by TLR expression and distribution has been discussed in section 1. 4.

There are few studies about TLR immune responses to rotavirus infection. Xu et al. [431] demonstrated that there was significantly higher expression of mRNA encoding TLR2, TLR3, TLR4, TLR7 and TLR8 in peripheral blood mononuclear cells (PBMCs) of 41 % (31/75) patients within the first 3 days of rotavirus-induced illness than those in healthy children. However, only TLR3 and TLR8 mRNA expressions were still significantly increased in 59 % (44/75) children with diarrhea after 3 days of illness. Consistently, the expression of IL-12p40 and IFN-γ mRNA in PBMCs of patients were significantly increased.

The same research team [432] also showed that rotavirus infection significantly increased the level of mRNA expression for TLR2, TLR3, TLR7 and TLR8 in HT-29 cells between 24 and 48 h measured by reverse transcription polymerase chain reaction (RT-PCR). Our study found that HRV induced TLR3 and TLR9 responses in spleen of Gn pigs measured by flow cytometry [259]. In addition, bovine rotavirus-infected calf tissues showed a strong activation of TLR3, IL-6 and p65, but down-regulation of IFN and proinflammatory cytokine associated pathways [433]. TLR3 might also be involved in the pathogenesis of rotavirus-induced enteritis because purified rotavirus dsRNA can trigger intestinal epithelial cells to secrete IL-15 through the TLR3 pathway [434] and rotavirus dsRNA induces severe apoptosis and diminishes wound repair in IEC cell lines, such as IEC-6, HT-29 and Caco-2, through the TLR3 signaling pathway [435].

1.5.2 Innate, anti-viral, Th1, Th2 and Treg cytokine responses

The patterns of cytokine production during rotavirus infection drive the immune responses. Rotavirus infection induces both Th1 and Th2 type cytokine responses, leading to the induction of both T cell and B cell/antibody mediated immunity. The first produced cytokines are proinflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, followed by Th1 cytokines, such as IL-2 and IFN-γ, and then the Th2 cytokines IL-4, IL-5, IL-13, and IL-10. Th1 and Th2 cytokines drive T cell and B cell differentiation and clonal expansion, respectively. Regulatory cytokines such as TGF-β and IL-10 secreted by Treg

cells control T cell responses during and after virus clearance to prevent chronic inflammation [436].

The severity of rotavirus-induced diarrhea correlated with higher serum TNF-α levels during the acute stage of illness. Children with rotavirus-induced diarrhea had increased levels of serum IFN-γ than uninfected children [437]. In addition, Jiang et al. [438] found that the levels of serum IL-6, IL-10, and IFN-γ in children with diarrhea were significantly higher than those from uninfected controls. Azim et al. [439] also found that the levels of serum IL-10 and TNF-α were higher in children with diarrhea compared to the controls and the IFN-γ levels were higher in children with persistent diarrhea than in those with acute diarrhea or the controls. One study [440] found that the IFN-γ responses were transient and were higher in rotavirus-exposed adults than in children and the IFN-γ responses in rotavirus-positive children with prior rotavirus exposure were higher than those without prior exposure. An increased production of IL-2 was found in 6/10 adults and an increase in NSP4-specific IFN-γ production in 8/10 adults were detected [441].

Rotavirus-infected human epithelial cell lines (Caco-2 or HT-29), mouse enterocytes and DCs also produce cytokines and chemokines, such as IFNs, GM-CSF, MIP-1, IP-10 [442, 443]. Stimulation of primary human peripheral pDCs with live or inactivated RRV induced IFN-α [344, 444]. Murine bone marrow-derived DCs have also been shown to secrete type I IFN and TNF-α following rhesus or bovine rotavirus infection, respectively [445, 446]. In the

Balb/c mice model, an increased expression of IFN-γ, IL-5 and IL-10 by spleen cells was induced by simian strain SA11, bovine strain RF or murine strain EHPw rotavirus. This increase was not observed in IL-2 or IL-4 [447].

A few studies were performed to investigate cytokine responses in the pig model [259, 361, 436] with a detailed study by Azevedo et al. [436]. A significantly higher percentage of virulent HRV infected pigs had IFN-γ and IL-10 responses in serum (67 and 70 %, respectively) than attenuated HRV pigs (39 and 29 %, respectively) or controls (0 %) [436]. Both VirHRV and AttHRV groups had significantly higher percentages of TNF-α, IL-6, and IL-4 immune responses than the controls [436]. For the kinetics of cytokine production, the levels of both TNF-α and IL-6 peaked earlier in serum of the VirHRV infected pigs compared to AttHRV infected pigs. VirHRV infected pigs also had higher and prolonged IFN-γ and IL-10 responses in serum and intestinal contents compared to AttHRV infected pigs. The number of IFN-γ secreting cells was also significantly higher in the ileum of VirHRV-infected pigs; however, the number of IL-10 secreting cells was significantly higher later in ileum and spleen of the AttHRV than in the VirHRV group, suggesting the higher cytokine levels early after infection with VirHRV and a delayed initiation of a Th2 response induced by AttHRV. IL-12 was detected in serum of all pigs with significantly increasing in both HRV infected pigs, indicating a role for IL-12 in the induction of immune responses to rotavirus infection. Liu et al. found that rotavirus-infected porcine cells IPEC-J2 can produce proinflammatory

cytokines, such as IL-6 [193].

1.5.3 B cell and antibody responses

B cells are necessary for long-term protection against rotavirus infection because B cell immunodeficient mice showed no protection against rotavirus reinfection [416]. Although animals and children undergo an antigenemic or viremic phase [35, 448, 449], only passive transfer of intestinally committed B cells into chronically rotavirus-infected T and B cell immunodeficient mice mediates an antiviral effect and clears the ongoing infection [450]. Neutralizing antibodies to VP4 and/or VP7 can directly block enterocyte infection with rotavirus when present in the gut lumen. Antibodies to NSP4 may block diarrhea, but not infection, via their anti-enterotoxin effects [91]. Rotavirus-specific serum IgG or IgM in sufficient quantities can reach the intestine to mediate protection against rotavirus in mice [451] and monkeys [452]. Anti-VP6 antibodies are not protective when administered passively to the intestinal cavity [65] and milk containing these antibodies is not protective to suckling mice [453], but active anti-VP6 antibodies in mice can mediate protection from rotavirus infection [65] or inhibit rotavirus transcription [454]. 2/6-VLPs can also induce active protection against rotavirus infection in rabbits by producing antibodies [67]. However, in the pig model of rotavirus diarrhea, active immunization with 2/6-VLPs did not induce protection [70] although vaccination with VP6 effectively boosted antibody responses and protection

rates in piglets after initial priming with an AttHRV vaccine [455, 456]. Also, in contrast to the findings in mice that passive transfer of milk antibodies against NSP4 was protective [91], no correlation was found between antibodies against NSP4 and protection in pigs [96]. The share of VP4 or VP7 serotypes between the immunizing rotavirus and the challenge virus seems to be required for protection in the pig model [457, 458].

Rotavirus-specific antibody-secreting cells (ASCs) are also investigated in the pig model [417, 459]. Gnotobiotic pigs infected with VirHRV had higher numbers of intestinal IgA and IgG rotavirus-specific primary and memory ASCs in lamina propria than those infected with AttHRV, consistent with higher protection rates against rotavirus challenge in VirHRV infected pigs. However, AttHRV inoculated pigs had higher numbers of memory B cells in spleen compared to VirHRV infected pigs. This suggests that the protection rates were positively correlated with the magnitude of ASCs responses in the lamina propria, which was also observed in a study with mice [460]. Intranasal boosting with two doses of 2/6-VLP enhanced the numbers of rotavirus-specific IgG and IgA ASCs in intestinal and systemic lymphoid tissues of oral AttHRV inoculated pigs pre- and postchallenge and also had the higher protection rates against virus shedding and diarrhea [461]. Probiotics LA also promoted the IgA and IgG antibody-secreting cell responses in ileum, and serum IgM, IgA and IgG antibody and virus neutralizing antibody titers compared to the AttHRV vaccinated alone pigs [29]. LA strain NCFM combined with *L. reuteri* (ATCC#

23272) also significantly enhanced total intestinal IgA secreting cell responses and total serum IgM and intestinal IgM and IgG titers [268].

In humans, the presence of IgG serum antibodies or high levels of neutralizing antibodies correlated with the resistance to diarrhea in adults [462, 463]. A recent study found that higher prechallenge titers of IgG antibody to homotypic VP7 (G1) and VP4 (P1A[8]) in adults largely provided the resistance to homotypic rotavirus reinfection [464]. Children with > 1:800 serum IgA titers had a lower chance of rotavirus infection and also were protected against diarrhea. However, children with > 1:6400 serum IgG titers were only protected against rotavirus infection but not diarrhea [465, 466]. This suggests that serum levels of IgA but not IgG levels correlated with protection against diarrhea in children [467]. The > 1:128 homotypic neutralizing antibody titers were also found to be protective in children [468, 469] and higher stool anti-rotavirus IgA antibody titers were associated with protection against infection and illness [470, 471].

1.5.4 Th1, Th2 and Treg responses

A large number of studies on the role of T cells in mediating protection against rotavirus infection have been conducted in gene knockout mice [384, 416, 472-474]. CD4+ T cells are essential for the development of more than 90 % of rotavirus-specific intestinal IgA [384]. These T cells were also shown to be the only needed lymphocytes to protect mice against rotavirus infection after the

mice were vaccinated with VP6 peptide vaccines [472, 473]. Rotavirus-specific CD8+ T cells are involved in the timely resolution of primary rotavirus infection in mice, and may also mediate partial protection against reinfection [360, 475, 476]. It is also demonstrated that B cell-deficient mice with CD8+ T cell depletion became chronically infected with murine rotavirus [360], and β2 microglobulin-/- or CD8+ depleted mice had a 1- to 4-day delay in clearance of primary rotavirus infection, suggesting the importance of both B cells and CD8+ T cells in protective immunity. Rotavirus infection in Balb/c mice induced the Th1 responses [477]. Protection rates correlated with a mucosal rotavirus-specific IgA and Th1 IFN-γ responses [478]. IL-6-deficient mice are more susceptible to both homologous and heterologous rotavirus infection.

In bovine, a slight increase of intraepithelial CD4+ and WC1 γδ T lymphocytes was induced especially in the distal small intestine after rotavirus infection, while a larger increasing of CD8+ cells were induced in the epithelium and lamina propria of the proximal, mid and distal small intestine [382].

In Gn pigs, one study [479] found that VirHRV infection induced significantly higher lymphoproliferative responses than the AttHRV. A more recent study from our lab [361] showed that HRV-specific IFN-γ producing T cells primarily reside in the ileum of infected pigs. The frequencies of intestinal IFN-γ producing T cells correlated with protection against VirHRV challenge. Studies of cytokine secreting cell responses in Gn pigs after rotavirus infection showed

that the numbers of IFN-γ secreting cells were significantly higher in the ileum of VirHRV-inoculated pigs than the AttHRV pigs at PID 28. Significantly higher numbers of IL-12 secreting cells were observed in the spleen of both HRV-infected pigs compared to the controls [436]. The numbers of IL-10 secreting cells were significantly higher in the ileum and spleen of the AttHRV inoculated pigs at PID 28 compared to the VirHRV pigs and controls. The numbers of IL-4 secreting cells were significantly higher in the ileum of pigs from both HRV groups compared to the controls at PID 5, PID 14, and PID 21 [436].

There are few studies on T cell immune responses to rotavirus infection in humans. One study showed that 8/11 healthy adults and 10/13 healthy children between 6 months and 5 years of age had evidence of rotavirus-specific lymphoproliferative activity [480]. In addition, 6/8 rotavirus-infected children demonstrated this activity 2 to 8 weeks after rotavirus infection in another study [481]. Children with rotavirus-induced diarrhea had undetectable or very low levels of IFN-γ producing CD4+ and CD8+ T cells in peripheral blood detected by flow cytometry [61]. In addition, healthy adults and children with acute rotavirus diarrhea had significantly lower frequencies of rotavirus-specific IFN-γ producing CD8+ and CD4+ T cells than symptomatically infected adults and rotavirus-exposed laboratory workers. A recent study showed that children with acute rotavirus gastroenteritis had low or undetectable levels of circulating IFN-γ, IL-13, IL-2, IL-10 or IL-17 producing T cells [389]. Taken together, the

observations in pigs and humans suggest that rotavirus induces strong T cell responses in the intestine, the site of virus replication, but it is a poor inducer of T cell responses in circulation.

For Treg cell immune responses to rotavirus, one study [388] with a murine model demonstrated that murine rotavirus infection induces an expansion of the Treg cell population, which determines the magnitude of the T cell-mediated immune response. The depletion of natural Treg cells led to increased CD4+ and CD8+ T cell responses to rotavirus, including increased proliferation and IFN-γ secretion, and also increased proliferation of CD19 B cells. However, Treg depletion did not affect diarrheal disease, virus shedding or rotavirus-specific IgA responses. Using PBMCs from health adults, a study showed that frequencies of IFN-γ+ rotavirus-specific T cells increased after removing CD25+ cells or blocking TGF-β with its natural inhibitor, latency-associated peptide (LAP) [389].

1.6 Intestinal mucosal immune system: structures and functions

1.6.1 Mucosal inductive and effector sites

The main intestinal mucosal immune system is gut-associated lymphoid tissues (GALT) which includes inductive sites (Peyer's patches and MLNs) and effector sites (lamina propria) [482].

Peyer's patches are intestinal lymphoid aggregates beneath a single layer of columnar cells: follicle-associated epithelium (FAE). Peyer's patches are made

up of B cell follicles, T cell regions between follicles, and numerous macrophages and DCs for antigen-presentation. Microfold cells (M cells) are derived from enterocytes, are situated throughout the FAE and are developed under the influence of lymphotoxin αβ [483]. M cells lack microvilli, hydrolytic enzymes and glycocalyx which are present on the absorptive epithelium [484]. Furthermore, an invaginated subdomain or intraepithelial pocket is formed at the basolateral membrane of M cells [485]. M cells also express cathepsin E and TLRs [484]. The major function of M cells is the transport of lumen antigens to the subepithelial lymphoid tissues, called the subepithelial dome (SED), and are capable of producing proinflammatory cytokines and chemokines [484]. Memory T cells (CD4+CD45RO+), DCs and both naive and memory B cells in SED interact with the M cells. DCs within the SED are capable of migrating within Peyer's patches from the basolateral surface of M cells to acquire antigens or migrating to the interfollicular T cell area, and even to far distant sites, including MLNs and lamina propria to regulate immune responses. Notably, DCs in lamina propria also can breach the intestinal epithelium to take up luminal antigens [486]. DCs can induce either oral tolerance or protective immune responses [487] based on special activated environments where they are situated. For example, CD103+ DCs from MLNs can convert intestinal naive T cells into Foxp3+ Treg cells [488]. MLNs are very important for controlling the population of intestinal commensals [489]. Murine DCs were only capable of detecting small numbers of live commensal

bacteria in Peyer's patches and MLNs following challenge with high doses of commensals [489]. DCs loaded with commensals do not penetrate beyond the MLNs as long as the MLNs are intact. Otherwise, the intestinal challenge with commensals caused cultivable commensal bacteria in spleen and enlarged the spleen on repeated challenge [489]. Therefore, MLNs form the barrier between the mucosal and the systemic immune system. In contrast, activated lymphocytes (T and B cells) can circulate systemically and populate sites far from the inductive sites [489]. The secreted dimeric IgA induced by commensal bacteria can pass through the epithelial cells and bind commensal bacteria to prevent them from passing through the intestinal mucosa [489].

The Peyer's patches are also a major site of IgA-producing B cell development under the influence of DCs, T cells and cytokines, including TGF-β and IL-10 [490]. IgA-producing B cells from Peyer's patches migrate first to the MLNs, then to the circulation through the lymphatic system, and finally into the lamina propria of the gut. This final step is regulated by the interaction of $\alpha_4\beta_7$ on B cells and cell adhesion molecule 1 (CAM1) on the high endothelial venules in the lamina propria. Chemokine CCL25 expressed on small intestinal epithelial cells drives this chemotaxis [491]. These B cells will mature into B1 and B2 IgA-producing plasma cells. B2 cells account for approximately 75 % of IgA-producing B cells in the gut. The other 25 % of IgA-producing cells in the gut can be derived from peritoneal B1 cells and are driven by commensal bacteria in a T cell-dependent manner. These cells are thought to be important

in modulating the mucosal immune responses to bacterial flora [492].

Secretory IgA is produced in the gut as dimers joined by the J-chain and normally binds the polymeric Ig receptor expressed on the basolateral aspect of the intestinal epithelial cells. This complex is then transported across the epithelial cells to the apical surface. The bound IgA is released from the polymeric Ig receptor by means of proteolytic cleavage, generating a secretory component with the dimeric IgA to enter the intestinal lumen and protecting IgA from degradation. Secretory IgA acts to prevent luminal antigens, microorganisms, and other foreign proteins from penetrating the intestinal surface, and can neutralize toxins and infectious organisms [493]. IgA can also regulate the composition of the microbial environment of the gut and limit local inflammation induced by pathogen associated molecular patterns, such as LPS [494].

T cells localize in the small intestine as a result of the selective expression of $\alpha_4\beta_7$ and CCR9. CD4+ and CD8+ T cells are found throughout the lamina propria, whereas CD8+ T cells are preferentially found in the epithelium. These T cells represent a heterogeneous population of effector cells, effector memory cells and Treg cells, which simultaneously provide help for IgA production by B cells and maintain tolerance to commensal bacteria and other antigens [495].

The lamina propria also has many functional aspects. Mast cells are abundant and important in the host response to parasite infection, and even play a role in innate immune responses to bacteria which breach the epithelial barrier [496].

Mast cells and lymphocytes in the lamina propria interface with the enteric nervous system, providing another pathway to influence mucosal immune responses [497]. Eosinophils are also a normal resident of the lamina propria of the stomach and small and large intestines and function in parasite infection, allergic responses, and in normal gut homeostasis [498]. Intraepithelial lymphocytes (IELs) reside above the basement membrane and between adjacent epithelial cells in the intestine, mainly including γδ TCR CD8+ T cells, two distinct subsets of αβ TCR cells (αβ TCR CD4+ and CD8+ T cells), and double-negative cells with a common feature of expressing CD8αα for all IEL subsets [499]. IELs mainly function to maintain the homeostasis of the intestinal epithelium [500] but also are involved in protective immune responses to certain pathogens and even in cancer surveillance [499]. Paneth cells also are present and mainly function in innate immune responses by producing antimicrobial peptides, α-defensins, lysozymes and secretory phospholipase A_2. Mice deficient in a paneth cell processing enzyme cannot produce mature α-defensins and subsequently were more susceptible to orally administered *S. typhimurium* than wild-type mice [501].

As have been discussed in section 1.4., intestinal epithelial cells are involved in the immune responses along with maintaining intestinal homeostasis, providing barrier function and transporting secretory IgA, and nutrient absorption. Intestinal epithelial cells can also act as APCs and produce a variety of cytokines and chemokines that can influence immune responses [193, 502].

Finally, intestinal epithelial cells can influence Treg cell expansion in the intestine [503].

1.6.2 Gnotobiotic animl models, gut microbiota and mucosal immune system

Gnotobiotic animal models are an important tool for studying the role of commensal microbiota on the development of the mucosal immune system [504-511]. Germfree animals are derived by surgical procedures near the time of birht and kept in isolator units. Germfree isolators provide a sterile environment free of bacteria, virus, fungus, etc. Once an agent, such as a virus, is introduced into the germfree isolator, it is no longer truly germfree, and is referred to as being gnotobiotic (Gn). However, the words germfree and gnotobiotic are used interchangeably in litetures. Germfree rodents, chickens, calves, pigs and goats are most often used. In germfree animals, there is accumulation of mucus with retention of water due to missing mucolytic bacteria, larger caecum, prolonged intestinal epithelial cell cycle, and peristalsis decrease. Germfree animals also have underdeveloped intestinal lymphatic constituents and contain the decreasing number of lymphatic gut-associated tissues [508, 510], preferentially affecting αβ TCR IELs [512]. The wound healing and pathogen infection preventing functions of gut γδ IELs are reduced in germfree mice and colonization with specific pathogen-free microbiota can recover these functions [513]. Also, germfree pigs, rats and rabbits delay the appearance of cells which secret natural antibodies against bacterial antigens

(such as LPS) and tissue antigens (such as rabbit appendix, Peyer's patches of rats, ileal patches of pigs) [510]. Germfree animals have less diversified Ig [510, 514-518].

Despite these differences from conventional animals and defects in germfree animals, Gn pigs offer distinct advantages for studies of the effects of probiotics on enteric virus infections and vaccines. Due to six layers of porcine placenta to prevent the transport of maternal antibodies, germfree piglets are deprived of colostrum and thus are a good model to evaluate immune responses to specific antigens [479]. There are three distinctive advantages of using Gn pig models to study the immunomodulating mechanism of lactobacilli: (1) neonatal pigs provide an immunologically naïve background that allows clear identification of the immune responses to a single pathogen or a single vaccine in hosts colonized with a clearly defined probiotic/commensal bacterial strain; (2) the gastrointestinal, nutritional, metabolic, and immunologic similarities between pigs and humans; and (3) relatively large numbers of immune cells can be harvested from each tissue of the pig allowing comprehensive studies of many immunological parameters to identify the mechanism of lactobacilli's immunomodulating effect. Moreover, only neonatal pigs and no conventional lab animals (mice, rats, guinea pigs, rabbits) are susceptible to diarrhea following HRV inoculation. The use of Gn pigs will prevent both interference and confounding factors from wild swine rotavirus contaminations that are inevitable in conventional pig facilities.

The gastrointestinal (GI) tract of humans consists of between 400 to 1000 bacterial species distributed among nine phyla with *Firmicutes, Bacteroidetes* and *Actinobacteria* representing around 75 % of the diversity [519]. The LA (*L. acidophilus* strain NCFM) we used in our study was isolated from a human source in the 1970s [520]. It belongs to the phylum *Firmicutes,* class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae*.

During the first few weeks of life, microbial succession in the GI tract of humans, pigs, chickens and calves is very similar, even though animal species are usually exposed to a greater number of bacteria from fecal and environmental sources compared to humans [521].

Commensal microbiota helps the host against pathogenic microorganisms. These bacteria provide the colonization resistance against attachment, multiplication and invasion of pathogenic microorganisms into epithelial cells and potential circulation in the host. Furthermore, they affect the mucosal immune system and mucosal immune responses. In the very early stage of postnatal period, the immune responses are biased toward Th2 and the Th1 modulated immune responses are lacking [522, 523]. Later, intestinal commensal microbiota stimulate the development of both local and systemic immune responses, preferentially Th1 immune responses [522, 523], but, further later, induce regulatory mechanisms to maintain homeostasis [524].

Intestinal colonization of germfree animals with commensal microbes significantly affects the development of mucosal and systemic immune systems.

It may promote integrity of the epithelial barrier by regulating tight junctions and protecting from injury with enhancing proliferation and cytoprotective protein production [205, 216, 525-527]. In addition, commensal bacteria regulate cell migration to keep the epithelial barrier [528]. Intestinal colonization also stimulates mucin secretion from goblet cells to limit intestinal infections by binding mucin to pathogens [529, 530]. Intestinal microbiota also drive the development of Th17 cells [531-535] and Treg cells [523, 536-540]. A few studies even found that segmented filamentous bacteria are important to drive the development of lamina propria's Th17 cells [533-535]. For Treg cell induction, different microbiota could have different mechanisms. For example, *Bifidobacterium fragilis* functioned directly on CD4 T cells to drive their Treg differentiation by increasing TGF-β2 production [538], whereas *Clostridium* spp. drive Treg differentiation via TGF-β1 produced by epithelial cells [539]. For B cells, intestinal colonization results in an increase of the Ig level, production of natural antibodies and some specific antibodies, antimicrobial peptides such as RegIIIγ and α-defensins [492, 541-543], and an increase of the overall immunological capacity [510]. In addition, it influences the migration pattern of lymphocytes into mucosal sites of rats [510, 511]. The commensals induced the migration of mast cells into the intestine [544]. Monocolonization of germfree pigs with a nonpathogenic *E. coli* strain induced heavy cellular infiltration into gut mucosa, formation of germinal centers in Peyer's patches, transient bacteria translocation into MLNs, and production of specific

antibacterial antibodies during the first few days [514, 545]. However, the local and systemic responses are inhibited by this monoassociation after 5 to 10 days [509, 514, 518, 545, 546], suggesting that the early postnatal ontogeny is essential for the development of the immune system.

1.6.3 Interactions among gut microbiota, rotavirus and mucosal immune system

The initial studies of the beneficial effects of probiotics on the mucosal immune responses to rotavirus-induced diarrhea were mainly descriptive. For example, there were clinical studies on the effect of probiotics on rotavirus-induced diarrhea which showed that milk containing *L. casei* DN 114 001 reduced the severity and duration of acute diarrhea in young children [286, 547, 548] and it had protective effects against SA11 rotavirus-induced diarrhea in a germfree sucking rat model [549]. LGG in an oral rehydration solution resulted in a shorter duration of disease in children with acute diarrhea [550]. However, not all lactobacilli strains are effective against rotavirus diarrhea [551]. The mechanism of the beneficial effects of probiotics on the mucosal immune responses to rotavirus needs further study, which was the goal of my dissertation research. Probiotics may inhibit the first step of viral infection, adsorption, by their steric hindrance and the improving barrier effects of mucus, glycocalyx and intercellular junctions. Probiotics could also block viral attachment by modulating the cellular apical glycosylation pattern and cellular signal transduction pathways involved in the viral cycle. For example, Freitas et al.

[552] found that *L. casei* strain DN114 001 and *B. thetaiotaomicron* protect epithelial cells from rotavirus infection partially by modulating the apical cellular glycosylation pattern. The modification of MEK, PKA, p38 MAPK and NF-κB by probiotics can also affect rotavirus multiplication and dissemination as evident by inhibitors of p38 MAPK, ERK and PI3K blocking the function of probiotics to restore Cl^- secretion [553]. Probiotics may also directly interfere with the cellular pathways involved in rotavirus pathogenesis. Rotavirus-infected enterocytes die through apoptosis [554] However, two polypeptides from LGG activate Akt to prevent cytokine-induced apoptosis and promote the growth of human intestinal epithelial cells [218]. Probiotics may also regulate cellular homeostasis by directly stimulating innate and/or adaptive immune responses and then limiting inflammation later. For example, *B. lactis* HN019 protects against rotavirus diarrhea by enhancing immune responses in a piglet model [555]. *Bifidobacterium breve* strain YIT4064 promotes the production of anti-rotavirus IgA antibodies in mice [556]. *S. thermophilus* and LA prevent the deleterious effects of TNF-α and IFN-γ [557]. Our study [29] found that LA significantly enhanced HRV-specific IFN-γ producing CD8 T cell responses in ileum and spleen, IgA and IgG antibody-secreting cell responses in ileum, and serum IgM, IgA and IgG antibody and virus neutralizing antibody titers in AttHRV-vaccinated pigs. A mixture of LA strain NCFM and *L. reuteri* (ATCC# 23272) significantly enhanced total intestinal IgA secreting cell responses and total serum IgM and intestinal IgM and IgG titers compared to AttHRV only

pigs, however, it didn't affect the magnitude of virus-specific B cell responses [268]. More studies are needed to improve our understanding of the effects of probiotics on immune responses to rotavirus infection.

1.6.4 Intestinal homeostasis

There are multiple mechanisms present in the intestinal immune system to mediate activation of Th1, Th2, and Th17 immune responses against intestinal pathogens, but prevent development of autoimmune disorders [558]. Additionally, these immune responses must remain tolerant of antigens from resident bacteria and food.

Intestinal epithelial cells sense the luminal antigens and express immunomodulatory factors to influence intestinal DCs and create a tolerogenic environment to modify the phenotype and function of DCs towards tolerogenic DCs [414]. Antigen-presenting cells in the intestine continuously sample intestinal antigens from both food and bacteria. Depending on the presented antigens, APCs (mainly DCs) mediate differential T cell activation, clonal expansion and differentiation, including effector T cells, Treg cells and γδ T cells to balance effector function and homeostasis.

1.6.5 The role of DCs in intestinal homeostasis

Each specific tissue has its own DCs locally [559-563]. The specific anatomical environment where immature DCs reside and encounter antigens dramatically influences the aspects of immune responses generated by the DCs.

The phenotypes of DCs in the intestine are diverse and determine the outcome of immune responses of T cells. When DCs encounter antigens together with maturation signals, activated immune responses follow; however, when they encounter apoptotic cells, commensal bacteria or dietary antigens, immune tolerance should be induced. The focus of this section is on how homeostasis is maintained. There are significant differences between costimulatory molecules, cytokine secretion and induced T cell responses of intestinal DCs from peripheral DCs. In mice, DCs isolated from the intestine express low levels of MHC II and costimulatory molecules compared to DCs from other tissues [564]. In response to pathogens, intestinal DCs secrete anti-inflammatory cytokines (TGF-β and IL-10) to induce the differentiation of Th2 cells or regulatory T cells [565]. Furthermore, intestinal DCs can induce B cells to undergo isotype switching to IgA to neutralize pathogens in the lumen. Human and mouse mucosal DCs express lower levels of TLRs compared to spleen or blood derived DCs [566, 567], which also indicates that DCs are involved in regulating the homeostasis.

IECs play a role in maintaining homeostasis by expressing immunomodulatory factors to influence the function of DCs. Butler et al. [414] showed that IECs are able to create a tolerogenic environment to modulate DCs towards tolerogenic DCs with secreted TGF-β. The thymic stromal lymphopoeitin (TSLP) released by IECs induces noninflammatory DCs with IL-10 production but not IL-12 [415]. TGF-β [488, 568, 569], and prostaglandin

E2 (PGE2) [570] are also involved in regulating intestinal DCs. IL-10 secreted by DCs is important in maintaining DCs in an immature state [571] and promoting the development of Treg cells [572]. Intestinal CD103+ DCs induce the development of Foxp3+ Treg cells depending on secreting TGF-β and the dietary metabolite retinoic acid (RA) [569, 573, 574].

1.6.6 The role of Treg cells in intestinal homeostasis

Powrie et al. [575] originally characterized the importance of nTreg cells in gut inflammation by transferring these Treg cells to suppress colonic inflammation by secreting TGF-β and IL-10. The importance of the nTreg cells are also demonstrated in patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome [576]. The iTreg cells are also important for regulating immune responses. Some studies showed that intestinal antigens can lead to the induction and expansion of both antigen-specific effector T cells and antigen-specific Foxp3+ Treg cells which prevented uncontrolled severe intestinal inflammation [577, 578]. Furthermore, CD8+ Treg cells have been identified to be involved in controlling mucosal immune responses and maintaining intestinal homeostasis. Brimnes et al. [579] suggested that the deficiency of CD8+ Treg cells in the lamina propria may lead to the development of IBD. Menager-Marcq et al. [580] demonstrated that CD8+CD28− Treg cells isolated from the spleen or the gut efficiently prevented the development of colitis. Chaput et al. [581] demonstrated that

CD8+CD25+Foxp3+ Treg cells may contribute to gut homeostasis. The intestinal expression of a self-antigen also led to the peripheral induction of antigen-specific CD8+Foxp3+ Treg cells *in vivo* [582].

1.6.7 The role of γδ T cells in intestinal homeostasis

In contrast to the systemic lymphoid system, the intestine contains a prominent T cell population expressing the γδ TCR. γδ T cells in the gut appear to regulate epithelial homeostasis. Activated intestinal intraepithelial γδ T cells express the epithelial cell growth factor and keratinocyte growth factor (KGF) and regulate epithelial homeostasis. Skin γδ T cells also produced KGF after activation [583]. Mice that lack intestinal intraepithelial γδ T cells develop severe diseases in two different IBD models owing to non KGF-1-mediated epithelial repair [500, 584]. KGF-1 secretion by intestinal intraepithelial γδ T cells was also analyzed in the mouse model of gut epithelial dysfunction. Villus atrophy in the small intestine is associated with a down-regulation of KGF-1 expression, whereas villus hypertrophy is associated with an up-regulation of KGF expression [585]. One study [586] demonstrated that IL-7 is crucial for the development and maintenance of intestinal intraepithelial γδ T cells and increases KGF-1 expression by intestinal γδ T cells.

Intestinal γδ T cells also regulate inflammatory responses to keep homeostasis. Transfer of intestinal γδ T cells to mice that lack γδ T cells relieves colitis by decreasing production of IFN-γ and TNF-α and increasing

production of TGF-β [584]. Intestinal γδ T cells also decrease IL-15 secretion by epithelial cells [584], and increase the production of IL-8 and IFN-γ inducible protein-10 [587]. In our study, porcine γδ T cells produce TGF-β and IL-10 in CD2+CD8+ subset to restore homeostasis. The function of each porcine γδ T subset was clearly identified for the first time in our studies (see Chapter 5).

1.7 Conclusions and future directions

Both two licensed vaccines, RotaTeq and Rotarix, have more than 85 % protective efficacy against moderate to severe rotavirus gastroenteritis in middle and high-income countries. After their introduction, the rotavirus-related healthcare visits are dramatically decreasing and herd protection is also observed in these countries. However, these two licensed vaccines have a substantially lower protective efficacy against moderate to severe rotavirus gastroenteritis in low income countries. The initial colonization process of human infants with commensal bacteria may be different between developed and developing countries with different hygiene standards thus may influence the efficacy of oral rotavirus vaccines, although there are several other environmental and host factors to affect the efficacy of vaccines as discussed previously. Using probiotics as an intervention approach to modulate gut microbiotia in human infants will likely improve rotavirus vaccine efficacy. Our results from the dose effect of LA on immune responses to the AttHRV

vaccine in Gn pigs demonstrated that relatively low dose LA enhanced immune responses to AttHRV vaccine, whereas high dose LA promoted Treg cell responses. This may partly explain why the efficacy of oral rotavirus vaccines are significantly lower in developing countries (higher bacteria load) compared to developed countries (lower bacteria load). My next research goal will focus on seeking optimal (intermediate) dose of probiotic LA that will have simultaneously immune stimulatory and anti-inflammatory effects and test the dose in Gn pigs as well as in conventionalized Gn pigs. It was recently reported that live probiotics influence the gut microbiota and the immune responses, whereas the dead cell components induce the anti-inflammatory responses [588]. Combining with the results from our LA dose response study, these findings suggest that it is important to define the exact titer of live probiotics in commercial probiotic products. Comparing immune responses induced by different ratios of live to dead probiotics is the objective of one of my future studies.

Our studies of TLR responses and γδ T cell functions in rotavirus infection broadened our understanding of immune responses induced by rotavirus and also opened the options for enhancing protective immunity against rotavirus infection by introducing ligands for TLR and/or γδ T cells. CD2+CD8+ γδ T cell subset initially has proinflammatory function to enhance immune responses and later anti-inflammatory function to limit inflammation and resolve caused damage. Therefore, an efficient agonist specific for CD2+CD8+ γδ T cell

subset will be sought in my future study and it may enhance both the anti-viral immune responses to rotavirus and the ability to maintain immune homeostasis during infection and after virus clearance.

1.8. References

1. Lundgren, O. and L. Svensson, *Pathogenesis of rotavirus diarrhea.* Microbes Infect, 2001. 3(13): p. 1145-56.

2. Parashar, U.D., et al., *Global mortality associated with rotavirus disease among children in 2004.* J Infect Dis, 2009. 200 Suppl 1: p. S9-S15.

3. Armah, G.E., et al., *Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: a randomised, double-blind, placebo-controlled trial.* Lancet, 2010. 376(9741): p. 606-14.

4. Zaman, K., et al., *Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia: a randomised, double-blind, placebo-controlled trial.* Lancet, 2010. 376(9741): p. 615-23.

5. O'Ryan, M. and A.C. Linhares, *Update on Rotarix: an oral human rotavirus vaccine.* Expert Rev Vaccines, 2009. 8(12): p. 1627-41.

6. O'Ryan, M.L., G. Hermosilla, and G. Osorio, *Rotavirus vaccines for the developing world.* Curr Opin Infect Dis, 2009. 22(5): p. 483-9.

7. Aggarwal, R., J. Sentz, and M.A. Miller, *Role of zinc administration in prevention of childhood diarrhea and respiratory illnesses: a meta-analysis.* Pediatrics, 2007. 119(6): p. 1120-30.

8. Sazawal, S., et al., *Zinc supplementation in infants born small for gestational age reduces mortality: a prospective, randomized, controlled trial.* Pediatrics, 2001. 108(6): p. 1280-6.

9. Patel, M., et al., *Oral rotavirus vaccines: how well will they work where they are needed most?* J Infect Dis, 2009. 200 Suppl 1: p. S39-48.

10. Posey, D.L., et al., *The effect of diarrhea on oral poliovirus vaccine failure*

in Brazil. J Infect Dis, 1997. 175 Suppl 1: p. S258-63.

11. Steele, A.D., et al., *Co-administration study in South African infants of a live-attenuated oral human rotavirus vaccine (RIX4414) and poliovirus vaccines.* Vaccine, 2010. 28(39): p. 6542-8.

12. Cunliffe, N.A., et al., *Epidemiology of rotavirus diarrhoea in Africa: a review to assess the need for rotavirus immunization.* Bull World Health Organ, 1998. 76(5): p. 525-37.

13. Lagos, R., et al., *Palatability, reactogenicity and immunogenicity of engineered live oral cholera vaccine CVD 103-HgR in Chilean infants and toddlers.* Pediatr Infect Dis J, 1999. 18(7): p. 624-30.

14. Mulholland, K., et al., *Randomised trial of Haemophilus influenzae type-b tetanus protein conjugate vaccine [corrected] for prevention of pneumonia and meningitis in Gambian infants.* Lancet, 1997. 349(9060): p. 1191-7.

15. Moon, S.S., et al., *Inhibitory effect of breast milk on infectivity of live oral rotavirus vaccines.* Pediatr Infect Dis J, 2010. 29(10): p. 919-23.

16. Glass, R.I. and B.J. Stoll, *The protective effect of human milk against diarrhea. A review of studies from Bangladesh.* Acta Paediatr Scand Suppl, 1989. 351: p. 131-6.

17. Rennels, M.B., *Influence of breast-feeding and oral poliovirus vaccine on the immunogenicity and efficacy of rotavirus vaccines.* J Infect Dis, 1996. 174 Suppl 1: p. S107-11.

18. Ruiz-Palacios, G.M., et al., *Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis.* N Engl J Med, 2006. 354(1): p. 11-22.

19. Vesikari, T., et al., *Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine.* N Engl J Med, 2006. 354(1): p. 23-33.

20. Glass, R.I., et al., *Immune response to rotavirus vaccines among breast-fed and nonbreast-fed children.* Adv Exp Med Biol, 1991. 310: p. 249-54.

21. Dennehy, P.H., et al., *Comparative evaluation of safety and immunogenicity of two dosages of an oral live attenuated human rotavirus vaccine.* Pediatr Infect Dis J, 2005. 24(6): p. 481-8.

22. Lawrence, R.M. and C.A. Pane, *Human breast milk: current concepts of immunology and infectious diseases.* Curr Probl Pediatr Adolesc Health Care, 2007. 37(1): p. 7-36.

23. Newburg, D.S., et al., *Role of human-milk lactadherin in protection against symptomatic rotavirus infection.* Lancet, 1998. 351(9110): p. 1160-4.

24. Ing, D.J., et al., *Immunogenicity of tetravalent rhesus rotavirus vaccine administered with buffer and oral polio vaccine.* Am J Dis Child, 1991. 145(8): p. 892-7.

25. Marteau, P.R., *Probiotics in clinical conditions.* Clin Rev Allergy Immunol, 2002. 22(3): p. 255-73.

26. Kaila, M., et al., *Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain.* Pediatr Res, 1992. 32(2): p. 141-4.

27. Olivares, M., et al., *Oral intake of Lactobacillus fermentum CECT5716 enhances the effects of influenza vaccination.* Nutrition, 2007. 23(3): p. 254-60.

28. Paineau, D., et al., *Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial.* FEMS Immunol Med Microbiol, 2008. 53(1): p. 107-13.

29. Zhang, W., et al., *Probiotic Lactobacillus acidophilus enhances the immunogenicity of an oral rotavirus vaccine in gnotobiotic pigs.* Vaccine, 2008. 26(29-30): p. 3655-61.

30. Winkler, P., et al., *Effect of a dietary supplement containing probiotic bacteria plus vitamins and minerals on common cold infections and cellular immune parameters.* Int J Clin Pharmacol Ther, 2005. 43(7): p. 318-26.

31. Mohamadzadeh, M., et al., *Targeting mucosal dendritic cells with microbial antigens from probiotic lactic acid bacteria.* Expert Rev Vaccines, 2008. 7(2): p. 163-74.

32. Isolauri, E., et al., *Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by Lactobacillus casei GG.* Vaccine, 1995. 13(3): p. 310-2.

33. Boge, T., et al., *A probiotic fermented dairy drink improves antibody response to influenza vaccination in the elderly in two randomised controlled trials.* Vaccine, 2009. 27(41): p. 5677-84.

34. Guerrero, C.A., A.Y. Santana, and O. Acosta, *Mouse intestinal villi as a model system for studies of rotavirus infection.* J Virol Methods, 2010. 168(1-2): p. 22-30.

35. Azevedo, M.S., et al., *Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus.* J Virol, 2005. 79(9): p. 5428-36.

36. Blutt, S.E. and M.E. Conner, *Rotavirus: to the gut and beyond!* Curr Opin Gastroenterol, 2007. 23(1): p. 39-43.

37. Blutt, S.E., et al., *Active viremia in rotavirus-infected mice.* J Virol, 2006. 80(13): p. 6702-5.

38. Blutt, S.E., et al., *Rotavirus antigenemia in children is associated with viremia.* PLoS Med, 2007. 4(4): p. e121.

39. Offit, P.A. and D.H. Rubin, *Viral diseases: infections of the gastrointestinal tract.* Compr Ther, 1982. 8(8): p. 21-6.

40. Carreno-Torres, J.J., et al., *Characterization of viroplasm formation during the early stages of rotavirus infection.* Virol J, 2010. 7: p. 350.

41. Pesavento, J.B., et al., *Rotavirus proteins: structure and assembly.* Curr Top Microbiol Immunol, 2006. 309: p. 189-219.

42. McClain, B., et al., *X-ray crystal structure of the rotavirus inner capsid particle at 3.8 A resolution.* J Mol Biol, 2010. 397(2): p. 587-99.

43. Patton, J.T., et al., *Coupling of rotavirus genome replication and capsid assembly.* Adv Virus Res, 2007. 69: p. 167-201.

44. Lu, X., et al., *Mechanism for coordinated RNA packaging and genome replication by rotavirus polymerase VP1.* Structure, 2008. 16(11): p. 1678-88.

45. McDonald, S.M. and J.T. Patton, *Rotavirus VP2 core shell regions critical for viral polymerase activation.* J Virol, 2011. 85(7): p. 3095-105.

46. Chen, D., et al., *Rotavirus open cores catalyze 5'-capping and methylation of exogenous RNA: evidence that VP3 is a methyltransferase.* Virology, 1999. 265(1): p. 120-30.

47. Hyser, J.M. and M.K. Estes, *Rotavirus vaccines and pathogenesis: 2008.* Curr Opin Gastroenterol, 2009. 25(1): p. 36-43.

48. Yoder, J.D., et al., *VP5* rearranges when rotavirus uncoats.* J Virol, 2009. 83(21): p. 11372-7.

49. Yoder, J.D. and P.R. Dormitzer, *Alternative intermolecular contacts underlie the rotavirus VP5* two- to three-fold rearrangement.* EMBO J, 2006. 25(7): p. 1559-68.

50. Lopez, S. and C.F. Arias, *Early steps in rotavirus cell entry.* Curr Top Microbiol Immunol, 2006. 309: p. 39-66.

51. Li, Y.J., et al., *Oral vaccination with the porcine rotavirus VP4 outer capsid protein expressed by Lactococcus lactis induces specific antibody production.* J Biomed Biotechnol, 2010. 2010: p. 708460.

52. Ward, R., *Mechanisms of protection against rotavirus infection and disease.* Pediatr Infect Dis J, 2009. 28(3 Suppl): p. S57-9.

53. Ruggeri, F.M., et al., *Antirotavirus immunoglobulin A neutralizes virus in vitro after transcytosis through epithelial cells and protects infant mice from diarrhea.* J Virol, 1998. 72(4): p. 2708-14.

54. Mackow, E.R., et al., *Immunization with baculovirus-expressed VP4 protein passively protects against simian and murine rotavirus challenge.* J Virol, 1990. 64(4): p. 1698-703.

55. Ward, R.L., et al., *Immunodominance of the VP4 neutralization protein of rotavirus in protective natural infections of young children.* J Virol, 1993. 67(1): p. 464-8.

56. Coulson, B.S., S.L. Londrigan, and D.J. Lee, *Rotavirus contains integrin ligand sequences and a disintegrin-like domain that are implicated in virus entry into cells.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 5389-94.

57. Hewish, M.J., Y. Takada, and B.S. Coulson, *Integrins alpha2beta1 and alpha4beta1 can mediate SA11 rotavirus attachment and entry into cells.* J

Virol, 2000. 74(1): p. 228-36.

58. Gilbert, J.M. and H.B. Greenberg, *Virus-like particle-induced fusion from without in tissue culture cells: role of outer-layer proteins VP4 and VP7.* J Virol, 1997. 71(6): p. 4555-63.

59. Ludert, J.E., et al., *Antibodies to rotavirus outer capsid glycoprotein VP7 neutralize infectivity by inhibiting virion decapsidation.* J Virol, 2002. 76(13): p. 6643-51.

60. Heath, R.R., et al., *Mapping of the target antigens of the rotavirus-specific cytotoxic T cell response.* J Gen Virol, 1997. 78 (Pt 5): p. 1065-75.

61. Jaimes, M.C., et al., *Frequencies of virus-specific CD4(+) and CD8(+) T lymphocytes secreting gamma interferon after acute natural rotavirus infection in children and adults.* J Virol, 2002. 76(10): p. 4741-9.

62. Conner, M.E., et al., *Virus-like particles as a rotavirus subunit vaccine.* J Infect Dis, 1996. 174 Suppl 1: p. S88-92.

63. Johansen, K., et al., *Serum IgA immune response to individual rotavirus polypeptides in young children with rotavirus infection.* Arch Virol, 1994. 138(3-4): p. 247-59.

64. Svensson, L., et al., *Serum antibody responses to individual viral polypeptides in human rotavirus infections.* J Gen Virol, 1987. 68 (Pt 3): p. 643-51.

65. Burns, J.W., et al., *Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity.* Science, 1996. 272(5258): p. 104-7.

66. Shuttleworth, G., D.C. Eckery, and P. Awram, *Oral and intraperitoneal immunization with rotavirus 2/6 virus-like particles stimulates a systemic and mucosal immune response in mice.* Arch Virol, 2005. 150(2): p. 341-9.

67. Ciarlet, M., et al., *Subunit rotavirus vaccine administered parenterally to rabbits induces active protective immunity.* J Virol, 1998. 72(11): p. 9233-46.

68. O'Neal, C.M., et al., *Rotavirus 2/6 viruslike particles administered intranasally with cholera toxin, Escherichia coli heat-labile toxin (LT), and LT-R192G induce protection from rotavirus challenge.* J Virol, 1998. 72(4):

p. 3390-3.

69. Schwartz-Cornil, I., et al., *Heterologous protection induced by the inner capsid proteins of rotavirus requires transcytosis of mucosal immunoglobulins.* J Virol, 2002. 76(16): p. 8110-7.

70. Yuan, L., et al., *Intranasal administration of 2/6-rotavirus-like particles with mutant Escherichia coli heat-labile toxin (LT-R192G) induces antibody-secreting cell responses but not protective immunity in gnotobiotic pigs.* J Virol, 2000. 74(19): p. 8843-53.

71. VanCott, J.L., et al., *Mice develop effective but delayed protective immune responses when immunized as neonates either intranasally with nonliving VP6/LT(R192G) or orally with live rhesus rotavirus vaccine candidates.* J Virol, 2006. 80(10): p. 4949-61.

72. Barro, M. and J.T. Patton, *Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3.* Proc Natl Acad Sci U S A, 2005. 102(11): p. 4114-9.

73. Pina-Vazquez, C., et al., *Post-translational regulation of rotavirus protein NSP1 expression in mammalian cells.* Arch Virol, 2007. 152(2): p. 345-68.

74. Graff, J.W., et al., *Interferon regulatory factor 3 is a cellular partner of rotavirus NSP1.* J Virol, 2002. 76(18): p. 9545-50.

75. Silvestri, L.S., Z.F. Taraporewala, and J.T. Patton, *Rotavirus replication: plus-sense templates for double-stranded RNA synthesis are made in viroplasms.* J Virol, 2004. 78(14): p. 7763-74.

76. Barro, M. and J.T. Patton, *Rotavirus NSP1 inhibits expression of type I interferon by antagonizing the function of interferon regulatory factors IRF3, IRF5, and IRF7.* J Virol, 2007. 81(9): p. 4473-81.

77. Feng, N., et al., *Roles of VP4 and NSP1 in determining the distinctive replication capacities of simian rotavirus RRV and bovine rotavirus UK in the mouse biliary tract.* J Virol, 2011. 85(6): p. 2686-94.

78. Arnold, M.M. and J.T. Patton, *Diversity of interferon antagonist activities mediated by NSP1 proteins of different rotavirus strains.* J Virol, 2011. 85(5): p. 1970-9.

79. Contin, R., et al., *Rotavirus replication requires a functional proteasome*

for effective assembly of viroplasms. J Virol, 2011. 85(6): p. 2781-92.

80. Piron, M., et al., *Identification of the RNA-binding, dimerization, and eIF4GI-binding domains of rotavirus nonstructural protein NSP3.* J Virol, 1999. 73(7): p. 5411-21.

81. Piron, M., et al., *Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F.* EMBO J, 1998. 17(19): p. 5811-21.

82. Torres-Vega, M.A., et al., *The C-terminal domain of rotavirus NSP5 is essential for its multimerization, hyperphosphorylation and interaction with NSP6.* J Gen Virol, 2000. 81(Pt 3): p. 821-30.

83. Sen, A., N. Sen, and E.R. Mackow, *The formation of viroplasm-like structures by the rotavirus NSP5 protein is calcium regulated and directed by a C-terminal helical domain.* J Virol, 2007. 81(21): p. 11758-67.

84. Ball, J.M., et al., *Rotavirus NSP4: a multifunctional viral enterotoxin.* Viral Immunol, 2005. 18(1): p. 27-40.

85. Kavanagh, O.V., et al., *Rotavirus enterotoxin NSP4 has mucosal adjuvant properties.* Vaccine, 2010. 28(18): p. 3106-11.

86. Kabcenell, A.K. and P.H. Atkinson, *Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11.* J Cell Biol, 1985. 101(4): p. 1270-80.

87. Tian, P., et al., *Rotavirus protein expression is important for virus assembly and pathogenesis.* Arch Virol Suppl, 1996. 12: p. 69-77.

88. Au, K.S., N.M. Mattion, and M.K. Estes, *A subviral particle binding domain on the rotavirus nonstructural glycoprotein NS28.* Virology, 1993. 194(2): p. 665-73.

89. Lopez, T., et al., *Silencing the morphogenesis of rotavirus.* J Virol, 2005. 79(1): p. 184-92.

90. Suzuki, H., *A hypothesis about the mechanism of assembly of double-shelled rotavirus particles.* Arch Virol Suppl, 1996. 12: p. 79-85.

91. Ball, J.M., et al., *Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein.* Science, 1996. 272(5258): p. 101-4.

92. Hyser, J.M., et al., *Rotavirus disrupts calcium homeostasis by NSP4 viroporin activity.* MBio, 2010. 1(5).

93. Hagbom, M., et al., *Rotavirus stimulates release of serotonin (5-HT) from human enterochromaffin cells and activates brain structures involved in nausea and vomiting.* PLoS Pathog, 2011. 7(7): p. e1002115.

94. Saunders, D., et al., *Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor.* J Exp Med, 1996. 184(6): p. 2185-96.

95. Iosef, C., et al., *Systemic and intestinal antibody responses to NSP4 enterotoxin of Wa human rotavirus in a gnotobiotic pig model of human rotavirus disease.* J Med Virol, 2002. 68(1): p. 119-28.

96. Yuan, L., et al., *Species-specific but not genotype-specific primary and secondary isotype-specific NSP4 antibody responses in gnotobiotic calves and piglets infected with homologous host bovine (NSP4[A]) or porcine (NSP4[B]) rotavirus.* Virology, 2004. 330(1): p. 92-104.

97. Ray, P.G., et al., *Rotavirus immunoglobulin levels among Indian mothers of two socio-economic groups and occurrence of rotavirus infections among their infants up to six months.* J Med Virol, 2007. 79(3): p. 341-9.

98. Linhares, A.C. and J.S. Bresee, *Rotavirus vaccines and vaccination in Latin America.* Rev Panam Salud Publica, 2000. 8(5): p. 305-31.

99. Greenberg, H.B. and M.K. Estes, *Rotaviruses: from pathogenesis to vaccination.* Gastroenterology, 2009. 136(6): p. 1939-51.

100. Liu, M., N.M. Mattion, and M.K. Estes, *Rotavirus VP3 expressed in insect cells possesses guanylyltransferase activity.* Virology, 1992. 188(1): p. 77-84.

101. Lorrot, M. and M. Vasseur, *How do the rotavirus NSP4 and bacterial enterotoxins lead differently to diarrhea?* Virol J, 2007. 4: p. 31.

102. Yuan, L., G.W. Stevenson, and L.J. Saif, eds. *Rotavirus and Reovirus*. Diseases of Swine, ed. J.J.Z. B. E. Straw, S. D'Allaire, D. J. Taylor Vol. 9th Edition. 2006. 435-454.

103. Davidson, G.P. and G.L. Barnes, *Structural and functional abnormalities of*

the small intestine in infants and young children with rotavirus enteritis. Acta Paediatr Scand, 1979. 68(2): p. 181-6.

104. Schnagl, R.D. and I.H. Holmes, *Characteristics of the genome of human infantile enteritis virus (Rotavirus).* J Virol, 1976. 19(1): p. 267-70.

105. Li, H., D.N. Sheppard, and M.J. Hug, *Transepithelial electrical measurements with the Ussing chamber.* J Cyst Fibros, 2004. 3 Suppl 2: p. 123-6.

106. Halaihel, N., et al., *Rotavirus infection impairs intestinal brush-border membrane Na(+)-solute cotransport activities in young rabbits.* Am J Physiol Gastrointest Liver Physiol, 2000. 279(3): p. G587-96.

107. Obert, G., I. Peiffer, and A.L. Servin, *Rotavirus-induced structural and functional alterations in tight junctions of polarized intestinal Caco-2 cell monolayers.* J Virol, 2000. 74(10): p. 4645-51.

108. Dickman, K.G., et al., *Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells.* Am J Physiol Gastrointest Liver Physiol, 2000. 279(4): p. G757-66.

109. Lundgren, O., et al., *Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhea.* Science, 2000. 287(5452): p. 491-5.

110. Isolauri, E., et al., *Diet during rotavirus enteritis affects jejunal permeability to macromolecules in suckling rats.* Pediatr Res, 1993. 33(6): p. 548-53.

111. Stintzing, G., et al., *Intestinal permeability in small children during and after rotavirus diarrhoea assessed with different-size polyethyleneglycols (PEG 400 and PEG 1000).* Acta Paediatr Scand, 1986. 75(6): p. 1005-9.

112. Hartley, J.L., M. Davenport, and D.A. Kelly, *Biliary atresia.* Lancet, 2009. 374(9702): p. 1704-13.

113. Bassett, M.D. and K.F. Murray, *Biliary atresia: recent progress.* J Clin Gastroenterol, 2008. 42(6): p. 720-9.

114. Wang, W., et al., *The Rhesus Rotavirus Gene Encoding VP4 Is a Major Determinant in the Pathogenesis of Biliary Atresia in Newborn Mice.* J Virol, 2011. 85(17): p. 9069-77.

115. Lu, B.R., et al., *alpha-enolase autoantibodies cross-reactive to viral proteins in a mouse model of biliary atresia.* Gastroenterology, 2010. 139(5): p. 1753-61.

116. Honeyman, M.C., et al., *Evidence for molecular mimicry between human T cell epitopes in rotavirus and pancreatic islet autoantigens.* J Immunol, 2010. 184(4): p. 2204-10.

117. Jafri, M., et al., *Cholangiocyte secretion of chemokines in experimental biliary atresia.* J Pediatr Surg, 2009. 44(3): p. 500-7.

118. Shivakumar, P., et al., *Neonatal NK cells target the mouse duct epithelium via Nkg2d and drive tissue-specific injury in experimental biliary atresia.* J Clin Invest, 2009. 119(8): p. 2281-90.

119. Santos, N. and Y. Hoshino, *Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine.* Rev Med Virol, 2005. 15(1): p. 29-56.

120. Gray, J., et al., *Rotavirus.* J Pediatr Gastroenterol Nutr, 2008. 46 Suppl 2: p. S24-31.

121. Perjesi, P., et al., *Comparison of effects of some cyclic chalcone analogues on selected mitochondrial functions.* Pharmazie, 2008. 63(12): p. 899-903.

122. Widdowson, M.A., et al., *Global rotavirus surveillance: determining the need and measuring the impact of rotavirus vaccines.* J Infect Dis, 2009. 200 Suppl 1: p. S1-8.

123. Gentsch, J.R., et al., *Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs.* J Infect Dis, 2005. 192 Suppl 1: p. S146-59.

124. Soenarto, Y., et al., *Burden of severe rotavirus diarrhea in indonesia.* J Infect Dis, 2009. 200 Suppl 1: p. S188-94.

125. Rahman, M., et al., *Evolutionary history and global spread of the emerging g12 human rotaviruses.* J Virol, 2007. 81(5): p. 2382-90.

126. Sherchand, J.B., et al., *Molecular epidemiology of rotavirus diarrhea among children aged < 5 years in nepal: predominance of emergent G12*

strains during 2 years. J Infect Dis, 2009. 200 Suppl 1: p. S182-7.

127. Castello, A.A., et al., *Characterization of genotype P[9]G12 rotavirus strains from Argentina: high similarity with Japanese and Korean G12 strains.* J Med Virol, 2009. 81(2): p. 371-81.

128. Cunliffe, N.A., et al., *Serotype g12 rotaviruses, Lilongwe, Malawi.* Emerg Infect Dis, 2009. 15(1): p. 87-90.

129. Leite, J.P., F.A. Carvalho-Costa, and A.C. Linhares, *Group A rotavirus genotypes and the ongoing Brazilian experience: a review.* Mem Inst Oswaldo Cruz, 2008. 103(8): p. 745-53.

130. Samajdar, S., et al., *Increase in prevalence of human group A rotavirus G9 strains as an important VP7 genotype among children in eastern India.* J Clin Virol, 2008. 43(3): p. 334-9.

131. Sharma, S., et al., *Emergence of G12 rotavirus strains in Delhi, India, in 2000 to 2007.* J Clin Microbiol, 2008. 46(4): p. 1343-8.

132. Khamrin, P., et al., *Molecular characterization of VP4, VP6, VP7, NSP4, and NSP5/6 genes identifies an unusual G3P[10] human rotavirus strain.* J Med Virol, 2009. 81(1): p. 176-82.

133. Theamboonlers, A., et al., *Molecular characterization of group A human rotaviruses in Bangkok and Buriram, Thailand during 2004-2006 reveals the predominance of G1P[8], G9P[8] and a rare G3P[19] strain.* Virus Genes, 2008. 36(2): p. 289-98.

134. Zade, J.K., P. Chhabra, and S.D. Chitambar, *Characterization of VP7 and VP4 genes of rotavirus strains: 1990-1994 and 2000-2002.* Epidemiol Infect, 2009. 137(7): p. 936-42.

135. Nakagomi, O. and T. Nakagomi, *Genetic diversity and similarity among mammalian rotaviruses in relation to interspecies transmission of rotavirus.* Arch Virol, 1991. 120(1-2): p. 43-55.

136. Adah, M.I., et al., *Close relationship between G8-serotype bovine and human rotaviruses isolated in Nigeria.* J Clin Microbiol, 2003. 41(8): p. 3945-50.

137. Gurgel, R.Q., et al., *Rotavirus genotypes circulating in Brazil before national rotavirus vaccination: a review.* J Clin Virol, 2008. 43(1): p. 1-8.

138. Steele, A.D. and B. Ivanoff, *Rotavirus strains circulating in Africa during 1996-1999: emergence of G9 strains and P[6] strains.* Vaccine, 2003. 21(5-6): p. 361-7.

139. Rahman, M., et al., *Prevalence of G2P[4] and G12P[6] rotavirus, Bangladesh.* Emerg Infect Dis, 2007. 13(1): p. 18-24.

140. Ramani, S. and G. Kang, *Burden of disease & molecular epidemiology of group A rotavirus infections in India.* Indian J Med Res, 2007. 125(5): p. 619-32.

141. Kim, H.J., et al., *Intestinal and extra-intestinal pathogenicity of a bovine reassortant rotavirus in calves and piglets.* Vet Microbiol, 2011. 152(3-4): p. 291-303.

142. Dennehy, P.H., *Rotavirus vaccines: an overview.* Clin Microbiol Rev, 2008. 21(1): p. 198-208.

143. Hoshino, Y., et al., *Identification of group A rotavirus genes associated with virulence of a porcine rotavirus and host range restriction of a human rotavirus in the gnotobiotic piglet model.* Virology, 1995. 209(1): p. 274-80.

144. Vesikari, T., et al., *Immunogenicity and safety of live oral attenuated bovine rotavirus vaccine strain RIT 4237 in adults and young children.* Lancet, 1983. 2(8354): p. 807-11.

145. Vesikari, T., et al., *Protection of infants against rotavirus diarrhoea by RIT 4237 attenuated bovine rotavirus strain vaccine.* Lancet, 1984. 1(8384): p. 977-81.

146. Vesikari, T., et al., *A comparative trial of rhesus monkey (RRV-1) and bovine (RIT 4237) oral rotavirus vaccines in young children.* J Infect Dis, 1986. 153(5): p. 832-9.

147. Lanata, C.F., et al., *Protection of Peruvian children against rotavirus diarrhea of specific serotypes by one, two, or three doses of the RIT 4237 attenuated bovine rotavirus vaccine.* J Infect Dis, 1989. 159(3): p. 452-9.

148. De Mol, P., et al., *Failure of live, attenuated oral rotavirus vaccine.* Lancet, 1986. 2(8498): p. 108.

149. Hanlon, P., et al., *Trial of an attenuated bovine rotavirus vaccine (RIT 4237) in Gambian infants.* Lancet, 1987. 1(8546): p. 1342-5.

150. Santosham, M., et al., *A field study of the safety and efficacy of two candidate rotavirus vaccines in a Native American population.* J Infect Dis, 1991. 163(3): p. 483-7.

151. Clark, H.F., et al., *Immune response of infants and children to low-passage bovine rotavirus (strain WC3).* Am J Dis Child, 1986. 140(4): p. 350-6.

152. Ciarlet, M., J.M. Hyser, and M.K. Estes, *Sequence analysis of the VP4, VP6, VP7, and NSP4 gene products of the bovine rotavirus WC3.* Virus Genes, 2002. 24(2): p. 107-18.

153. Clark, H.F., et al., *Protective effect of WC3 vaccine against rotavirus diarrhea in infants during a predominantly serotype 1 rotavirus season.* J Infect Dis, 1988. 158(3): p. 570-87.

154. Bernstein, D.I., et al., *Evaluation of WC3 rotavirus vaccine and correlates of protection in healthy infants.* J Infect Dis, 1990. 162(5): p. 1055-62.

155. Georges-Courbot, M.C., et al., *Evaluation of the efficacy of a low-passage bovine rotavirus (strain WC3) vaccine in children in Central Africa.* Res Virol, 1991. 142(5): p. 405-11.

156. Vesikari, T., et al., *Rhesus Rotavirus candidate vaccine. Clinical trial in children vaccinated between 2 and 5 months of age.* Am J Dis Child, 1990. 144(3): p. 285-9.

157. Gothefors, L., et al., *Prolonged efficacy of rhesus rotavirus vaccine in Swedish children.* J Infect Dis, 1989. 159(4): p. 753-7.

158. Flores, J., et al., *Protection against severe rotavirus diarrhoea by rhesus rotavirus vaccine in Venezuelan infants.* Lancet, 1987. 1(8538): p. 882-4.

159. Perez-Schael, I., et al., *Clinical studies of a quadrivalent rotavirus vaccine in Venezuelan infants.* J Clin Microbiol, 1990. 28(3): p. 553-8.

160. Ciarlet, M. and F. Schodel, *Development of a rotavirus vaccine: clinical safety, immunogenicity, and efficacy of the pentavalent rotavirus vaccine, RotaTeq.* Vaccine, 2009. 27 Suppl 6: p. G72-81.

161. Rennels, M.B., et al., *Safety and efficacy of high-dose rhesus-human*

reassortant rotavirus vaccines--report of the National Multicenter Trial. United States Rotavirus Vaccine Efficacy Group. Pediatrics, 1996. 97(1): p. 7-13.

162. Santosham, M., et al., *Efficacy and safety of high-dose rhesus-human reassortant rotavirus vaccine in Native American populations.* J Pediatr, 1997. 131(4): p. 632-8.

163. Joensuu, J., et al., *Randomised placebo-controlled trial of rhesus-human reassortant rotavirus vaccine for prevention of severe rotavirus gastroenteritis.* Lancet, 1997. 350(9086): p. 1205-9.

164. Perez-Schael, I., et al., *Efficacy of the rhesus rotavirus-based quadrivalent vaccine in infants and young children in Venezuela.* N Engl J Med, 1997. 337(17): p. 1181-7.

165. Rippinger, C.M., J.T. Patton, and S.M. McDonald, *Complete genome sequence analysis of candidate human rotavirus vaccine strains RV3 and 116E.* Virology, 2010. 405(1): p. 201-13.

166. Heaton, P.M. and M. Ciarlet, *Vaccines: the pentavalent rotavirus vaccine: discovery to licensure and beyond.* Clin Infect Dis, 2007. 45(12): p. 1618-24.

167. De Vos, B., et al., *A rotavirus vaccine for prophylaxis of infants against rotavirus gastroenteritis.* Pediatr Infect Dis J, 2004. 23(10 Suppl): p. S179-82.

168. Tate, J.E., et al., *Uptake, impact, and effectiveness of rotavirus vaccination in the United States: review of the first 3 years of postlicensure data.* Pediatr Infect Dis J, 2011. 30(1 Suppl): p. S56-60.

169. Yen, C., et al., *Diarrhea-associated hospitalizations among US children over 2 rotavirus seasons after vaccine introduction.* Pediatrics, 2011. 127(1): p. e9-e15.

170. Hull, J.J., et al., *United States rotavirus strain surveillance from 2005 to 2008: genotype prevalence before and after vaccine introduction.* Pediatr Infect Dis J, 2011. 30(1 Suppl): p. S42-7.

171. Lanzieri, T.M., et al., *Impact of rotavirus vaccination on childhood deaths from diarrhea in Brazil.* Int J Infect Dis, 2011. 15(3): p. e206-10.

172. Lopman, B.A., et al., *Infant rotavirus vaccination may provide indirect protection to older children and adults in the United States.* J Infect Dis, 2011. 204(7): p. 980-6.

173. Macartney, K.K., et al., *Decline in rotavirus hospitalisations following introduction of Australia's national rotavirus immunisation programme.* J Paediatr Child Health, 2011. 47(5): p. 266-70.

174. Paulke-Korinek, M., et al., *Herd immunity after two years of the universal mass vaccination program against rotavirus gastroenteritis in Austria.* Vaccine, 2011. 29(15): p. 2791-6.

175. Gray, J., *Rotavirus vaccines: safety, efficacy and public health impact.* J Intern Med, 2011. 270(3): p. 206-14.

176. John, T.J., *Problems with oral poliovaccine in India.* Indian Pediatr, 1972. 9(5): p. 252-6.

177. Grassly, N.C., et al., *New strategies for the elimination of polio from India.* Science, 2006. 314(5802): p. 1150-3.

178. Grassly, N.C., et al., *Mucosal immunity after vaccination with monovalent and trivalent oral poliovirus vaccine in India.* J Infect Dis, 2009. 200(5): p. 794-801.

179. Levine, M.M., et al., *Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR.* Lancet, 1988. 2(8609): p. 467-70.

180. Huang, P.W., et al., *Sialic acid independent human rotaviruses may recognize histo-blood group antigens as receptors.* 30th Annual Meeting, American Society for Virology, 2011: p. 224-225.

181. Roth, D.E., S.A. Richard, and R.E. Black, *Zinc supplementation for the prevention of acute lower respiratory infection in children in developing countries: meta-analysis and meta-regression of randomized trials.* Int J Epidemiol, 2010. 39(3): p. 795-808.

182. Cucchiara, S., et al., *New therapeutic approach in the management of intestinal disease: probiotics in intestinal disease in paediatric age.* Dig Liver Dis, 2002. 34 Suppl 2: p. S44-7.

183. Spiekermann, G.M. and W.A. Walker, *Oral tolerance and its role in*

clinical disease. J Pediatr Gastroenterol Nutr, 2001. 32(3): p. 237-55.

184. Bashir, M.E., et al., *An enteric helminth infection protects against an allergic response to dietary antigen.* J Immunol, 2002. 169(6): p. 3284-92.

185. Bashir, M.E., et al., *Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy.* J Immunol, 2004. 172(11): p. 6978-87.

186. Sudo, N., et al., *The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction.* J Immunol, 1997. 159(4): p. 1739-45.

187. Braat, H., et al., *Lactobacillus rhamnosus induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function.* Am J Clin Nutr, 2004. 80(6): p. 1618-25.

188. Cunliffe, N.A., et al., *Effect of concomitant HIV infection on presentation and outcome of rotavirus gastroenteritis in Malawian children.* Lancet, 2001. 358(9281): p. 550-5.

189. Kang, G., et al., *Epidemiological profile of rotaviral infection in India: challenges for the 21st century.* J Infect Dis, 2005. 192 Suppl 1: p. S120-6.

190. Bresee, J.S., et al., *Update on rotavirus vaccines.* Pediatr Infect Dis J, 2005. 24(11): p. 947-52.

191. Menard, O., et al., *Gnotobiotic mouse immune response induced by Bifidobacterium sp. strains isolated from infants.* Appl Environ Microbiol, 2008. 74(3): p. 660-6.

192. Mohan, R., et al., *Effects of Bifidobacterium lactis Bb12 supplementation on body weight, fecal pH, acetate, lactate, calprotectin, and IgA in preterm infants.* Pediatr Res, 2008. 64(4): p. 418-22.

193. Liu, F., et al., *Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics.* Viral Immunol, 2010. 23(2): p. 135-49.

194. Wagner, R.D., et al., *Variable biotherapeutic effects of Lactobacillus acidophilus isolates on orogastric and systemic candidiasis in immunodeficient mice.* Rev Iberoam Micol, 1998. 15(4): p. 271-6.

195. So, J.S., et al., *Lactobacillus casei potentiates induction of oral tolerance in experimental arthritis.* Mol Immunol, 2008. 46(1): p. 172-80.

196. So, J.S., et al., *Lactobacillus casei suppresses experimental arthritis by down-regulating T helper 1 effector functions.* Mol Immunol, 2008. 45(9): p. 2690-9.

197. Van Overtvelt, L., et al., *Lactic acid bacteria as adjuvants for sublingual allergy vaccines.* Vaccine, 2010. 28(17): p. 2986-92.

198. Farnworth, E.R., *The evidence to support health claims for probiotics.* J Nutr, 2008. 138(6): p. 1250S-4S.

199. Fang, S.B., et al., *Dose-dependent effect of Lactobacillus rhamnosus on quantitative reduction of faecal rotavirus shedding in children.* J Trop Pediatr, 2009. 55(5): p. 297-301.

200. Guandalini, S., *Probiotics for children with diarrhea: an update.* J Clin Gastroenterol, 2008. 42 Suppl 2: p. S53-7.

201. Gill, H.S., et al., *Enhancement of immunity in the elderly by dietary supplementation with the probiotic Bifidobacterium lactis HN019.* Am J Clin Nutr, 2001. 74(6): p. 833-9.

202. Chiang, B.L., et al., *Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (Bifidobacterium lactis HN019): optimization and definition of cellular immune responses.* Eur J Clin Nutr, 2000. 54(11): p. 849-55.

203. Konstantinov, S.R., et al., *S layer protein A of Lactobacillus acidophilus NCFM regulates immature dendritic cell and T cell functions.* Proc Natl Acad Sci U S A, 2008. 105(49): p. 19474-9.

204. Oelschlaeger, T.A., *Mechanisms of probiotic actions - A review.* Int J Med Microbiol, 2010. 300(1): p. 57-62.

205. Hooper, L.V., et al., *Molecular analysis of commensal host-microbial relationships in the intestine.* Science, 2001. 291(5505): p. 881-4.

206. Nanthakumar, N.N., et al., *The role of indigenous microflora in the development of murine intestinal fucosyl- and sialyltransferases.* FASEB J, 2003. 17(1): p. 44-6.

207. Stappenbeck, T.S., L.V. Hooper, and J.I. Gordon, *Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells.* Proc Natl Acad Sci U S A, 2002. 99(24): p. 15451-5.

208. Fitzpatrick, L.R., et al., *In vitro and in vivo effects of the probiotic Escherichia coli strain M-17: immunomodulation and attenuation of murine colitis.* Br J Nutr, 2008. 100(3): p. 530-41.

209. Mack, D.R., et al., *Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro.* Gut, 2003. 52(6): p. 827-33.

210. Mack, D.R., et al., *Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression.* Am J Physiol, 1999. 276(4 Pt 1): p. G941-50.

211. Nanda Kumar, N.S., et al., *Probiotic administration alters the gut flora and attenuates colitis in mice administered dextran sodium sulfate.* J Gastroenterol Hepatol, 2008. 23(12): p. 1834-9.

212. Otte, J.M. and D.K. Podolsky, *Functional modulation of enterocytes by gram-positive and gram-negative microorganisms.* Am J Physiol Gastrointest Liver Physiol, 2004. 286(4): p. G613-26.

213. Parassol, N., et al., *Lactobacillus casei DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic Escherichia coli-infected T84 cells.* Res Microbiol, 2005. 156(2): p. 256-62.

214. Resta-Lenert, S. and K.E. Barrett, *Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC).* Gut, 2003. 52(7): p. 988-97.

215. Sartor, R.B., *Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis.* Nat Clin Pract Gastroenterol Hepatol, 2006. 3(7): p. 390-407.

216. Ukena, S.N., et al., *Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity.* PLoS One, 2007. 2(12): p. e1308.

217. Vetrano, S., et al., *Colifagina, a novel preparation of 8 lysed bacteria ameliorates experimental colitis.* Int J Immunopathol Pharmacol, 2008. 21(2): p. 401-7.

218. Yan, F., et al., *Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth.* Gastroenterology, 2007. 132(2): p. 562-75.

219. Yan, F. and D.B. Polk, *Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells.* J Biol Chem, 2002. 277(52): p. 50959-65.

220. Zyrek, A.A., et al., *Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair.* Cell Microbiol, 2007. 9(3): p. 804-16.

221. Chung, Y.W., et al., *Lactobacillus casei prevents the development of dextran sulphate sodium-induced colitis in Toll-like receptor 4 mutant mice.* Clin Exp Immunol, 2008. 151(1): p. 182-9.

222. Lee, H.S., et al., *Lactic acid bacteria inhibit proinflammatory cytokine expression and bacterial glycosaminoglycan degradation activity in dextran sulfate sodium-induced colitic mice.* Int Immunopharmacol, 2008. 8(4): p. 574-80.

223. Pena, J.A. and J. Versalovic, *Lactobacillus rhamnosus GG decreases TNF-alpha production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism.* Cell Microbiol, 2003. 5(4): p. 277-85.

224. Schultz, M., et al., *Immunomodulatory consequences of oral administration of Lactobacillus rhamnosus strain GG in healthy volunteers.* J Dairy Res, 2003. 70(2): p. 165-73.

225. Borruel, N., et al., *Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria.* Gut, 2002. 51(5): p. 659-64.

226. Haller, D., et al., *IL-10 producing CD14low monocytes inhibit lymphocyte-dependent activation of intestinal epithelial cells by commensal bacteria.* Microbiol Immunol, 2002. 46(3): p. 195-205.

227. Saksena, S., et al., *Upregulation of P-glycoprotein by Probiotics in Intestinal Epithelial Cells and in the Dextran Sulfate Sodium Model of Colitis in Mice.* Am J Physiol Gastrointest Liver Physiol, 2011.

228. Mukai, T., et al., *Inhibition of binding of Helicobacter pylori to the glycolipid receptors by probiotic Lactobacillus reuteri.* FEMS Immunol Med Microbiol, 2002. 32(2): p. 105-10.

229. Xu, J., et al., *A genomic view of the human-Bacteroides thetaiotaomicron symbiosis.* Science, 2003. 299(5615): p. 2074-6.

230. Freitas, M., et al., *Microbial-host interactions specifically control the glycosylation pattern in intestinal mouse mucosa.* Histochem Cell Biol, 2002. 118(2): p. 149-61.

231. Alakomi, H.L., et al., *Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane.* Appl Environ Microbiol, 2000. 66(5): p. 2001-5.

232. De Keersmaecker, S.C., et al., *Strong antimicrobial activity of Lactobacillus rhamnosus GG against Salmonella typhimurium is due to accumulation of lactic acid.* FEMS Microbiol Lett, 2006. 259(1): p. 89-96.

233. Makras, L., et al., *Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards Salmonella enterica serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds.* Res Microbiol, 2006. 157(3): p. 241-7.

234. Wehkamp, J., et al., *NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium.* Infect Immun, 2004. 72(10): p. 5750-8.

235. Schlee, M., et al., *Induction of human beta-defensin 2 by the probiotic Escherichia coli Nissle 1917 is mediated through flagellin.* Infect Immun, 2007. 75(5): p. 2399-407.

236. Corr, S.C., et al., *Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118.* Proc Natl Acad Sci U S A, 2007. 104(18): p. 7617-21.

237. Cleusix, V., et al., *Glycerol induces reuterin production and decreases Escherichia coli population in an in vitro model of colonic fermentation with immobilized human feces.* FEMS Microbiol Ecol, 2008. 63(1): p. 56-64.

238. Heikkila, M.P. and P.E. Saris, *Inhibition of Staphylococcus aureus by the*

commensal bacteria of human milk. J Appl Microbiol, 2003. 95(3): p. 471-8.

239. Hooper, L.V., et al., *Angiogenins: a new class of microbicidal proteins involved in innate immunity.* Nat Immunol, 2003. 4(3): p. 269-73.

240. Sherman, P.M., et al., *Probiotics reduce enterohemorrhagic Escherichia coli O157:H7- and enteropathogenic E. coli O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements.* Infect Immun, 2005. 73(8): p. 5183-8.

241. Collado, M.C., J. Meriluoto, and S. Salminen, *Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus.* Lett Appl Microbiol, 2007. 45(4): p. 454-60.

242. Lin, C.K., et al., *Lactobacillus acidophilus LAP5 able to inhibit the Salmonella choleraesuis invasion to the human Caco-2 epithelial cell.* Anaerobe, 2008. 14(5): p. 251-5.

243. Fuller, R., *Probiotics in man and animals.* J Appl Bacteriol, 1989. 66(5): p. 365-78.

244. Spanhaak, S., R. Havenaar, and G. Schaafsma, *The effect of consumption of milk fermented by Lactobacillus casei strain Shirota on the intestinal microflora and immune parameters in humans.* Eur J Clin Nutr, 1998. 52(12): p. 899-907.

245. Yamano, T., et al., *Improvement of the human intestinal flora by ingestion of the probiotic strain Lactobacillus johnsonii La1.* Br J Nutr, 2006. 95(2): p. 303-12.

246. Ishikawa, H., et al., *Randomized controlled trial of the effect of bifidobacteria-fermented milk on ulcerative colitis.* J Am Coll Nutr, 2003. 22(1): p. 56-63.

247. Kuhbacher, T., et al., *Bacterial and fungal microbiota in relation to probiotic therapy (VSL#3) in pouchitis.* Gut, 2006. 55(6): p. 833-41.

248. Gaudier, E., et al., *The VSL# 3 probiotic mixture modifies microflora but does not heal chronic dextran-sodium sulfate-induced colitis or reinforce the mucus barrier in mice.* J Nutr, 2005. 135(12): p. 2753-61.

249. Boudeau, J., et al., *Inhibitory effect of probiotic Escherichia coli strain*

Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive E. coli strains isolated from patients with Crohn's disease. Aliment Pharmacol Ther, 2003. 18(1): p. 45-56.

250. Altenhoefer, A., et al., *The probiotic Escherichia coli strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens.* FEMS Immunol Med Microbiol, 2004. 40(3): p. 223-9.

251. Mukai, T., et al., *Binding of Bifidobacterium bifidum and Lactobacillus reuteri to the carbohydrate moieties of intestinal glycolipids recognized by peanut agglutinin.* Int J Food Microbiol, 2004. 90(3): p. 357-62.

252. Sun, J., et al., *Factors involved in binding of Lactobacillus plantarum Lp6 to rat small intestinal mucus.* Lett Appl Microbiol, 2007. 44(1): p. 79-85.

253. Ingrassia, I., A. Leplingard, and A. Darfeuille-Michaud, *Lactobacillus casei DN-114 001 inhibits the ability of adherent-invasive Escherichia coli isolated from Crohn's disease patients to adhere to and to invade intestinal epithelial cells.* Appl Environ Microbiol, 2005. 71(6): p. 2880-7.

254. Cario, E., *Bacterial interactions with cells of the intestinal mucosa: Toll-like receptors and NOD2.* Gut, 2005. 54(8): p. 1182-93.

255. Rachmilewitz, D., et al., *Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis.* Gastroenterology, 2004. 126(2): p. 520-8.

256. Grabig, A., et al., *Escherichia coli strain Nissle 1917 ameliorates experimental colitis via toll-like receptor 2- and toll-like receptor 4-dependent pathways.* Infect Immun, 2006. 74(7): p. 4075-82.

257. Kamada, N., et al., *Nonpathogenic Escherichia coli strain Nissle 1917 inhibits signal transduction in intestinal epithelial cells.* Infect Immun, 2008. 76(1): p. 214-20.

258. Tobita, K., H. Yanaka, and H. Otani, *Heat-treated Lactobacillus crispatus KT strains reduce allergic symptoms in mice.* J Agric Food Chem, 2009. 57(12): p. 5586-90.

259. Wen, K., et al., *Toll-like receptor and innate cytokine responses induced by lactobacilli colonization and human rotavirus infection in gnotobiotic pigs.* Vet Immunol Immunopathol, 2009. 127(3-4): p. 304-15.

260. Smits, H.H., et al., *Commensal Gram-negative bacteria prime human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development.* Eur J Immunol, 2004. 34(5): p. 1371-80.

261. Karlsson, H., et al., *Pattern of cytokine responses to gram-positive and gram-negative commensal bacteria is profoundly changed when monocytes differentiate into dendritic cells.* Infect Immun, 2004. 72(5): p. 2671-8.

262. Braat, H., et al., *Dichotomy between Lactobacillus rhamnosus and Klebsiella pneumoniae on dendritic cell phenotype and function.* J Mol Med, 2004. 82(3): p. 197-205.

263. Becker, C., et al., *Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells.* J Clin Invest, 2003. 112(5): p. 693-706.

264. Drakes, M., T. Blanchard, and S. Czinn, *Bacterial probiotic modulation of dendritic cells.* Infect Immun, 2004. 72(6): p. 3299-309.

265. Kwon, H.K., et al., *Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders.* Proc Natl Acad Sci U S A, 2010. 107(5): p. 2159-64.

266. Hart, A.L., et al., *Modulation of human dendritic cell phenotype and function by probiotic bacteria.* Gut, 2004. 53(11): p. 1602-9.

267. Smits, H.H., et al., *Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin.* J Allergy Clin Immunol, 2005. 115(6): p. 1260-7.

268. Zhang, W., et al., *Influence of probiotic Lactobacilli colonization on neonatal B cell responses in a gnotobiotic pig model of human rotavirus infection and disease.* Vet Immunol Immunopathol, 2008. 122(1-2): p. 175-81.

269. Salzman, N.H., et al., *Enteric defensins are essential regulators of intestinal microbial ecology.* Nat Immunol, 2010. 11(1): p. 76-83.

270. Collado, M.C., L. Grzeskowiak, and S. Salminen, *Probiotic strains and their combination inhibit in vitro adhesion of pathogens to pig intestinal mucosa.* Curr Microbiol, 2007. 55(3): p. 260-5.

271. Botes, M., et al., *Adhesion of the probiotic strains Enterococcus mundtii ST4SA and Lactobacillus plantarum 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments.* Arch Microbiol, 2008. 190(5): p. 573-84.

272. Golowczyc, M.A., et al., *Protective action of Lactobacillus kefir carrying S-layer protein against Salmonella enterica serovar Enteritidis.* Int J Food Microbiol, 2007. 118(3): p. 264-73.

273. Asahara, T., et al., *Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157:H7.* Infect Immun, 2004. 72(4): p. 2240-7.

274. Carey, C.M., et al., *The effect of probiotics and organic acids on Shiga-toxin 2 gene expression in enterohemorrhagic Escherichia coli O157:H7.* J Microbiol Methods, 2008. 73(2): p. 125-32.

275. Weinberg, E.D., *The Lactobacillus anomaly: total iron abstinence.* Perspect Biol Med, 1997. 40(4): p. 578-83.

276. Elli, M., et al., *Iron requirement of Lactobacillus spp. in completely chemically defined growth media.* J Appl Microbiol, 2000. 88(4): p. 695-703.

277. Grozdanov, L., et al., *Analysis of the genome structure of the nonpathogenic probiotic Escherichia coli strain Nissle 1917.* J Bacteriol, 2004. 186(16): p. 5432-41.

278. Grosse, C., et al., *A new ferrous iron-uptake transporter, EfeU (YcdN), from Escherichia coli.* Mol Microbiol, 2006. 62(1): p. 120-31.

279. Szajewska, H., et al., *Efficacy of Lactobacillus GG in prevention of nosocomial diarrhea in infants.* J Pediatr, 2001. 138(3): p. 361-5.

280. Mastretta, E., et al., *Effect of Lactobacillus GG and breast-feeding in the prevention of rotavirus nosocomial infection.* J Pediatr Gastroenterol Nutr, 2002. 35(4): p. 527-31.

281. Saavedra, J.M., et al., *Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhoea and shedding of rotavirus.* Lancet, 1994. 344(8929): p. 1046-9.

282. Chouraqui, J.P., L.D. Van Egroo, and M.C. Fichot, *Acidified milk formula supplemented with bifidobacterium lactis: impact on infant diarrhea in residential care settings.* J Pediatr Gastroenterol Nutr, 2004. 38(3): p. 288-92.

283. Oberhelman, R.A., et al., *A placebo-controlled trial of Lactobacillus GG to prevent diarrhea in undernourished Peruvian children.* J Pediatr, 1999. 134(1): p. 15-20.

284. Hatakka, K., et al., *Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial.* BMJ, 2001. 322(7298): p. 1327.

285. Thibault, H., C. Aubert-Jacquin, and O. Goulet, *Effects of long-term consumption of a fermented infant formula (with Bifidobacterium breve c50 and Streptococcus thermophilus 065) on acute diarrhea in healthy infants.* J Pediatr Gastroenterol Nutr, 2004. 39(2): p. 147-52.

286. Pedone, C.A., et al., *Multicentric study of the effect of milk fermented by Lactobacillus casei on the incidence of diarrhoea.* Int J Clin Pract, 2000. 54(9): p. 568-71.

287. Saavedra, J.M., et al., *Long-term consumption of infant formulas containing live probiotic bacteria: tolerance and safety.* Am J Clin Nutr, 2004. 79(2): p. 261-7.

288. Weizman, Z., G. Asli, and A. Alsheikh, *Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents.* Pediatrics, 2005. 115(1): p. 5-9.

289. Shornikova, A.V., et al., *Lactobacillus reuteri as a therapeutic agent in acute diarrhea in young children.* J Pediatr Gastroenterol Nutr, 1997. 24(4): p. 399-404.

290. Perdigon, G., R. Fuller, and R. Raya, *Lactic acid bacteria and their effect on the immune system.* Curr Issues Intest Microbiol, 2001. 2(1): p. 27-42.

291. Wells, J.M. and A. Mercenier, *Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria.* Nat Rev Microbiol, 2008. 6(5): p. 349-62.

292. Torii, A., et al., *Lactobacillus Acidophilus strain L-92 regulates the production of Th1 cytokine as well as Th2 cytokines.* Allergol Int, 2007.

56(3): p. 293-301.

293. Galdeano, C.M. and G. Perdigon, *The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity.* Clin Vaccine Immunol, 2006. 13(2): p. 219-26.

294. Rosenfeldt, V., et al., *Effect of probiotic Lactobacillus strains in children with atopic dermatitis.* J Allergy Clin Immunol, 2003. 111(2): p. 389-95.

295. Marschan, E., et al., *Probiotics in infancy induce protective immune profiles that are characteristic for chronic low-grade inflammation.* Clin Exp Allergy, 2008. 38(4): p. 611-8.

296. de Vrese, M., et al., *Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination.* Eur J Nutr, 2005. 44(7): p. 406-13.

297. Link-Amster, H., et al., *Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake.* FEMS Immunol Med Microbiol, 1994. 10(1): p. 55-63.

298. Repa, A., et al., *Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy.* Vaccine, 2003. 22(1): p. 87-95.

299. Sashihara, T., N. Sueki, and S. Ikegami, *An analysis of the effectiveness of heat-killed lactic acid bacteria in alleviating allergic diseases.* J Dairy Sci, 2006. 89(8): p. 2846-55.

300. Kekkonen, R.A., et al., *Probiotic Leuconostoc mesenteroides ssp. cremoris and Streptococcus thermophilus induce IL-12 and IFN-gamma production.* World J Gastroenterol, 2008. 14(8): p. 1192-203.

301. van Furth, R. and M.M. Diesselhoff-den Dulk, *Dual origin of mouse spleen macrophages.* J Exp Med, 1984. 160(5): p. 1273-83.

302. van Furth, R. and Z.A. Cohn, *The origin and kinetics of mononuclear phagocytes.* J Exp Med, 1968. 128(3): p. 415-35.

303. Crofton, R.W., M.M. Diesselhoff-den Dulk, and R. van Furth, *The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state.* J Exp Med, 1978. 148(1): p. 1-17.

304. Matute-Bello, G., et al., *Optimal timing to repopulation of resident alveolar macrophages with donor cells following total body irradiation and bone marrow transplantation in mice.* J Immunol Methods, 2004. 292(1-2): p. 25-34.

305. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* J Exp Med, 2007. 204(12): p. 3037-47.

306. Arnold, L., et al., *Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis.* J Exp Med, 2007. 204(5): p. 1057-69.

307. Sawyer, R.T., P.H. Strausbauch, and A. Volkman, *Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89.* Lab Invest, 1982. 46(2): p. 165-70.

308. Tarling, J.D., H.S. Lin, and S. Hsu, *Self-renewal of pulmonary alveolar macrophages: evidence from radiation chimera studies.* J Leukoc Biol, 1987. 42(5): p. 443-6.

309. Landsman, L., C. Varol, and S. Jung, *Distinct differentiation potential of blood monocyte subsets in the lung.* J Immunol, 2007. 178(4): p. 2000-7.

310. Wijffels, J.F., et al., *Macrophage subpopulations in the mouse spleen renewed by local proliferation.* Immunobiology, 1994. 191(1): p. 52-64.

311. Mildner, A., et al., *Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions.* Nat Neurosci, 2007. 10(12): p. 1544-53.

312. Smith, A.M., et al., *Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease.* J Exp Med, 2009. 206(9): p. 1883-97.

313. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. 8(12): p. 958-69.

314. Kreider, T., et al., *Alternatively activated macrophages in helminth infections.* Curr Opin Immunol, 2007. 19(4): p. 448-53.

315. Gerber, J.S. and D.M. Mosser, *Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors.* J Immunol, 2001. 166(11):

p. 6861-8.

316. Zhang, W., et al., *Lactic acid bacterial colonization and human rotavirus infection influence distribution and frequencies of monocytes/macrophages and dendritic cells in neonatal gnotobiotic pigs.* Vet Immunol Immunopathol, 2008. 121(3-4): p. 222-31.

317. Mohanty, S.K., et al., *Macrophages are targeted by rotavirus in experimental biliary atresia and induce neutrophil chemotaxis by Mip2/Cxcl2.* Pediatr Res, 2010. 67(4): p. 345-51.

318. Fenaux, M., et al., *Extraintestinal spread and replication of a homologous EC rotavirus strain and a heterologous rhesus rotavirus in BALB/c mice.* J Virol, 2006. 80(11): p. 5219-32.

319. Crawford, S.E., et al., *Rotavirus viremia and extraintestinal viral infection in the neonatal rat model.* J Virol, 2006. 80(10): p. 4820-32.

320. Siegal, F.P., et al., *The nature of the principal type 1 interferon-producing cells in human blood.* Science, 1999. 284(5421): p. 1835-7.

321. Liu, Y.J., *IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors.* Annu Rev Immunol, 2005. 23: p. 275-306.

322. Jego, G., et al., *Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6.* Immunity, 2003. 19(2): p. 225-34.

323. Fonteneau, J.F., et al., *Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity.* Blood, 2003. 101(9): p. 3520-6.

324. Di Pucchio, T., et al., *Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I.* Nat Immunol, 2008. 9(5): p. 551-7.

325. Hawiger, D., et al., *Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo.* J Exp Med, 2001. 194(6): p. 769-79.

326. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig, *Tolerogenic dendritic cells.* Annu Rev Immunol, 2003. 21: p. 685-711.

327. Volkmann, A., T. Zal, and B. Stockinger, *Antigen-presenting cells in the thymus that can negatively select MHC class II-restricted T cells recognizing a circulating self antigen.* J Immunol, 1997. 158(2): p. 693-706.

328. Zal, T., A. Volkmann, and B. Stockinger, *Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen.* J Exp Med, 1994. 180(6): p. 2089-99.

329. Fairchild, P.J. and J.M. Austyn, *Thymic dendritic cells: phenotype and function.* Int Rev Immunol, 1990. 6(2-3): p. 187-96.

330. Hawiger, D., et al., *Immunological unresponsiveness characterized by increased expression of CD5 on peripheral T cells induced by dendritic cells in vivo.* Immunity, 2004. 20(6): p. 695-705.

331. Jonuleit, H., et al., *Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells.* J Exp Med, 2000. 192(9): p. 1213-22.

332. Wing, K. and S. Sakaguchi, *Regulatory T cells exert checks and balances on self tolerance and autoimmunity.* Nat Immunol, 2010. 11(1): p. 7-13.

333. Zheng, Y., et al., *Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate.* Nature, 2010. 463(7282): p. 808-12.

334. Akbari, O., R.H. DeKruyff, and D.T. Umetsu, *Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen.* Nat Immunol, 2001. 2(8): p. 725-31.

335. Finkelman, F.D., et al., *Dendritic cells can present antigen in vivo in a tolerogenic or immunogenic fashion.* J Immunol, 1996. 157(4): p. 1406-14.

336. Brimnes, M.K., et al., *Influenza virus-induced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein.* J Exp Med, 2003. 198(1): p. 133-44.

337. Jego, G., et al., *Dendritic cells control B cell growth and differentiation.* Curr Dir Autoimmun, 2005. 8: p. 124-39.

338. Qi, H., et al., *Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells.* Science, 2006. 312(5780): p. 1672-6.

339. Zhong, G., C. Reis e Sousa, and R.N. Germain, *Antigen-unspecific B cells and lymphoid dendritic cells both show extensive surface expression of processed antigen-major histocompatibility complex class II complexes after soluble protein exposure in vivo or in vitro.* J Exp Med, 1997. 186(5): p. 673-82.

340. Wykes, M., et al., *Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response.* J Immunol, 1998. 161(3): p. 1313-9.

341. Bergtold, A., et al., *Cell surface recycling of internalized antigen permits dendritic cell priming of B cells.* Immunity, 2005. 23(5): p. 503-14.

342. Batista, F.D. and N.E. Harwood, *The who, how and where of antigen presentation to B cells.* Nat Rev Immunol, 2009. 9(1): p. 15-27.

343. Gonzalez, A.M., et al., *Innate immune responses to human rotavirus in the neonatal gnotobiotic piglet disease model.* Immunology, 2010. 131(2): p. 242-56.

344. Deal, E.M., et al., *Rotavirus structural proteins and dsRNA are required for the human primary plasmacytoid dendritic cell IFNalpha response.* PLoS Pathog, 2010. 6(6): p. e1000931.

345. Lopez-Guerrero, D.V., et al., *Rotavirus infection activates dendritic cells from Peyer's patches in adult mice.* J Virol, 2010. 84(4): p. 1856-66.

346. Godfrey, D.I., et al., *NKT cells: what's in a name?* Nat Rev Immunol, 2004. 4(3): p. 231-7.

347. Sallusto, F. and A. Lanzavecchia, *Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity.* Eur J Immunol, 2009. 39(8): p. 2076-82.

348. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity.* J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S33-40.

349. Unutmaz, D., *RORC2: the master of human Th17 cell programming.* Eur J Immunol, 2009. 39(6): p. 1452-5.

350. Hong, T., et al., *A mathematical model for the reciprocal differentiation of T helper 17 cells and induced regulatory T cells.* PLoS Comput Biol, 2011. 7(7): p. e1002122.

351. Lochner, M., et al., *In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells.* J Exp Med, 2008. 205(6): p. 1381-93.

352. Pescovitz, M.D., et al., *Summary of workshop findings for antibodies reacting with porcine T-cells and activation antigens: results from the Second International Swine CD Workshop.* Vet Immunol Immunopathol, 1998. 60(3-4): p. 251-60.

353. Yang, H. and R.M. Parkhouse, *Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues.* Immunology, 1996. 89(1): p. 76-83.

354. Saalmuller, A., T. Werner, and V. Fachinger, *T-helper cells from naive to committed.* Vet Immunol Immunopathol, 2002. 87(3-4): p. 137-45.

355. Yang, H. and R.M. Parkhouse, *Differential expression of CD8 epitopes amongst porcine CD8-positive functional lymphocyte subsets.* Immunology, 1997. 92(1): p. 45-52.

356. Zuckermann, F.A. and R.J. Husmann, *Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells.* Immunology, 1996. 87(3): p. 500-12.

357. Saalmuller, A., F. Weiland, and M.J. Reddehase, *Resting porcine T lymphocytes expressing class II major histocompatibility antigen.* Immunobiology, 1991. 183(1-2): p. 102-14.

358. Charerntantanakul, W. and J.A. Roth, *Biology of porcine T lymphocytes.* Anim Health Res Rev, 2006. 7(1-2): p. 81-96.

359. Ward, R.L., *Rotavirus vaccines: how they work or don't work.* Expert Rev Mol Med, 2008. 10: p. e5.

360. Franco, M.A., C. Tin, and H.B. Greenberg, *CD8+ T cells can mediate almost complete short-term and partial long-term immunity to rotavirus in mice.* J Virol, 1997. 71(5): p. 4165-70.

361. Yuan, L., et al., *Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs.* Vaccine, 2008. 26(26): p. 3322-31.

362. Chien, Y.H. and Y. Konigshofer, *Antigen recognition by gammadelta T cells.* Immunol Rev, 2007. 215: p. 46-58.

363. Grant, E.P., et al., *Fine specificity of TCR complementarity-determining region residues and lipid antigen hydrophilic moieties in the recognition of a CD1-lipid complex.* J Immunol, 2002. 168(8): p. 3933-40.

364. Wu, J., V. Groh, and T. Spies, *T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells.* J Immunol, 2002. 169(3): p. 1236-40.

365. Prinz, I., et al., *Visualization of the earliest steps of gammadelta T cell development in the adult thymus.* Nat Immunol, 2006. 7(9): p. 995-1003.

366. Jensen, K.D., et al., *Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma.* Immunity, 2008. 29(1): p. 90-100.

367. Ley, K., E. Smith, and M.A. Stark, *IL-17A-producing neutrophil-regulatory Tn lymphocytes.* Immunol Res, 2006. 34(3): p. 229-42.

368. Stark, M.A., et al., *Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17.* Immunity, 2005. 22(3): p. 285-94.

369. O'Brien, R.L., C.L. Roark, and W.K. Born, *IL-17-producing gammadelta T cells.* Eur J Immunol, 2009. 39(3): p. 662-6.

370. Azuara, V., et al., *A novel subset of adult gamma delta thymocytes that secretes a distinct pattern of cytokines and expresses a very restricted T cell receptor repertoire.* Eur J Immunol, 1997. 27(2): p. 544-53.

371. Gerber, D.J., et al., *IL-4-producing gamma delta T cells that express a very restricted TCR repertoire are preferentially localized in liver and spleen.* J Immunol, 1999. 163(6): p. 3076-82.

372. Stepanova, H., et al., *Early postnatal development of the immune system in*

piglets: the redistribution of T lymphocyte subsets. Cell Immunol, 2007. 249(2): p. 73-9.

373. Gerner, W., T. Kaser, and A. Saalmuller, *Porcine T lymphocytes and NK cells--an update.* Dev Comp Immunol, 2009. 33(3): p. 310-20.

374. Saalmuller, A., W. Hirt, and M.J. Reddehase, *Porcine gamma/delta T lymphocyte subsets differing in their propensity to home to lymphoid tissue.* Eur J Immunol, 1990. 20(10): p. 2343-6.

375. Takamatsu, H.H., et al., *Porcine gammadelta T cells: possible roles on the innate and adaptive immune responses following virus infection.* Vet Immunol Immunopathol, 2006. 112(1-2): p. 49-61.

376. Takamatsu, H.H., M.S. Denyer, and T.E. Wileman, *A sub-population of circulating porcine gammadelta T cells can act as professional antigen presenting cells.* Vet Immunol Immunopathol, 2002. 87(3-4): p. 223-4.

377. Olin, M.R., et al., *Gammadelta lymphocyte response to porcine reproductive and respiratory syndrome virus.* Viral Immunol, 2005. 18(3): p. 490-9.

378. Hedges, J.F., et al., *Differential mRNA expression in circulating gammadelta T lymphocyte subsets defines unique tissue-specific functions.* J Leukoc Biol, 2003. 73(2): p. 306-14.

379. Hayday, A. and R. Tigelaar, *Immunoregulation in the tissues by gammadelta T cells.* Nat Rev Immunol, 2003. 3(3): p. 233-42.

380. Tramonti, D., et al., *Evidence for the opposing roles of different gamma delta T cell subsets in macrophage homeostasis.* Eur J Immunol, 2006. 36(7): p. 1729-38.

381. Ziegler, H.K., *The role of gamma/delta T cells in immunity to infection and regulation of inflammation.* Immunol Res, 2004. 29(1-3): p. 293-302.

382. Parsons, K.R., et al., *Number and distribution of T lymphocytes in the small intestinal mucosa of calves inoculated with rotavirus.* Vet Immunol Immunopathol, 1993. 39(4): p. 355-64.

383. Oldham, G., et al., *In vivo role of lymphocyte subpopulations in the control of virus excretion and mucosal antibody responses of cattle infected with rotavirus.* J Virol, 1993. 67(8): p. 5012-9.

384. Franco, M.A. and H.B. Greenberg, *Immunity to rotavirus in T cell deficient mice.* Virology, 1997. 238(2): p. 169-79.

385. Sakaguchi, S., et al., *Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease.* Immunol Rev, 2006. 212: p. 8-27.

386. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.* J Exp Med, 2001. 194(5): p. 629-44.

387. Curotto de Lafaille, M.A. and J.J. Lafaille, *Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor?* Immunity, 2009. 30(5): p. 626-35.

388. Kim, B., et al., *The influence of CD4+ CD25+ Foxp3+ regulatory T cells on the immune response to rotavirus infection.* Vaccine, 2008. 26(44): p. 5601-11.

389. Mesa, M.C., et al., *A TGF-beta mediated regulatory mechanism modulates the T cell immune response to rotavirus in adults but not in children.* Virology, 2010. 399(1): p. 77-86.

390. Miethke, A.G., et al., *Post-natal paucity of regulatory T cells and control of NK cell activation in experimental biliary atresia.* J Hepatol, 2010. 52(5): p. 718-26.

391. Durandy, A., et al., *Pathophysiology of B-cell intrinsic immunoglobulin class switch recombination deficiencies.* Adv Immunol, 2007. 94: p. 275-306.

392. Tangye, S.G. and D.M. Tarlinton, *Memory B cells: effectors of long-lived immune responses.* Eur J Immunol, 2009. 39(8): p. 2065-75.

393. Fehr, T., et al., *T cell-independent type I antibody response against B cell epitopes expressed repetitively on recombinant virus particles.* Proc Natl Acad Sci U S A, 1998. 95(16): p. 9477-81.

394. Kendall, E.A., et al., *Development of immunoglobulin M memory to both a T-cell-independent and a T-cell-dependent antigen following infection with Vibrio cholerae O1 in Bangladesh.* Infect Immun, 2010. 78(1): p. 253-9.

395. Sinkora, M. and J.E. Butler, *The ontogeny of the porcine immune system.* Dev Comp Immunol, 2009. 33(3): p. 273-83.

396. Porter, P., *Immunoglobulins in bovine mammary secretions. Quantitative changes in early lactation and absorption by the neonatal calf.* Immunology, 1972. 23(2): p. 225-38.

397. Porter, P. and D.E. Noakes, *Immunoglobulins in sow mammary secretions throughout lactation and their significance as anti-(Escherichia coli) antibodies to the young pig.* Biochem J, 1969. 113(2): p. 6P-7P.

398. Lecce, J.G. and D.O. Morgan, *Effect of dietary regimen on cessation of intestinal absorption of large molecules (closure) in the neonatal pig and lamb.* J Nutr, 1962. 78: p. 263-8.

399. Vaerman, J.P., J.B. Arbuckle, and J.F. Heremans, *Immunoglobulin A in the pig. II. Sow's colostral and milk IgA: quantitative studies and molecular size estimation.* Int Arch Allergy Appl Immunol, 1970. 39(2-3): p. 323-33.

400. Allen, W.D. and P. Porter, *The relative distribution of IgM and IgA cells in intestinal mucosa and lymphoid tissues of the young unweaned pig and their significance in ontogenesis of secretory immunity.* Immunology, 1973. 24(3): p. 493-501.

401. Shen, L. and J.R. Turner, *Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed.* Am J Physiol Gastrointest Liver Physiol, 2006. 290(4): p. G577-82.

402. McAuley, J.L., et al., *MUC1 cell surface mucin is a critical element of the mucosal barrier to infection.* J Clin Invest, 2007. 117(8): p. 2313-24.

403. Salzman, N.H., et al., *Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin.* Nature, 2003. 422(6931): p. 522-6.

404. Abreu, M.T., et al., *Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide.* J Immunol, 2001. 167(3): p. 1609-16.

405. Lotz, M., et al., *Postnatal acquisition of endotoxin tolerance in intestinal*

epithelial cells. J Exp Med, 2006. 203(4): p. 973-84.

406. Melmed, G., et al., *Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut.* J Immunol, 2003. 170(3): p. 1406-15.

407. Medzhitov, R., *Recognition of microorganisms and activation of the immune response.* Nature, 2007. 449(7164): p. 819-26.

408. Otte, J.M., E. Cario, and D.K. Podolsky, *Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells.* Gastroenterology, 2004. 126(4): p. 1054-70.

409. Shao, L., D. Serrano, and L. Mayer, *The role of epithelial cells in immune regulation in the gut.* Semin Immunol, 2001. 13(3): p. 163-76.

410. Cruickshank, S.M., et al., *Colonic epithelial cell mediated suppression of CD4 T cell activation.* Gut, 2004. 53(5): p. 678-84.

411. Mayer, L., et al., *Expression of class II molecules on intestinal epithelial cells in humans. Differences between normal and inflammatory bowel disease.* Gastroenterology, 1991. 100(1): p. 3-12.

412. Westendorf, A.M., et al., *CD4+Foxp3+ regulatory T cell expansion induced by antigen-driven interaction with intestinal epithelial cells independent of local dendritic cells.* Gut, 2009. 58(2): p. 211-9.

413. Iwasaki, A. and B.L. Kelsall, *Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine.* J Exp Med, 2000. 191(8): p. 1381-94.

414. Butler, M., et al., *Modulation of dendritic cell phenotype and function in an in vitro model of the intestinal epithelium.* Eur J Immunol, 2006. 36(4): p. 864-74.

415. Rimoldi, M., et al., *Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells.* Nat Immunol, 2005. 6(5): p. 507-14.

416. Franco, M.A. and H.B. Greenberg, *Immunity to rotavirus infection in mice.* J Infect Dis, 1999. 179 Suppl 3: p. S466-9.

417. Yuan, L., et al., *Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease.* J Virol, 1996. 70(5): p. 3075-83.

418. Yuan, L. and L.J. Saif, *Induction of mucosal immune responses and protection against enteric viruses: rotavirus infection of gnotobiotic pigs as a model.* Vet Immunol Immunopathol, 2002. 87(3-4): p. 147-60.

419. Mendicino, M., et al., *Generation of antibody- and B cell-deficient pigs by targeted disruption of the J-region gene segment of the heavy chain locus.* Transgenic Res, 2010.

420. Kaisho, T. and S. Akira, *Toll-like receptor function and signaling.* J Allergy Clin Immunol, 2006. 117(5): p. 979-87; quiz 988.

421. Kim, S.H., et al., *Distinct protein kinase a anchoring proteins direct prostaglandin E2 modulation of toll-like receptor signaling in alveolar macrophages.* J Biol Chem, 2011.

422. Mitchell, D., et al., *Toll-like receptor-mediated adjuvanticity and immunomodulation in dendritic cells: Implications for peptide vaccines.* Hum Vaccin, 2011. 7.

423. Booth, J., et al., *Modulation of B cell responses by Toll-like receptors.* Cell Tissue Res, 2011. 343(1): p. 131-40.

424. Kulkarni, R., S. Behboudi, and S. Sharif, *Insights into the role of Toll-like receptors in modulation of T cell responses.* Cell Tissue Res, 2011. 343(1): p. 141-52.

425. Peri, F., et al., *Exploring the LPS/TLR4 signal pathway with small molecules.* Biochem Soc Trans, 2010. 38(5): p. 1390-5.

426. West, A.P., A.A. Koblansky, and S. Ghosh, *Recognition and signaling by toll-like receptors.* Annu Rev Cell Dev Biol, 2006. 22: p. 409-37.

427. Gay, N.J. and M. Gangloff, *Structure and function of Toll receptors and their ligands.* Annu Rev Biochem, 2007. 76: p. 141-65.

428. Beutler, B., et al., *Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large.* Annu Rev Immunol, 2006. 24: p. 353-89.

429. Sandor, F. and M. Buc, *Toll-like receptors. II. Distribution and pathways involved in TLR signalling.* Folia Biol (Praha), 2005. 51(6): p. 188-97.

430. Seth, R.B., L. Sun, and Z.J. Chen, *Antiviral innate immunity pathways.* Cell Res, 2006. 16(2): p. 141-7.

431. Xu, J., et al., *Expression of Toll-like receptors and their association with cytokine responses in peripheral blood mononuclear cells of children with acute rotavirus diarrhoea.* Clin Exp Immunol, 2006. 144(3): p. 376-81.

432. Xu, J., et al., *Rotavirus and coxsackievirus infection activated different profiles of toll-like receptors and chemokines in intestinal epithelial cells.* Inflamm Res, 2009. 58(9): p. 585-92.

433. Aich, P., et al., *Comparative analysis of innate immune responses following infection of newborn calves with bovine rotavirus and bovine coronavirus.* J Gen Virol, 2007. 88(Pt 10): p. 2749-61.

434. Zhou, R., et al., *Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice.* J Immunol, 2007. 178(7): p. 4548-56.

435. Sato, A., et al., *Rotavirus double-stranded RNA induces apoptosis and diminishes wound repair in rat intestinal epithelial cells.* J Gastroenterol Hepatol, 2006. 21(3): p. 521-30.

436. Azevedo, M.S., et al., *Cytokine responses in gnotobiotic pigs after infection with virulent or attenuated human rotavirus.* J Virol, 2006. 80(1): p. 372-82.

437. Azim, T., et al., *Rotavirus-specific subclass antibody and cytokine responses in Bangladeshi children with rotavirus diarrhoea.* J Med Virol, 2003. 69(2): p. 286-95.

438. Jiang, B., et al., *Cytokines as mediators for or effectors against rotavirus disease in children.* Clin Diagn Lab Immunol, 2003. 10(6): p. 995-1001.

439. Azim, T., et al., *Immune response of children who develop persistent diarrhea following rotavirus infection.* Clin Diagn Lab Immunol, 1999. 6(5): p. 690-5.

440. Malik, J., et al., *Evaluation of IFN-gamma response to rotavirus and non-structural protein NSP4 of rotavirus in children following severe rotavirus diarrhea.* J Clin Virol, 2008. 43(2): p. 202-6.

441. Johansen, K., et al., *Humoral and cell-mediated immune responses in humans to the NSP4 enterotoxin of rotavirus.* J Med Virol, 1999. 59(3): p. 369-77.

442. Rollo, E.E., et al., *The epithelial cell response to rotavirus infection.* J Immunol, 1999. 163(8): p. 4442-52.

443. Casola, A., et al., *Rotavirus infection of cultured intestinal epithelial cells induces secretion of CXC and CC chemokines.* Gastroenterology, 1998. 114(5): p. 947-55.

444. Mesa, M.C., et al., *Interaction of rotavirus with human peripheral blood mononuclear cells: plasmacytoid dendritic cells play a role in stimulating memory rotavirus specific T cells in vitro.* Virology, 2007. 366(1): p. 174-84.

445. Douagi, I., et al., *Role of interferon regulatory factor 3 in type I interferon responses in rotavirus-infected dendritic cells and fibroblasts.* J Virol, 2007. 81(6): p. 2758-68.

446. Istrate, C., et al., *Bone marrow dendritic cells internalize live RF-81 bovine rotavirus and rotavirus-like particles (RF 2/6-GFP-VLP and RF 8*2/6/7-VLP) but are only activated by live bovine rotavirus.* Scand J Immunol, 2007. 65(6): p. 494-502.

447. Fromantin, C., et al., *Oral delivery of homologous and heterologous strains of rotavirus to BALB/c mice induces the same profile of cytokine production by spleen cells.* Virology, 1998. 244(2): p. 252-60.

448. Blutt, S.E., et al., *Rotavirus antigenaemia and viraemia: a common event?* Lancet, 2003. 362(9394): p. 1445-9.

449. Chiappini, E., et al., *Viraemia is a common finding in immunocompetent children with rotavirus infection.* J Med Virol, 2005. 76(2): p. 265-7.

450. Williams, M.B., et al., *The memory B cell subset responsible for the secretory IgA response and protective humoral immunity to rotavirus expresses the intestinal homing receptor, alpha4beta7.* J Immunol, 1998. 161(8): p. 4227-35.

451. O'Neal, C.M., G.R. Harriman, and M.E. Conner, *Protection of the villus epithelial cells of the small intestine from rotavirus infection does not*

require immunoglobulin A. J Virol, 2000. 74(9): p. 4102-9.

452. Westerman, L.E., et al., *Serum IgG mediates mucosal immunity against rotavirus infection.* Proc Natl Acad Sci U S A, 2005. 102(20): p. 7268-73.

453. Coste, A., et al., *Nasal immunization of mice with virus-like particles protects offspring against rotavirus diarrhea.* J Virol, 2000. 74(19): p. 8966-71.

454. Feng, N., et al., *Inhibition of rotavirus replication by a non-neutralizing, rotavirus VP6-specific IgA mAb.* J Clin Invest, 2002. 109(9): p. 1203-13.

455. Gonzalez, A.M., et al., *Antibody responses to human rotavirus (HRV) in gnotobiotic pigs following a new prime/boost vaccine strategy using oral attenuated HRV priming and intranasal VP2/6 rotavirus-like particle (VLP) boosting with ISCOM.* Clin Exp Immunol, 2004. 135(3): p. 361-72.

456. Azevedo, M.S., et al., *Magnitude of serum and intestinal antibody responses induced by sequential replicating and nonreplicating rotavirus vaccines in gnotobiotic pigs and correlation with protection.* Clin Diagn Lab Immunol, 2004. 11(1): p. 12-20.

457. Bohl, E.H., K.W. Theil, and L.J. Saif, *Isolation and serotyping of porcine rotaviruses and antigenic comparison with other rotaviruses.* J Clin Microbiol, 1984. 19(2): p. 105-11.

458. Hoshino, Y., et al., *Infection immunity of piglets to either VP3 or VP7 outer capsid protein confers resistance to challenge with a virulent rotavirus bearing the corresponding antigen.* J Virol, 1988. 62(3): p. 744-8.

459. Yuan, L., et al., *Protective immunity and antibody-secreting cell responses elicited by combined oral attenuated Wa human rotavirus and intranasal Wa 2/6-VLPs with mutant Escherichia coli heat-labile toxin in gnotobiotic pigs.* J Virol, 2001. 75(19): p. 9229-38.

460. Feng, N., et al., *Comparison of mucosal and systemic humoral immune responses and subsequent protection in mice orally inoculated with a homologous or a heterologous rotavirus.* J Virol, 1994. 68(12): p. 7766-73.

461. Azevedo, M.S., et al., *An oral versus intranasal prime/boost regimen using attenuated human rotavirus or VP2 and VP6 virus-like particles with immunostimulating complexes influences protection and antibody-secreting cell responses to rotavirus in a neonatal gnotobiotic pig model.* Clin

Vaccine Immunol, 2010. 17(3): p. 420-8.

462. Kapikian, A.Z., et al., *Oral administration of human rotavirus to volunteers: induction of illness and correlates of resistance.* J Infect Dis, 1983. 147(1): p. 95-106.

463. Green, K.Y. and A.Z. Kapikian, *Identification of VP7 epitopes associated with protection against human rotavirus illness or shedding in volunteers.* J Virol, 1992. 66(1): p. 548-53.

464. Yuan, L., et al., *Resistance to rotavirus infection in adult volunteers challenged with a virulent G1P1A[8] virus correlated with serum immunoglobulin G antibodies to homotypic viral proteins 7 and 4.* J Infect Dis, 2009. 200(9): p. 1443-51.

465. Velazquez, F.R., et al., *Rotavirus infections in infants as protection against subsequent infections.* N Engl J Med, 1996. 335(14): p. 1022-8.

466. Velazquez, F.R., et al., *Serum antibody as a marker of protection against natural rotavirus infection and disease.* J Infect Dis, 2000. 182(6): p. 1602-9.

467. Hjelt, K., et al., *Protective effect of preexisting rotavirus-specific immunoglobulin A against naturally acquired rotavirus infection in children.* J Med Virol, 1987. 21(1): p. 39-47.

468. Chiba, S., et al., *Protective effect of naturally acquired homotypic and heterotypic rotavirus antibodies.* Lancet, 1986. 2(8504): p. 417-21.

469. O'Ryan, M.L., et al., *Acquisition of serum isotype-specific and G type-specific antirotavirus antibodies among children in day care centers.* Pediatr Infect Dis J, 1994. 13(10): p. 890-5.

470. Matson, D.O., et al., *Fecal antibody responses to symptomatic and asymptomatic rotavirus infections.* J Infect Dis, 1993. 167(3): p. 577-83.

471. Coulson, B.S., et al., *Role of coproantibody in clinical protection of children during reinfection with rotavirus.* J Clin Microbiol, 1992. 30(7): p. 1678-84.

472. McNeal, M.M., et al., *CD4 T cells are the only lymphocytes needed to protect mice against rotavirus shedding after intranasal immunization with a chimeric VP6 protein and the adjuvant LT(R192G).* J Virol, 2002. 76(2):

p. 560-8.

473. McNeal, M.M., et al., *Protection against rotavirus shedding after intranasal immunization of mice with a chimeric VP6 protein does not require intestinal IgA.* Virology, 2006. 346(2): p. 338-47.

474. McNeal, M.M., M.N. Rae, and R.L. Ward, *Evidence that resolution of rotavirus infection in mice is due to both CD4 and CD8 cell-dependent activities.* J Virol, 1997. 71(11): p. 8735-42.

475. Franco, M.A. and H.B. Greenberg, *Role of B cells and cytotoxic T lymphocytes in clearance of and immunity to rotavirus infection in mice.* J Virol, 1995. 69(12): p. 7800-6.

476. McNeal, M.M., et al., *Effector functions of antibody and CD8+ cells in resolution of rotavirus infection and protection against reinfection in mice.* Virology, 1995. 214(2): p. 387-97.

477. Chen, S.C., et al., *Immunity obtained by gene-gun inoculation of a rotavirus DNA vaccine to the abdominal epidermis or anorectal epithelium.* Vaccine, 1999. 17(23-24): p. 3171-6.

478. VanCott, J.L., et al., *Protective immunity to rotavirus shedding in the absence of interleukin-6: Th1 cells and immunoglobulin A develop normally.* J Virol, 2000. 74(11): p. 5250-6.

479. Ward, L.A., et al., *Development of mucosal and systemic lymphoproliferative responses and protective immunity to human group A rotaviruses in a gnotobiotic pig model.* Clin Diagn Lab Immunol, 1996. 3(3): p. 342-50.

480. Offit, P.A., et al., *Rotavirus-specific helper T cell responses in newborns, infants, children, and adults.* J Infect Dis, 1992. 165(6): p. 1107-11.

481. Offit, P.A., et al., *Rotavirus-specific humoral and cellular immune response after primary, symptomatic infection.* J Infect Dis, 1993. 167(6): p. 1436-40.

482. Mason, K.L., et al., *Overview of gut immunology.* Adv Exp Med Biol, 2008. 635: p. 1-14.

483. Debard, N., et al., *Effect of mature lymphocytes and lymphotoxin on the development of the follicle-associated epithelium and M cells in mouse*

Peyer's patches. Gastroenterology, 2001. 120(5): p. 1173-82.

484. Miller, H., et al., *Intestinal M cells: the fallible sentinels?* World J Gastroenterol, 2007. 13(10): p. 1477-86.

485. Neutra, M.R., N.J. Mantis, and J.P. Kraehenbuhl, *Collaboration of epithelial cells with organized mucosal lymphoid tissues.* Nat Immunol, 2001. 2(11): p. 1004-9.

486. Niess, J.H. and H.C. Reinecker, *Dendritic cells: the commanders-in-chief of mucosal immune defenses.* Curr Opin Gastroenterol, 2006. 22(4): p. 354-60.

487. Bilsborough, J. and J.L. Viney, *Gastrointestinal dendritic cells play a role in immunity, tolerance, and disease.* Gastroenterology, 2004. 127(1): p. 300-9.

488. Coombes, J.L., et al., *A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism.* J Exp Med, 2007. 204(8): p. 1757-64.

489. Macpherson, A.J., M.B. Geuking, and K.D. McCoy, *Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria.* Immunology, 2005. 115(2): p. 153-62.

490. Fagarasan, S. and T. Honjo, *Intestinal IgA synthesis: regulation of front-line body defences.* Nat Rev Immunol, 2003. 3(1): p. 63-72.

491. Bowman, E.P., et al., *The intestinal chemokine thymus-expressed chemokine (CCL25) attracts IgA antibody-secreting cells.* J Exp Med, 2002. 195(2): p. 269-75.

492. Macpherson, A.J., et al., *A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria.* Science, 2000. 288(5474): p. 2222-6.

493. Mestecky, J., M.W. Russell, and C.O. Elson, *Intestinal IgA: novel views on its function in the defence of the largest mucosal surface.* Gut, 1999. 44(1): p. 2-5.

494. Fagarasan, S. and T. Honjo, *Regulation of IgA synthesis at mucosal surfaces.* Curr Opin Immunol, 2004. 16(3): p. 277-83.

495. Mowat, A.M., *Anatomical basis of tolerance and immunity to intestinal antigens.* Nat Rev Immunol, 2003. 3(4): p. 331-41.

496. Marshall, J.S., *Mast-cell responses to pathogens.* Nat Rev Immunol, 2004. 4(10): p. 787-99.

497. Koon, H.W. and C. Pothoulakis, *Immunomodulatory properties of substance P: the gastrointestinal system as a model.* Ann N Y Acad Sci, 2006. 1088: p. 23-40.

498. Rothenberg, M.E. and S.P. Hogan, *The eosinophil.* Annu Rev Immunol, 2006. 24: p. 147-74.

499. Cheroutre, H., *Starting at the beginning: new perspectives on the biology of mucosal T cells.* Annu Rev Immunol, 2004. 22: p. 217-46.

500. Chen, Y., et al., *Protection of the intestinal mucosa by intraepithelial gamma delta T cells.* Proc Natl Acad Sci U S A, 2002. 99(22): p. 14338-43.

501. Wilson, C.L., et al., *Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense.* Science, 1999. 286(5437): p. 113-7.

502. Dahan, S., et al., *Epithelia: lymphocyte interactions in the gut.* Immunol Rev, 2007. 215: p. 243-53.

503. Allez, M., et al., *Expansion of CD8+ T cells with regulatory function after interaction with intestinal epithelial cells.* Gastroenterology, 2002. 123(5): p. 1516-26.

504. Stepankova, R., *Rearing germfree rats and rabbits.* Folia Microbiol (Praha), 1979. 24(1): p. 11-5.

505. Pleasants, J.R., *Rearing germfree cesarean-born rats, mice, and rabbits through weaning.* Ann N Y Acad Sci, 1959. 78: p. 116-26.

506. Cebra, J.J., *Influences of microbiota on intestinal immune system development.* Am J Clin Nutr, 1999. 69(5): p. 1046S-1051S.

507. Umesaki, Y., et al., *Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system.* Infect Immun, 1999. 67(7): p. 3504-11.

508. Pleasants, J.R., M.H. Johnson, and B.S. Wostmann, *Adequacy of chemically defined, water-soluble diet for germfree BALB/c mice through successive generations and litters.* J Nutr, 1986. 116(10): p. 1949-64.

509. Sterzl, J., L. Mandel, and R. Stepankova, *The use of gnotobiological models for the studies of immune mechanisms.* Nahrung, 1987. 31(5-6): p. 599-608.

510. Tlaskalova-Hogenova, H., et al., *Development of immunological capacity under germfree and conventional conditions.* Ann N Y Acad Sci, 1983. 409: p. 96-113.

511. Vetvicka, V., H. Tlaskalova-Hogenova, and R. Stepankova, *Effects of microflora antigens on lymphocyte migration patterns in germfree and conventional rats.* Folia Biol (Praha), 1983. 29(6): p. 412-8.

512. Stepankova, R., et al., *Differences in development of lymphocyte subpopulations from gut-associated lymphatic tissue (GALT) of germfree and conventional rats: effect of aging.* Folia Microbiol (Praha), 1998. 43(5): p. 531-4.

513. Ismail, A.S., C.L. Behrendt, and L.V. Hooper, *Reciprocal interactions between commensal bacteria and gamma delta intraepithelial lymphocytes during mucosal injury.* J Immunol, 2009. 182(5): p. 3047-54.

514. Tlaskalova, H., et al., *The immune response of germfree piglets after peroral monocontamination with living Escherichia coli strain 086. I. The fate of antigen, dynamics and site of antibody formation, nature of antibodies and formation of heterohaemagglutinins.* Folia Biol (Praha), 1970. 16(3): p. 177-87.

515. Tlaskalova-Hogenova, H. and R. Stepankova, *Development of antibody formation in germfree and conventionally reared rabbits: the role of intestinal lymphoid tissue in antibody formation to E. coli antigens.* Folia Biol (Praha), 1980. 26(2): p. 81-93.

516. Butler, J.E., et al., *Antibody repertoire development in fetal and newborn piglets, III. Colonization of the gastrointestinal tract selectively diversifies the preimmune repertoire in mucosal lymphoid tissues.* Immunology, 2000. 100(1): p. 119-30.

517. Bos, N.A., H.Q. Jiang, and J.J. Cebra, *T cell control of the gut IgA response against commensal bacteria.* Gut, 2001. 48(6): p. 762-4.

518. Tlaskalova-Hogenova, H., J. Cerna, and L. Mandel, *Peroral immunization of germfree piglets: appearance of antibody-forming cells and antibodies of different isotypes.* Scand J Immunol, 1981. 13(5): p. 467-72.

519. Peris-Bondia, F., et al., *The active human gut microbiota differs from the total microbiota.* PLoS One, 2011. 6(7): p. e22448.

520. Gilliland, S.E., M.L. Speck, and C.G. Morgan, *Detection of Lactobacillus acidophilus in feces of humans, pigs, and chickens.* Appl Microbiol, 1975. 30(4): p. 541-5.

521. Bauer, E., et al., *Influence of the gastrointestinal microbiota on development of the immune system in young animals.* Curr Issues Intest Microbiol, 2006. 7(2): p. 35-51.

522. Smith, P.M. and W.S. Garrett, *The gut microbiota and mucosal T cells.* Front Microbiol, 2011. 2: p. 111.

523. McLoughlin, R.M. and K.H. Mills, *Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma.* J Allergy Clin Immunol, 2011. 127(5): p. 1097-107; quiz 1108-9.

524. Tlaskalova-Hogenova, H., et al., *Mucosal immunity: its role in defense and allergy.* Int Arch Allergy Immunol, 2002. 128(2): p. 77-89.

525. O'Hara, A.M. and F. Shanahan, *The gut flora as a forgotten organ.* EMBO Rep, 2006. 7(7): p. 688-93.

526. Ewaschuk, J.B., et al., *Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function.* Am J Physiol Gastrointest Liver Physiol, 2008. 295(5): p. G1025-34.

527. Rakoff-Nahoum, S., et al., *Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis.* Cell, 2004. 118(2): p. 229-41.

528. Swanson, P.A., 2nd, et al., *Enteric commensal bacteria potentiate epithelial restitution via reactive oxygen species-mediated inactivation of focal adhesion kinase phosphatases.* Proc Natl Acad Sci U S A, 2011. 108(21): p. 8803-8.

529. Linden, S.K., T.H. Florin, and M.A. McGuckin, *Mucin dynamics in*

intestinal bacterial infection. PLoS One, 2008. 3(12): p. e3952.

530. Linden, S.K., et al., *MUC1 limits Helicobacter pylori infection both by steric hindrance and by acting as a releasable decoy.* PLoS Pathog, 2009. 5(10): p. e1000617.

531. Atarashi, K., et al., *ATP drives lamina propria T(H)17 cell differentiation.* Nature, 2008. 455(7214): p. 808-12.

532. Ivanov, II, et al., *Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine.* Cell Host Microbe, 2008. 4(4): p. 337-49.

533. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. 139(3): p. 485-98.

534. Gaboriau-Routhiau, V., et al., *The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses.* Immunity, 2009. 31(4): p. 677-89.

535. Atarashi, K., T. Tanoue, and K. Honda, *Induction of lamina propria Th17 cells by intestinal commensal bacteria.* Vaccine, 2010. 28(50): p. 8036-8.

536. Cong, Y., et al., *Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora.* J Immunol, 2002. 169(11): p. 6112-9.

537. Maynard, C.L., et al., *Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10.* Nat Immunol, 2007. 8(9): p. 931-41.

538. Round, J.L. and S.K. Mazmanian, *Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.* Proc Natl Acad Sci U S A, 2010. 107(27): p. 12204-9.

539. Atarashi, K., et al., *Induction of colonic regulatory T cells by indigenous Clostridium species.* Science, 2011. 331(6015): p. 337-41.

540. Kunisawa, J. and H. Kiyono, *Peaceful mutualism in the gut: revealing key commensal bacteria for the creation and maintenance of immunological homeostasis.* Cell Host Microbe, 2011. 9(2): p. 83-4.

541. Cash, H.L., et al., *Symbiotic bacteria direct expression of an intestinal*

bactericidal lectin. Science, 2006. 313(5790): p. 1126-30.

542. Ayabe, T., et al., *Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria.* Nat Immunol, 2000. 1(2): p. 113-8.

543. He, B., et al., *Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL.* Immunity, 2007. 26(6): p. 812-26.

544. Kunii, J., et al., *Commensal bacteria promote migration of mast cells into the intestine.* Immunobiology, 2011. 216(6): p. 692-7.

545. Cukrowska, B., et al., *Specific antibody and immunoglobulin responses after intestinal colonization of germfree piglets with non-pathogenic Escherichia coli O86.* Immunobiology, 2001. 204(4): p. 425-33.

546. Cukrowska, B., et al., *Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic E. coli strain Nissle 1917.* Scand J Immunol, 2002. 55(2): p. 204-9.

547. Agarwal, K.N. and S.K. Bhasin, *Feasibility studies to control acute diarrhoea in children by feeding fermented milk preparations Actimel and Indian Dahi.* Eur J Clin Nutr, 2002. 56 Suppl 4: p. S56-9.

548. Pedone, C.A., et al., *The effect of supplementation with milk fermented by Lactobacillus casei (strain DN-114 001) on acute diarrhoea in children attending day care centres.* Int J Clin Pract, 1999. 53(3): p. 179-84.

549. Guerin-Danan, C., et al., *Food supplementation with milk fermented by Lactobacillus casei DN-114 001 protects suckling rats from rotavirus-associated diarrhea.* J Nutr, 2001. 131(1): p. 111-7.

550. Guandalini, S., et al., *Lactobacillus GG administered in oral rehydration solution to children with acute diarrhea: a multicenter European trial.* J Pediatr Gastroenterol Nutr, 2000. 30(1): p. 54-60.

551. Sarker, S.A., et al., *Lactobacillus paracasei strain ST11 has no effect on rotavirus but ameliorates the outcome of nonrotavirus diarrhea in children from Bangladesh.* Pediatrics, 2005. 116(2): p. e221-8.

552. Freitas, M., et al., *Host-pathogens cross-talk. Indigenous bacteria and probiotics also play the game.* Biol Cell, 2003. 95(8): p. 503-6.

553. Rossen, J.W., et al., *Inhibition of cyclooxygenase activity reduces rotavirus infection at a postbinding step.* J Virol, 2004. 78(18): p. 9721-30.

554. Boshuizen, J.A., et al., *Changes in small intestinal homeostasis, morphology, and gene expression during rotavirus infection of infant mice.* J Virol, 2003. 77(24): p. 13005-16.

555. Shu, Q., F. Qu, and H.S. Gill, *Probiotic treatment using Bifidobacterium lactis HN019 reduces weanling diarrhea associated with rotavirus and Escherichia coli infection in a piglet model.* J Pediatr Gastroenterol Nutr, 2001. 33(2): p. 171-7.

556. Yasui, H., et al., *Immunomodulatory function of lactic acid bacteria.* Antonie Van Leeuwenhoek, 1999. 76(1-4): p. 383-9.

557. Resta-Lenert, S. and K.E. Barrett, *Probiotics and commensals reverse TNF-alpha- and IFN-gamma-induced dysfunction in human intestinal epithelial cells.* Gastroenterology, 2006. 130(3): p. 731-46.

558. Podolsky, D.K., *Inflammatory bowel disease.* N Engl J Med, 2002. 347(6): p. 417-29.

559. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nat Rev Immunol, 2007. 7(1): p. 19-30.

560. Coombes, J.L. and F. Powrie, *Dendritic cells in intestinal immune regulation.* Nat Rev Immunol, 2008. 8(6): p. 435-46.

561. Iwasaki, A., *Mucosal dendritic cells.* Annu Rev Immunol, 2007. 25: p. 381-418.

562. Johansson, C. and B.L. Kelsall, *Phenotype and function of intestinal dendritic cells.* Semin Immunol, 2005. 17(4): p. 284-94.

563. Kelsall, B.L. and F. Leon, *Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease.* Immunol Rev, 2005. 206: p. 132-48.

564. Bell, S.J., et al., *Migration and maturation of human colonic dendritic cells.* J Immunol, 2001. 166(8): p. 4958-67.

565. Sato, A., et al., *CD11b+ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells.* J Immunol, 2003. 171(7): p. 3684-

90.

566. Hart, A.L., et al., *Characteristics of intestinal dendritic cells in inflammatory bowel diseases.* Gastroenterology, 2005. 129(1): p. 50-65.

567. Takenaka, S., et al., *Dendritic cells derived from murine colonic mucosa have unique functional and phenotypic characteristics.* J Immunol, 2007. 178(12): p. 7984-93.

568. Li, M.O., S. Sanjabi, and R.A. Flavell, *Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms.* Immunity, 2006. 25(3): p. 455-71.

569. Corthesy, B., *Roundtrip ticket for secretory IgA: role in mucosal homeostasis?* J Immunol, 2007. 178(1): p. 27-32.

570. Harizi, H., et al., *Cyclooxygenase-2-issued prostaglandin e(2) enhances the production of endogenous IL-10, which down-regulates dendritic cell functions.* J Immunol, 2002. 168(5): p. 2255-63.

571. Igietseme, J.U., et al., *Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development.* J Immunol, 2000. 164(8): p. 4212-9.

572. Groux, H., et al., *A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells.* J Immunol, 1999. 162(3): p. 1723-9.

573. Benson, M.J., et al., *All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation.* J Exp Med, 2007. 204(8): p. 1765-74.

574. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid.* J Exp Med, 2007. 204(8): p. 1775-85.

575. Powrie, F., et al., *Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice.* Int Immunol, 1993. 5(11): p. 1461-71.

576. Bacchetta, R., et al., *Defective regulatory and effector T cell functions in*

patients with FOXP3 mutations. J Clin Invest, 2006. 116(6): p. 1713-22.

577. Westendorf, A.M., et al., *CD4+ T cell mediated intestinal immunity: chronic inflammation versus immune regulation.* Gut, 2005. 54(1): p. 60-9.

578. Westendorf, A.M., et al., *Intestinal epithelial antigen induces CD4+ T cells with regulatory phenotype in a transgenic autoimmune mouse model.* Ann N Y Acad Sci, 2006. 1072: p. 401-6.

579. Brimnes, J., et al., *Defects in CD8+ regulatory T cells in the lamina propria of patients with inflammatory bowel disease.* J Immunol, 2005. 174(9): p. 5814-22.

580. Menager-Marcq, I., et al., *CD8+CD28- regulatory T lymphocytes prevent experimental inflammatory bowel disease in mice.* Gastroenterology, 2006. 131(6): p. 1775-85.

581. Chaput, N., et al., *Identification of CD8+CD25+Foxp3+ suppressive T cells in colorectal cancer tissue.* Gut, 2009. 58(4): p. 520-9.

582. Westendorf, A.M., et al., *Autoimmune-mediated intestinal inflammation-impact and regulation of antigen-specific CD8+ T cells.* Gastroenterology, 2006. 131(2): p. 510-24.

583. Boismenu, R. and W.L. Havran, *Modulation of epithelial cell growth by intraepithelial gamma delta T cells.* Science, 1994. 266(5188): p. 1253-5.

584. Inagaki-Ohara, K., et al., *Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation.* J Immunol, 2004. 173(2): p. 1390-8.

585. Yang, H., et al., *Intestinal intraepithelial lymphocyte gamma delta-T cell-derived keratinocyte growth factor modulates epithelial growth in the mouse.* J Immunol, 2004. 172(7): p. 4151-8.

586. Yang, H., A.U. Spencer, and D.H. Teitelbaum, *Interleukin-7 administration alters intestinal intraepithelial lymphocyte phenotype and function in vivo.* Cytokine, 2005. 31(6): p. 419-28.

587. Shibahara, T., et al., *Alteration of intestinal epithelial function by intraepithelial lymphocyte homing.* J Gastroenterol, 2005. 40(9): p. 878-86.

588. Deshpande, G., S. Rao, and S. Patole, *Progress in the field of probiotics:*

year 2011. Curr Opin Gastroenterol, 2011. 27(1): p. 13-8.

CHAPTER 2

Toll-like receptor and innate cytokine responses induced by lactobacilli colonization and human rotavirus infection in gnotobiotic pigs

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2.1 Summary

Toll-like receptors (TLRs) play an important role in the recognition of microbes by host sentinel cells that leads to the subsequent innate and adaptive immune responses. In this study, we evaluated the patterns of TLR2-, TLR3- and TLR9-expressing antigen-presenting cells (APCs) in spleen and blood of gnotobiotic (Gn) pigs after colonization with a mixture of two strains of lactic acid bacteria (LAB), *Lactobacillus acidophilus* strain NCFM and *L. reuteri* (ATCC# 23272) or infection with the virulent human rotavirus (HRV) Wa strain. We also assessed the influence of LAB on TLR and serum innate cytokine responses induced by HRV. Distributions of subpopulations of APCs [CD14+/-SWC3+CD11R1- monocytes/macrophages and CD14+/-SWC3+CD11R1+ cDCs] were described in our previous report. We demonstrated that LAB induced strong TLR2-expressing APC responses in blood and spleen, HRV induced a TLR3 response in spleen, and TLR9 responses were induced by either HRV (in spleen) or LAB (in blood). LAB and HRV have an additive effect on TLR2- and TLR9-expressing APC responses, consistent with the adjuvant effect of LAB. Overall, the frequencies of TLR-expressing CD14+ APCs were higher than CD14- APCs. LAB alone also induced high levels of IL-12 in serum, but it

had a suppressive effect on the TLR3- and TLR9-expressing CD14- APC responses in spleen and the serum IFN-α response induced by HRV. These results elucidated the systemic TLR2-, TLR3-, and TLR9-expressing monocyte/macrophage and cDC responses after HRV infection, LAB colonization, and the two combined. Our findings facilitate the understanding of the mechanism of LAB's adjuvant effect on rotavirus vaccines and the diverse innate and adaptive immune responses induced by commensal LAB colonization versus rotavirus infection and the interactions between them.

2.2 Introduction

TLRs, a type of pattern-recognition receptor (PRR), play an important role in viral antigen recognition, innate immunity and in bridging innate and adaptive immune responses [1]. TLRs are expressed on many cell types, but mainly on professional APCs, e.g. monocytes/macrophages and DCs. TLR activation induces type I interferon production through several signaling pathways that lead to anti-viral and proinflammatory cytokine responses and induction of adaptive immune responses [2, 3]. Different microbe-associated molecular patterns (MAMPs) are recognized by different TLRs [4]. TLR2, in association with TLR1 and/or TLR6, recognizes peptidoglycans, lipopeptides, and lipoteichoic acids from Gram-positive bacteria [5]. TLR3 recognizes dsRNA, which is found in rotavirus genome and many other viruses during their replication cycles (e.g. negative-stranded RNA viruses), and synthetic

polyinosine-polycytidylic acid (polyI:C). TLR9 recognizes unmethylated CpG DNA found abundantly in bacterial and viral genomes [6]. Both TLR3 and TLR9 play crucial role in defense against viral infection *in vivo* **[**7**].** Alterations in TLR expression levels in peripheral blood monocuclear cells (PBMCs) have been reported in various viral infections [8-13] and have been directly correlated with plasma viral load [10] or associated with the severity of disease outcomes [9, 12].

Rotaviruses are the single most important etiologic agent of severe gastroenteritis in infants and young children worldwide [14]. Rotavirus virion has a nonenveloped, triple-layered capsid structure that surrounds the genome, which is composed of 11 segments of dsRNA. Rotavirus replicates mainly in the mature epithelial cells of the small intestinal villi and causes villous atrophy [15]. However, recent studies confirmed that rotavirus infection is not restricted to the intestinal tract. Rotavirus infection has an acute phase of viremia [16, 17]. Thus, TLR-expressing APC responses in systemic lymphoid tissues may contribute to the anti-viral immunity or pathogenesis in rotavirus infection. Patients with acute rotavirus infection had elevated mean levels of TLR2, TLR3, TLR4, TLR7, TLR8 mRNA expression in PBMCs within 3 days of onset of the disease [18].

Lactobacillus spp., Gram-positive rod-shaped bacteria, are normal components of the healthy human and pig intestinal microflora. LAB, including lactobacilli are widely evaluated as probiotics in animals and humans [19] and

have been shown to significantly stimulate gut epithelial cell proliferation [20], enhance innate and acquired immunity in young lab animals (mice, rats) and children [21, 22] and suppress intestinal inflammation [23]. Several LAB strains have been shown to reduce the severity of acute rotavirus gastroenteritis in children [24, 25]. The mechanisms of LAB's beneficial effects on human and animal health are the subject of many ongoing studies. Our studies are focused on LAB's effect on innate and adaptive immune responses to rotavirus infection [26, 27] and the adjuvant effect on rotavirus vaccines [28]. The adjuvant effect of several LAB strains has been documented in humans and in pigs [28-32]; however, the mechanism is undefined. It is known that many LAB strains are full of CpG islands in the genome [33]; therefore, lactobacilli may exert an immunostimulating effect via activation of TLR9 on APCs.

The objectives of the present study were to (1) evaluate TLR2-, TLR3- and TLR9-expressing APC responses in rotavirus infection or LAB colonization in systemic lymphoid tissues of neonatal Gn pigs; and (2) to assess the influence of LAB on TLR2, TLR3 and TLR9, and innate cytokine responses to rotavirus infection. The Gn pig model of HRV infection and diarrhea has been well defined in our previous studies [34, 35]. Early and late cytokine responses (e.g. IFN-γ, IL-4, IL-6, IL-10, IL-12, TGF-β1, and TNF-α) in serum of Gn pigs infected with the virulent Wa HRV have been reported [36]. In this study, we evaluated the effect of LAB on early cytokine responses as they are indicators of activation of innate immune cells via PRR. Besides being a

differentiation/maturation marker of monocytes/macrophages and DCs [37-39], CD14 is also a PRR and plays a role in the innate immune response. It directly interacts with intracellular TLR3 and enhances dsRNA-mediated TLR3 activation by aiding uptake of dsRNA into cells [40]. Because the monocytes (while in blood circulation) and macrophages (after entering spleen) are defined by the same cell markers, we refer to them as monocytes/macrophages in both locations [27]. It was previously found that LAB significantly reduced the total, but not CD14+, frequencies of monocytes/macrophages and cDCs in spleen of LAB plus HRV pigs compared to HRV-only pigs [27]. The influences of LAB on frequencies of TLR2-, TLR3- and TLR9-expressing CD14+ versus CD14- monocytes/macrophages and DCs in the spleen and blood of HRV infected Gn pigs were evaluated in this study to clarify the effect of LAB on different APC subpopulations in systemic lymphoid tissues.

2.3 Materials and methods

Bacteria. The *Lactobacillus reuteri* (ATCC# 23272) and *L. acidophilus* strain NCFM (ATCC, Manassas, VA, USA) were used in this study. Both LAB strains were propagated in Lactobacilli MRS broth (Weber, Hamilton, NJ, USA). Each strain was identified and then the big stock was prepared, aliquoted and stored in -80 $^{\circ}$C. When used, one vial was thawed and diluted to the needed titers. LAB inoculums were prepared and titrated as previously described in detail [27]. The two LAB inoculums with known titers were diluted to the specified

colonly forming unit (CFU)/ml in 0.1 % peptone water (BD Biosciences) and mixed in equal amounts on the day of feeding.

Virus. The virulent Wa strain (G1P1A[8]) HRV was passaged through Gn pigs and the pooled intestinal contents from the 23rd passage were used for inoculation at a dose of 1×10^5 fluorescent focus-forming units (FFU). The 50 % infectious dose (ID_{50}) of the Wa HRV in pigs was determined as approximately 1 FFU [15]. Virus fecal shedding was detected by a cell-culture immunofluorescent (CCIF) assay and HRV antigen in serum was detected with an antigen capture enzyme-linked immunosorbent assay (ELISA) as previously described [41, 42].

Inoculation of Gn pigs. Near-term pigs were derived by surgery from two sows (Large White) and maintained in germfree isolator units as described [43]. All pigs were confirmed as seronegative for rotavirus antibodies and germfree prior to LAB and HRV exposure. Gn pigs (both males and females) were randomly assigned to four treatment groups with four pigs in each group as follows: (1) LAB colonization plus HRV infection (LAB+HRV+), (2) HRV only (LAB-HRV+), (3) LAB only (LAB+HRV-) or (4) mock control (LAB-HRV-). Pigs in LAB+ groups were orally dosed at 3, 5, 7 and 9 days of age with 10^3, 10^4, 10^5 and 10^6 CFU, respectively, of a 1:1 mixture of *L. acidophilus* and *L. reuteri* in 2 ml of 0.1 % peptone water. The total dose of lactobacilli provided to each pig was 1.1×10^6 CFU in 4 feedings. Non-LAB-fed pigs were given an equal volume of 0.1 % peptone water. At 5 days of age, pigs in HRV+ groups

were orally inoculated with 10^5 FFU virulent Wa HRV in 5 ml of Dulbecco's Modified Eagle's Medium (DMEM). Non-infected pigs were given an equal volume of diluent. Pigs were given 5 ml of 100 mM sodium bicarbonate to reduce gastric acidity 20 min before HRV inoculation. Post-HRV-inoculation, pigs were examined daily for clinical signs, including % with diarrhea, duration of diarrhea and diarrhea scores as described [15, 44]. Rectal swabs were collected daily for HRV and lactobacilli shedding. Pigs were euthanized at day 5 post-HRV inoculation (1 day after the last/7 days after the first LAB feeding) to isolate mononuclear cells (MNCs) from spleen and peripheral blood. MNCs were isolated as previously described [44] and stained with antibodies to porcine cell markers and TLR antibodies directly, without in vitro stimulation, on the same day of MNC isolation.

Enumeration of LAB. Each rectal swab was diluted in 4 ml of 0.1 % peptone water (~1:10) and a 100 μl aliquot was diluted in 900 μl of peptone water and plated onto MRS agar. The plates were incubated in sealed BBL Gaspak jars (Fisher, Hanover Park, IL) containing Anaerogen packs for 24 hrs at 37 °C. The number of CFU on plates with 20 to 200 colonies were enumerated and recorded. LAB shedding was expressed as CFU/ml. Bacteremia was assessed by plating pig sera onto MRS agar plates and incubated in the same way as for LAB enumeration.

Staining cells for flow cytometry analysis. The MNCs (2×10^6 cells/tube) were first stained with antibodies to porcine monocyte/macrophage and cDC

markers (SWC3, CD11R1 and CD14), followed by antibodies to TLR2, TLR3 and TLR9, respectively. Except when specifically noted, MNCs were washed once with a staining buffer (prepared according to BD Pharmingen BrdU Flow Kits Instruction Manual) and incubated for 15 min at room temperature (RT) at each step. Cells were first stained with mouse anti-porcine CD14 (IgG2b, Fitzgerald, clone MIL-2) and mouse anti-porcine CD11R1 (IgG1, Serotech: MCA1220, clone MIL4). After washing at 500 × g for 5 min at 4 ^{0}C, the secondary fluorescent conjugated antibodies, fluorescein isothiocyanate (FITC) conjugated rat anti-mouse IgG2b (IgG2a, BD pharmingen, clone R12-3) and allophycocyanin (APC) conjugated rat anti-mouse IgG1 (IgG1, BD pharmingen, clone X56), were added. After washing the cells twice, biotin conjugated mouse anti-porcine SWC3a (IgG1, Southern Biotech, clone 74-22-15) was added followed by streptavidin conjugated with peridinine chlorophyll protein (PerCP). For staining of TLR2, which is expressed on the cell surface, phycoerythrin (PE) conjugated mouse anti-human TLR2 (mouse IgG1, eBioscience, clone T2.5) and the PE conjugated mouse IgG1 isotype control were added, respectively, to the TLR2 and its corresponding isotype-matched control tubes. For staining intracellular TLR3, TLR9 and their corresponding isotype controls, after staining with surface markers (SWC3, CD11R1 and CD14), cells were permeabilized with BD cytofix/cytoperm buffer (BD pharmingen) for 15 min at RT. In TLR3 and TLR9 tubes, PE conjugated mouse anti-human TLR3 (mouse IgG1, eBioscience, clone TLR3.7) and PE conjugated

rat anti-human TLR9 (rat IgG2a, eBioscience, clone eB72-1665) antibodies were added, respectively. In the corresponding isotype-matched control tubes, PE conjugated mouse IgG1 isotype control and PE conjugated rat IgG2a isotype control were added, respectively. Cells were incubated, washed, fixed and resuspended in staining buffer and kept in dark at 4 ^{0}C before flow cytometry analysis. All antibodies were titrated and used at optimal concentrations. Analysis of the stained cells was performed using a 4-color FACSCalibur flow cytometer (Becton Dickinson) and at least 20,000 cells were acquired. Data analysis was performed using CellQuest^{TM} Pro (Becton Dickinson) or FlowJo 7.2.2 (Tree Star, Inc) software. Data are presented as mean frequencies of CD14+ or CD14- TLR-expressing monocytes/macrophages and cDCs. Any non-specific staining occurring in the isotype-matched control tubes was subtracted from the frequencies of TLR+ cells.

Detection of serum cytokine levels by ELISA. Blood samples were collected from pigs at euthanasia (post-inoculation day (PID) 5). Sera were processed (without heat inactivation) and stored at -20 °C until tested. The ELISA for detection of porcine IFN-γ, IL-4, IL-6, IL-10, IL-12, IFN-α, TGF-β, and TNF-α were conducted using anti-swine cytokine antibodies as previously described [36]. Sensitivities of the ELISA were 7.5 pg/ml for IFN-γ, IL-4, IL-10, and IL-12; 15 pg/ml for IL-6, TNF-α, TGF-β; and 75 pg/ml for IFN-α.

Statistical analysis. Non-parametric Kruskal-Wallis rank sum test was

performed to compare frequencies of TLR-expressing cells in each cell subpopulation in spleen and blood among groups and the serum cytokine concentrations at PID 5 among groups. When differences among these groups were detected, the same test was used in a pairwise fashion to clarify the nature of the differences. Spearman's rank correlation test was used for assessment of correlation between frequencies of TLR-expressing cDCs or monocytes/macrophages and cytokine concentrations in serum. Statistical significance was assessed at $p < 0.05$ throughout. All statistical analyses were performed using the SAS program (SAS Institute, NC, USA).

2.4 Results

LAB fecal counts, HRV infection and clinical signs. From post-HRV inoculation day 0 to 5, the average fecal LAB counts in LAB+ groups ranged between 5.9×10^6 to 8.1×10^7 CFU/ml, which were greater than the count of LAB in the original feeding inoculums (10^3 to 10^6 CFU/ml). The mean LAB count in the LAB+HRV+ group was significantly higher than the LAB+HRV- group on PID 5 (data not shown). The bacterial strains in the intestinal contents of the LAB-fed pigs were examined by plating serially diluted samples on selective MRS agar plates for *Lactobacillus* and culturing anaerobically at 37 ^{o}C overnight. The bacterial colonies on MRS agar plates from the intestinal content samples had identical morphology as the colonies from the original LAB inoculum. The serially diluted intestinal contents and daily rectal swab

samples were also plated on regular blood agar plates and cultured aerobically at 37 ^{o}C overnight. No bacterial growth was detected on the blood agar plates from any of the pigs, confirming that no extraneous bacterial contamination occurred.

The kinetics and magnitude of virus fecal and nasal shedding and antigenemia after the virulent Wa HRV inoculation of Gn pigs have been characterized previously [17]. HRV infection in this study was confirmed by detection of HRV titers in intestinal contents by CCIF and HRV antigen in serum by ELISA from all the pigs in the HRV+ groups (none in the HRV- groups) at PID 5. All the HRV+ pigs developed antigenemia, but none of the LAB+ pigs developed bacteremia (data not shown). There were no significant differences in HRV titers in intestinal contents (mean peak titer 6.4×10^4 vs 3.3×10^4 FFU/ml) or antigen-ELISA OD values in serum (data not shown) between the two HRV+ groups, suggesting that LAB colonization did not reduce rotavirus intestinal replication or prevent viremia. The duration and severity of diarrhea between the LAB+HRV+ and LAB-HRV+ groups did not differ significantly (data not shown), thus intestinal colonization by the mixture of *L. acidophilus* and *L. reuteri* did not reduce rotavirus diarrhea.

Detection of TLR expression in porcine APCs by flow cytometry using anti-human TLR antibodies. The cross reactivity and specificity of anti-human TLR2, TLR3 and TLR9 antibodies were evaluated for detection of porcine TLRs in MNCs by flow cytometry. Sufficient cross reactivity between

human and porcine TLR antibodies was indicated by the strong fluorescent intensity and the specificity was indicated by the clear separation of the histograms of isotype-matched irrelevant control antibody-stained MNCs from the TLR antibody-stained MNC (data not shown). The cross reactivity of the antibodies can be explained by the high amino acid sequence homologies between human and porcine TLRs. There is a 78.1 % for TLR2, 84.2 % for TLR3, and 82.0 % for TLR9 amino acid sequence identity, respectively, between humans and pigs based on comparing the predicted amino acid sequences from NCBI data base (TLR2 porcine NP_998926 vs. human AAH33756; TLR3 porcine ABB92547 vs. human NP_003256; and TLR9 porcine NP_999123 vs. human AAQ89443).

Rabbit anti-pig TLR2 and TLR9 polyclonal antibodies became available (Cosmo, Bio, Co) later in the study. We compared the sensitivity of the anti-human TLR cross reactive antibodies with the anti-pig TLR polyclonal antibodies using normal conventional pig blood MNCs. The MNCs were stimulated in vitro with CpG OND or peptidoglycan. As shown in Fig. 2.1, similar frequencies of SWC3+TLR2+ and SWC3+TRL9+ monocytes were detected by anti-human or anti-pig TLR antibodies. We compared various working dilutions of the antibodies using blood monocytes from three pigs (Fig. 2.1 shows the representative frequencies). The differences in frequency were less than 0.5 % and were not statistically significant. These results confirmed that the anti-human TLR antibodies are applicable for study of TLR responses

in pigs. To date, anti-pig TLR3 is still not commercially available. However, because TLR3 has a higher amino acid sequence similarity than TLR2 and TLR9 between humans and pigs, similar results are expected, if anti-pig TLR antibodies become available in the future.

Detection of frequencies of TLR-expressing cDCs (SWC3+CD11R1+) and monocytes/macrophages (SWC3+CD11R1-) in the four treatment groups is depicted by the representative dot plots in Fig. 2.2 using TLR3 in spleen as an example. The frequencies and tissue distribution of porcine cDCs and monocytes/macrophages in the four groups have been described in a previous publication [27]. As shown in Fig. 2.2 (dot plot at lower right corner), the MNCs stained with antibodies to cell markers and the isotype-matched irrelevant control antibody for TLR3 had zero percent TLR3+ cells, confirming the specificity of the antibody. The mean frequencies of TLR2-, TLR3- and TLR9-expressing CD14+ and CD14- monocytes/macrophages and cDCs are summarized in Fig. 2.3 (spleen; note the difference in y-axis scales between CD14+ and CD14- APCs) and Fig. 2.4 (blood; note the 10-fold difference in y-axis scales between TLR2-expressing CD14+ APCs and the others). Overall, higher frequencies of TLR-expressing CD14+ APCs than CD14- APCs were detected. High variability within treatment groups was observed for frequencies of TLR-expressing APCs in both spleen and blood. A matrix table was constructed based on both the frequencies and statistical comparison results among the four pig groups to depict the effect of treatment on frequencies of

TLR-expressing APCs and serum cytokine levels (Table 2.1).

TLR2-expressing CD14+ APCs were induced by either LAB or HRV and LAB plus HRV had an additive effect. As shown in Fig. 2.3, no or minimal frequencies of TLR-expressing monocytes/macrophages or cDCs were detected in spleen of mock control pigs (LAB-HRV-). LAB plus HRV (LAB+HRV+), HRV infection alone (LAB-HRV+), and LAB colonization alone (LAB+HRV-) induced significantly higher frequencies of CD14+ TLR2-expressing monocytes/macrophages and cDCs than the mock controls, indicating the involvement of TLR2 in recognition of LAB colonization and HRV infection. In contrast to the CD14+ APCs, CD14- monocytes/macrophages and cDCs had minimal or no TLR2 expression, except for the low TLR2 expression on cDCs in the HRV only group.

TLR3-expressing CD14+ and CD14- APCs were induced by HRV whereas LAB had an antagonistic effect with HRV on frequencies of TLR3-expressing CD14- APCs. HRV alone induced 2- to 3-fold higher frequencies of TLR3-expressing CD14+ APCs compared to the mock control group. LAB plus HRV induced significantly higher frequencies of TLR3-expressing CD14+ APCs than mock controls, and they were similar to the HRV-only group. In contrast, frequencies of TLR3-expressing CD14- monocytes/macropahges and cDCs in the LAB plus HRV group were significantly lower than those in the HRV-only group, suggesting an antagonistic effect of LAB on the HRV-induced TLR3 response. LAB alone induced minimal frequencies of TLR3-expressing

CD14+ and CD14- cDCs in spleen, coinciding with the low cytokine responses (except for IL-12) induced by LAB alone (Fig. 2.5). Still, frequencies of TLR3-expressing monocytes/macrophages in LAB-only group were, albeit low, significantly higher than mock controls. This is unexpected because LAB does not contain known TLR3 ligands.

TLR9-expressing CD14+ and CD14- APCs were induced by HRV whereas LAB had an antagonistic effect with HRV on frequencies of TLR9-expressing CD14- APCs. The patterns of TLR9-expressing CD14+ and CD14- APCs in the HRV+ groups were similar to those of TLR3 with slightly lower frequencies (Fig. 2.3, Table 2.1), suggesting that TLR9 is also involved in the HRV-induced innate immune responses. LAB alone induced minimal TLR9-expressing CD14+ and CD14- APCs in spleen.

In summary, similar frequencies of TLR3-expressing CD14+ APCs were detected in spleen of the HRV infected Gn pigs with or without LAB colonization (Fig. 2.3 and Table 2.1), suggesting that HRV infection is mainly responsible for the increased frequencies of TLR3-expressing APCs. LAB plus HRV enhanced (not statistically significant) the frequencies of TLR2- and TLR9-expressing CD14+ APCs compared to LAB or HRV alone, suggesting an additive effect between LAB and HRV. The most striking observation in spleen is that the frequencies of TLR3- and TLR9-expressing CD14- APCs were significantly reduced in the LAB plus HRV group compared to the HRV-only group, thus LAB had a suppressive effect on the TLR responses to HRV in a

subset of systemic APCs.

High frequencies of TLR2-expressing CD14+ APCs were induced by LAB (cDCs) and LAB plus HRV (cDCs and monocytes/macrophages). In blood, minimal to no TLR-expressing CD14+ or CD14- monocytes/macrophages were detected in mock inoculated pigs (Fig. 2.4). However, low frequencies of TLR2- and TLR3-expressing CD14+ and CD14- cDCs were detected, which likely reflect the baseline constitutive expression of the TLRs by blood cDCs. LAB plus HRV and LAB alone induced the highest mean frequencies of TLR2-expressing CD14+ cDCs in blood compared to all the others (note the y-axis scale difference) and they were significantly higher (6 to 21-fold) than the HRV-only and mock control groups.

LAB plus HRV, but not LAB alone, induced significantly higher frequencies of CD14+ TLR2-expressing monocytes/macrophages compared to the HRV-only and mock control groups. This additive effect was also observed for CD14- TLR2-expressing cDCs. HRV alone did not induce significant TLR-expressing APCs in blood. Frequencies of TLR2-expressing CD14- monocytes/macrophages and TLR3-expressing CD14+ and CD14- APCs were low to minimal and with high variability, and did not differ significantly among the four groups.

TLR9-expressing CD14+ APCs were induced by LAB (cDCs) and LAB plus HRV (cDCs and monocytes/macrophages). The pattern of TLR9-expressing CD14+ APCs was similar to the pattern of TLR2, but at an

approximately 10-fold lower magnitude, with the two LAB+ groups inducing significantly higher frequencies of TLR9-expressing CD14+ cDCs compared to the HRV-only and mock control groups. LAB plus HRV, but not HRV or LAB alone, induced significantly higher frequencies of TLR9-expressing CD14+ monocytes/macrophages compared to the mock controls. Frequencies of TLR9-expressing CD14- APCs were low and did not differ significantly among the groups.

In summary, high frequencies of TLR2-expressing CD14+ cDCs in blood were induced by LAB, or LAB plus HRV. Significant TLR9-expressing CD14+ cDCs were also detected in the two LAB+ groups, but not in the HRV-only group (Fig. 2.4 and Table 2.1). Overall, LAB plus HRV induced higher or significantly higher frequencies of TLR2- and TLR9-expressing-CD14+ monocytes/macrophages and cDCs and TLR2-expressing CD14- cDCs than HRV alone. These data indicate that TLR2- and TLR9-expressing cDCs in blood were mainly induced by LAB; however LAB and HRV had an additive effect on frequencies of TLR2- and TLR9-expressing cDCs.

LAB induced the highest serum IL-12 level, HRV induced the highest IFN-α and the lowest TGF-β levels, and LAB plus HRV induced significantly lower IFN-α levels than HRV. The mean concentrations of anti-viral cytokine (IFN-α), proinflammatory cytokines (IL-12), Th1 cytokine (IFN-γ), Th2 cytokine (IL-4), and T regulatory cell (Treg) cytokines (IL-10 and TGF-β) at PID 5 in the sera of pigs are summarized in Fig. 2.5. The concentrations of

IFN-α in the HRV-only group were significantly higher than the LAB plus HRV and the LAB-only groups and they were all significantly higher than the mock control group, indicating a downregulating effect of LAB on the IFN-α response induced by HRV. On the other hand, the concentrations of IL-12 in the LAB-only group were significantly higher than the LAB plus HRV and the HRV-only groups and they were all significantly higher than the mock control group. The concentrations of TGF-β in the HRV-only group were significantly lower than the LAB plus HRV and the LAB-only groups and they were all significantly lower than the mock control group, thus LAB may play a role in maintaining the TGF-β levels in serum. The concentrations of IFN-γ, IL-4 and IL-10 did not differ significantly among groups at PID 5, but there was a trend for higher concentrations of IFN-γ, IL-4 and IL-10 in the HRV+ groups (Fig. 2.5). The mean IFN-γ and IL-4 concentrations were 4- to 11-fold higher in the LAB plus HRV group compared to the HRV-only group. The IL-6 and TNF-α levels were low or undetectable (data not shown).

Serum IFN-α concentrations significantly correlated with frequencies of TLR3- and TLR9-expressing CD14- APCs in spleen. Spearman's rank correlation analysis was performed to identify the correlations between frequencies of TLR2-, TLR3- and TLR9-expressing APCs in spleen or blood and the concentrations of serum cytokines among the four treatment groups. Significant positive correlations were found between IFN-α concentrations and frequencies of TLR3-expressing CD14- cDC ($r = 0.6151$, $p = 0.0112$) and

monocytes/macrophages (r = 0.6372, p = 0.0079) and TLR9-expressing CD14- cDC (r = 0.6398, p = 0.0076) and monocytes/macrophages (r = 0.5417, p = 0.0302) in spleen. These correlations suggest that the CD14- APCs in spleen may contribute significantly to the levels of IFN-α in serum.

2.5 Discussion

This study is the continuation of our previous report [27] on the effects of probiotic LAB colonization and HRV infection on development of the innate immune responses in neonatal Gn pigs. In this study, we evaluated the frequencies of systemic TLR2-, TLR3- and TLR9-expressing CD14+ and CD14- APCs in Gn pigs colonized with LAB, infected with HRV, or both. Previously, the TLR expression in pigs has been studied only at the mRNA level in tissues by using real-time quantitative PCR because antibodies against porcine TLRs are not currently available [45-47]. Using cross reactive anti-human TLR antibodies enabled us to evaluate the expression pattern at the protein level for cell surface (TLR2) and intracellular (TLR3, TLR9) TLR expression in the defined porcine APC subpopulations.

LAB intestinal colonization alone had a profound stimulating effect on early postnatal development of the systemic innate immune cells, as evidenced by the significantly increased total frequencies of monocytes/macrophages and cDCs and the CD14+ monocyte/macrophage frequencies in spleen [27], the significantly increased TLR2- and TLR9-expressing CD14+ cDCs in blood, and

the significantly elevated serum pro-Th1 cytokine (IL-12) level. The induction of TLR2- and TLR9-expressing cDC and IL-12 responses can be explained, at least partially, by the presence of TLR2 and TLR9 ligands (peptidoglycan in the cell wall and CpG motifs in the genome for TLR2 and TLR9, respectively) in the LAB. We identified 6405 CpG islands in the whole genome of *L. acidophilus* NCFM [48] using EMBOSS CpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpglot/index.html). Induction of TLR2- and TLR9-expressing cDC and IL-12 responses may provide the mechanism for the adjuvanticity of LAB in enhancing cellular and humoral immune responses induced by influenza virus, poliovirus and rotavirus vaccines and rotavirus or *Salmonella typhi* Ty21a infections [28-32]. Our results are consistent with other studies, e.g. (1) feeding *L. casei* increased TLR2 expression in Peyer's patches in mice [49]; and (2) *L. paracasei* and *L. salivarius* significantly increased the production of IL-12 in PBMCs of healthy adults [50].

An additive effect between LAB and HRV in activating TLR2- and TLR9-expressing CD14+ APCs in spleen and CD14- cDCs in blood was observed. On the other hand, the increased frequencies of TLR2- and TLR9-expressing APCs in LAB plus HRV pigs may simply be due to the significantly higher counts of LAB, which may translate to an increased magnitude of TLR agonists available to stimulate the host immune system. However, because the frequencies of total APCs or CD14+ APCs in the LAB plus HRV pigs did not differ from the LAB-only pigs in blood, and the total APCs in spleen of the LAB plus HRV pigs were

significantly lower than the LAB-only group [27], it is not likely that the higher LAB count in the LAB plus HRV group played a more pertinent role in the significant increases of TLR2- and TLR9-expressing APC frequencies than did the co-infection of LAB and HRV.

HRV infection of the Gn pigs, with or without LAB colonization, induced similar frequencies of TLR3- and TLR9-expressing CD14+ APCs in spleen, indicating that HRV is chiefly responsible for the increases of TLR3- and TLR9-expressing CD14+ APCs. Notably, in LAB plus HRV pigs, the frequencies of TLR3- and TLR9-expressing CD14- APCs in spleen were significantly reduced compared to the HRV-only pigs. The reduced TLR3- and TLR9-expressing CD14- APC responses correlated significantly with the reduced serum IFN-α level in this pig group. Our previous study also showed that the LAB plus HRV pigs had significantly reduced total frequencies of APCs in spleen, reduced total frequencies of monocytes/macrophages in ileum, and significantly reduced frequencies of CD14+ monocytes/macrophages in ileum than the HRV-only pigs [27]. Because the viremia level and fecal virus shedding titers did not differ significantly among the HRV-infected pigs with or without LAB, the reductions are not likely due to reduced HRV replication, although some studies have indicated that probiotic feeding can reduce rotavirus shedding [22, 51, 52]. Thus, the results may suggest that LAB colonization has a downregulatory effect on subpopulations of APCs in HRV-infected pigs. Mechanisms and implications of such an effect of LAB on innate immune

responses to HRV infection require further investigation.

Both CD14+ and CD14- APCs expressing TLR2, TLR3 and TLR9 were induced in spleen by HRV infection; however, the TLR-expressing APC responses in blood were very different from that of spleen. Frequencies of TLR2-, TLR3- and TLR9-expressing APCs in blood of the HRV-only pigs were similar to the control (LAB-HRV-) pigs. The lack of TLR responses in blood in the HRV-only pigs is consistent with our previous findings showing that HRV infection did not increase frequencies of total APCs or CD14+ cDCs, and significantly reduced the total frequencies of CD14+ monocytes/macrophages at PID 5 in blood [27]. The potential reasons for the considerable differences between the APC response in spleen and blood may be because (1) samples were collected at only one time point (PID 5), TLR-expressing APC responses occurring in the blood at an earlier time point might have been missed; or (2) splenic and circulating APCs are diverse APC populations and they react to MAMP stimulation from LAB and HRV differently. To date, the only two previous studies of TLR responses induced by rotavirus infection were conducted by measuring TLR mRNA levels in intestinal tissues [53] or PBMCs [18]. Bovine rotavirus induced strong TLR3 mRNA expression in the intestinal tissue of newborn calves at 18 hrs post-inoculation [53]. Rotavirus infected children had significantly elevated expression of TLR2, TLR3, TLR8 mRNA levels in PBMCs within 3 days of diarrhea onset and the elevated TLR mRNA expression lasted for 7-14 days [18]. The time frame for sample collection in

the children overlapped with that in Gn pigs. Thus, the lack of TLR responses in blood APCs of the HRV-only pigs may not be solely due to the single sample collection time point; however, it is important to investigate the TLR responses to HRV at an earlier time point (PID 2-3) in the future study.

Induction of TLR2-expressing APCs in spleen of Gn pigs by HRV infection is an interesting finding because the ligands for TLR2 have not been identified previously in rotavirus. Likewise were the TLR2, TLR4, TLR7 and TLR8 mRNA responses in rotavirus infected children [18]. Further studies are needed to understand these observations. Rotavirus dsRNA are known to be recognized only by TLR3 [54-56]. A recent study by Zhou et al [56] demonstrated that recognition of rotavirus dsRNA by TLR3 on intestinal epithelial cells triggers the secretion of IL-15, which functions to increase the percentage of intestinal intraepithelial lymphocytes and enhances their cytotoxicity in mice. Another study of rats [55] also showed that rotavirus dsRNA induced severe apoptosis and diminished wound repair in intestinal epithelial cells through TLR3 activation. Hence, TLR3 may be involved in the pathogenesis of rotavirus gastroenteritis. Study of the TLR3 response induced by HRV and LAB in the intestinal epithelial and lymphoid tissues is the objective of our ongoing investigation.

Whether TLR9 is involved in rotavirus infection and immunity has not been reported before. The patterns of TLR9-expressing CD14+ APCs were similar to those of TLR3 in spleen and to those of TLR2 in blood. These data provide

incidental evidence that TLR9 is involved in the innate immune responses induced by both LAB and HRV. RNA viruses are found to have a low presence of CpG dinucleotides in their genomes [57]. We identified 52 CpG islands in the whole genome of the virulent Wa HRV (Yuan, unpublished data) using EMBOSS CpGPlot. The presence of CpG motifs may explain the HRV-induced TLR9-expressing APC responses. TLR9-expressing APC responses were induced by either LAB or HRV depending on the cell type and location. LAB alone induced a significant TLR9-expressing CD14+ cDC response in blood, whereas HRV alone induced significant TLR9-expressing CD14- (and some CD14+) monocyte/macrophage and cDC responses in spleen. LAB plus HRV induced the highest frequencies of TLR9-expressing CD14+ monocytes/macrophages in spleen and blood, which is consistent with the adjuvant effect of LAB on HRV vaccine-induced B and T cell immune responses [28].

The reduced IFN-α levels significantly correlated with the reduced frequencies of TLR3- and TLR9-expressing CD14- APCs in spleen and blood, which may suggest that the activity of CD14- APCs contributes significantly to the IFN-α response. LAB had significant influence on IFN-α and TGF-β serum responses induced by HRV infection. LAB plus HRV induced significantly lower levels of serum IFN-α and higher levels of TGF-β than HRV alone, suggesting the regulatory effect of LAB. However, LAB plus HRV pigs had 4- to 11-fold higher serum IFN-γ and IL-4 concentrations (not significantly due to

high variability) at PID 5 compared to the HRV-only pigs, which is again consistent with the adjuvant effect of LAB on HRV-induced immune responses. In the previous study of cytokine responses to HRV in Gn pigs [36], serum TNF-α, IFN-γ, IL-4 and IL-10 levels after HRV infection peaked at PID5 , whereas IL-6 and IL-12 peaked earlier at PID 1-3 and TGF-β peaked later at PID 14. IFN-α levels were not measured previously. We selected PID 5 in this study to correspond with the peaks of most cytokines after HRV infection in Gn pigs. The single sampling time of PID 5 did not affect detection of significant differences in IL-12 and TGF-β responses, but may explain the lack of IL-6 response (but not the lack of TNF-α response). Future studies should include more time points for detection of TLR and innate cytokine responses to better understand the kinetics of innate immune responses induced by LAB, HRV and the two combined.

In summary, this study elucidated the systemic TLR2-, TLR3-, and TLR9-expressing monocyte/macrophage and cDC responses after LAB colonization, HRV infection and the two combined. Our findings facilitate the understanding of the mechanism for LAB's adjuvant effect on rotavirus vaccines and the diverse innate and adaptive immune responses induced by commensal LAB colonization versus rotavirus infection and the interactions between them.

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2.7 References

1. Kaisho, T. and S. Akira, *Toll-like receptor function and signaling.* J Allergy Clin Immunol, 2006. 117(5): p. 979-87; quiz 988.

2. Sandor, F. and M. Buc, *Toll-like receptors. II. Distribution and pathways involved in TLR signalling.* Folia Biol (Praha), 2005. 51(6): p. 188-97.

3. Seth, R.B., L. Sun, and Z.J. Chen, *Antiviral innate immunity pathways.* Cell Res, 2006. 16(2): p. 141-7.

4. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. 4(7): p. 499-511.

5. Modlin, R.L., *Mammalian toll-like receptors.* Ann Allergy Asthma Immunol, 2002. 88(6): p. 543-7; quiz 548-50, 583.

6. Werling, D. and T.W. Jungi, *TOLL-like receptors linking innate and adaptive immune response.* Vet Immunol Immunopathol, 2003. 91(1): p. 1-12.

7. Tabeta, K., et al., *Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection.* Proc Natl Acad Sci U S A, 2004. 101(10): p. 3516-21.

8. Chen, Z., et al., *Expression profiles and function of Toll-like receptors 2 and 4 in peripheral blood mononuclear cells of chronic hepatitis B patients.* Clin Immunol, 2008.

9. de Kruif, M.D., et al., *Differential gene expression changes in children with severe dengue virus infections.* PLoS Negl Trop Dis, 2008. 2(4): p. e215.

10. Lester, R.T., et al., *Toll-like receptor expression and responsiveness are increased in viraemic HIV-1 infection.* Aids, 2008. 22(6): p. 685-94.

11. Sato, K., et al., *Expression of Toll-like receptors in chronic hepatitis C virus infection.* J Gastroenterol Hepatol, 2007. 22(10): p. 1627-32.

12. Xu, J., et al., *[Expression of Toll-like receptors in mononuclear cells from children with acute rotavirus diarrhea]*. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 2007. 21(1): p. 38-40.

13. Xu, N., et al., *Toll-like receptor 7 and 9 expression in peripheral blood mononuclear cells from patients with chronic hepatitis B and related hepatocellular carcinoma.* Acta Pharmacol Sin, 2008. 29(2): p. 239-44.

14. Parashar, U.D., et al., *Rotavirus and severe childhood diarrhea.* Emerg Infect Dis, 2006. 12(2): p. 304-6.

15. Ward, L.A., et al., *Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs.* J Gen Virol, 1996. 77 (Pt 7): p. 1431-41.

16. Blutt, S.E. and M.E. Conner, *Rotavirus: to the gut and beyond!* Curr Opin Gastroenterol, 2007. 23(1): p. 39-43.

17. Azevedo, M.S., et al., *Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus.* J Virol, 2005. 79(9): p. 5428-36.

18. Xu, J., et al., *Expression of Toll-like receptors and their association with cytokine responses in peripheral blood mononuclear cells of children with acute rotavirus diarrhoea.* Clin Exp Immunol, 2006. 144(3): p. 376-81.

19. Vaughan, E.E., et al., *The intestinal LABs.* Antonie Van Leeuwenhoek, 2002. 82(1-4): p. 341-52.

20. Ichikawa, H., et al., *Probiotic bacteria stimulate gut epithelial cell proliferation in rat.* Dig Dis Sci, 1999. 44(10): p. 2119-23.

21. Herias, M.V., et al., *Immunomodulatory effects of Lactobacillus plantarum colonizing the intestine of gnotobiotic rats.* Clin Exp Immunol, 1999. 116(2): p. 283-90.

22. Yasui, H., et al., *Immunomodulatory function of lactic acid bacteria.* Antonie Van Leeuwenhoek, 1999. 76(1-4): p. 383-9.

23. Zocco, M.A., et al., *Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis.* Aliment Pharmacol Ther, 2006. 23(11): p. 1567-74.

24. Majamaa, H., et al., *Lactic acid bacteria in the treatment of acute rotavirus*

gastroenteritis. J Pediatr Gastroenterol Nutr, 1995. 20(3): p. 333-8.

25. Shornikova, A.V., et al., *Bacteriotherapy with Lactobacillus reuteri in rotavirus gastroenteritis.* Pediatr Infect Dis J, 1997. 16(12): p. 1103-7.

26. Zhang, W., et al., *Influence of probiotic Lactobacilli colonization on neonatal B cell responses in a gnotobiotic pig model of human rotavirus infection and disease.* Vet Immunol Immunopathol, 2008. 122(1-2): p. 175-81.

27. Zhang, W., et al., *Lactic acid bacterial colonization and human rotavirus infection influence distribution and frequencies of monocytes/macrophages and dendritic cells in neonatal gnotobiotic pigs.* Vet Immunol Immunopathol, 2008. 121(3-4): p. 222-31.

28. Zhang, W., et al., *Probiotic Lactobacillus acidophilus enhances the immunogenicity of an oral rotavirus vaccine in gnotobiotic pigs.* Vaccine 2008. 26: p. 3655-3661.

29. Link-Amster, H., et al., *Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake.* FEMS Immunol Med Microbiol, 1994. 10(1): p. 55-63.

30. Olivares, M., et al., *Oral intake of Lactobacillus fermentum CECT5716 enhances the effects of influenza vaccination.* Nutrition, 2007. 23(3): p. 254-60.

31. de Vrese, M., et al., *Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination.* Eur J Nutr, 2005. 44(7): p. 406-13.

32. Kaila, M., et al., *Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain.* Pediatr Res, 1992. 32(2): p. 141-4.

33. Rachmilewitz, D., et al., *Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis.* Gastroenterology, 2004. 126(2): p. 520-8.

34. Saif, L.J., et al., *The gnotobiotic piglet as a model for studies of disease pathogenesis and immunity to human rotaviruses.* Arch Virol Suppl, 1996. 12: p. 153-61.

35. Yuan, L. and L.J. Saif, *Induction of mucosal immune responses and protection against enteric viruses: rotavirus infection of gnotobiotic pigs as a model.* Vet Immunol Immunopathol, 2002. 87(3-4): p. 147-60.

36. Azevedo, M.S., et al., *Cytokine responses in gnotobiotic pigs after infection with virulent or attenuated human rotavirus.* J Virol, 2006. 80(1): p. 372-82.

37. Carrasco, C.P., et al., *Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties.* Immunology, 2001. 104(2): p. 175-84.

38. Paillot, R., et al., *Functional and phenotypic characterization of distinct porcine dendritic cells derived from peripheral blood monocytes.* Immunology, 2001. 102(4): p. 396-404.

39. Summerfield, A., et al., *Porcine peripheral blood dendritic cells and natural interferon-producing cells.* Immunology, 2003. 110(4): p. 440-9.

40. Lee, H.K., et al., *Double-stranded RNA-mediated TLR3 activation is enhanced by CD14.* Immunity, 2006. 24(2): p. 153-63.

41. Bohl, E.H., et al., *Porcine pararotavirus: detection, differentiation from rotavirus, and pathogenesis in gnotobiotic pigs.* J Clin Microbiol, 1982. 15(2): p. 312-9.

42. Hoblet, K.H., et al., *Efficacy of an orally administered modified-live porcine-origin rotavirus vaccine against postweaning diarrhea in pigs.* Am J Vet Res, 1986. 47(8): p. 1697-703.

43. Meyer, R.C., E.H. Bohl, and E.M. Kohler, *Procurement and Maintenance of Germfree Seine for Microbiological Investigations.* Appl Microbiol, 1964. 12: p. 295-300.

44. Yuan, L., et al., *Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease.* J Virol, 1996. 70(5): p. 3075-83.

45. Burkey, T.E., et al., *Expression of Toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with Salmonella enterica serovar Typhimurium or serovar Choleraesuis.* Vet Immunol Immunopathol, 2007. 115(3-4): p. 309-19.

46. Tohno, M., et al., *Toll-like receptor 2 and 9 are expressed and functional in gut-associated lymphoid tissues of presuckling newborn swine.* Vet Res, 2006. 37(6): p. 791-812.

47. Willing, B.P. and A.G. Van Kessel, *Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig.* J Anim Sci, 2007. 85(12): p. 3256-66.

48. Altermann, E., et al., *Complete genome sequence of the probiotic lactic acid bacterium Lactobacillus acidophilus NCFM.* Proc Natl Acad Sci U S A, 2005. 102(11): p. 3906-12.

49. Galdeano, C.M. and G. Perdigon, *The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity.* Clin Vaccine Immunol, 2006. 13(2): p. 219-26.

50. Castellazzi, A.M., et al., *In vitro activation of mononuclear cells by two probiotics: Lactobacillus paracasei I 1688, Lactobacillus salivarius I 1794, and their mixture (PSMIX).* Immunol Invest, 2007. 36(4): p. 413-21.

51. Mao, M., et al., *Effect of a lactose-free milk formula supplemented with bifidobacteria and streptococci on the recovery from acute diarrhoea.* Asia Pac J Clin Nutr, 2008. 17(1): p. 30-4.

52. Saavedra, J.M., et al., *Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhoea and shedding of rotavirus.* Lancet, 1994. 344(8929): p. 1046-9.

53. Aich, P., et al., *Comparative analysis of innate immune responses following infection of newborn calves with bovine rotavirus and bovine coronavirus.* J Gen Virol, 2007. 88(Pt 10): p. 2749-61.

54. Matsumoto, M., et al., *Subcellular localization of Toll-like receptor 3 in human dendritic cells.* J Immunol, 2003. 171(6): p. 3154-62.

55. Sato, A., et al., *Rotavirus double-stranded RNA induces apoptosis and diminishes wound repair in rat intestinal epithelial cells.* J Gastroenterol Hepatol, 2006. 21(3): p. 521-30.

56. Zhou, R., et al., *Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice.* J Immunol, 2007. 178(7): p. 4548-56.

57. Greenbaum, B.D., et al., *Patterns of evolution and host gene mimicry in influenza and other RNA viruses.* PLoS Pathog, 2008. 4(6): p. e1000079.

58. Watarai, H., et al., *PDC-TREM, a plasmacytoid dendritic cell-specific receptor, is responsible for augmented production of type I interferon.* Proc Natl Acad Sci U S A, 2008. 105(8): p. 2993-8.

59. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. 301(5633): p. 640-3.

Fig. 2.1. Comparisons between anti-human and anti-pig TLR antibodies for detection of TLR2 and TLR9 expression in pig blood monocytes. The frequencies of SWC3+TLR2+ and SWC3+TLR9+ monocytes detected by the different TLR antibodies are labeled on each dot plot. Dot plots show the representative of results from three pigs. Blood MNCs isolated from three normal conventional pigs were stimulated in vitro with CpG OND D19 (1 μM) [*58*] or B.subtilis peptidoglycan (10 μg/ml) [*59*] for 48 hrs before staining. The MNCs were stained with SWC3a-biotin and streptavidin-PerCP and followed by the PE conjugated anti-human TLR2 and TLR9, respectively (see Material and Methods) or followed by the rabbit anti-pig TLR2 and TLR9 polyclonal antibodies (Cosmo Bio Co, Japan) respectively, and then a PE conjugated goat anti-rabbit IgG (Open Biosystems).

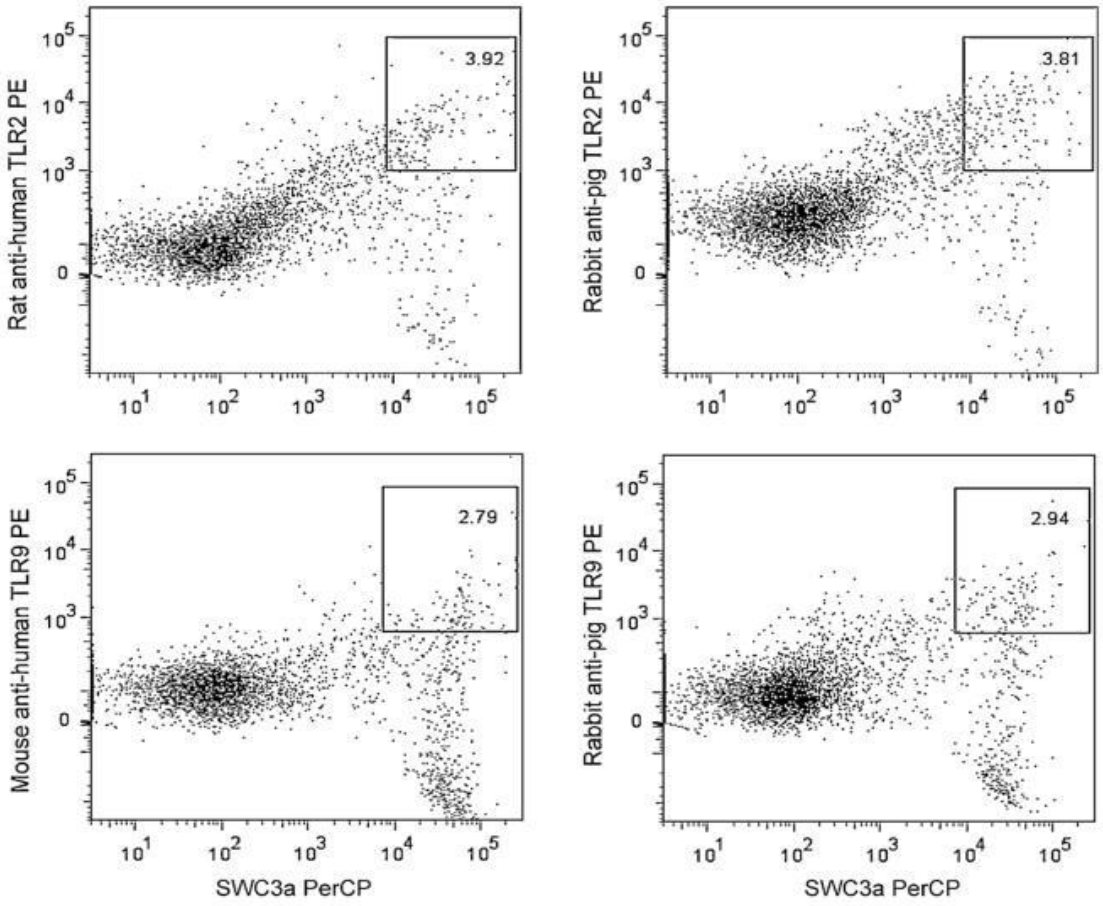
Rat anti-human TLR2 PE
3.92
Rabbit anti-pig TLR2 PE
3.81
Mouse anti-human TLR9 PE
2.79
Rabbit anti-pig TLR9 PE
2.94
SWC3a PerCP
SWC3a PerCP

Fig. 2.1

Fig. 2.2. Example of frequencies of TLR3-expressing splenic monocytes/macrophages of Gn pigs from each treatment group. Treatment group is labeled on each dot plot: LAB+HRV+, pigs were inoculated with LAB and virulent Wa strain HRV; LAB-HRV+, pigs were inoculated with HRV only; LAB+HRV-, pigs were inoculated with LAB only; and LAB-HRV-, pigs were mock inoculated. Monocytes/macrophages were defined as CD14+/-SWC3+CD11R1- [27]. CD14/TLR3 dot plots were performed within the SWC3+CD11R1- subpopulation. The dot plot at lower left corner shows a TLR3 isotype control staining which included antibodies to all the cell markers (SWC3, CD11R1 and CD14), except that the antibodies to TLR3 were replaced by the isotype-matched antigen-irrelevant control antibodies.

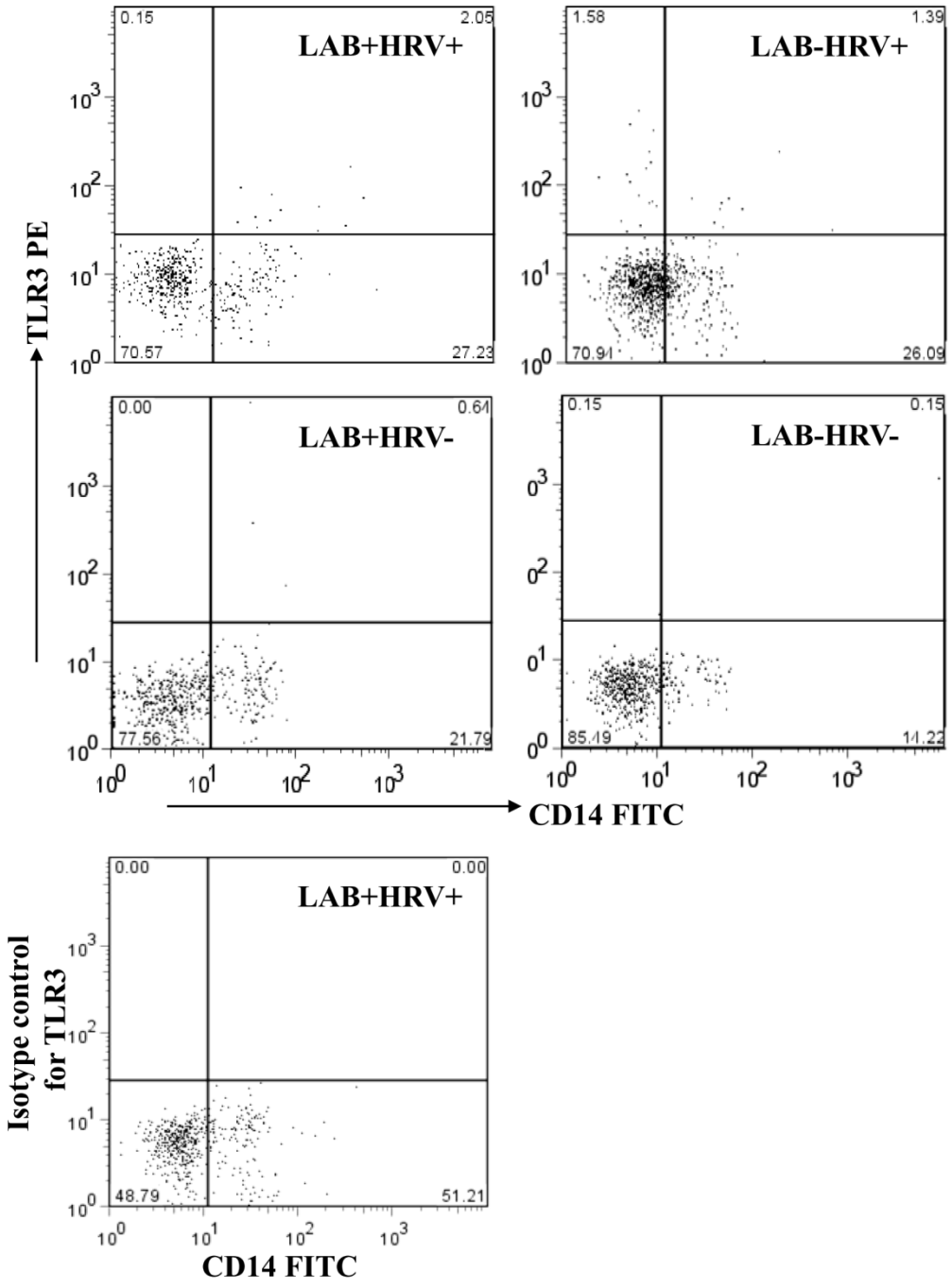
LAB+HRV+
LAB-HRV+
LAB+HRV-
LAB-HRV-
TLR3 PE
CD14 FITC
0.15
2.05
70.57
27.23
1.58
1.39
70.94
26.09
0.00
0.61
77.56
21.79
0.15
0.15
85.49
14.22
Isotype control for TLR3
0.00
0.00
48.79
51.21

Fig. 2.2

Fig. 2.3. Frequencies of TLR2-, TLR3-, and TLR9-expressing CD14+ and CD14- APCs in spleen of Gn pigs at PID 5. Gn pigs were inoculated with LAB and virulent Wa strain HRV (LAB+HRV+), HRV only (LAB-HRV+), LAB only (LAB+HRV-) or mock (LAB-HRV-). The y-axis is the mean frequencies (%) of CD14+ or CD14- TLR-expressing cells among monocytes/macrophages or cDCs. Note the difference in y-axis scales between CD14+ and CD14- APCs. The error bars represent standard error of the mean (n = 4). The letters A, B, and C indicate the results of significance testing for difference between treatments. Unshared letters indicate significant difference between treatment groups on frequencies of the TLR-expressing APCs (Kruskal-Wallis rank sum test, $P < 0.05$), while shared letters indicate no significant difference.

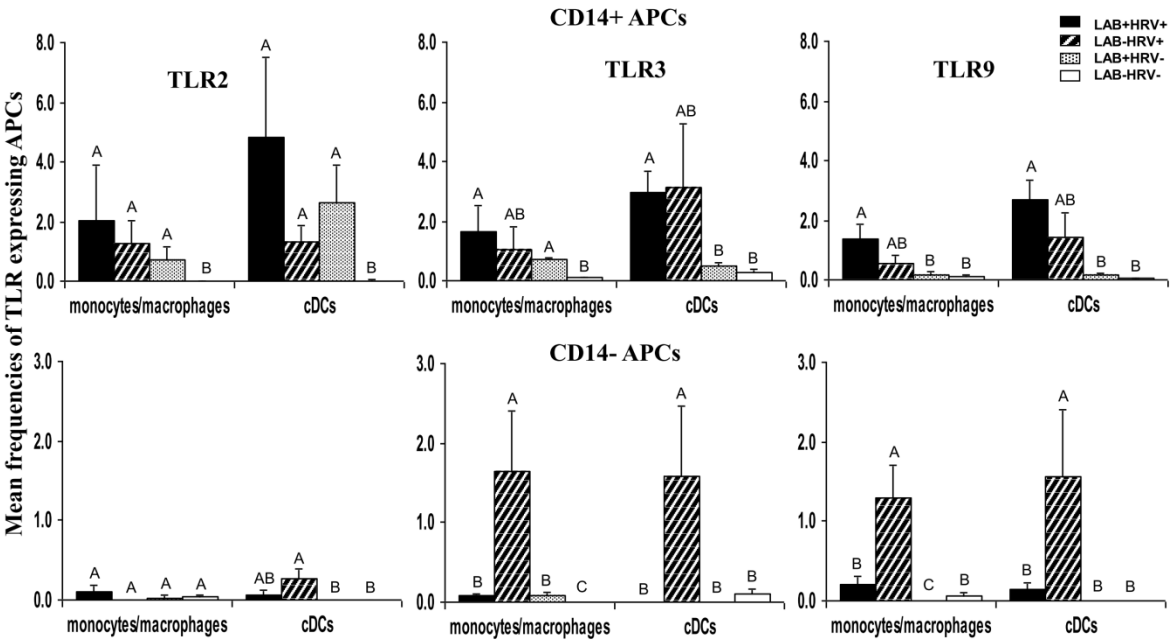
CD14+ APCs
TLR2
TLR3
TLR9
LAB+HRV+
LAB-HRV+
LAB+HRV-
LAB-HRV-
Mean frequencies of TLR expressing APCs
CD14- APCs
monocytes/macrophages
cDCs

Fig. 2.3

Fig. 2.4. Frequencies of TLR2-, TLR3-, and TLR9-expressing CD14+ and CD14- APCs in blood of Gn pigs at PID 5. Gn pigs were inoculated with LAB and virulent Wa strain HRV (LAB+HRV+), HRV only (LAB-HRV+), LAB only (LAB+HRV-) or mock (LAB-HRV-). The y-axis is the mean frequencies (%) of CD14+ or CD14- TLR-expressing cells among monocytes/macrophages or cDCs. Note the difference in y-axis scales between CD14+ and CD14- TLR2-expressing APCs. The error bars represent standard error of the mean (n = 4). For letters A, B, C, see figure legend for Fig. 2.3.

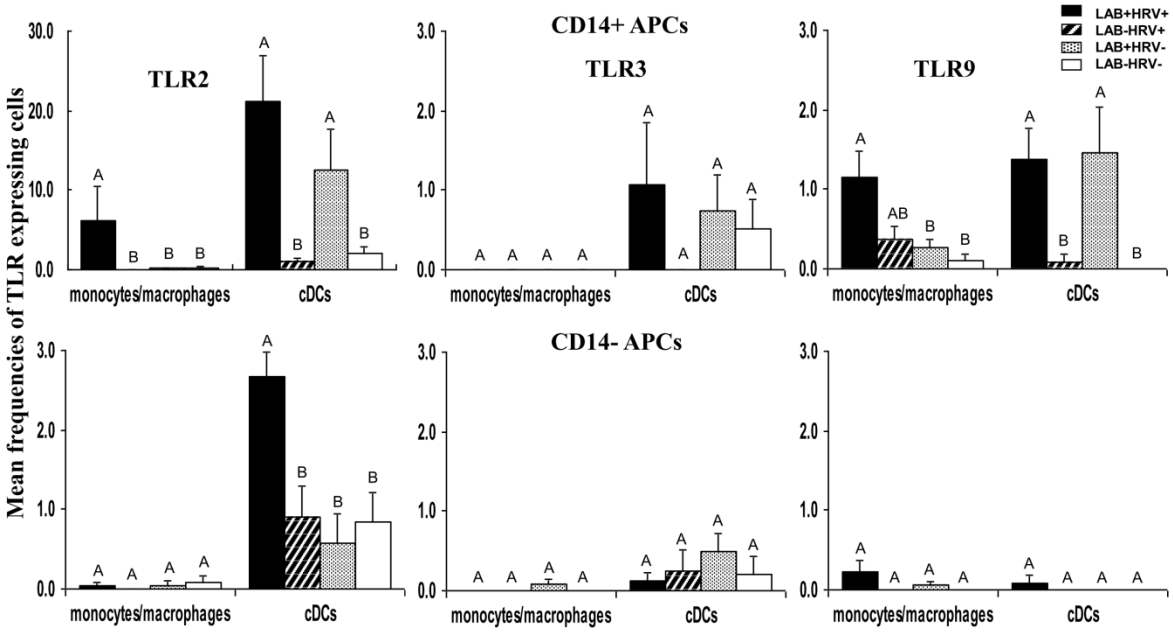
CD14+ APCs
CD14- APCs
TLR2
TLR3
TLR9
LAB+HRV+
LAB-HRV+
LAB+HRV-
LAB-HRV-
Mean frequencies of TLR expressing cells
monocytes/macrophages
cDCs

Fig. 2.4

Fig. 2.5. Cytokine levels in serum of Gn pigs at PID 5. Gn pigs were inoculated with LAB and virulent Wa strain HRV (LAB+HRV+), HRV only (LAB-HRV+), LAB only (LAB+HRV-) or mock (LAB-HRV-). Cytokine concentrations were measured by ELISA. The error bars represent standard error of the mean (n = 4). For letters A, B, C, see figure legend for Fig. 2.3.

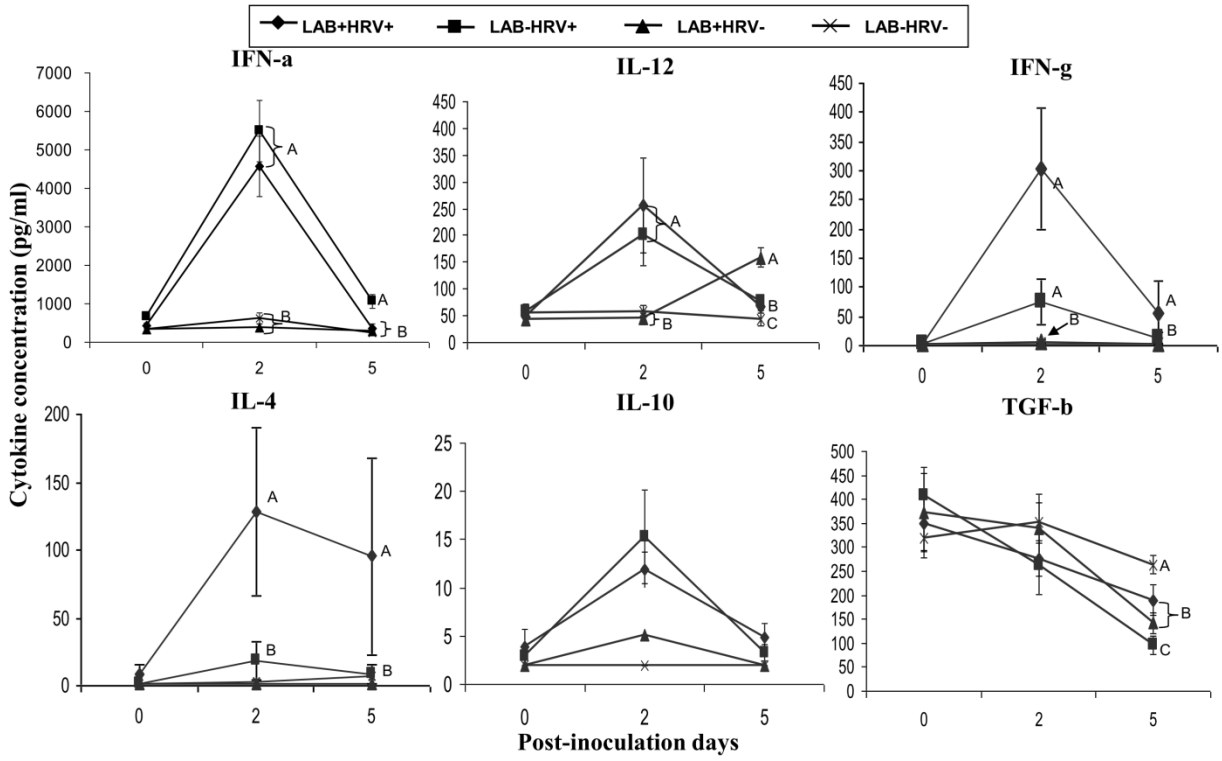
LAB+HRV+
LAB-HRV+
LAB+HRV-
LAB-HRV-
IFN-a
IL-12
IFN-g
IL-4
IL-10
TGF-b
Cytokine concentration (pg/ml)
Post-inoculation days

Fig. 2.5

Table 2.1. Effect of treatment on frequencies of TLR-expressing APCs in spleen and blood and serum cytokine concentrations

Treatment	Spleen APCs	TLR2	TLR3	TLR9	Blood APCs	TLR2	TLR3	TLR9	Cytokines in serum	
									IFN-α	**+**
LAB plus HRV	CD14+ MP[a]	**++**[bc]	**+**	**+**	CD14+ MP	**+++++**	-	**+**	IL-12	**+**
	CD14- MP	+/-	**+/-**	+/-	CD14- MP	-	-	+/-	TGF-β	**--**
	CD14+ cDCs	**+++**	**++**	**++**	CD14+ cDCs	**++++++**	+/-	**+**	IFN-γ	+
	CD14- cDCs	+/-	-	+/-	CD14- cDCs	**++**	-	-	IL-4	+
									IFN-α	**++++**
HRV-only	CD14+ MP	**+**	+	+/-	CD14+ MP	-	-	+/-	IL-12	**+**
	CD14- MP	-	**+**	**+**	CD14- MP	-	-	-	TGF-β	**-----**
	CD14+ cDCs	**+**	+++	+	CD14+ cDCs	-	-	-	IFN-γ	+/-
	CD14- cDCs	**+/-**	**+**	**+**	CD14- cDCs	-	-	-	IL-4	+/-
									IFN-α	**+**
LAB-only	CD14+ MP	**+/-**	**+/-**	+/-	CD14+ MP	-	-	+/-	IL-12	**++++**
	CD14- MP	-	**+/-**	**--**	CD14- MP	-	-	-	TGF-β	**---**
	CD14+ cDCs	**++**	+/-	+/-	CD14+ cDCs	**+++++**	+/-	**+**	IFN-γ	-
	CD14- cDCs	-	-	-	CD14- cDCs	-	+/-	-	IL-4	-

a. MP, monocytes/macrophages.

b. Plus and minus signs indicate various degree of effect of the treatment groups, from increased frequencies of APCs or cytokine concentrations (++) to equal (-) or lower than the baseline values (--) of the mock control (LAB-HRV-) group.

c. Values differing significantly from the mock control group are in boldface (Kruskal-Wallis rank sum test; $p<0.05$).

CHAPTER 3

High dose and low dose Lactobacilli acidophilus exerted opposite immune modulating effects on T cell immune responses induced by an oral human rotavirus vaccine in gnotobiotic pigs

Ke Wen, Guohua Li, Tammy Bui, Fangning Liu, Yanru Li, Jacob Kocher, Lin Lin, Xingdong Yang, and Lijuan Yuan

3.1 Summary

We studied dose effects of probiotic *Lactobacillus acidophilus* strain NCFM (LA) on T cell immune responses in a gnotobiotic pig model. Frequencies of IFN-γ producing CD4+ and CD8+ T cell and IL-10 and TGF-β producing natural and induced Treg cell responses were determined in the intestinal and systemic lymphoid tissues of gnotobiotic pigs vaccinated with an oral human rotavirus vaccine in conjunction with low dose (accumulative total dose 2.11×10^6 colony forming unit [CFU]) or high dose (accumulative total dose 2.22×10^9 CFU) or without LA feeding. Low dose LA strongly and significantly promoted Th1 type and down-regulated Treg cell immune responses as evidenced by the higher frequencies of virus-specific and non-specific IFN-γ producing T cells, and the decreased frequencies of Treg cells and their TGF-β and IL-10 production in all the tissues compared to the high dose LA group and control group. To the contrary, high dose LA increased the frequencies of Treg cells and TGF-β production in most of the tissues compared to the control group. The dose effects of LA on Th1 and Treg cell immune responses were similar in the intestinal and systemic lymphoid tissues and were independent

from the vaccination. Thus, the same probiotic strain in different doses can either promote or suppress Th1 or Treg cell immune responses. These findings have significant implications in the use of probiotic lactobacilli as immunostimulatory versus immunoregulatory agents. Probiotics can be ineffective or even detrimental if not used at the optimal dosage for the appropriate purposes.

3.2 Introduction

Intestinal commensal bacteria and probiotics, including lactobacilli, help maintain gut homeostasis by balancing proinflammatory and antiinflammatory mucosal responses. Many *Lactobacillus* strains are known to have immune regulatory functions and have been tested clinically or experimentally for therapeutic effects on controlling inflammatory diseases, autoimmune diseases and allergies, such as using *L. gasseri, L. fermentum* and *L. salivarius* for inflammatory bowel disease [1, 2], *L. casei* for rheumatoid arthritis [3, 4], *Lactobacillus rhamnosus* strain GG (LGG) for eczema [5], and *L. fermentum* for atopic dermatitis [6]. However, the same *Lactobacillus* strains have sometimes been shown to have opposite immune modulatory functions by different studies. *L. casei* was reported by one study to be a pure Th1 inducer [7] while it was reported by another study to promote Treg cell response and suppress Th 1 type responses [3]. LA, *L. gasseri, L. fermentum, L. casei*, *L. plantarum, L. johnsonii,* and *L. rhamnosus* were all reported to stimulate human or murine

DCs to produce increased levels of proinflammatory cytokines (IL-2, IL-12, TNF-α) that favored Th1 and cytotoxic T cell polarization, and decreased levels of the regulatory cytokine TGF-β [7-13]. Such immunostimulatory effects are common characteristics to vaccine adjuvants. Adjuvanticity of various *Lactobacillus* strains in enhancing cellular and/or humoral immune responses has been reported in studies of influenza, polio, rotavirus and cholera vaccines and rotavirus and *Salmonella typhi* Ty21a infections [14-21].

Although the strain-specific effects of probiotics in up- or down-regulating proinflammatory immune responses [22] or in inducing Treg cell responses [23] have been well recognized, the dose effects of probiotics on such immune responses are not clearly understood. A recent study on the dose-dependent immunomodulation of human DCs by the probiotic *L. rhamnosus* strain Lcr35 showed that very different profiles of gene expression were induced with different doses [24]. The authors suggested that depending on the doses ingested and their frequency, the effects of probiotics could be very different and suitable for the treatment of different diseases via pro- or anti-inflammatory responses [24].

The objective of the present study is to evaluate the dose effects of immunomodulation of probiotic LA on T cell responses and protection induced by an oral rotavirus vaccine. Our overall goal is to identify the LA dose that is most effective in potentiating the vaccine-induced protective immunity. Gnotobiotic (Gn) pigs monoassociated with LA provide an ideal animal model

for the study. The distinctive advantages of using the Gn pig model to study the immunomodulating mechanism of different doses of lactobacilli include: (1) the highly recognized similarities between human and porcine intestinal physiology and mucosal immune system; (2) the gnotobiotic status prevents confounding factors from commensal microflora that are present in conventionally reared animals or in humans; and, (3) unlike Gn mice, Gn pigs are devoid of maternal antibodies thus providing an immunologically naïve background that allows clear identification of the immune responses to a single vaccine in hosts colonized with a qualitatively and quantitatively defined probiotic bacterial strain. The Gn pig model of human rotavirus (HRV) infection and probiotic colonization has been well established in our previous studies [17, 25, 26].

The prominent cell types involved in transducing immunomodulating signals from probiotics to protective effector/memory T and B cell immune responses include epithelial cells, macrophages, DCs and Treg cells. In this study, we investigated the effects of high dose (10^3 to 10^9 CFU/dose for 14 days with 10-fold incremental dose increase every other day) and low dose LA (10^3, 10^4, 10^5, 10^6, and 10^6 CFU/dose at 3, 5, 7, 9 and 11 days of age) feeding on virus-specific IFN-γ producing CD4+ and CD8+ T cell responses, and IL-10 or TGF-β producing nTreg and iTreg cell responses in Gn pigs vaccinated with an oral attenuated HRV (AttHRV) vaccine and challenged with the virulent HRV (VirHRV). In parallel, we also studied the dose effects of LA on B cell and DC responses *in vivo* in Gn pigs and on cytokine profiles of intestinal epithelial

cells *in vitro* using the IPEC-J2 cell line model [27] of porcine rotavirus infection.

There is accumulating evidence that probiotics' immunomodulating effects are not limited to the gut. Oral administration of *L. fermentum* strain CECT5716 potentiated the Th1 type response in blood and virus-neutralizing antibody levels in serum of adults vaccinated parentally with an influenza vaccine [15]. LA feeding significantly increased IFN-γ producing CD8+ T cell responses to AttHRV vaccine not only in the intestine but also in spleen and blood in Gn pigs [17]. An important question we also aim to address is whether varying oral doses of probiotics differentially modulate the intestinal versus systemic immune responses. We measured the T cell responses in the intestinal (ileum) and systemic (spleen) lymphoid tissues and blood of Gn pigs and associated enhanced intestinal Th1 type response with increased protection in the low dose LA group. We demonstrated that high dose and low dose LA can exert qualitatively opposite immune modulating effects on Th1 cell and Treg cell responses with and without rotavirus vaccines and the effects were similar at the intestinal and systemic sites.

3.3 Materials and methods

Virus. The cell culture-adapted Wa strain (G1P1A[8]) HRV, derived from the 34th passage in African green monkey kidney cells (MA104), was used as the AttHRV vaccine to inoculate Gn pigs at a dose of 5×10^7 fluorescent focus-

forming unit (FFU) [28]. The AttHRV was also used as detector antigens in the enzyme-linked immunosorent assay (ELISA) and as stimulating antigens in the intracellular IFN-γ staining assay as described previously [29].

The VirHRV Wa strain was passaged through Gn pigs and the pooled intestinal contents from the 23th passage were used to challenge Gn pigs at a dose of 1×10^5 FFU. The median infentious dose (ID_{50}) of the VirHRV in Gn pigs were determined as approximately 1 FFU [30].

Bacteria. LA NCFM strain (also known as NCK56, kindly provided by Dr. Ahmed Yousef, Department of Food Science and Technology, The Ohio State University) was used in this study and was propagated in lactobacilli MRS broth (Weber, Hamilton, NJ, USA). A large stock was prepared so that the amount of LA is enough for the entire study. The stock was aliquoted and stored in -80 °C. When used, one vial was thawed and diluted in 0.1 % peptone water to the needed titers. LA inoculums were prepared and titrated as we previously described [26]. The enumeration of LA in Gn pig fecal samples was performed throughout the experiment to confirm colonization as previously described [26].

Treatment groups and inoculation of Gn pigs. Near-term pigs were derived by hysterectomy and maintained in germfree isolator units as described [31]. All pigs were confirmed seronegative for rotavirus antibodies and germfree prior to LA and AttHRV exposure. Gn pigs (both males and females) were randomly assigned to six treatment groups (n = 8-13): (1) high dose LA plus AttHRV (HiLA+AttHRV+), (2) low dose LA plus AttHRV (LoLA+AttHRV+),

(3) AttHRV only (LA-AttHRV+), (4) high dose LA only (HiLA+), (5) low dose LA only (LoLA+), and (6) control (LA-AttHRV-). Pigs in HiLA+ groups were orally dosed daily with 10^3 to 10^9 CFU/dose of LA in 3 ml of 0.1 % peptone water (BD Biosciences) for 14 days with 10-fold incremental dose increase every other day from 3-16 days of age (Table 3. 1). Pigs in LoLA+ groups were orally dosed with 10^3, 10^4, 10^5, 10^6, and 10^6 CFU/dose of LA on 3, 5, 7, 9 and 11 days of age, respectively. Pigs in non-LA fed groups (LA-AttHRV+ and LA-AttHRV-) were given an equal volume of 0.1 % peptone water. Pigs in AttHRV+ groups were orally inoculated with two doses of 5×10^7 FFU AttHRV diluted in 5 ml of Dulbecco's Modified Eagle's Medium (DMEM) at 5 and 15 days of age [post-inoculation day (PID) 0 and 10, respectively]. At PID 28, subsets of pigs from all treatment groups were orally challenged with 10^5 FFU VirHRV [30]. Pigs were given 5 ml of 100 mM sodium bicarbonate to reduce gastric acidity 20 min before HRV inoculation. Pigs were examined daily postchallenge for clinical signs, including prevalence, duration and severity of diarrhea as described [28], and rectal swabs were collected daily for HRV shedding. Pigs were euthanized on PID 28 (postchallenge day [PCD] 0) or PID 35 (PCD 7) to isolate mononuclear cells (MNCs) from ileum, spleen and peripheral blood [28]. Briefly, MNCs were extracted from the ileum by using EDTA twice (to collect intraepithelial lymphocytes (IEL)) and collagenase twice and enriched by discontinuous Percoll gradient, from the spleen by mechanical separation and enriched by discontinuous Percoll gradient, and from blood by

using Ficoll-Paque^{TM} plus. All animal experimental procedures were conducted in accordance with protocols approved by Institutional Animal Care and Use Committees of Virginia Polytechnic Institute and State University.

Detection of rotavirus and LA shedding and assessment of rotavirus diarrhea. Rectal swabs were collected on PID 5, 10, 21, and 28 for enumeration of LA shedding and collected weekly for sterility test as described previously [25]. Rectal swabs were also collected for 7 days after VirHRV challenge to assess rotavirus shedding and diarrhea. Virus shedding was detected by ELISA and cell-culture immunofluorescent (CCIF) assay in processed rectal swab fluids as described previously [32]. The severity of diarrhea was assessed using fecal consistency scores as we previously described [28].

Intracellular cytokine staining and flow cytometry analysis of IFN-γ producing CD4+ and CD8+ T cells. Flow cytometry was used to determine frequencies of HRV-specific and non-specific IFN-γ producing CD4+ and CD8+ T cells in ileum, IEL, spleen and blood of Gn pigs as we previously described [29]. Briefly, the purified MNCs from all tissues were resuspended in complete medium consisting of RPMI-1640 (Gibco, BRL) supplemented with 8 % fetal bovine serum, 20 mM HEPES (*N*-2-hydroxyethyl-piperazine-Nk-2-ethanesulphonic acid), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 μg/ml of gentamicin, 10 μg/ml of ampicillin and 50 mM 2-mercaptoethanol (E-RPMI). The cells were restimulated *in vitro* with

semi-purified AttHRV antigen (12 μg/ml), positive control PHA (10 μg/ml) or mock stimulated (E-RPMI only) for 17 hrs at 37 °C. Brefeldin A (10 μg/ml; Sigma) was added for the last 5 hrs to block secretion of cytokines produced by the T cells. For flow cytometry, all antibodies and reagents were titrated and used at optimal concentrations. The appropriate isotype-matched irrelevant antibody controls were included for MNCs from each tissue in each staining as negative controls to set the quadrant markers for the bivariate dot plots [29]. The second set of controls was stained with all the antibodies to surface markers and the cytokine antibody was replaced with the isotype control antibody. At least 100,000 cells were acquired on a FACSAria flow cytometer (BD Biosciences). Data were analyzed using FlowJo 7.2.2 software (Tree Star, Ashland, Oregon). The frequencies of IFN-γ+CD4+ and IFN-γ+CD8+ T cells were expressed as percentages among total CD3+ T cells. All mean frequencies are reported after subtraction of the background frequencies.

Intracellular cytokine staining and flow cytometry analysis of TGF-β and IL-10 producing nTreg and iTreg cells. The MNCs were stained freshly on the same day of MNC isolation. The MNCs (2×10^6 cells/tube) were first stained at 4 °C for 15 min with FITC conjugated mouse anti-porcine CD4α (IgG2b, BD Pharmingen), SpectralRed™ (SPRD) conjugated mouse anti-porcine CD8α (IgG2a, BD Pharmingen), and mouse anti-porcine CD25 (IgG1, AbDSerotec), followed by APC conjugated rat anti-mouse IgG1 (IgG1, BD pharmingen). After staining of cell surface markers, the MNCs were

permeabilized with FoxP3 (transcription factor forkhead box P3) Staining Buffer Set (eBiosciences) at 4 °C for 30 min. Then the cells were washed with FoxP3 Staining Buffer Set and stained with the phycoerythrin-cyanine tandem fluorochrome (PE-Cy7) conjugated rat anti-mouse/rat FoxP3 (IgG2a, eBioscience), biotin conjugated mouse anti-porcine IL-10 (IgG1, Cell Sciences), and PE conjugated mouse anti-human TGF-β1 (IgG1, R&D Systems), followed by staining with streptavidin conjugated pacific blue (Invitrogen) at 4 °C for 30 min. The rat anti-mouse/rat-FoxP3 antibody cross-reacts with porcine FoxP3, allowing the identification of porcine Treg cells [33]. The frequencies of CD4+CD25+FoxP3+ (natural) and CD4+CD25-FoxP3+ (induced) Treg cells were expressed as percentages among total MNCs. The frequencies of IL-10+ and TGF-β+ cells were expressed as percentages among CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+ Treg cells, respectively. All mean frequencies are reported after subtraction of the background frequencies.

Statistical analysis. Non-parametric Kruskal-Wallis rank sum test was performed to compare frequencies of T cell subsets in different tissues among treatment groups at each time point. When differences among the groups were detected, the same test was used in a pairwise fashion to identify the nature of the differences. One-way analysis of variance (ANOVA-general linear model), followed by Duncan's multiple range test, were used to compare mean duration of virus shedding and diarrhea, and mean cumulative fecal consistency scores. All statistical significance was assessed at $p < 0.05$.

3.4 Results

Intestinal bacterial load reached equilibrium at PID 10 in both low and high dose LA fed Gn pigs. LA colonization in all LA-fed Gn pigs were confirmed by LA enumeration in rectal swab samples collected on PID 5, 10, 21, and 28 (10, 15, 26 and 33 days of age, respectively). The high dose LA fed pigs shed significantly higher titers of LA than the low dose group at PID 5 (Table 3.1). From PID 10 on, the LA mean shedding titers in both high and low dose LA groups were maintained between 2.2×10^5 and 8.4×10^6 CFU/ml. There was no significant difference in LA shedding titers between the high and low dose LA groups at any time point except PID 5. The Gn pigs in low LA dose groups received the last LA feeding (10^6 CFU) on PID 6 whereas the Gn pigs in high LA dose groups received the last LA feeding (10^9 CFU) on PID 11 (Table 3.1). Therefore, after colonization, the intestinal bacterial load reached equilibrium quickly and the number of LA the Gn pigs received later did not influence the LA fecal shedding. There was a trend for increased titers of LA shedding in the low dose LA group and decreased titers in the high dose LA group, but the differences at PID 10, 21 and 28 were not statistically significant.

Representative responses in four treatment groups detected by flow cytometry analyses. Flow cytometry analyses of HRV-specific IFN-γ+CD4+ T cells, nTreg cells (CD4+CD25+FoxP3+), iTreg cells (CD4+CD25-FoxP3+), and TGF-β producing iTreg cells in spleen of Gn pigs from treatment groups HiLA+AttHRV, LoLA+AttHRV+, LA-AttHRV+ and LA-AttHRV- are

illustrated in Fig. 3.1. Analyses of TGF-β producing nTreg and IL-10 producing nTreg and iTreg cells followed the same gating strategy (not shown). Fig. 3.1A shows representative dot plots of the HRV-specific IFN-γ+CD4+ T cells in the four treatment groups and the isotype control. LoLA+AttHRV+ pigs had the highest frequencies of IFN-γ+CD4+ T cells in spleen at PID35 (PCD 7) compared to other treatment groups. Detailed comparison of IFN-γ producing CD4+ and CD8+ T cell responses in the intestinal and systemic lymphoid tissues and blood among the treatment groups are presented in Figs. 3.2 and Fig. 3.3. Fig3.1B shows representative dot plots of nTreg and iTreg cells among MNCs in the four treatment groups and the isotype control. Fig. 3.1C shows the frequencies of TGF-β producing cells among iTreg cells. The Gn pigs in the HiLA+AttHRV+ group had the highest frequencies of nTreg and iTreg cells and TGF-β producing iTreg cells in spleen compared to Gn pigs in other treatment groups. Detailed comparison of Treg cell responses in the intestinal and systemic lymphoid tissues and blood among the treatment groups are illustrated in Figs. 3.4-3.9.

Low dose LA enhanced HRV-specific and non-specific IFN-γ producing T cell responses. The magnitude of HRV-specific IFN-γ producing T cell responses in Gn pigs was differentially modulated by low versus high dose LA at both prechallenge (PID 28 [PCD 0]) and postchallenge (PID 35 [PCD 7]). As shown in Fig. 3.2, high dose LA did not enhance the HRV-specific IFN-γ producing CD4+ and CD8+ T cell responses in the HiLA+HRV+ pigs compared

to AttHRV only pigs. In contrast, LoLA+AttHRV+ pigs had significantly higher frequencies of HRV-specific IFN-γ+CD8+ T cells in ileum (11- and 5-fold higher pre- and post-challenge, respectively), IEL (6-fold higher prechallenge), spleen (4-fold higher prechallenge) and blood (20-fold higher postchallenge) compared to the AttHRV only pigs. The LoLA+AttHRV+ pigs also had significantly higher frequencies of HRV-specific IFN-γ+CD4+ T cells in blood (3-fold higher for both pre- and post-challenge) compared to the AttHRV only pigs. Consequently, the AttHRV vaccine with high dose LA induced overall lower HRV-specific IFN-γ producing CD4+ and CD8+ T cell responses in all tissues of Gn pigs than the AttHRV vaccine with low dose LA, pre- and post-challenge. The frequencies of IFN-γ+CD8+ T cells in ileum, IEL, and blood pre- and post-challenge and IFN-γ+CD4+ T cells in ileum and blood prechallenge and in spleen and blood postchallenge of the LoLA+AttHRV+ pigs were significantly higher (ranging from 4- to 24-fold) than the HiLA+AttHRV+ pigs.

In the low dose and high dose LA only groups (Fig. 3.3), the non-specific IFN-γ producing CD4+ and CD8+ T cell responses (detected from mock-stimulated MNCs) also differed significantly, with low dose LA inducing higher or significantly higher frequencies of IFN-γ+CD4+ and IFN-γ+CD8+ T cells in most tissues compared to the high dose LA fed group and the non-LA fed control group pre- and post-challenge. The results indicate that the dose effect of LA on the IFN-γ producing T cell response is independent of AttHRV vaccine

or VirHRV challenge. Low dose, but not high dose, LA acted as a general immunostimulator to promote the development of IFN-γ producing T cells in the intestinal and systemic lymphoid tissues and blood.

Low dose LA strongly and significantly reduced the frequencies of intestinal and systemic iTreg cells. Because the magnitude of virus-specific T cell responses are modulated by both nTreg (CD4+CD25+FoxP3+) and iTreg (CD4+CD25-FoxP3+) cells, we compared frequencies of the Treg cells induced by high and low dose LA in the vaccinated pigs (HiLA+AttHRV+ versus LoLA+AttHRV+) pre- and post-challenge. LoLA+AttHRV+ pigs had significantly lower frequencies of iTreg cells (ranging from 11- to 90-fold lower) in all the tissues compared to HiLA+AttHRV+ pigs pre- and post-challenge (Fig. 3.4 bottom panel). The dose effects of LA on nTreg cells were similar to iTreg cells but at a reduced magnitude (note the difference in y axis scales in Fig. 3.4). LoLA+AttHRV+ pigs had lower or significantly lower frequencies of nTreg cells (2- to 3-fold lower) in spleen and blood compared to HiLA+AttHRV+ pigs pre- and post-challenge. Conversely, low dose LA induced slightly higher, but significant, nTreg cell responses in the intestine (2- to 3-fold higher) compared to HiLA+AttHRV+ pigs prechallenge and did not have a significant effect on intestinal nTreg cells postchallenge (Fig. 3.4 top panel).

In the pigs fed high or low dose LA without AttHRV vaccine, LA had similar dose effects on the Treg cell responses compared to the AttHRV vaccinated pigs

(Fig. 3.5). Low dose LA fed pigs had significantly reduced frequencies of nTreg in IEL and spleen, iTreg cells in ileum, IEL and spleen prechallenge, and iTreg cells in ileum, IEL and spleen postchallenge compared to the non-LA fed controls. On the other hand, high dose LA fed pigs had significantly higher frequencies of iTreg cells in spleen prechallenge and in ileum, spleen and blood postchallenge compared to the low dose LA and non-LA fed control groups. High dose LA group also had significantly higher frequencies of nTreg cells in ileum and spleen postchallenge compared to the low LA and non-LA control groups. Consistent with the dose effect of LA on the IFN-γ producing T cell response, these results showed that the dose effect of LA on Treg cells is independent of AttHRV vaccine or VirHRV challenge. The dose effect on iTreg cells was substantially stronger postchallenge than prechallenge (note the difference in y axis scales of the bottom panel in Fig. 3.5).

High dose LA induced higher TGF-β producing Treg cell responses than low dose LA. Because Treg cells exert regulatory functions through mechanisms involving TGF-β, we compared frequencies of the Treg cell subsets that produced TGF-β (by intracellular staining) between the low and high dose LA groups. The HiLA+AttHRV+ pigs had higher or significantly higher frequencies of TGF-β producing nTreg and iTreg cells than the LoLA+AttHRV+ pigs in all tissues pre- and post-challenge (Fig. 3.6). The difference was greater between the LA dose groups in iTreg cells than nTreg cells, especially postchallenge.

In the pigs fed high or low dose LA without AttHRV vaccine (Fig. 3.7), low dose LA fed pigs had significantly reduced frequencies of TGF-β producing iTreg cells in ileum, spleen and blood prechallenge compared to the non-LA control pigs (Fig. 3.7A bottom panel). Postchallenge (Fig. 3.7B), frequencies of TGF-β producing nTreg cells in IEL and TGF-β producing iTreg cell in ileum were significantly lower in the low dose group compared to the non-LA control pigs. Interestingly, the frequencies of TGF-β+ iTreg cells in the high dose group were significantly higher in IEL; while significantly lower in ileum, compared to the non-LA control group. There was a trend that high dose LA increased frequencies of both TGF-β+ nTreg and iTreg cells in all tissues except spleen prechallenge; while decreased the frequency of both TGF-β+ nTreg and iTreg cells in all tissues except IEL postchallenge. However, these differences between high dose LA and non-LA control groups were not statistically significant due to the high variability of the data.

Taken together, low dose LA, with or without AttHRV vaccine, down-regulated the TGF-β production in nTreg or iTreg cells in the intestinal or systemic lymphoid tissues. With few exceptions, high dose LA did not significantly influence the TGF-β production in Treg cells.

High dose LA induced higher IL-10 producing Treg cell responses than low dose LA. Because IL-10 is the most important regulatory cytokine in virus infection and immunity; increased IL-10 production has been associated with reduced anti-viral immunity, we compared frequencies of the Treg cell subsets

that produced IL-10 (by intracellular staining) between the low and high dose LA groups. The high dose LA induced overall higher frequencies of IL-10 producing nTreg and iTreg cells in all tissues (except for IL-10 producing iTreg cells in IEL postchallenge) than the low dose LA pre- and post-challenge (Fig. 3.8).

In the pigs fed with high or low dose LA without AttHRV vaccine, low dose LA significantly reduced the frequencies of IL-10 producing nTreg cells in ileum and IL-10 producing iTreg cells in blood prechallenge (Fig. 3.9A) and IL-10 producing nTreg cells in IEL and blood postchallenge (Fig. 3.9B) compared to the non-LA fed control pigs. There was a trend for higher frequencies of IL-10 producing iTreg in the high dose LA group prechallenge, but lower frequencies of IL-10 producing nTreg and iTreg cells postchallenge compared to the non-LA fed controls. However, the differences were not statistically significant.

Low dose LA slightly enhanced protection conferred by the AttHRV vaccine against rotavirus diarrhea upon VirHRV challenge. To examine the effects of low and high dose LA on improving the protection conferred by the AttHRV vaccine, subsets of Gn pigs from each treatment group were challenged with VirHRV at PID 28. Clinical signs and virus shedding were monitored for 7 days postchallenge (Table 3.2). After VirHRV challenge, although the proportion of pigs that developed virus shedding and diarrhea did not differ significantly among the three AttHRV vaccinated groups (31-50 %, data not

shown), the LoLA+AttHRV+ group had the shortest mean durations of fecal virus shedding and diarrhea and the lowest mean cumulative fecal consistency scores among all the treatment groups. The durations of diarrhea in the LoLA+AttHRV+ pigs were significantly shorter compared to the LA-AttHRV+ and the mock-vaccinated control pigs. The durations of virus shedding in the LoLA+AttHRV+ pigs were significantly shorter compared to the HiLA+AttHRV+ and the mock control pigs. The mean cumulative fecal consistency scores in all the pigs in the LoLA+AttHRV+ and LA-AttHRV+ groups (8.4 and 9.0, respectively) were significantly lower than the control group, indicating significant protection against the severity of diarrhea. Although the fecal scores in the HiLA+AttHRV+ group were lower than the control group (12.5 versus 16.9) they did not differ significantly. Thus, low dose LA slightly but clearly improved the protection conferred by the AttHRV vaccine against rotavirus diarrhea. In contrast, high dose LA reduced the protection conferred by the AttHRV vaccine as indicated by the significantly longer mean duration of virus shedding (3.8 versus 1.3 days) and higher mean cumulative fecal scores compared to the AttHRV only pigs.

3.5 Discussion

In this study, we demonstrated that the low dose LA feeding regimen promoted Th1 type immune responses and reduced the frequencies and functions (regulatory cytokine TGF-β and IL-10 production) of Treg cells

whereas the high dose LA feeding regimen promoted the development of Treg cell responses in Gn pigs vaccinated with the AttHRV vaccine. Corresponding to the differentially altered Th1 and Treg cell responses, low dose LA slightly but clearly improved the protection conferred by the AttHRV vaccine against rotavirus diarrhea whereas high dose LA significantly prolonged the virus shedding (for 2.5 days) compared to the AttHRV vaccine alone upon challenge with the VirHRV. Thus dose effects of LA on rotavirus vaccine-induced protective immunity were highly significant. The same probiotic LA strain at low or high dose exerted opposite modulating effects on the T cell immune responses induced by the oral AttHRV vaccine.

Our previous studies have demonstrated that the low dose LA was sufficient to colonize the intestine of germfree pigs [17, 25]. However, it was unexpected in this study that the extra amounts of LA ingested by the HiLA+ pigs did not result in higher numbers of LA fecal shedding beyond PID 5. Innate immune responses induced by the LA (i.e., α-defensins secreted by Paneth cells, neutrophil and γδ T cell responses) may play important roles in controlling the loads of colonizing bacteria in the gut [34]. Studies of Gn mice inoculated with *L. johnsonii* or *L. paracasei* at the same dose showed that the two *Lactobacillus* strains shed in very different titers in feces, suggesting that different *Lactobacillus* strains colonize the gut in different amounts [35]. The study also showed that bacterial counts from fecal samples in the killing days were similar to the counts from the luminal contents of the colon, indicating that fecal

bacterial counts reflect the relative bacterial loads in the gut [35]. In our study, although the additional high dose LA the HiLA+ pigs received from PID 6-11 did not increase the intestinal bacterial load, the effects between the high and low dose LA on the Th1 and Treg cell, especially iTreg cell, immune responses measured 3-4 weeks after PID 5 (PID 28 and PCD 7) were strikingly different. Therefore the initially different LA doses that the intestinal innate immune cells (epithelial cells, macrophages, DCs, nTreg cells, etc.) interacted with before PID 10 had determined the profile of the adaptive Th1 and iTreg cell responses developed later on.

It has been suggested that low and high dose microbe-associated molecular pattern (MAMP), i.e. bacterial LPS from *E. coli* and peptidoglycan from LA, engages different receptor conformations, and/or differentially distributes to sub-cellular locations and subsequently, activates different downstream pathways [36]. The effect of low dose LPS was strikingly different as compared to that of high dose LPS on macrophage cell functions: low dose LPS induced a strong inflammatory response in macrophages. It is plausible that similar interaction occurs between the MAMP from LA and innate immune cells in the gut. Future studies are needed to identify the molecular mechanisms of the dose responses of different MAMP.

To our knowledge, this study is the first *in vivo* study to investigate the dose effects of probiotics on immune responses and the protective efficacy of an oral viral vaccine. Other studies have shown similar qualitative, but not quantitative,

differences in the immunological effects between two dosages of probiotics without vaccination. In a double-blind, placebo-controlled, randomized clinical trial, two different doses of *L. plantarum* strain CECT7315/7316 were given to elderly subjects for 12 weeks (low dose = 5×10^8 CFU/day and high dose = 5×10^9 CFU/day) [37]. After treatment, high probiotic dose resulted in significant increases in the frequencies of CD8+CD25+ T cells and NK cells, while low probiotic dose increased CD4+CD25+ T cells, B cells, and antigen presenting cells in peripheral blood. Although the study did not examine the function of the different T cell populations, the authors suggested that low dose probiotics might be useful as vaccine adjuvant while higher dose might be useful to prevent infections [37]. Another recent study showed that low concentrations ($< 1 \times 10^6$ CFU/ml) of *Lactobacillus* and *Bifidobacterium* mixtures enhanced IFN-γ production and inhibited IL-4 production in mitogen-activated murine and human splenic T cells, whereas high concentrations ($\geq 1 \times 10^6$ CFU/ml) inhibited mitogen-induced T cell proliferation [38].

Low dose LA promoted Th1 and down-regulated Treg cell responses whereas high dose LA promoted Treg cell responses in Gn pigs (with or without AttHRV vaccine). These findings may explain some of the controversies that the same probiotic strains used by different research groups in animal studies showed opposite immunomodulatory functions. For example, administration of *L. casei* suppressed pro-inflammatory cytokine expression by CD4+ T cells and up-regulated IL-10 and TGF-β levels in rats [3, 4]. To the contrary, Van Overtvelt

et al. found that *L. casei* was a pure Th1 inducer in mice In addition to the difference in animal species, the *L. casei* doses used by the different studies differed significantly, with much higher doses used in So et al.'s studies [3, 4]. In So et al.'s studies the amount of *L. casei* was 5×10^9 or 2×10^{10} CFU/dose per rat, three times per week for 11-12 weeks. In Van Overtvelt et al.'s study, the amount of *L. casei* was 2×10^8 CFU/dose per mouse, twice per week for 8 weeks [7]. Additional caution concerning the dosages is needed in comparing and interpreting results from different probiotic studies.

The dose effect of LA on immune responses to the AttHRV vaccine in Gn pigs may also partly explain why the efficacies of oral rotavirus vaccines are significantly lower in developing countries compared to developed countries. The two licensed rotavirus vaccines, RotaTeq and Rotarix have a protective efficacy of > 85 % against moderate to severe rotavirus gastroenteritis in middle and high-income countries [39]. However, the protective efficacy of RotaTeq vaccine is only 39.3 % against severe rotavirus gastroenteritis in developing countries in sub-Saharan Africa [40] and 48.3 % in developing countries in Asia [41]. Rotarix vaccine showed a similar disparity in efficacy in developing countries in Africa [42]. During the initial colonization of human infants, exposure to high dose commensal bacteria (common in countries with lower hygiene standards) would have a suppressive effect on Th1 type responses and promote Treg cell responses, thus leading to the lowered protective immunity after rotavirus vaccination.

We reported previously that protection rates against rotavirus infection and diarrhea are correlated with virus-specific intestinal IFN-γ producing T cell and IgA antibody-secreting cell responses at PID 28 in Gn pigs [28, 29]. A balanced Th1 and Th2 type response is needed for the optimal protective immunity against rotavirus. Although low dose LA further reduced the duration of diarrhea in the AttHRV-vaccinated pigs postchallenge, neither low nor high dose LA significantly altered protection rate against rotavirus challenge (proportions of pigs that were infected and developed diarrhea after challenge). The differences in protection conferred by the HiLA+AttHRV+ versus LoLA+AttHRV vaccine against rotavirus shedding and diarrhea were not as substantial as the differences in the magnitudes of the intestinal IFN-γ producing CD8+ T cell responses between the two treatment groups. Because virus-specific intestinal IgA antibody-secreting cell responses probably play a more important role in rotavirus protective immunity than the IFN-γ producing CD8+ T cell responses [28, 29], the dose effect of LA on virus-specific antibody-secreting cell responses also need to be taken into consideration in understanding the differences in the protection conferred by the AttHRV vaccine with high or low dose LA. Indeed, low and high dose LA differentially altered the magnitude of virus-specific intestinal IgA antibody-secreting cell responses, with the LoLA+AttHRV+ vaccine inducing lower antibody-secreting cell responses than the HiLA+AttHRV+ (data not shown). To improve the AttHRV vaccine efficacy, an intermediate dose of LA may be optimal to promote a

balanced Th1 and Th2 response and without increased Treg cell responses.

Probiotics are increasingly used to improve human health, alleviate disease symptoms and to enhance vaccine efficacies. Our findings suggest that probiotics can be ineffective or even detrimental if not used at the optimal dosage for the appropriate purposes, highlighting the importance of dose selection in probiotic studies. Dose effects of each probiotic product should be fully investigated in clinical trials before use for improving human health or treating diseases. The underlying mechanisms of dose effects of probiotics require further study.

3.6 Acknowledgements

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3.7 References

1. Geier, M.S., et al., *Lactobacillus fermentum BR11, a potential new probiotic, alleviates symptoms of colitis induced by dextran sulfate sodium (DSS) in rats.* Int J Food Microbiol, 2007. 114(3): p. 267-74.

2. Carroll, I.M., et al., *Anti-inflammatory properties of Lactobacillus gasseri expressing manganese superoxide dismutase using the interleukin 10-deficient mouse model of colitis.* Am J Physiol Gastrointest Liver Physiol, 2007. 293(4): p. G729-38.

3. So, J.S., et al., *Lactobacillus casei potentiates induction of oral tolerance in experimental arthritis.* Mol Immunol, 2008. 46(1): p. 172-80.

4. So, J.S., et al., *Lactobacillus casei suppresses experimental arthritis by down-regulating T helper 1 effector functions.* Mol Immunol, 2008. 45(9):

p. 2690-9.

5. Wickens, K., et al., *A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial.* J Allergy Clin Immunol, 2008. 122(4): p. 788-94.

6. Weston, S., et al., *Effects of probiotics on atopic dermatitis: a randomised controlled trial.* Arch Dis Child, 2005. 90(9): p. 892-7.

7. Van Overtvelt, L., et al., *Lactic acid bacteria as adjuvants for sublingual allergy vaccines.* Vaccine, 2010. 28(17): p. 2986-92.

8. Vitini, E., et al., *Gut mucosal immunostimulation by lactic acid bacteria.* Biocell, 2000. 24(3): p. 223-32.

9. Yazdi, M.H., et al., *Oral administration of Lactobacillus acidophilus induces IL-12 production in spleen cell culture of BALB/c mice bearing transplanted breast tumour.* Br J Nutr, 2010. 104(2): p. 227-32.

10. Weiss, G., et al., *Lactobacillus acidophilus induces virus immune defence genes in murine dendritic cells by a Toll-like receptor-2-dependent mechanism.* Immunology, 2010. 131(2): p. 268-81.

11. Chiba, Y., et al., *Well-controlled proinflammatory cytokine responses of Peyer's patch cells to probiotic Lactobacillus casei.* Immunology, 2010. 130(3): p. 352-62.

12. Christensen, H.R., H. Frokiaer, and J.J. Pestka, *Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells.* J Immunol, 2002. 168(1): p. 171-8.

13. Mohamadzadeh, M., et al., *Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization.* Proc Natl Acad Sci U S A, 2005. 102(8): p. 2880-5.

14. Kaila, M., et al., *Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain.* Pediatr Res, 1992. 32(2): p. 141-4.

15. Olivares, M., et al., *Oral intake of Lactobacillus fermentum CECT5716 enhances the effects of influenza vaccination.* Nutrition, 2007. 23(3): p. 254-60.

16. Paineau, D., et al., *Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial.* FEMS Immunol Med Microbiol, 2008. 53(1): p. 107-13.

17. Zhang, W., et al., *Probiotic Lactobacillus acidophilus enhances the immunogenicity of an oral rotavirus vaccine in gnotobiotic pigs.* Vaccine, 2008. 26(29-30): p. 3655-61.

18. Winkler, P., et al., *Effect of a dietary supplement containing probiotic bacteria plus vitamins and minerals on common cold infections and cellular immune parameters.* Int J Clin Pharmacol Ther, 2005. 43(7): p. 318-26.

19. Mohamadzadeh, M., et al., *Targeting mucosal dendritic cells with microbial antigens from probiotic lactic acid bacteria.* Expert Rev Vaccines, 2008. 7(2): p. 163-74.

20. Isolauri, E., et al., *Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by Lactobacillus casei GG.* Vaccine, 1995. 13(3): p. 310-2.

21. Boge, T., et al., *A probiotic fermented dairy drink improves antibody response to influenza vaccination in the elderly in two randomised controlled trials.* Vaccine, 2009. 27(41): p. 5677-84.

22. Wagner, R.D., et al., *Variable biotherapeutic effects of Lactobacillus acidophilus isolates on orogastric and systemic candidiasis in immunodeficient mice.* Rev Iberoam Micol, 1998. 15(4): p. 271-6.

23. de Roock, S., et al., *Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans.* Clin Exp Allergy, 2010. 40(1): p. 103-10.

24. Evrard, B., et al., *Dose-dependent immunomodulation of human dendritic cells by the probiotic Lactobacillus rhamnosus Lcr35.* PLoS One, 2011. 6(4): p. e18735.

25. Zhang, W., et al., *Influence of probiotic Lactobacilli colonization on neonatal B cell responses in a gnotobiotic pig model of human rotavirus infection and disease.* Vet Immunol Immunopathol, 2008. 122(1-2): p. 175-81.

26. Zhang, W., et al., *Lactic acid bacterial colonization and human rotavirus*

infection influence distribution and frequencies of monocytes/macrophages and dendritic cells in neonatal gnotobiotic pigs. Vet Immunol Immunopathol, 2008. 121(3-4): p. 222-31.

27. Liu, F., et al., *Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics.* Viral Immunol, 2010. 23(2): p. 135-49.

28. Yuan, L., et al., *Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease.* J Virol, 1996. 70(5): p. 3075-83.

29. Yuan, L., et al., *Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs.* Vaccine, 2008. 26(26): p. 3322-31.

30. Ward, L.A., et al., *Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs.* J Gen Virol, 1996. 77 (Pt 7): p. 1431-41.

31. Meyer, R.C., E.H. Bohl, and E.M. Kohler, *Procurement and Maintenance of Germfree Seine for Microbiological Investigations.* Appl Microbiol, 1964. 12: p. 295-300.

32. Azevedo, M.S., et al., *Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus.* J Virol, 2005. 79(9): p. 5428-36.

33. Kaser, T., et al., *Detection of Foxp3 protein expression in porcine T lymphocytes.* Vet Immunol Immunopathol, 2008. 125(1-2): p. 92-101.

34. Ayabe, T., et al., *Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria.* Nat Immunol, 2000. 1(2): p. 113-8.

35. Ibnou-Zekri, N., et al., *Divergent patterns of colonization and immune response elicited from two intestinal Lactobacillus strains that display similar properties in vitro.* Infect Immun, 2003. 71(1): p. 428-36.

36. Maitra, U., et al., *Low-dose endotoxin induces inflammation by selectively removing nuclear receptors and activating CCAAT/enhancer-binding protein delta.* J Immunol, 2011. 186(7): p. 4467-73.

37. Mane, J., et al., *A mixture of Lactobacillus plantarum CECT 7315 and CECT 7316 enhances systemic immunity in elderly subjects. A dose-response, double-blind, placebo-controlled, randomized pilot trial.* Nutr Hosp, 2011. 26(1): p. 228-35.

38. Li, C.Y., et al., *Immunomodulatory Effects of Lactobacillus and Bifidobacterium on Both Murine and Human Mitogen-Activated T Cells.* Int Arch Allergy Immunol, 2011. 156(2): p. 128-136.

39. O'Ryan, M.L., G. Hermosilla, and G. Osorio, *Rotavirus vaccines for the developing world.* Curr Opin Infect Dis, 2009. 22(5): p. 483-9.

40. Armah, G.E., et al., *Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: a randomised, double-blind, placebo-controlled trial.* Lancet, 2010. 376(9741): p. 606-14.

41. Zaman, K., et al., *Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia: a randomised, double-blind, placebo-controlled trial.* Lancet, 2010. 376(9741): p. 615-23.

42. O'Ryan, M. and A.C. Linhares, *Update on Rotarix: an oral human rotavirus vaccine.* Expert Rev Vaccines, 2009. 8(12): p. 1627-41.

Fig. 3.1. Representatives of IFN-γ producing CD3+CD4+ T cells, nTreg and iTreg cells and TGF-β producing iTreg cells in spleen of Gn pigs fed high or low dose LA, vaccinated with AttHRV and challenged with VirHRV at PID35/PCD 7. MNCs were isolated from pigs infected with high dose LA plus AttHRV (HiLA+AttHRV+), low dose LA plus AttHRV (LoLA+AttHRV+), AttHRV only (LA-AttHRV+), or mock-inoculated controls (LA-AttHRV-). A representative dot plot was shown for each inoculation group and isotype control staining. The numbers at the upper right corners of dot plots of panel (A) are the frequencies of HRV-specific IFN-γ+CD4+ T cells among CD3+ T cells. The numbers at the upper left and right corners of dot plots of panel (B) are the frequencies of iTreg cells (CD4+CD25-FoxP3+) and nTreg cells (CD4+CD25-FoxP3+) among MNCs, respectively. The numbers in the rectangles in dot plots of panel (C) are the frequencies of TGF-β+CD4+CD25-FoxP3+ among iTreg cells. The data were summarized by subtracting the frequencies of non-specific cross-reaction shown in the isotype control staining from the frequencies of samples.

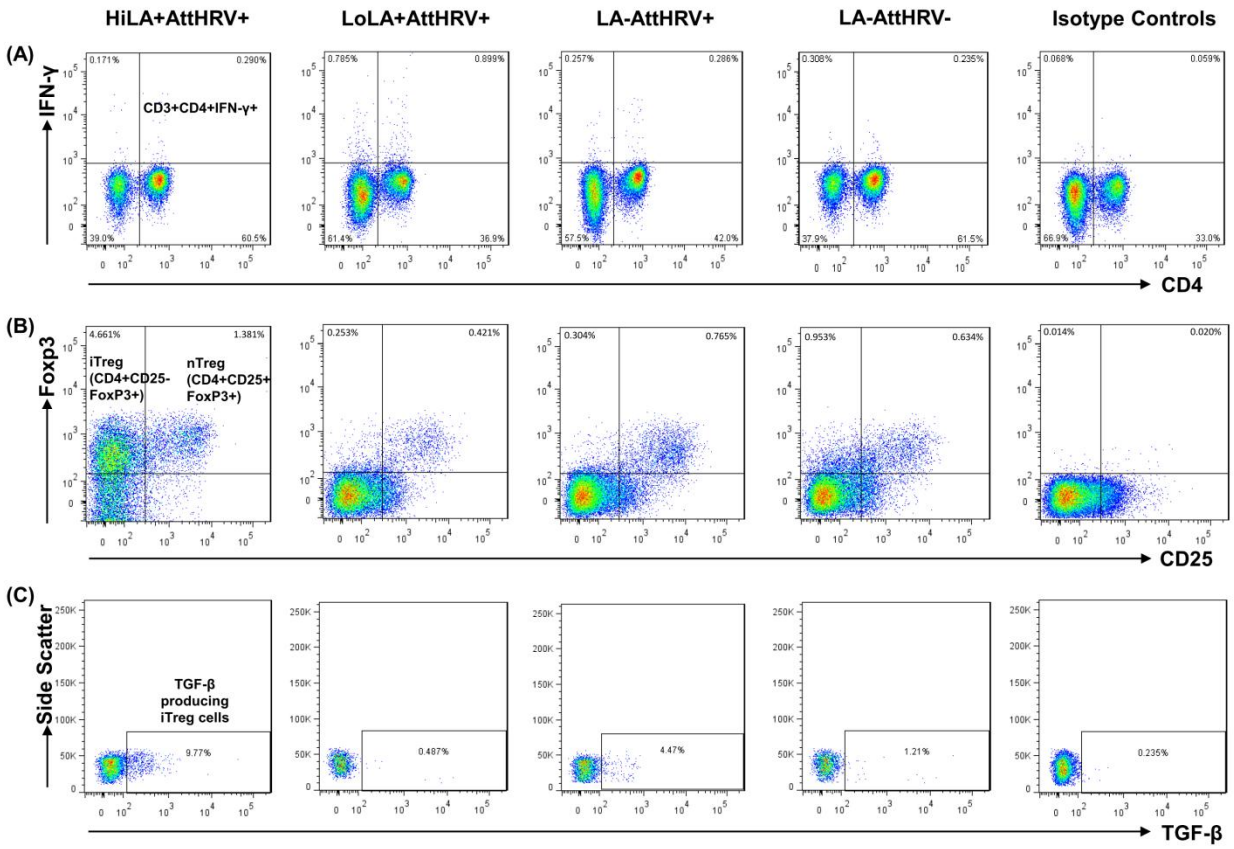
HiLA+AttHRV+
LoLA+AttHRV+
LA-AttHRV+
LA-AttHRV-
Isotype Controls
(A)
IFN-γ
CD3+CD4+IFN-γ+
0.171%
0.290%
39.0%
60.5%
0.785%
0.899%
61.4%
36.9%
0.257%
0.286%
57.5%
42.0%
0.308%
0.235%
37.9%
61.5%
0.068%
0.059%
66.9%
33.0%
CD4
(B)
Foxp3
iTreg
(CD4+CD25-
FoxP3+)
nTreg
(CD4+CD25+
FoxP3+)
4.661%
1.381%
0.253%
0.421%
0.304%
0.765%
0.953%
0.634%
0.014%
0.020%
CD25
(C)
Side Scatter
TGF-β
producing
iTreg cells
9.77%
0.487%
4.47%
1.21%
0.235%
TGF-β

Fig. 3.1

Fig. 3.2. Virus-specific IFN-γ producing T cell responses in Gn pigs vaccinated with AttHRV with or without high or low dose LA and the control. MNCs were stimulated with semi-purified AttHRV antigen *in vitro* for 17 hrs. Brefeldin A was added for the last 5 hrs to block secretion of cytokines produced by the T cells. IFN-γ production was detected by intracellular staining and flow cytometry. Data are presented as mean frequency ± standard error of the mean (n = 3-13). The top two figures show the frequencies of IFN-γ+CD3+CD4+ T cells and the bottom two show those of IFN-γ+CD3+CD8+ T cells. Two figures in panel (A) show the prechallenge data and those in panel (B) show the postchallenge data. Different letters on top of bars indicate significant differences in frequencies among groups for the same cell type and tissue (Kruskal–Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

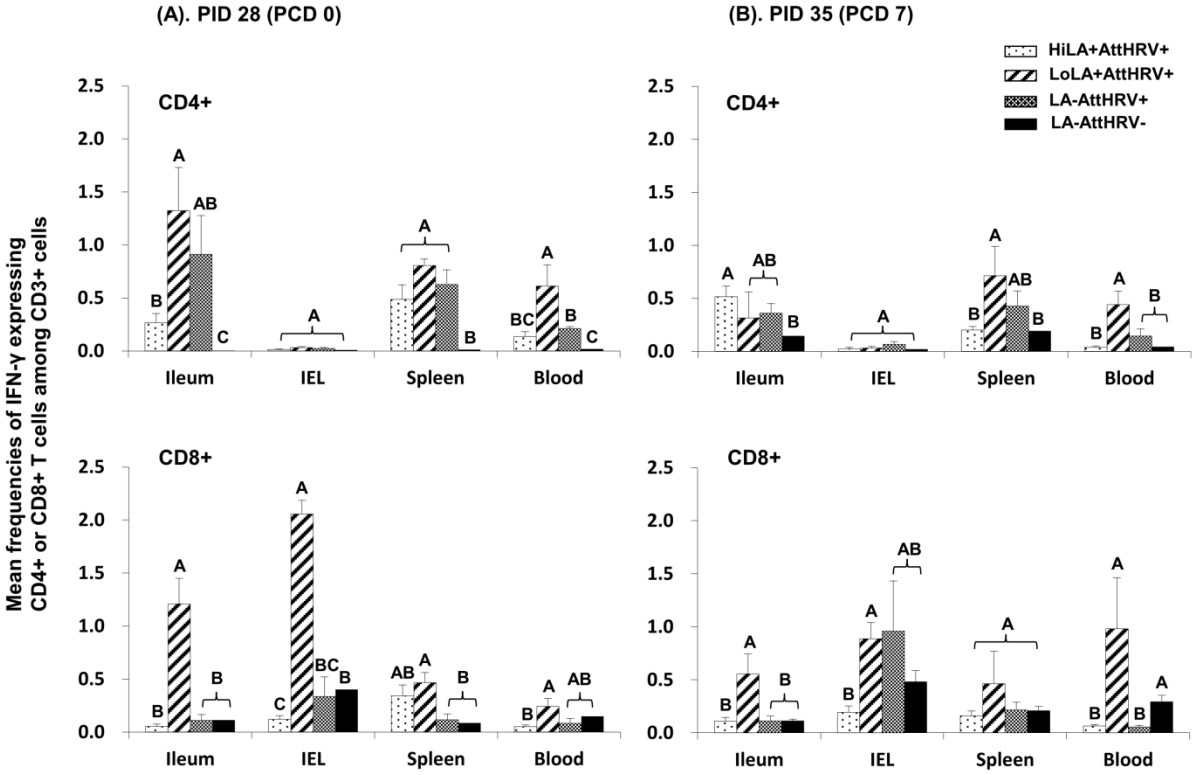
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
HiLA+AttHRV+
LoLA+AttHRV+
LA-AttHRV+
LA-AttHRV-
Mean frequencies of IFN-γ expressing CD4+ or CD8+ T cells among CD3+ cells
CD4+
CD8+
Ileum
IEL
Spleen
Blood

Fig. 3.2

Fig. 3.3. Non-specific IFN-γ producing T cell responses in Gn pigs fed with high dose, low dose LA and the control. MNCs were mock stimulated in vitro for 17 hrs. Data are presented as mean frequency ± standard error of the mean (n = 3-8). See Fig. 3.2 legend for panel description and statistical analysis.

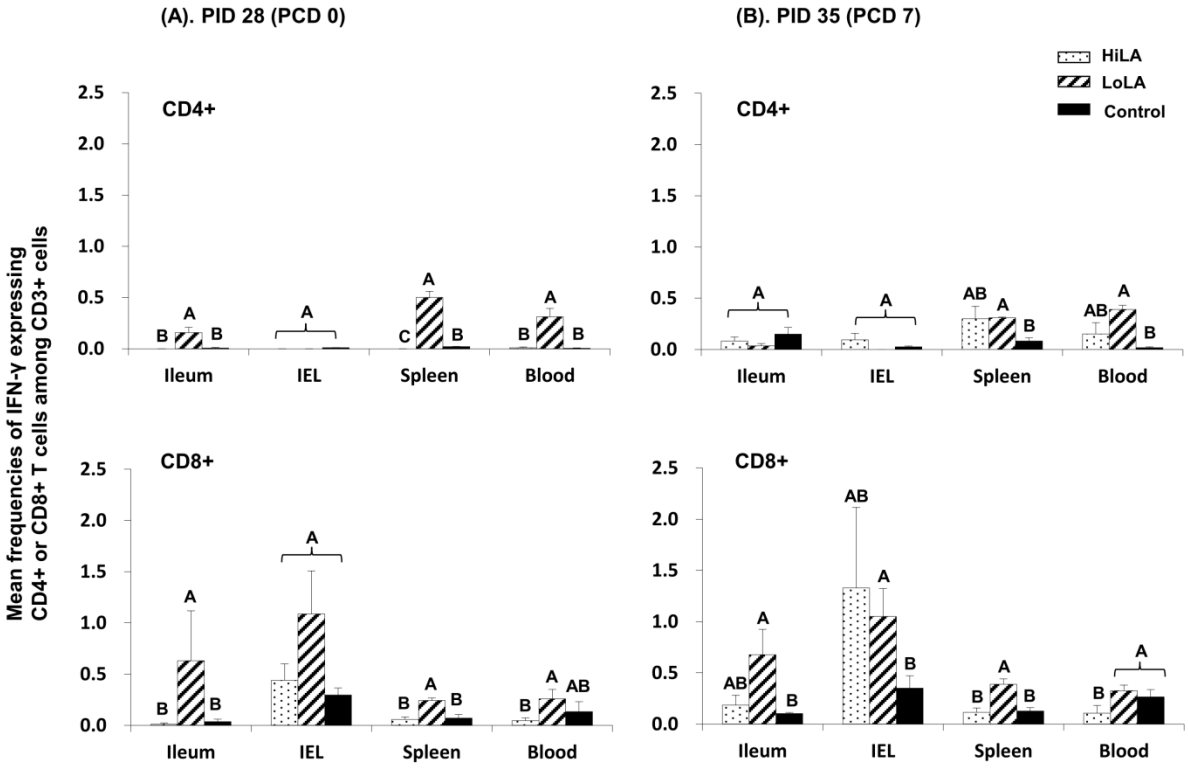
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
HiLA
LoLA
Control
Mean frequencies of IFN-γ expressing CD4+ or CD8+ T cells among CD3+ cells
CD4+
CD8+
Ileum
IEL
Spleen
Blood

Fig. 3.3

Fig. 3.4. Frequencies of nTreg (CD4+CD25+FoxP3) and iTreg (CD4+CD25-FoxP3+) cells among total MNCs from AttHRV-vaccinated Gn pigs fed with high or low dose LA. MNCs were stained freshly without in vitro stimulation. Data are presented as mean frequency ± standard error of the mean (n = 3-9). The top two figures show the frequencies of nTreg cells and the bottom two show those of iTreg cells. See Fig. 3.2 legend for panel description and statistical analysis.

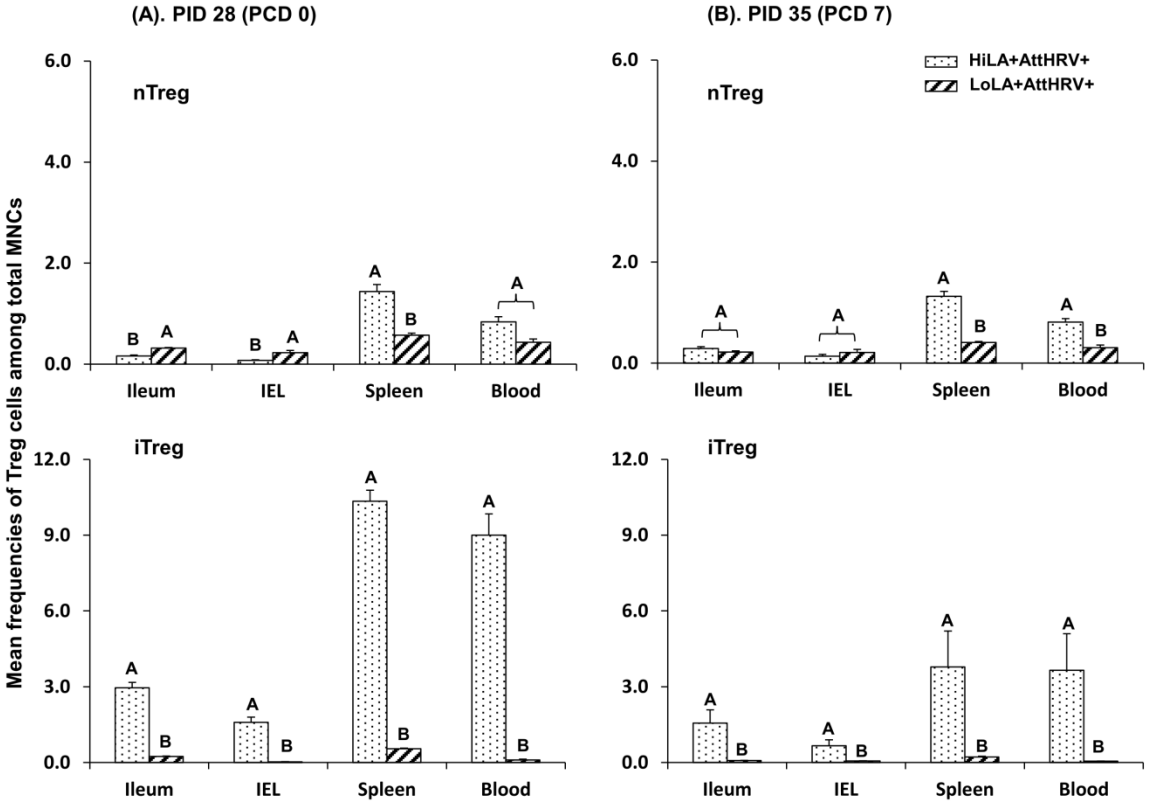
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
HiLA+AttHRV+
LoLA+AttHRV+
nTreg
iTreg
Mean frequencies of Treg cells among total MNCs
Ileum
IEL
Spleen
Blood

Fig. 3.4

Fig. 3.5. Frequencies of nTreg (CD4+CD25+FoxP3) and iTreg (CD4+CD25-FoxP3+) cells among total MNCs from Gn pigs fed with high dose, low dose LA and the control. MNCs were stained freshly without in vitro stimulation. The top two figures show the frequencies of nTreg cells and the bottom two show those of iTreg cells. Data are presented as mean frequency ± standard error of the mean (n = 3-4). See Fig. 3.2 legend for panel description and statistical analysis.

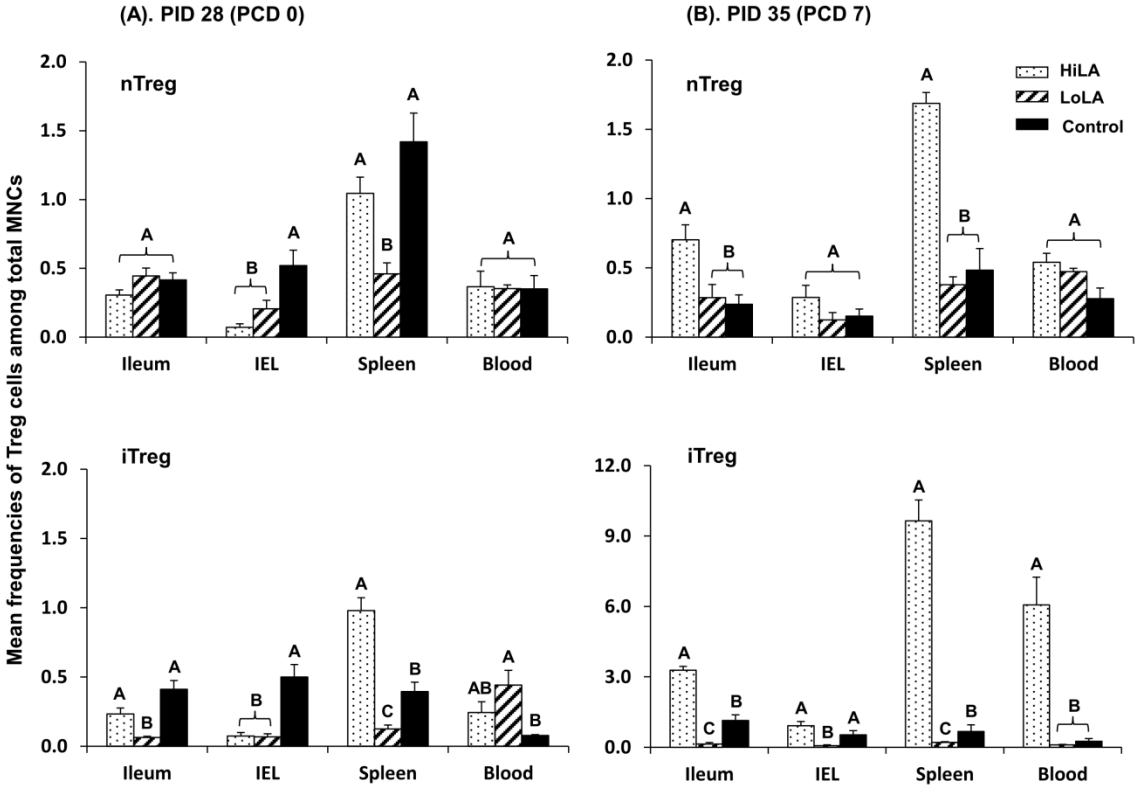
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
Mean frequencies of Treg cells among total MNCs
nTreg
iTreg
HiLA
LoLA
Control
Ileum
IEL
Spleen
Blood

Fig. 3.5

Fig. 3.6. TGF-β expressing nTreg and iTreg cells responses in AttHRV-vaccinated Gn pigs fed with high or low dose LA. MNCs were stained freshly without in vitro stimulation. Data are presented as mean frequency ± standard error of the mean (n = 3-9). The top two figures show the frequencies of TGF-β+ cells among nTreg cells and the bottom two show the frequencies of TGF-β+ cells among iTreg cells. See Fig. 3.2 legend for panel description and statistical analysis.

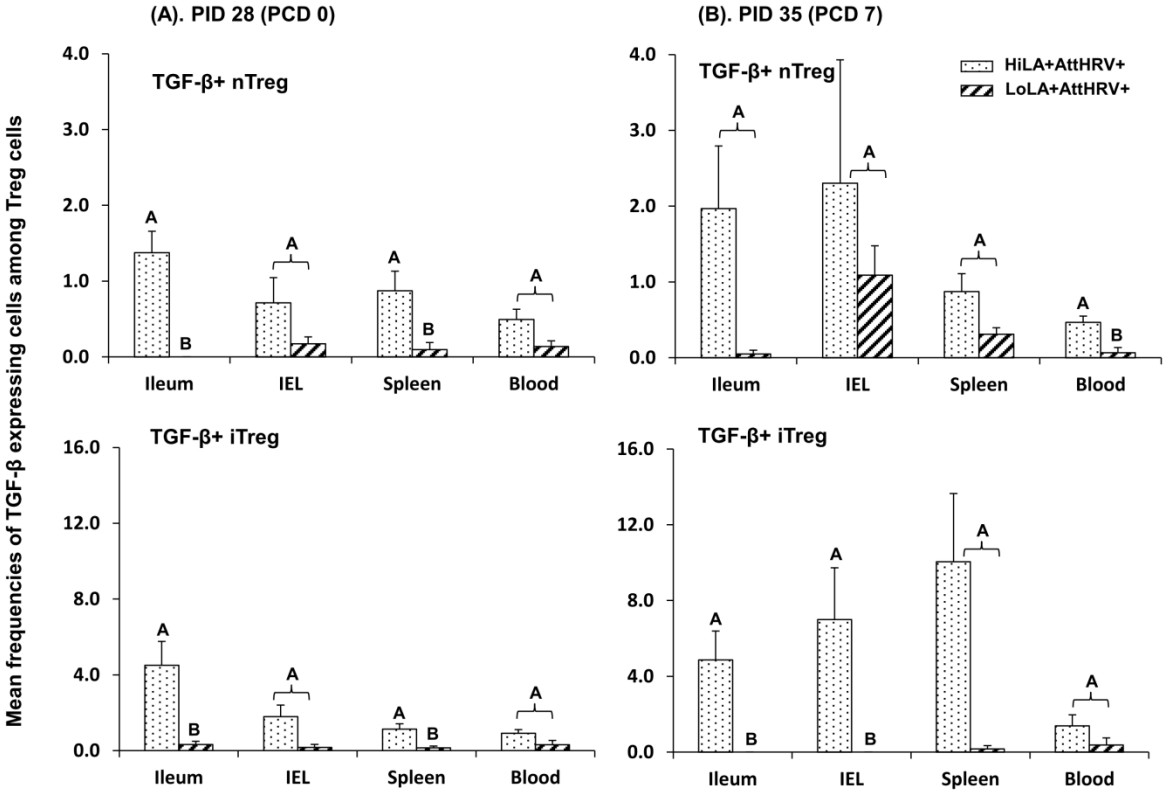
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
Mean frequencies of TGF-β expressing cells among Treg cells
TGF-β+ nTreg
TGF-β+ iTreg
HiLA+AttHRV+
LoLA+AttHRV+
Ileum
IEL
Spleen
Blood
4.0
3.0
2.0
1.0
0.0
16.0
12.0
8.0
4.0
0.0
A
B

Fig. 3.6

Fig. 3.7. TGF-β expressing nTreg and iTreg cells responses in Gn pigs fed with high dose, low dose LA and the control. MNCs were stained freshly without in vitro stimulation. Data are presented as mean frequency ± standard error of the mean (n = 3-4). The top two figures show the frequencies of TGF-β+ cells among nTreg cells and the bottom two show the frequencies of TGF-β+ cells among iTreg cells. See Fig. 3.2 legend for panel description and statistical analysis.

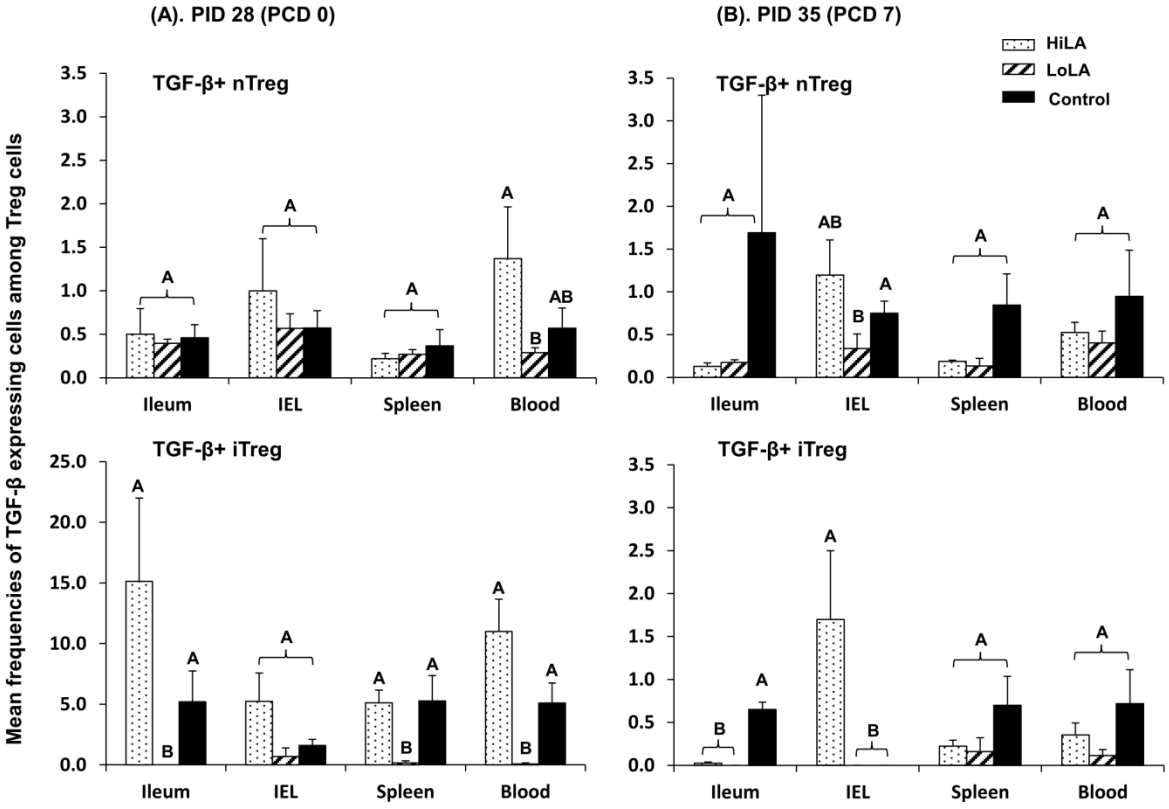
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
HiLA
LoLA
Control
Mean frequencies of TGF-β expressing cells among Treg cells
TGF-β+ nTreg
TGF-β+ iTreg
Ileum
IEL
Spleen
Blood

Fig. 3.7

Fig. 3.8. IL-10 expressing nTreg and iTreg cell responses in AttHRV-vaccinated Gn pigs fed with high or low dose LA. MNCs were stained freshly without in vitro stimulation. Data are presented as mean frequency ± standard error of the mean (n = 3-9). The top two figures show the frequencies of IL-10+ cells among nTreg cells and the bottom two show the frequencies of IL-10+ cells among iTreg cells. See Fig. 3.2 legend for panel description and statistical analysis.

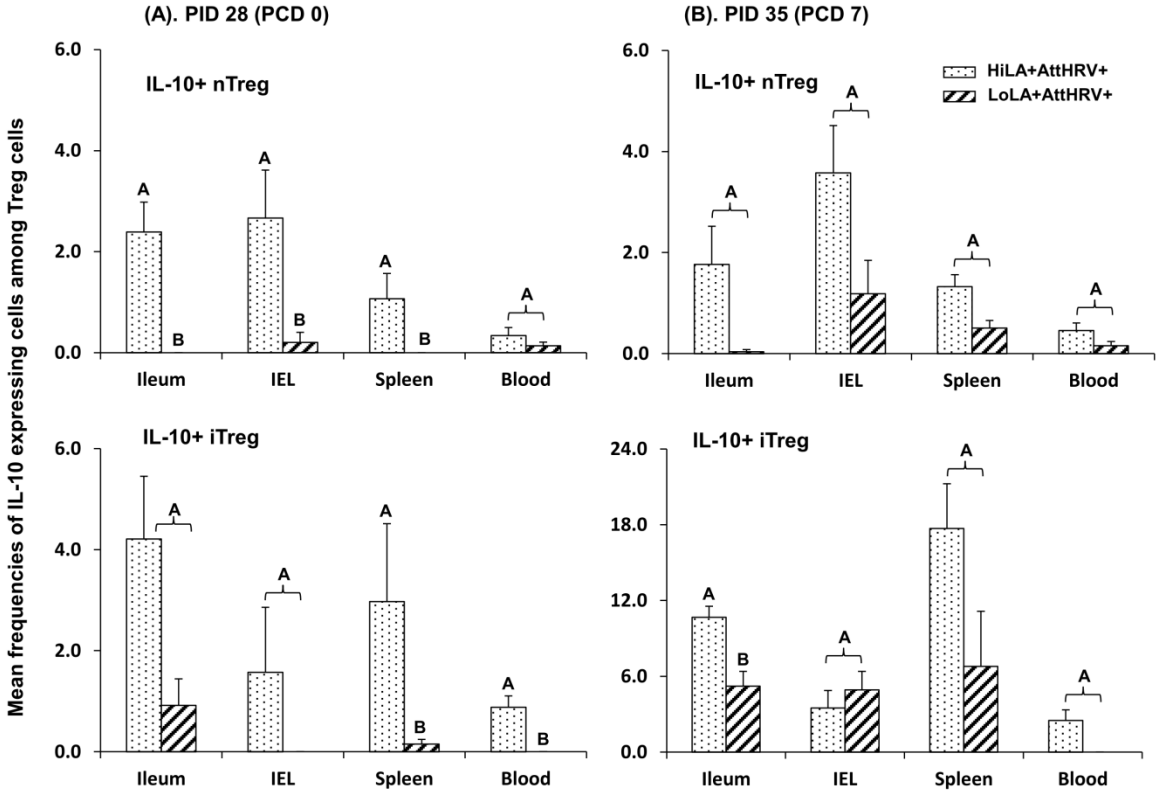
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
IL-10+ nTreg
IL-10+ iTreg
HiLA+AttHRV+
LoLA+AttHRV+
Mean frequencies of IL-10 expressing cells among Treg cells
Ileum
IEL
Spleen
Blood
6.0
4.0
2.0
0.0
24.0
18.0
12.0
6.0
A
B

Fig. 3.8

Fig. 3.9. IL-10 expressing nTreg and iTreg cell responses in Gn pigs fed with high dose, low dose LA and the control. MNCs were stained freshly without in vitro stimulation. Data are presented as mean frequency ± standard error of the mean (n = 3-4). The top two figures show the frequencies of IL-10+ cells among nTreg cells and the bottom two show the frequencies of IL-10+ cells among iTreg cells. See Fig. 3.2 legend for panel description and statistical analysis.

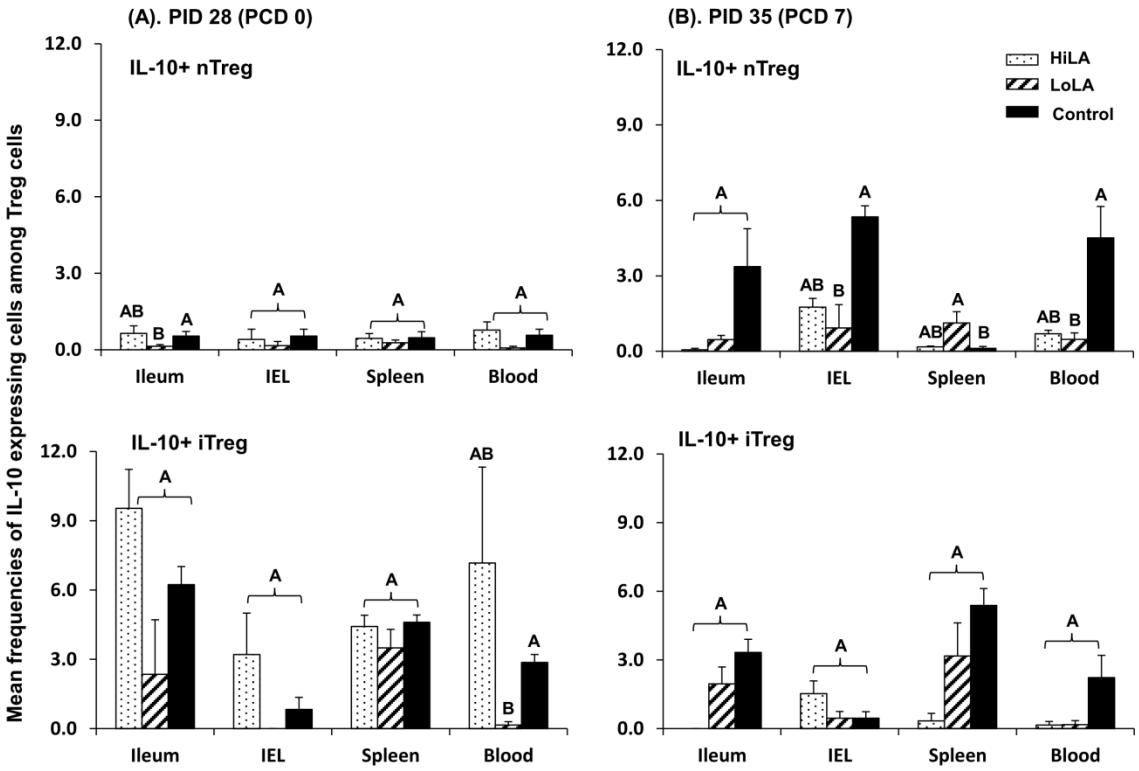
(A). PID 28 (PCD 0)
(B). PID 35 (PCD 7)
IL-10+ nTreg
IL-10+ iTreg
HiLA
LoLA
Control
Mean frequencies of IL-10 expressing cells among Treg cells
12.0
9.0
6.0
3.0
0.0
Ileum
IEL
Spleen
Blood
AB
A
B

Fig. 3.9

Table 3.1. Probiotic LA high dose and low dose feeding regimens and fecal LA shedding

Age (PPD)[a]	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	26	33	Accumulative total dose
PID[b]	-5	-4	-3	-2	-1	**0**	1	2	3	4	**5**	6	7	8	9	**10**	11	**21**	**28**	
HiLA+ dose (CFU)				10^3	10^3	10^4	10^4	10^5	10^5	10^6	10^6	10^7	10^7	10^8	10^8	10^9	10^9			2.22×10^9
LoLA+ dose (CFU)				10^3		10^4		10^5		10^6		10^6								2.11×10^6
HiLA+AttHRV+[c]											6.15 ± 0.12 *					5.74 ± 0.20		5.43 ± 0.17	5.20 ± 0.20	
LoLA+AttHRV+[c]											5.87 ± 0.39					6.15 ± 0.43		6.31 ± 0.44	6.30 ± 0.46	

[a]PPD, post-partum day.

[b]PID, post primary AttHRV inoculation day.

[c]Mean fecal LA count (log10 CFU/ml) ± standard error of the mean (n =3-6).

* Indicates significant difference compared to the low dose LA group (Kruskal-Wallis test on log transformed data, $p<0.05$).

Table 3.2. Protection against rotavirus shedding and diarrhea after VirHRV challenge

Treatment groups	N	Mean duration of virus shedding (days)	Mean duration of diarrhea (days) [a]	Mean cumulative fecal score [b]
HiLA+AttHRV+	13	3.8 (0.3[c]) [A d]	4.3 (0.7) [AB]	12.5 (1.4) [AB]
LoLA+AttHRV+	8	1.0 (0) [B]	2.4 (0.7) [B]	8.4 (1.3) [B]
LA-AttHRV+	12	1.3 (0.2) [B]	4.6 (0.5) [A]	9.0 (1.8) [B]
LA-AttHRV-	8	4.4 (0.7) [A]	5.8 (0.3) [A]	16.9 (1.3) [A]

[a]Pigs with daily fecal scores of ≥2 were considered diarrheic.

Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semiliquid; and 3, liquid.

[b]Mean cumulative score calculation included all the pigs for 7 days postchallenge in each group.

[c]Standard error of the mean.

[d] Means in the same column with different superscript letters (A, B) differ significantly (GLM, $p \leq 0.05$); shared letters indicate no significant difference.

CHAPTER 4

Development of γδ T cell subset responses in gnotobiotic pigs infected with human rotaviruses and colonized with probiotic lactobacilli

Ke Wen, Guohua Li, Wei Zhang, Marli SP Azevedo, Linda J Saif, Fangning Liu, Tammy Bui, Ahmed Yousef, and Lijuan Yuan

4.1 Summary

γδ T cell responses are induced by various viral and bacterial infections. Different γδ T cells contribute to activation and regulation of the inflammatory response and to epithelial repair. How γδ T cells respond to rotavirus infection and how the colonization of probiotics influences the γδ T cell response were unknown. In this study, we evaluated by multicolor flow cytometry the frequencies and distribution of total γδ T cells and three major subsets (CD2-CD8-, CD2+CD8- and CD2+CD8+) in ileum, spleen and blood of gnotobiotic (Gn) pigs at early (3-5 days) and late phases (28 days) after rotavirus infection. The Gn pigs were inoculated with the virulent human rotavirus Wa strain and colonized with a mixture of two strains of probiotics *Lactobacillus acidophilus* strain NCFM and *Lactobacillus reuteri* (ATCC# 23272). In naive pigs, the highest frequency of total γδ T cells was found in blood, followed by spleen and ileum at the early age (8-10 days old) whereas in older pigs (32 days of age) the highest frequency of total γδ T cells was found in ileum and spleen followed by blood. Rotavirus infection significantly increased frequencies of intestinal total γδ T cells and the putatively regulatory CD2+CD8+ γδ T cell subset and

decreased frequencies of the putatively proinflammatory CD8- subsets in ileum, spleen and blood at post-inoculation day (PID) 3 or 5. The three γδ T cell subsets distributed and responded differently after rotavirus infection and/or lactobacilli colonization. The CD2+CD8+ subset contributed the most to the expansion of total γδ T cells after rotavirus infection in ileum because more than 77 % of the total γδ T cells there were CD2+CD8+ cells. There was an additive effect between lactobacilli and rotavirus in inducing total γδ T cell expansion in ileum at PID 5. The overall effect of lactobacilli colonization versus rotavirus infection on frequencies of the CD2+CD8+ γδ T cell subset in ileum was similar; however, rotavirus-infected pigs maintained significantly higher frequencies of CD8- subsets in ileum than lactobacilli-colonized pigs. The dynamic γδ T cell responses suggest that γδ T cell subsets may play important roles in different stages of immune responses after rotavirus infection and probiotic colonization. The knowledge on the kinetics and distribution patterns of γδ T cell subsets in naïve pigs and after rotavirus infection or lactobacilli colonization provides the foundation for further mechanistic studies of their functions.

4.2 Introduction

γδ T cells represent a subset of T cells that possess a distinct T cell receptor (TCR) from αβ T cells. Accumulating evidence suggests that γδ T cells are components of both innate and adaptive immunity against various viral and

bacterial infections [1-3] and they are especially important in early responses against infections at epithelial surfaces [4]. γδ T cell responses to several viral infections in pigs have been reported (i.e., PRRSV and FMDV [5, 6]). There are no reports, however, on γδ T cell responses to rotaviruses which are one of the most important viral pathogens infecting epithelial cells of the small intestine and causing diarrhea in neonatal and newly weaned pigs [7], and infants and young children worldwide [8].

In pigs, γδ T cells form a major T cell subpopulation in peripheral blood of the young [9]. It was shown that CD2+CD8+ and CD2+CD8- γδ T cells preferentially reside in lymphoid tissues, while CD2-CD8- γδ T cells dominate in blood [10]. The percentages and distribution of total γδ T cells and three subsets (CD2+CD8+, CD2+CD8- and CD2-CD8-) in blood and secondary lymphoid tissues (spleen, popliteal lymph nodes, mesenteric lymph nodes and tonsil) of young pigs have been reported [11, 12]. However, an analysis of γδ T cells in the pig small intestine is still lacking and the γδ T cell responses after enteric viral infections have not been studied. Our goals in this study were to extend our knowledge of the development of γδ T cells in the small intestine and to elucidate the kinetics of total and the subset γδ T cell responses after rotavirus infection. Ileum was selected because it is the major site of rotavirus replication and the major induction site in gut-associated lymphoid tissues.

Studies of bovine and murine γδ T cells have suggested that different γδ T cell subsets may have opposite functions and can be pro-inflammatory (CD8-

subsets), regulatory (CD8+ subset) [13], or promote epithelial healing [14]. γδ T cells demonstrated both pro-inflammatory and anti-inflammatory roles in response to infection [15, 16]. In pigs, functions of different γδ T cell subsets have not been reported.

Lactic acid bacteria (LAB), including lactobacilli, are widely evaluated as probiotics in animals and humans [17] and have been shown to significantly stimulate gut epithelial cell proliferation [18], enhance innate and acquired immunity in young lab animals (mice, rats) and children [19, 20] and suppress intestinal inflammation [21]. Several LAB strains have been shown to reduce the severity of acute rotavirus gastroenteritis in children [22, 23] and enhance the immunogenicity of rotavirus vaccines [24, 25]. However, the mechanisms are not fully understood. Our previous study showed that Gn pigs infected with virulent human rotavirus (VirHRV) and fed with a mixture of *Lactobacillus acidophilus* strain NCFM (LA) and *L. reuteri* (ATCC# 23272) strains developed significantly higher total intestinal IgA secreting cell responses and total intestinal IgM and IgG titers than the Gn pigs without the LAB feeding [26]. In this study, we first elucidated γδ T cell responses to rotavirus infection in neonatal Gn pigs during the acute phase of rotavirus infection. We then compared γδ T cell responses with or without LAB colonization at the acute phase and 4 weeks after rotavirus inoculation to examine the effect of colonization of LAB on the development of γδ T cell responses to rotavirus. Our hypotheses are (1) that a robust γδ T cell response is induced by rotavirus

infection; (2) different subsets of γδ T cells may respond differently in different anatomical sites to rotavirus infection; and (3) among the many immune modulating effects, LAB have stimulating or regulating effects on different γδ T cell subsets. The gnotobiotic status of Gn pigs used in this study assured that the effects of specific probiotic and rotavirus strains on γδ T cell responses were not confounded by other microbes present in conventionally reared pigs.

4.3 Materials and methods

Virus. The Wa strain (G1P1A[8]) VirHRV were passaged through Gn pigs and the pooled intestinal contents from the 27th passage were used for inoculation at a dose of 1×10^5 fluorescent focus-forming unit (FFU). The median infectious dose (ID_{50}) of the VirHRV in Gn pigs was determined as approximately 1 FFU [27].

The cell-culture adapted Wa strain AttHRV, derived from the 34th passage in African green monkey kidney cells (MA104), was used as detecting antigens in enzyme-linked immunosorbent assay (ELISA). Virus fecal shedding was detected by cell-culture immunofluorescent (CCIF) assay and an antigen ELISA as previously described [28].

Bacteria. The *Lactobacillus acidophilus* strain NCFM and *L. reuteri* (ATCC 23272) (ATCC, Manassas, VA, USA) were used in this study. Both LAB strains were propagated in *Lactobacilli* MRS broth (Weber, Hamilton, NJ, USA). Each strain was identified and then the big stock was prepared, aliquoted and stored

in -80 °C. When used, one vial was thawed and diluted to the needed titers. LAB inoculums were prepared and titrated as previously described [29]. The two LAB inoculums with known titers were diluted to the specified colony forming unit (CFU)/ml in 0.1 % peptone water (BD Biosciences) and mixed in equal amounts on the day of feeding. Enumeration of LAB in fecal samples was performed as we previously described [29].

Inoculation of Gn pigs. Near-term pigs of Landrace and Large White cross breed were derived from pregnant sows by surgery and maintained in germfree isolator units as described [30]. Pigs were fed with commercial ultra-high temperature (UHT)-treated sterile milk. All pigs were confirmed germfree prior to LAB and VirHRV exposure. For the study of kinetics of early γδ T cell responses in naïve and VirHRV-infected pigs, Gn pigs (both males and females) were randomly assigned to VirHRV-inoculated group and mock-inoculated group with seven [PID 0 and 3] to eight (PID 5) pigs euthanized on each time point to isolate mononuclear cells (MNCs) from ileum, spleen and peripheral blood [31]. Briefly, the MNCs were extracted from the ileum by using EDTA and collagenase and enriched by discontinuous Percoll gradient, from the spleen by mechanical separation and enriched by discontinuous Percoll gradient, and from blood by using Ficoll-Paque^{TM} plus. Inoculation of pigs with VirHRV was performed orally at 5 days of age (PID 0).

For the study of γδ T cell responses to rotavirus infection and LAB colonization, Gn pigs were assigned to four treatment groups with four to eight

pigs euthanized on each time point at PID 5 (n = 4-8) and 28 (n = 6): (1) Mock controls (LAB-VirHRV-), (2) LAB only (LAB+VirHRV-), (3) VirHRV only (LAB-VirHRV+), or (4) LAB colonization plus VirHRV infection (LAB+VirHRV+). Pigs in LAB+ groups were orally dosed at 3, 5, 7, 9 and 11 days of age with 10^3, 10^4, 10^5, 10^6 and 10^6 CFU, respectively, of a 1:1 mixture of *L. acidophilus* and *L. reuteri* in 3 ml of 0.1 % peptone water. The total dose of lactobacilli received by each pig was 2.1×10^6 CFU in 5 feedings. Non-LAB-fed pigs were given an equal volume of 0.1 % peptone water. At 5 days of age, pigs in VirHRV+ groups were orally inoculated with 10^5 FFU virulent Wa HRV in 5 ml of Dulbecco's Modified Eagle's Medium (DMEM). Non-infected pigs were given an equal volume of diluent. Pigs were given 5 ml of 100 mM sodium bicarbonate to reduce gastric acidity 20 min before VirHRV inoculation. Post-VirHRV-inoculation, pigs were examined daily for clinical signs, including prevalence, duration and severity of diarrhea as described [31]. Rectal swabs were collected daily for HRV and lactobacilli shedding. All animal experimental procedures were conducted in accordance with protocols approved by Institutional Animal Care and Use Committees of The Ohio State University and Virginia Polytechnic Institute and State University.

Staining cells for flow cytometry analysis. The MNCs (2×10^6 cells/tube) were stained on the same day of MNC isolation without in vitro stimulation. MNCs were incubated for 15 min at 4 ^{o}C at each step and then washed once with the staining buffer (prepared according to BD Pharmingen BrdU Flow Kits

Instruction Manual) and centrifuged at 500 × g for 5 min at 4 °C. Because fluorescence conjugated porcine Tcr1-N4 and CD2 antibodies were not commercially available, we used unconjugated primary antibodies and then fluorescence conjugated secondary antibodies to detect porcine γδ T cells. MNCs were first stained with the mouse anti-pig Tcr1-N4 (IgG1, VMRD, PGBL22A), an antibody that defines porcine γδ T cells [32] and mouse anti-pig CD2 (IgG3, VMRD, PG168A), followed by APC conjugated rat anti-mouse IgG1 (IgG1, BD pharmingen, clone X56) and FITC conjugated rat anti-mouse IgG3 (IgM, Southern Biotech, clone LO-MG3). SPRD conjugated mouse anti-pig CD8α (IgG2a, Southern Biotech, clone 76-2-11) were added together with the primary antibodies. The secondary antibodies against different mouse IgG isotypes used in this study do not cross-react with each other based on the manufacturer statements. In the corresponding control tubes, only two secondary fluorescence conjugated antibodies and SPRD mouse IgG2a isotype control (Southern Biotech, clone HOPC-1) were added to the cells. All antibodies were titrated and used at optimal concentrations. Analysis of the stained cells was performed using a FACSCalibur or a FACSAria flow cytometer (Becton Dickinson) and at least 20,000 cells were acquired. Data analysis was performed using CellQuest™ Pro (Becton Dickinson) or FlowJo 7.2.2 (Tree Star, Inc) software. Data are presented as mean frequencies of total γδ T cells among MNCs and mean frequencies of CD2+CD8+, CD2+CD8- or CD2-CD8- γδ T cells among total γδ T cells. Any non-specific staining

occurring in the control tubes was subtracted from the corresponding samples.

Statistical analysis. Non-parametric Kruskal-Wallis rank sum test was performed to compare frequencies of total γδ T cells or the three subsets in ileum, spleen, and blood among groups at each time point. When differences among these groups were detected, the same test was used in a pairwise fashion to clarify the nature of the differences. Frequencies of γδ T cells were compared between time points by Analysis of Covariance (ANCOVA) using R programs. All statistical significance was assessed at $p < 0.05$.

4.4 Results

VirHRV infection and LAB colonization. HRV infection in the Gn pigs were confirmed by detection of HRV titers by CCIF and HRV antigen by ELISA in rectal swab samples and intestinal contents from all the pigs in the HRV+ groups (none in the HRV- groups) at PID 3-5. The kinetics and magnitude of virus fecal and nasal shedding and antigenemia after the Wa strain VirHRV inoculation of Gn pigs have been characterized previously [27, 28]. The VirHRV shedding titers and LAB fecal counts (confirming rotavirus infection and LAB colonization) in the Gn pigs in this study were found to be similar to those studies we described in previous reports [26, 33].

Detection of total γδ T cells and three subsets in ileum, spleen, and blood by flow cytometry. Detection of frequencies of total γδ T cells among MNCs and the subsets defined by CD2 and CD8α expression among total γδ T cells is

depicted in the representative dot plots for ileum, spleen, and blood using VirHRV-inoculation pigs at PID 5 as an example (Fig. 4.1). Frequencies of total γδ T cells and the three major subsets CD2+CD8+, CD2-CD8- and CD2+CD8- γδ T cells differed substantially among ileum, spleen and blood. The frequencies of total γδ T cells were calculated as the percentage among total MNCs and the frequencies of each γδ T cell subset as the percentage among total γδ T cells.

Total γδ T cell responses to VirHRV infection at PID 0, PID 3 and PID 5. Frequencies of total and three γδ T cell subsets in each tissue of mock-infected Gn pigs of 8 (n = 3) and 10 (n = 4) days of age were compared using non-parametric Kruskal-Wallis rank analysis. There were no statistically significant differences in frequencies of total γδ T cells and each subset in mock-infected Gn pigs between 8 and 10 days of age, therefore the data were combined and expressed as PID 0 for the statistical analysis and summarized in Figs. 5.2 and 5.3. In the mock-infected Gn pigs, the highest mean frequencies of total γδ T cells were found in blood followed by spleen and ileum (Fig. 4.2). In blood of VirHRV inoculated pigs, frequencies of total γδ T cells increased significantly from PID 0 to PID 3 and then returned to pre-infection level at PID 5, demonstrating an innate immune response (Fig. 4.2). In ileum, the frequencies of total γδ T cells were significantly higher at both PID 3 and PID 5 compared to PID 0 (Fig. 4.2). Thus, the kinetics of γδ T cell responses in different tissues

(blood versus intestine) at early stage after rotavirus infection are different. The data suggest activation and proliferation of γδ T cells in both the intestine and blood by PID 3 followed by the activated γδ T cells leaving blood and entering the site of rotavirus replication (ileum) by PID 5.

γδ T cell subset responses to VirHRV infection at PID 0, PID 3 and PID 5. Each γδ T cell subset has a unique distribution pattern in different lymphoid tissues in normal neonatal pigs (Fig. 4.3). At PID 0, the predominant γδ T cell subset in blood is the CD2-CD8- (85 %) and in ileum is the CD2-CD8- (48 %) followed by the CD2+CD8+ (39 %). In spleen, all the three subsets have similar frequencies (27-39 %). The highest mean frequencies of CD2-CD8- subset was found in blood, CD2+CD8+ in ileum, and CD2+CD8- in spleen.

Rotavirus infection significantly increased frequencies of CD2+CD8+ subset in ileum at PID 3 and PID 5 compared to PID 0, in spleen at PID 3 compared to PID 0, and in blood at PID 5 compared to PID 0 and PID 3 (Fig. 4.3). However, VirHRV down-regulated CD2+CD8- subset in spleen at PID 5 compared to PID 0, CD2-CD8- subset in ileum at PID 3 and PID 5 compared to PID 0 and CD2-CD8- subset in blood at PID 5 compared to PID 0 and PID 3 (Fig. 4.3). In summary, rotavirus infection increased frequencies of the putatively regulatory γδ T cells (CD8+ subset) and decreased frequencies of the putatively pro-inflammatory γδ T cells (CD8- subsets) in ileum, spleen and blood at 3-5 days post rotavirus infection.

Total γδ T cell responses at PID 5 and PID 28. Table 4.1 compares the frequencies of total γδ T cells in ileum, spleen and blood at PID 5 versus PID 28 among the four treatment groups of pigs. In mock-infected pigs (LAB-VirHRV- group) at 8-10 days of age (shown under PID 5), the highest mean frequency of total γδ T cells was found in blood (16.6 %), followed by spleen (9.5 %) and ileum (4.9 %). However, in mock-infected pigs at 32 days of age (shown under PID 28), mean frequencies of total γδ T cells in ileum and spleen were higher than in blood. At PID 28, frequencies of total γδ T cells in the mock-infected pigs increased significantly in ileum and spleen compared to PID 5.

Comparing the four treatment groups at PID 5, frequencies of total γδ T cells in LAB only (LAB+VirHRV-) and VirHRV only (LAB-VirHRV+) pigs were significantly higher in the ileum but were slightly lower in the blood than the mock-infected pigs (Table 4.1). Frequencies of total γδ T cells in ileum of LAB+VirHRV+ pigs were higher or significantly higher than those of the other groups, but they were lower or significantly lower in blood. There was no significant difference in frequencies of total γδ T cells in spleen among the four treatment groups at PID 5. These data suggest that there was proliferation of γδ T cells after either rotavirus infection or LAB colonization in the intestine which is the site of VirHRV infection, and LAB colonization. LAB and VirHRV had an additive effect in stimulating the proliferation of total γδ T cells in ileum of the LAB+VirHRV+ pigs. Apparently, γδ T cells in spleen are not activated

by VirHRV infection or LAB colonization at PID 5.

At PID 28, frequencies of total γδ T cells in ileum of LAB+VirHRV-, LAB-VirHRV+ and LAB+VirHRV+ pigs were significantly lower compared to the mock-infected pigs. In blood, the frequencies of total γδ T cells in the LAB-VirHRV+ pigs were also significantly lower than the mock-infected pigs. There was no significant difference in frequencies of total γδ T cells in spleen among the four treatment groups at PID 28.

Frequencies of total γδ T cells differed significantly between PID 5 and PID 28 in ileum and spleen of all the pig groups, and in blood of the LAB-VirHRV+ pigs (Table 4.1). Frequencies of total γδ T cells in the LAB+VirHRV-, LAB-VirHRV+ and LAB+VirHRV+ pigs decreased significantly in ileum but increased significantly in spleen when compared between PID 5 and PID 28. In blood of the LAB-VirHRV+ pigs, frequencies of total γδ T cells decreased significantly from PID 5 to 28. In summary, LAB colonization and/or rotavirus infection induced significant expansion of intestinal total γδ T cells at PID 5 but then the frequencies reduced to levels significantly lower than the mock-infected control pigs by PID 28. Frequencies of total γδ T cells in spleen were not significantly influenced by LAB colonization or rotavirus infection but significantly increased with aging from 8-10 to 32 days of age.

γδ T cell subset responses at PID 5 and PID 28. The mean frequencies of γδ T cell subsets among total γδ T cells in ileum, spleen and blood of the four

treatment groups of pigs at PID 5 and PID 28 are depicted in Fig. 4.4. At PID 5, CD2+CD8+ subset was the predominant subset in ileum after LAB colonization and/or rotavirus infection. There were significant increases of the CD2+CD8+ γδ T cell frequencies in the ileum of LAB only, VirHRV only and LAB+VirHRV+ pigs at PID 5. CD2-CD8- subset was the predominant γδ T cell subset in blood. After LAB colonization and/or VirHRV infection, there were significant decreases of the CD2-CD8- γδ T cell frequencies in blood and also in ileum. The highest mean frequencies of CD2+CD8- subset were found in spleen of mock-infected pigs. The frequencies of CD2+CD8- γδ T cells in spleen decreased significantly in VirHRV infected pigs (LAB-VirHRV+ and LAB+VirHRV+) and also in ileum and blood of LAB colonized pigs (LAB+VirHRV- and LAB+VirHRV+) at PID 5. Important to note, in the ileum the frequencies of the two CD8- subsets were significantly higher in the VirHRV alone pigs than the LAB alone pigs whereas the CD8- subsets decreased to near undetectable levels in ileum, spleen and blood of the LAB+VirHRV+ pigs at PID 5. In summary, the overall effect of LAB colonization versus rotavirus infection on frequencies of the regulatory CD2+CD8+ γδ T cell subset was similar in ileum and spleen at PID 5, but their effects on the putatively pro-inflammatory CD8- subsets in ileum (and CD2+CD8- subset in blood) were different. Rotavirus infected pigs maintained significantly higher CD8- subsets than the LAB colonized pigs.

The distribution of γδ T cell subsets at PID 28 in all tissues differs substantially from that of PID 5 (Fig. 4.4). The frequencies of CD2-CD8- and CD2+CD8-γδ T cell subsets in ileum and CD2+CD8- and CD2+CD8+ subsets in spleen of the LAB colonized and/or VirHRV infected pigs were significantly higher than the mock-infected pigs. There were no significant differences among the four treatment groups in any of the γδ T cell subset in blood at PID 28, although there was a trend for higher frequencies of CD2-CD8- subset in the LAB colonized and/or VirHRV infected pigs compared to the mock-infected pigs.

4.5 Discussion

To understand how γδ T cells respond to rotavirus infection, including the responses of each γδ T cell subset, and how colonization of probiotic bacteria modulates the γδ T responses, we studied the magnitude and kinetics of total γδ T cell responses and the distribution of the three subsets in ileum, spleen and blood in Gn pigs of early age (8 or 10 days) and older age (32 days) with or without LAB colonization and rotavirus infection. It is known that γδ T cells have the highest abundance in intraepithelial lymphocytes (IELs) in humans and mice [34]; however, IELs were not examined in this study due to low cell yield.

In Gn pigs, γδ T cells were most abundant in blood followed by spleen and intestine at PID 0-3 (8-10 days of age), which is in agreement with the previous report by Stepanova et al [12]. Rotavirus infection significantly expanded total

γδ T cells in blood and ileum at PID3 and γδ T frequencies continued to increase in ileum but decreased in blood from PID 3 to PID 5, suggesting proliferation of γδ T cells in ileum and blood, and potential recruitment of γδ T cells from blood to ileum. Because we did not track the trafficking of γδ T cells from blood to ileum or collect the data of absolute numbers of γδ T cells in ileum, spleen and blood at each time point, we could not know for sure if there were changes in absolute γδ T cell numbers. Proliferation of total γδ T cells in ileum and recruitment from blood to ileum (the site of rotavirus infection) were again suggested in the LAB colonized pigs with or without rotavirus infection at PID 5 because significant increase in the frequencies of total γδ T cells in ileum and decrease in blood were also observed in LAB+VirHRV- and LAB+VirHRV+ pigs. There was a clear additive effect between LAB and rotavirus in inducing total γδ T cell expansion in ileum and in recruiting γδ T cells from blood as the frequencies in LAB+VirHRV+ pigs increased the most (5-fold) in ileum and decreased the most (2.5-fold) in blood by PID 5 compared to LAB only and VirHRV only pigs. Worliczek et al [35] also reported that *Isospora suis* infection in neonatal pigs reduced the numbers of γδ T cells in blood, spleen and mesenteric lymph nodes and strongly increased the numbers in jejunum at PID 6-17, suggesting migration of γδ T cells from blood and secondary lymphoid organs to the site of infection.

Frequencies of total γδ T cells in spleen of all the pig groups and in ileum of the control pigs (LAB-VirHRV-) increased significantly from PID 5 to PID 28,

suggesting age-dependent development [12]. However, frequencies of total γδ T cells in ileum of all the rotavirus infected and/or LAB colonized pigs (LAB-VirHRV+, LAB+VirHRV-, and LAB+VirHRV+) and in blood of the VirHRV-only pigs decreased significantly by PID 28. Reduction of total CD4+ and CD8+ T cells (lymphopenia) after rotavirus infection in pigs and humans has been reported previously [36, 37]. But it is interesting to see that LAB colonization alone also significantly reduced total γδ T cell frequencies in ileum. The mechanism and immunologic significance of the observation require further studies. It was reported that the anti-inflammatory probiotic *Escherichia coli* strain Nissle 1017, but not *Lactobacillus* and *Bifidobacterium* strains contained in the SymbioLact® Comp., induced apoptosis and necrosis in activated γδ T cells in vitro [38]. Another study reported that γδ T cells were significantly reduced from the colonic epithelial layer in *Brachyspira hyodysenteriae* (causes swine dysentery) infected pigs at PID 15 [39]. It is possible that after the innate immune response phase, the activated γδ T cells migrated from gut to other secondary lymphoid organs, such as spleen, or underwent apoptosis. Consistent with our observation that frequencies of total γδ T cells in blood decreased from PID 5 to PID 28 in the control pigs, γδ T cell amounts in blood of human, cattle and goats decrease with age [32, 40, 41]. On the contrary, Stepanova et al reported decreases of γδ T cells in pig blood between 1 day to 2 weeks of age and then elevations from 2 weeks to 6 months. The authors suggested that the elevation of γδ T cells in blood observed in the

study was caused by a factor other than age alone [12].

Three γδ T cell subsets, CD2+CD8+, CD2+CD8-, and CD2-CD8- distributed differently in intestinal and systemic lymphoid tissues and responded differently after rotavirus infection and/or LAB colonization in Gn pigs during the innate immune response phase. Among the three subsets, CD2+CD8+ subset likely contributed the most to the expansion of total γδ T cells after rotavirus infection in ileum because more than 77 % of the total γδ T cells in ileum were CD2+CD8+ cells at PID 5. This subset has been suggested to have anti-viral and anti-inflammatory functions. In studies of PRRSV and FMDV infection and vaccination in pigs, CD8+ γδ T cells were shown to have antigen-specific cytolysis activity and expressed IFN-γ [6, 34, 42]. On the other hand, gene expression studies showed that bovine CD8+ γδ T cells were involved in promoting quiescence whereas CD8- γδ T cells expressed pro-inflammatory genes [13]. In the present study, we focused on evaluating the kinetics and magnitude of γδ T cell subset responses. Our subsequent study (chapter 5) showed that CD8- γδ T cells enhanced CD4+ T cell proliferation and IFN-γ production whereas CD8+ γδ T cells increased IL-10 and TGF-β production in the co-culture with CD4+ T cells. Apparently, γδ T cells may have diverse immune functions and should be further subdivided into different functional γδ T cell subsets.

Over the time course of rotavirus infection in Gn pigs, the increase of CD2+CD8+ γδ T cells in ileum at PID 3-5 corresponded to the beginning of

clearance of the virus infection and the recovery of the villous atrophy of the small intestine [27]. After VirHRV inoculation, the virus shedding peaked at PID 2, started to decline by PID 3 and was cleared by PID 7. The villous atrophy was most severe at PID 2-3 and completely recovered by PID 7. It is likely that CD2+CD8+ γδ T cells in ileum played a major role in the epithelial cell repair and also contributed to the virus clearance because production of both TGF-β and IFN-γ by CD2+CD8+ γδ T cells have been detected in Gn pigs after rotavirus infection (See Chapter 5).

The CD2-CD8- γδ T cell is the most abundant subset in Gn pig blood at both PID 5 and PID 28 in all the four treatment groups, except for the LAB+VirHRV+ pigs at PID 5. The CD8- γδ T cells have been suggested to be highly activated, proliferative, and inflammatory in cattle [13]. However, after rotavirus infection (and/or LAB colonization), frequencies of CD2-CD8- subset significantly reduced at PID 5 in both blood and ileum. Thus this γδ T cell subset is not likely to play the role as anti-viral effector cells. The effect of LAB alone on the CD2-CD8- γδ T cell subset response in ileum and blood was slightly more pronounced than rotavirus alone. The function of this subset in the immune responses to rotavirus infection or LAB colonization needs further study. The observed additive effect of rotavirus infection and LAB colonization on the severe depletion of CD2-CD8- and CD2+CD8- γδ T cells in ileum, spleen and blood of LAB+VirHRV+ pigs at PID 5 is intriguing. Because CD2+CD8+ γδ T cell frequencies in ileum and spleen and the total γδ T cells in

ileum increased significantly in the LAB+VirHRV+ pigs compared to VirHRV only pigs, it is tempting to postulate that the CD8- γδ T cells acquired CD8 marker upon activation and differentiated into CD2+CD8+ γδ T cells and migrated to the site of infection, the ileum. In addition, LAB plus VirHRV may have provided the most suitable stimulations for the differentiation and migration.

γδ T cells are also suggested to be involved in memory response [9, 43]. At PID 28, CD2+CD8- and CD2+CD8+ subsets in spleen and CD2-CD8- subset in ileum were significantly higher in the LAB colonized and/or rotavirus infection pigs than the controls, thus these γδ T cells are potential memory γδ T cells. A re-infection/challenge study is needed to identify the role of γδ T cells in memory T cell responses.

In summary, the present study in Gn pigs demonstrated that γδ T cells responded vigorously to rotavirus infection and the majority of responding γδ T cells was the CD2+CD8+ subset in ileum. This finding is consistent with the studies using BoWC1+ lymphocyte depletion in gnotobiotic calves [44] and γδ T cell knockout mice [45] showing that γδ T cells were not important in limiting primary rotavirus infection, because CD2+CD8+ γδ T cells are known to be regulatory [13] or promote epithelial healing [14]. Although the exact function of each γδ T cell subset in innate and adaptive immunity remains to be determined in more in-depth studies, this present study illustrated for the first

time the γδ T cell subset responses to rotavirus infection and LAB colonization. The dynamic γδ T cell responses we observed indicate that γδ T cells are important components in responses to rotavirus infection and LAB colonization. The knowledge on the kinetics and distribution patterns of total and each γδ T cell subset in the intestinal and systemic lymphoid tissues in Gn pigs provides the foundation for further mechanistic studies of the functional characteristics of γδ T cells in their role in the development or regulation of immune responses to rotavirus infection and in the immune modulating effect of LAB on rotavirus infection and vaccines.

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4.7 References

1. Pitard, V., et al., *Long-term expansion of effector/memory Vdelta2-gammadelta T cells is a specific blood signature of CMV infection.* Blood, 2008. 112(4): p. 1317-24.

2. Devilder, M.C., et al., *Early triggering of exclusive IFN-gamma responses of human Vgamma9Vdelta2 T cells by TLR-activated myeloid and plasmacytoid dendritic cells.* J Immunol, 2009. 183(6): p. 3625-33.

3. Wang, T., et al., *Gamma delta T cells facilitate adaptive immunity against*

West Nile virus infection in mice. J Immunol, 2006. 177(3): p. 1825-32.

4. Hedges, J.F., et al., *Mucosal lymphatic-derived gammadelta T cells respond early to experimental Salmonella enterocolitis by increasing expression of IL-2R alpha.* Cell Immunol, 2007. 246(1): p. 8-16.

5. Olin, M.R., et al., *Gammadelta lymphocyte response to porcine reproductive and respiratory syndrome virus.* Viral Immunol, 2005. 18(3): p. 490-9.

6. Takamatsu, H.H., et al., *Porcine gammadelta T cells: possible roles on the innate and adaptive immune responses following virus infection.* Vet Immunol Immunopathol, 2006. 112(1-2): p. 49-61.

7. Holland, R.E., *Some infectious causes of diarrhea in young farm animals.* Clin Microbiol Rev, 1990. 3(4): p. 345-75.

8. Parashar, U.D., et al., *Rotavirus and severe childhood diarrhea.* Emerg Infect Dis, 2006. 12(2): p. 304-6.

9. Gerner, W., T. Kaser, and A. Saalmuller, *Porcine T lymphocytes and NK cells--an update.* Dev Comp Immunol, 2009. 33(3): p. 310-20.

10. Saalmuller, A., W. Hirt, and M.J. Reddehase, *Porcine gamma/delta T lymphocyte subsets differing in their propensity to home to lymphoid tissue.* Eur J Immunol, 1990. 20(10): p. 2343-6.

11. Yang, H. and R.M. Parkhouse, *Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues.* Immunology, 1996. 89(1): p. 76-83.

12. Stepanova, H., et al., *Early postnatal development of the immune system in piglets: the redistribution of T lymphocyte subsets.* Cell Immunol, 2007. 249(2): p. 73-9.

13. Hedges, J.F., et al., *Differential mRNA expression in circulating gammadelta T lymphocyte subsets defines unique tissue-specific functions.* J Leukoc Biol, 2003. 73(2): p. 306-14.

14. Hayday, A. and R. Tigelaar, *Immunoregulation in the tissues by gammadelta T cells.* Nat Rev Immunol, 2003. 3(3): p. 233-42.

15. Tramonti, D., et al., *Evidence for the opposing roles of different gamma*

delta T cell subsets in macrophage homeostasis. Eur J Immunol, 2006. 36(7): p. 1729-38.

16. Ziegler, H.K., *The role of gamma/delta T cells in immunity to infection and regulation of inflammation.* Immunol Res, 2004. 29(1-3): p. 293-302.

17. Vaughan, E.E., et al., *The intestinal LABs.* Antonie Van Leeuwenhoek, 2002. 82(1-4): p. 341-52.

18. Ichikawa, H., et al., *Probiotic bacteria stimulate gut epithelial cell proliferation in rat.* Dig Dis Sci, 1999. 44(10): p. 2119-23.

19. Herias, M.V., et al., *Immunomodulatory effects of Lactobacillus plantarum colonizing the intestine of gnotobiotic rats.* Clin Exp Immunol, 1999. 116(2): p. 283-90.

20. Yasui, H., et al., *Immunomodulatory function of lactic acid bacteria.* Antonie Van Leeuwenhoek, 1999. 76(1-4): p. 383-9.

21. Zocco, M.A., et al., *Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis.* Aliment Pharmacol Ther, 2006. 23(11): p. 1567-74.

22. Majamaa, H., et al., *Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis.* J Pediatr Gastroenterol Nutr, 1995. 20(3): p. 333-8.

23. Shornikova, A.V., et al., *Bacteriotherapy with Lactobacillus reuteri in rotavirus gastroenteritis.* Pediatr Infect Dis J, 1997. 16(12): p. 1103-7.

24. Zhang, W., et al., *Probiotic Lactobacillus acidophilus enhances the immunogenicity of an oral rotavirus vaccine in gnotobiotic pigs.* Vaccine, 2008. 26(29-30): p. 3655-61.

25. Isolauri, E., et al., *Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by Lactobacillus casei GG.* Vaccine, 1995. 13(3): p. 310-2.

26. Zhang, W., et al., *Influence of probiotic Lactobacilli colonization on neonatal B cell responses in a gnotobiotic pig model of human rotavirus infection and disease.* Vet Immunol Immunopathol, 2008. 122(1-2): p. 175-81.

27. Ward, L.A., et al., *Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs.* J Gen Virol, 1996.

77 (Pt 7): p. 1431-41.

28. Azevedo, M.S., et al., *Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus.* J Virol, 2005. 79(9): p. 5428-36.

29. Zhang, W., et al., *Lactic acid bacterial colonization and human rotavirus infection influence distribution and frequencies of monocytes/macrophages and dendritic cells in neonatal gnotobiotic pigs.* Vet Immunol Immunopathol, 2008. 121(3-4): p. 222-31.

30. Meyer, R.C., E.H. Bohl, and E.M. Kohler, *Procurement and Maintenance of Germfree Seine for Microbiological Investigations.* Appl Microbiol, 1964. 12: p. 295-300.

31. Yuan, L., et al., *Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease.* J Virol, 1996. 70(5): p. 3075-83.

32. Davis, W.C., et al., *Analysis of monoclonal antibodies that recognize gamma delta T/null cells.* Vet Immunol Immunopathol, 1998. 60(3-4): p. 305-16.

33. Wen, K., et al., *Toll-like receptor and innate cytokine responses induced by lactobacilli colonization and human rotavirus infection in gnotobiotic pigs.* Vet Immunol Immunopathol, 2009. 127(3-4): p. 304-15.

34. Thielke, K.H., et al., *Proliferating intestinal gamma/delta T cells recirculate rapidly and are a major source of the gamma/delta T cell pool in the peripheral blood.* Eur J Immunol, 2003. 33(6): p. 1649-56.

35. Worliczek, H.L., et al., *Changes in lymphocyte populations in suckling piglets during primary infections with Isospora suis.* Parasite Immunol. 32(4): p. 232-44.

36. Yuan, L., et al., *Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs.* Vaccine, 2008. 26(26): p. 3322-31.

37. Wang, Y., et al., *Rotavirus infection alters peripheral T-cell homeostasis in children with acute diarrhea.* J Virol, 2007. 81(8): p. 3904-12.

38. Guzy, C., et al., *The probiotic Escherichia coli strain Nissle 1917 induces gammadelta T cell apoptosis via caspase- and FasL-dependent pathways.* Int Immunol, 2008. 20(7): p. 829-40.

39. Hontecillas, R., et al., *CD4+ T-cell responses and distribution at the colonic mucosa during Brachyspira hyodysenteriae-induced colitis in pigs.* Immunology, 2005. 115(1): p. 127-35.

40. Wilson, R.A., et al., *T-cell subsets in blood and lymphoid tissues obtained from fetal calves, maturing calves, and adult bovine.* Vet Immunol Immunopathol, 1996. 53(1-2): p. 49-60.

41. Caro, M.R., et al., *Postnatal evolution of lymphocyte subpopulations in peripheral blood and lymphoid organs in the goat.* Res Vet Sci, 1998. 65(2): p. 145-8.

42. Lopez Fuertes, L., et al., *Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection.* Virus Res, 1999. 64(1): p. 33-42.

43. Blumerman, S.L., C.T. Herzig, and C.L. Baldwin, *WC1+ gammadelta T cell memory population is induced by killed bacterial vaccine.* Eur J Immunol, 2007. 37(5): p. 1204-16.

44. Oldham, G., et al., *In vivo role of lymphocyte subpopulations in the control of virus excretion and mucosal antibody responses of cattle infected with rotavirus.* J Virol, 1993. 67(8): p. 5012-9.

45. Franco, M.A. and H.B. Greenberg, *Immunity to rotavirus in T cell deficient mice.* Virology, 1997. 238(2): p. 169-79.

Fig. 4.1. Detection of total γδ T cells and subsets in ileum, spleen and blood with flow cytometry. Gn pigs were inoculated with virulent Wa HRV. The MNCs were isolated upon euthanasia at PID 5 and stained with primary antibodies to porcine γδ T cell marker (Tcr1-N4), CD2, and CD8, and secondary antibodies conjugated to APC, FITC and PerCP, respectively. The MNCs were gated based on forward scatter and side scatter profile (A) followed by gating on Tcr1-N4+ T cells (B). The frequencies (%) of total γδ T cells among MNCs are shown on the dot plots. Bivariate dot plots were drawn to define CD2+CD8-, CD2+CD8+ and CD2-CD8- γδ T cell subpopulations within Tcr1-N4+ T cells (C). The frequencies of γδ T cell subsets are labeled on the corner of each quadrant.

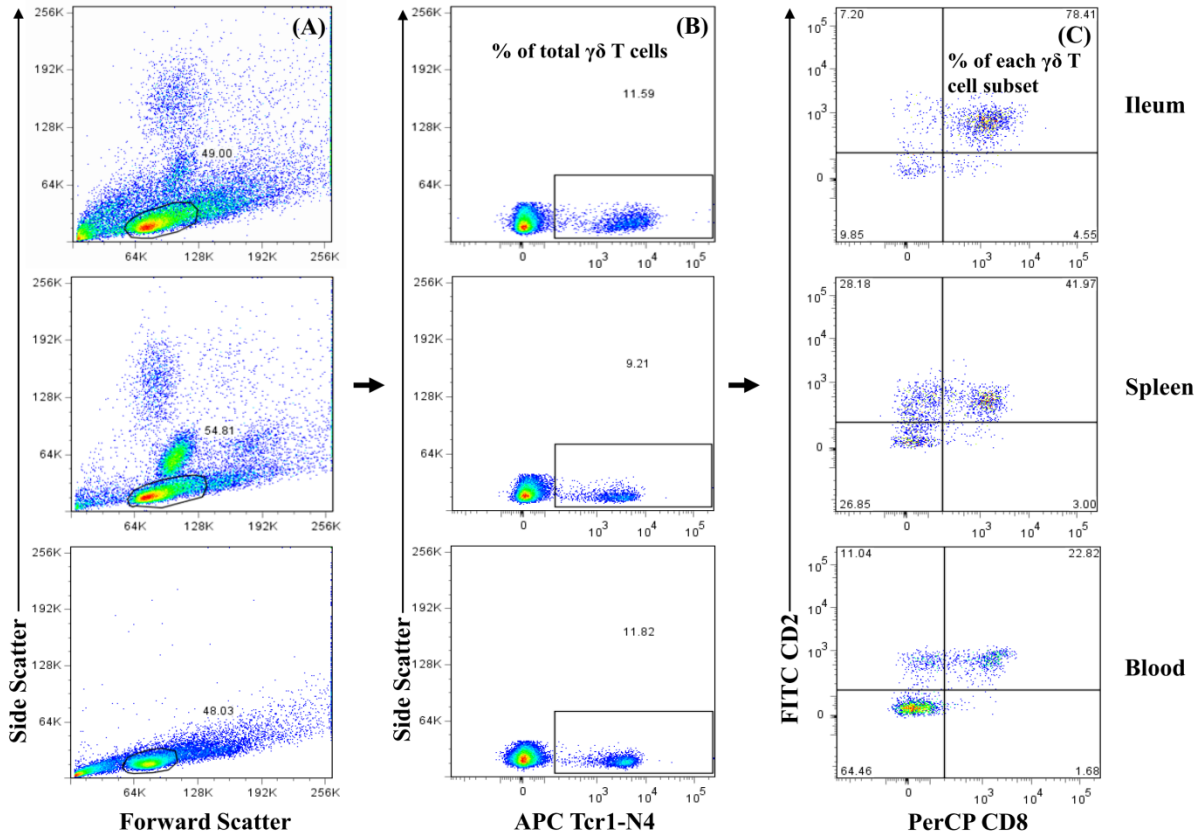
(A)
(B)
(C)
% of total γδ T cells
% of each γδ T cell subset
Ileum
Spleen
Blood
Side Scatter
Forward Scatter
APC Tcr1-N4
FITC CD2
PerCP CD8
49.00
54.81
48.03
11.59
9.21
11.82
7.20
78.41
9.85
4.55
28.18
41.97
26.85
3.00
11.04
22.82
64.46
1.68

Fig. 4.1

Fig. 4.2. Frequencies of total γδ T cells in VirHRV-infected Gn pigs. Gn pigs were inoculated with virulent Wa HRV or mock inoculated and euthanized on PID 0, 3 and 5, respectively. The total γδ T cells were detected as shown in Fig. 4.1. The y-axis is the mean frequencies (%) of total γδ T cells among MNCs. The error bars represent standard error of the mean. The capital letters A, B, and C indicate the results of significance testing for differences among PID 0, 3 and 5. Unshared letters indicate significant difference among PID 0, 3 and 5 on frequencies of the total γδ T cells (Kruskal–Wallis rank sum test, $p < 0.05$, $n = 7\text{-}8$), while shared letters indicate no significant difference.

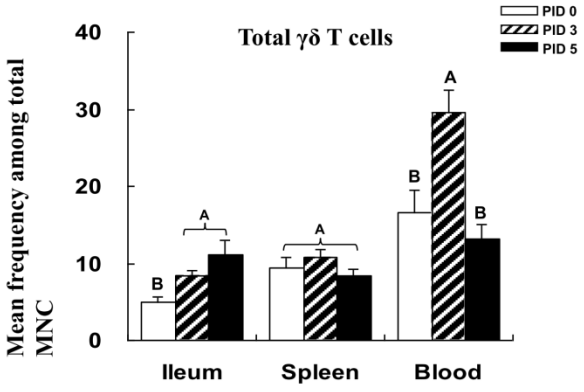
Total γδ T cells
PID 0
PID 3
PID 5
Mean frequency among total MNC
40
30
20
10
0
A
A
B
B
A
B
Ileum
Spleen
Blood

Fig. 4.2

Fig. 4.3. Frequencies of γδ T cell subsets in VirHRV-infected Gn pigs. The γδ T cell subsets from the VirHRV-infected pigs were detected as shown in Fig. 4.1. The y-axis is the mean frequencies (%) of each of three subsets among total γδ T cells. The error bars represent standard error of the mean. The capital letters A, B, and C indicate the results of significance testing for differences among PID 0, 3 and 5. Unshared letters indicate significant difference among PID 0, 3 and 5 on frequencies of the three subsets (Kruskal–Wallis rank sum test, $p < 0.05$, $n = 7\text{-}8$), while shared letters indicate no significant difference.

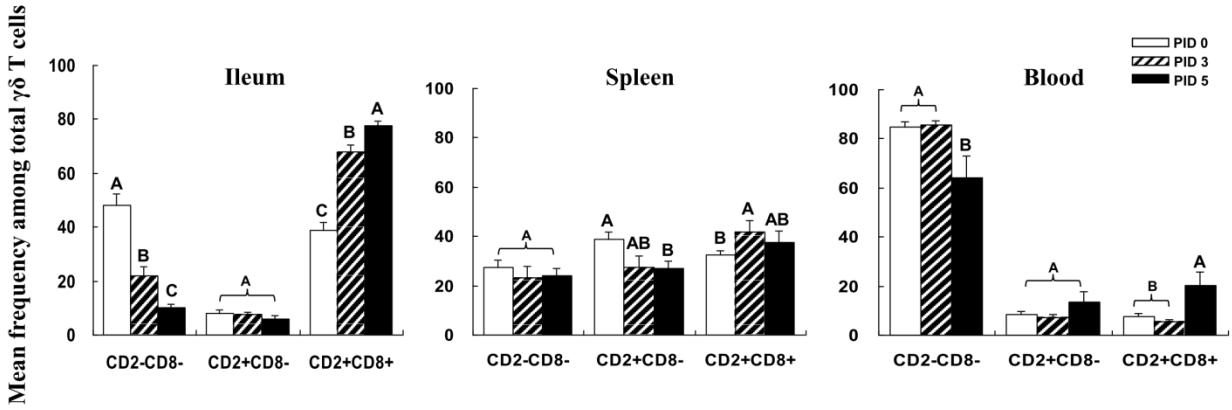
Mean frequency among total γδ T cells
Ileum
Spleen
Blood
PID 0
PID 3
PID 5
CD2-CD8-
CD2+CD8-
CD2+CD8+

Fig. 4.3

Fig. 4.4. Mean frequencies of γδ T cell subsets in Gn pigs with or without LAB and infected with VirHRV at PID 5 and 28. Gn pigs were inoculated with LAB and virulent Wa strain HRV (LAB+VirHRV+), HRV only (LAB-VirHRV+), LAB only (LAB+VirHRV-) or mock (LAB-VirHRV-). The frequencies of γδ T cell subsets were measured on PID 5 and 28. The y-axis is the mean frequencies (%) of three subsets among total γδ T cells. The error bars represent standard error of the mean. The capital letters A, B, and C indicate the results of significance testing for differences among four treatment groups (Kruskal–Wallis rank sum test, $p < 0.05$, $n = 4$-8), while shared letters indicate no significant difference.

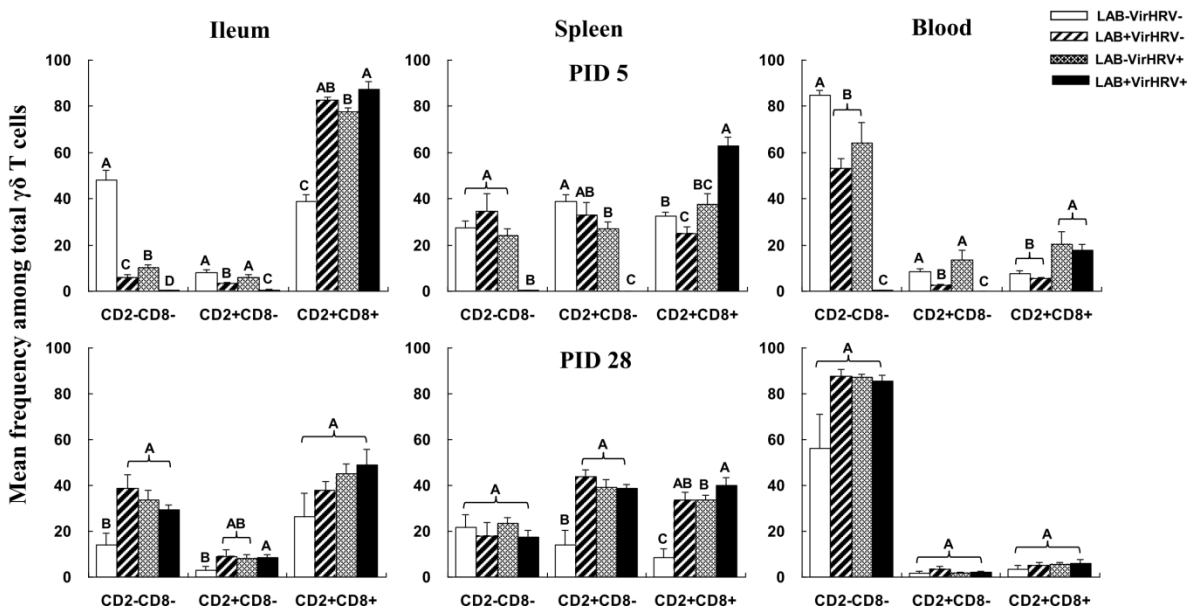
Ileum
Spleen
Blood
PID 5
PID 28
LAB-VirHRV-
LAB+VirHRV-
LAB-VirHRV+
LAB+VirHRV+
Mean frequency among total γδ T cells
CD2-CD8-
CD2+CD8-
CD2+CD8+

Fig. 4.4

Table 4.1. Total γδ T cell responses among treatment groups at PID 5 and PID 28

Treatment group	Mean frequencies of total γδ T cells among MNC							
	PID 5				PID 28			
	n	Ileum	Spleen	Blood	n	Ileum	Spleen	Blood
LAB-VirHRV-	7	4.9^{C*} (0.7)**	9.5^{A} (1.2)	16.6^{A} (3.0)	6	$17.2^{A\dagger}$ (6.0)	$16.7^{A\dagger}$ (2.5)	9.7^{A} (1.5)
LAB+VirHRV-	4	19.3^{AB} (5.2)	7.9^{A} (0.9)	11.8^{AB} (1.6)	6	$6.9^{B\dagger}$ (0.9)	$16.5^{A\dagger}$ (2.5)	8.3^{AB} (1.5)
LAB-VirHRV+	8	11.2^{B} (1.8)	8.5^{A} (0.8)	13.2^{A} (1.9)	6	$4.7^{C\dagger}$ (0.3)	$13.9^{A\dagger}$ (1.0)	$5.3^{B\dagger}$ (0.5)
LAB+VirHRV+	4	24.9^{A} (1.8)	9.1^{A} (0.6)	6.6^{B} (1.0)	6	$6.1^{B\dagger}$ (0.5)	$15.1^{A\dagger}$ (1.9)	7.3^{AB} (1.5)

* Means in the same column with different superscript letters (A, B, and C) differ significantly (Kruskal-Wallis rank sum test; $p<0.05$).

** Standard error of the mean.

†. Indicates significant difference when compared to PID 5 for the same tissue in the same group (ANCOVA; $p<0.05$).

CHAPTER 5

Characterization of immune modulating functions of porcine γδ T cell subsets

Ke Wen, Tammy Bui, Guohua Li, Fangning Liu, Yanru Li, Jacob Kocher, Lijuan Yuan

5.1 Summary

We characterized the immune modulating functions of porcine γδ T cell subsets in gnotobiotic pigs and in sort-purified lymphocyte autologous co-cultures. CD2+CD8- and CD2-CD8- γδ T cells have mainly proinflammatory function as evidenced by directly secreting IFN-γ or promoting CD4+ αβ T cell proliferation and IFN-γ production, whereas CD2+CD8+ γδ T cells mainly exert regulatory T cell function by expressing FoxP3, directly secreting IL-10 and TGF-β or increasing IL-10 and TGF-β production by CD4+ and CD8+ αβ T cells. γδ T cells responded to rotavirus infection by increasing TLR2, TLR3, and TLR9 expression and IFN-γ and/or TGF-β production. The CD8- subsets can differentiate into CD8+ subset by acquiring CD8 expression with or without isopentenyl pyrophosphate (IPP) stimulation, explaining at least in part, the apparently dual functions of CD2+CD8+ and CD2+CD8- subsets. Thus, the CD8+ and CD8- γδ T cell subsets contributed differently to the anti-virus immunity and to the maintaining and restoring intestinal and systemic homeostasis.

5.2 Introduction

Studies of γδ T cells in humans, mice and cattle have revealed the diverse immune functions of different γδ T cell subsets; however the immune functions of porcine γδ T cell subsets have not been clearly identified. Pig is the most important large animal models for human biomedical research and used as the donor for xenotrasplantation, therefore it is important to characterize the immune modulating functions of porcine γδ T cell subsets. All mammalian species studied share two principal subsets of γδ T cells in terms of their distributions. One subset is predominant in tissues including mucosal and skin surfaces (Vδ1 in humans and mice; WC1-CD8+ in cattle, and CD2+CD8+ in pigs) and the other subset is predominant in the blood (Vδ2 in humans; Vγ1 in mice, WC1+ in cattle, and CD2-CD8- in pigs) [1-5]. Functionally, γδ T cells in humans, mice and cattle have also been divided into two principal subsets: the proinflammatory and antiinflammatory subsets [6]. Circulatory γδ T cells are mainly proinflammatory and act as primary responders for invading pathogens, including viruses, bacteria and parasites; whereas tissue-specific γδ T cells have proinflammatory and antiinflammatory dual functions depending on the anatomic locations and the microenvironment. γδ T cells located in mucosal and skin surfaces are largely antiinflammatory and contribute to the maintenance of the epithelial integrity.

Phenotypically, porcine γδ T cells are defined by the monoclonal anti-porcine antibody Tcr1-N4 (clone PGBL22A) that recognizes a determinant on a constant region of porcine TCR δ chain [7]. The total γδ T cells are divided into three

subsets based on the surface expression of CD2 and CD8αα (CD2+CD8+, CD2+CD8- and CD2-CD8-) [8]. Our previous study focusing on porcine γδ T cell subset distribution and kinetics in responses to enteric virus infection showed that rotavirus infection significantly increased frequencies of CD2+CD8+ γδ T cell subset and decreased the CD8- subsets in ileum, spleen and blood of Gn pigs at post-inoculation day (PID) 3-5 [4]. However, the exact immune functions of each porcine γδ T cell subset are yet to be identified.

γδ T cells can respond directly to microbe-associated molecular patterns (MAMPs) without any help from antigen-presenting cells (APCs) by increasing MAMP receptor expressions, such as Toll-like receptors (TLRs) [9]. Freshly isolated human γδ T cells could be stimulated via the T cell receptor (TCR) ligand in the presence of Poly (I:C) without the presence of APCs to enhance IFN-γ production [10], which indicated that human γδ T cells express the Poly (I:C) receptor TLR3. Also, human Vγ2Vδ2 T cells expressed TLR2 mRNA and produced IFN-γ under the stimulation of TLR2 ligand [11]. Pietschmann et al [12] compared the different TLR expression patterns among human Vδ1 and Vδ2 T cells and found that TLR2, TLR3 and TLR6 proteins were detected in both Vδ1 and Vδ2 T cells. Mouse γδ T cells were also found to express TLR2 and TLR4 [13]. However, it is unknown if porcine γδ T cells express TLRs and whether TLR expressing γδ T cells are involved in the immune responses to rotavirus infection.

The objective of the present study is to characterize immunological functions

of the three porcine γδ T cell subsets in the face of enteric virus infections. The frequencies of TLR2, TLR3, TLR9 and FoxP3 expression, and IFN-γ and TGF-β production among the three γδ T cell subsets in gnotobiotic (Gn) pigs were determined after human rotavirus (HRV) inoculation. The cytokine production profiles of the three γδ T cell subsets after phosphoantigen IPP stimulation and the influence of each γδ T cell subset on CD4+ or CD8+ αβ T cell cytokine production and proliferation in sort-purified autologous co-cultures were examined by using enzyme-linked immunosorbent assay (ELISA) and multi-color flow cytometry assays. Our findings indicate that similar to other mammalian species studied previously (humans, mice and cattle), porcine γδ T cells express TLRs and can be divided into two major functional subsets, the proinflammatory (CD2+/-CD8-) and the antiinflammatory (CD2+CD8+) subsets. CD2+/-CD8- γδ T cells can exert anti-viral effector cell functions by producing IFN-γ and promoting the proliferation and IFN-γ production by CD4+ αβ T cells. CD2+CD8+ γδ T cells from the intestine and spleen have characteristics of regulatory cell functions (express FoxP3 and produce TGF-β and IL-10); CD2+CD8+ γδ T cells also have anti-viral functions (produce IFN-γ) in early stage of rotavirus infection.

5.3 Materials and methods

Viruses and inoculums. The virulent Wa strain HRV (G1P1A[8]) was passaged through Gn pigs and the pooled intestinal contents from the 27th

passage were used for inoculation or challenge of Gn pigs at a dose of 1×10^5 fluorescent focus-forming unit (FFU). The median infectious dose (ID_{50}) of the HRV in Gn pigs was approximately 1 FFU [14]. The HRV infection in Gn pigs were confirmed by fecal virus shedding using ELISA and cell-culture immunofluorescent (CCIF) assays as previously described [14].

The cell-culture adapted attenuated Wa strain HRV, derived from the 34^{th} passage in African green monkey kidney cells (MA104) [15], was used for inoculation of a subgroup of Gn pigs at 5×10^7 FFU/dose and was used to prepare semi-purified Wa HRV antigen by centrifugation through a 40 % sucrose cushion as described [16]. The semi-purified Wa HRV antigen was used in the CD4+ T cell proliferation assay in the co-culture studies.

Inoculation of gnotobiotic pigs. Near-term pigs of Landrace and Big White cross breed were derived from pregnant sows by surgery and maintained in germfree isolator units as described [17]. Pigs were fed with commercial ultra-high temperature (UHT)-treated sterile milk. All pigs were confirmed germfree prior to rotavirus or norovirus exposure. Pigs were given 5 ml of 100 mM sodium bicarbonate to reduce gastric acidity 20 min before virus inoculation. All animal experimental procedures were conducted in accordance with protocols approved by Institutional Animal Care and Use Committees of Virginia Polytechnic Institute and State University.

For the study of TLR expressing γδ T cell responses [PID 0 (n = 7), 3 (n = 7) and 5 (n = 4)] and IFN-γ [PID 0 (n = 7), 3 (n = 4) and 5 (n = 4)] or TGF-β [PID

0 (n = 3) and 5 (n = 4)] producing γδ T cell responses, Gn pigs (both males and females) were randomly assigned to the HRV and mock control groups. At 5 days of age (PID 0), Gn pigs in HRV groups were orally inoculated with 1×10^5 FFU virulent Wa HRV in 5 ml of Dulbecco's Modified Eagle's Medium (DMEM). Control pigs were given an equal volume of the diluent. The pigs were euthanized on PID 0, 3 and 5 to isolate mononuclear cells (MNCs) from ileum, spleen and peripheral blood as described [15].

In the following studies, Gn pigs were inoculated with rotavirus or norovirus and colonized with or without a probiotic lactobacilli strain. Those Gn pigs were shared with other research projects. MNCs from those Gn pigs were used in the present study because the inoculations and ages do not affect the interpretation of the data regarding the function of each γδ T cell subset and the use of animal resource was maximized. In the studies of FoxP3 expression among γδ T cell subsets and APC function of γδ T cells, Gn pigs were monoassociated with (FoxP3 expression study, n = 3) or without (APC function study, n = 3) the *lactobacilli acidophilus* NCFM strain as we previously described [18], inoculated with two oral doses of attenuated Wa HRV at 5×10^7 FFU/dose in 5 ml of DMEM at 5 (PID 0) and 15 (PID 10) days of age, challenged with 1×10^5 FFU of virulent Wa HRV on PID 28, and euthanized on PID 35 (PCD 7). MNCs from ileum, spleen and peripheral blood were isolated.

In the co-culture studies, human norovirus inoculated Gn pigs were used for

acquiring sort-purified γδ T cell subsets and αβ T cells. Gn pigs were orally inoculated at the age of 33 days with 2.74×10^4 to 2.74×10^6 copies of viral RNA (determined by real-time quantitative PCR) of a norovirus GII.4 strain (human stool sample 092895 suspension from Dr. Xi Jiang, Cincinnati Children's Hospital Medical Center, OH). All the Gn pigs were infected by norovirus, which was confirmed by fecal virus shedding using RT-PCR and real-time quantitative PCR (Bui and Yuan, unpublished data). MNCs from spleen and IEL were isolated from the pigs at PID3 or PID 4 for sort-purification of γδ T cell subsets and αβ T cells.

Staining cells for flow cytometry analysis. For Staining TLR expressing γδ T cells, MNCs (2×10^6 cells/tube) were stained on the same day of MNC isolation without in vitro stimulation [19]. For FoxP3 expression by γδ T cells, MNCs were incubated for 5 hrs with Brefeldin A (10 ug/ml, Sigma) in E-RPMI (see dtailed compoents in Chapter 3) at 37 ^{0}C. For IFN-γ or TGF-β production by γδ T cells, the MNCs were incubated in E-RPMI for 17 and 48 hrs, respectively, at 37 ^{0}C and Brefeldin A was added for the last 5 hrs as described previously [20].

For all staining, except when specifically noted, MNCs were incubated with antibodies for 15 min at 4 ^{o}C at each step and then washed once between steps with the staining buffer (prepared according to BD Pharmingen BrdU Flow Kits Instruction Manual) and centrifuged at 500 × g for 5 min at 4 ^{o}C. The staining of γδ T cell subsets has been described previously [4]. Briefly, MNCs were first

stained with mouse anti-porcine Tcr1-N4 (IgG1, VMRD, PGBL22A), mouse anti-porcine CD2 (IgG3, VMRD, PG168A) and SPRD conjugated mouse anti-porcine CD8α (IgG2a, Southern Biotech, 76-2-11) antibodies followed by APC conjugated rat anti-mouse IgG1 (IgG1, BD pharmingen, A85-1) and FITC conjugated rat anti-mouse IgG3 (IgM, Southern Biotech, LO-MG3). Intracellular TLR (TLR3 and TLR9) and extracellular TLR (TLR2) staining was performed as previously described [19]. PE conjugated mouse anti-porcine IFN-γ (IgG1, BD pharmingen, P2G10) and PE conjugated mouse anti-human TGF-β1 (IgG1, R&D systems, 27232) antibodies were used to detect intracellular IFN-γ and TGF-β production, respectively, as described [20].

For staining of FoxP3 expressing γδ T cells, after surface staining of Tcr1-N4, CD2 and CD8, MNCs were permeabilized with FoxP3 Staining Buffer Set (eBiosciences, 00-5523) for 30 min at 4 ^{o}C and were washed with FoxP3 Staining Buffer Set (eBiosciences, 00-5523) followed by staining with PE-Cy7 conjugated rat anti-mouse/rat FoxP3 (IgG2a, eBioscience, FJK-16s) for 30 min at 4 ^{o}C.

First sets of negative controls included the secondary antibodies APC conjugated rat anti-mouse IgG1 (IgG1, BD pharmingen, A85-1) (for Tcr1-N4) and FITC conjugated rat anti-mouse IgG3 (IgM, Southern Biotech, LO-MG3) (for CD2), and the following isotype-matched irrelevant control antibodies: SRPD conjugated mouse IgG2a isotype control (Southern Biotech, HOPC-1) (for CD8); PE conjugated mouse IgG1 isotype control (eBioscience, P3.6.2.1)

(for TLR2, TLR3, IFN-γ and TGF-β); PE conjugated rat IgG2a isotype control (eBioscience, 12-4321) (for TLR9); and PE-Cy7 conjugated rat IgG2a isotype control (eBioscience, 25-4321) (for FoxP3). The negative controls were included in each staining to set the quadrant markers for the bivariate dot plots. The second sets of control tubes were stained with all the antibodies to define γδ T cell subsets and the isotype-matched irrelevant control antibodies for detecting the non-specific reactions for TLRs, cytokines and FoxP3. All antibodies were titrated to provide the optimal signal to noise ratio.

Analysis of the stained cells was performed using a FACSAria flow cytometer (Becton Dickinson) and at least 100,000 cells were acquired. Data analysis was performed using FlowJo 7.2.2 (Tree Star, Inc) software. Data are presented as mean frequencies of TLRs, IFN-γ or TGF-β expressing/producing γδ T cells among each γδ T cell subset, and FoxP3 expressing γδ T cell subsets among total γδ T cells. The frequencies of sample tubes were subtracted by the frequencies of non-specific reactions in the second sets of control tubes for each tissue.

Sort-purification of γδ T subsets and CD4+ and CD8+ αβ T cells. For the in vitro cell co-culture studies, MNCs from spleen and IEL were collected from Gn pigs (n = 6) infected with human norovirus at PID 3 or 4. The three γδ T cell subsets and CD4+ and CD8+ αβ T cells were enriched from the MNCs by magnetic antibody cell sorting (MACS) and fluorescence-activated cell sorting (FACS) technology. Streptavidin conjugated Dynabeads (Invitrogen, 656.01) was primed before use according to the manufacturer's instruction and then

incubated with biotinylated rat anti-mouse IgG1 antibody (IgG1, BD pharmingen, A85-1) at 4 ^{o}C for 1 hr followed by washing 4 times with the staining buffer (prepared according to BD Pharmingen BrdU Flow Kits Instruction Manual). MNCs were stained with mouse anti-porcine Tcr1-N4 (IgG1, VMRD, PGBL22A) or mouse anti-porcine CD3 (IgG1, Southern Biotech, PPT3) at 4 ^{o}C for 30 min, followed by adding the prepared Dynabeads and incubation at room temperature for 30 min. Total γδ T cells and CD3+ T cells were then enriched using the magnetic separation rack (Invitrogen) according to the manufacturer's instructions.

Enriched total γδ T cells and CD3+ T cells were further sorted into three γδ T cell subsets, and CD3+CD4+ and CD3+CD8+ αβ T cells, respectively by FACS. Mouse anti-porcine CD2 (IgG3, VMRD, PG168A), FITC conjugated rat anti-mouse IgG3 (IgM, Southern Biotech, LO-MG3) and SPRD conjugated mouse anti-porcine CD8α (IgG2a, Southern Biotech, 76-2-11) antibodies were used for sorting γδ T cell subsets. PE conjugated mouse anti-porcine CD4 (IgG2b, BD pharmingen, 74-12-4) and SPRD conjugated mouse anti-porcine CD8α were used for separating CD3+CD4+ and CD3+CD8+ αβ T cells. The purity of each T subset was determined by flow cytometry and only cells with > 98 % purity were used in the subsequent in vitro culture or autologous lymphocytes co-culture studies.

In vitro culture with IPP and IL-2 stimulation. To determine the cytokine production profile of each γδ T cell subset, each sort-purified γδ T cell subset

(1.25×10^4 cells in 0.8 ml) were cultured for 5 days in E-RPMI media at 37 °C in 5 % CO_2 humidified air with the stimulation of 7.5 μg/ml of IPP plus 1.25 μg/ml of IL-2 or mock stimulated. During the last 2 days, fresh media containing 7.5 μg/ml of IPP plus 1.25 μg/ml of IL-2 were added. The supernatants were collected and stored at -80 °C for measuring cytokine concentrations by ELISA later and the MNCs were harvested, washed with the staining buffer and stained with the following antibodies: SPRD conjugated mouse anti-porcine CD8α (IgG2a, Southern Biotech, 76-2-11) and mouse anti-porcine Tcr1-N4 (IgG1, VMRD, PGBL22A) followed by APC conjugated rat anti-mouse IgG1 (IgG1, BD pharmingen, A85-1). Flow cytometry analysis was performed to determine the frequencies of CD8+ γδ T cells among total γδ T cells using a FACSAria flow cytometer (Becton Dickinson) and FlowJo 7.2.2 (Tree Star, Inc) software.

In vitro co-culture and αβ T cell proliferation assay. To determine the influence of each γδ T cell subset on αβ T cell proliferation and cytokine production, sort-purified splenic CD3+CD4+ or CD3+CD8+ αβ T cells (1.25×10^4) were co-cultured at 1:1 ratio with each splenic γδ T cell subset (or without) for 5 days in E-RPMI media (total 2.5×10^4 cells in 0.8 ml) in 5 % CO_2 at 37 °C with the stimulation of 12 μg/ml phytohaemagglutinin (PHA) or with 12 μg/ml of the semi-purified Wa HRV [20]. During the last 2 days, 0.2 ml of fresh media containing 12 μg/ml of PHA or Wa HRV antigen was added. 10μg/ml of bromodeoxyuridine (BrdU) was added to the cell cultures for the last 18hrs. The supernatants were collected and stored at -80 °C for measuring cytokine

concentrations by ELISA later and the MNCs were harvested, washed with the staining buffer and stained with the following antibodies: SPRD conjugated mouse anti-porcine CD8α (IgG2a, Southern Biotech, 76-2-11), PE conjugated mouse anti-porcine CD4 (IgG2b, BD pharmingen, 74-12-4) and mouse anti-porcine CD3 (IgG1, Southern Biotech, PPT3) followed by APC conjugated rat anti-mouse IgG1 (IgG1, BD pharmingen, A85-1) and FITC conjugated anti-BrdU (BD pharmingen, 557891) as described [20]. Flow cytometry analysis was performed to determine the frequencies of BrdU+CD3+CD4+ and BrdU+CD3+CD8+ αβ T cells using a FACSAria flow cytometer (Becton Dickinson) and FlowJo 7.2.2 (Tree Star, Inc) software. The proliferating T cell responses were presented as the frequencies of BrdU+CD4+ or BrdU+CD8+ αβ T cells among total CD3+ MNCs.

Cytokine assays. Supernatants from the in vitro culture or co-cultures were examined for IFN-γ, IL-10, TGF-β1 and IL-17 concentrations by ELISA as described [21] or following the manufacturer's instructions (the IL-17 kit, Cat# E101-807, Bethyl Laboratories, Inc.). The OD value was measured at 450 nm using a spectrophotometer (Tecan Group Ltd.). The standard curves were calculated using a computer-generated two-parameter curve-fit for each cytokine. The minimal detection concentrations were 7.8 pg/ml for IFN-γ and IL-10, 15.6 pg/ml for TGF-β1 and 18.8 pg/ml for IL-17.

Statistical analysis. Non-parametric Kruskal-Wallis rank sum test was performed to compare frequencies of TLRs, cytokines and FoxP3

expressing/producing γδ T cell subsets in each tissue, concentrations of IFN-γ, IL-10 and TGF-β in the cell culture supernatants and frequencies of proliferating CD4+ and CD8+ αβ T cells in the co-cultures. When differences among these groups were detected, the same test was used in a pairwise fashion to clarify the nature of the differences. Correlations between TLR expression and cytokine production were analyzed using Spearman's correlation coefficiency. All statistical analyses were performed using SAS program 9.2 (SAS Institute, NC, USA). All statistical significance was assessed at $p < 0.05$.

5.4 Results

TLR expression by all three γδ T cell subsets increased significantly after HRV infection. To assess whether and which γδ T cell subsets respond to rotavirus infection, we investigated the frequencies of TLR2, TLR3 and TLR9 expression by each γδ T cell subset from ileum, spleen and blood of Gn pigs inoculated with HRV. We have previously demonstrated that HRV inoculation induced increased TLR2, TLR3 and TLR9 expression on CD14+ macrophages and conventional dendritic cells in spleen of Gn pigs [19].

As shown in the representative dot plots in Fig. 5.1, CD2+CD8+ is the predominant γδ T cell subset in ileum of HRV-infected Gn pigs, followed by CD2-CD8- and CD2+CD8- subsets. Frequencies of TLR2 and TLR3 expressing CD2+CD8+ γδ T cells in ileum were substantially higher (Fig. 5.1B and 1D) in HRV-infected pigs compared to the mock-inoculated pigs (Fig. 5.1C and 1E).

Mean frequencies of TLR2, TLR3 and TLR9 expressing γδ T cell subsets in ileum, TLR2 and TLR3 expressing γδ T cell subsets in spleen and TLR9 expressing γδ T cell subsets in blood of the HRV-infected Gn pigs at PID 0, 3 and 5 were summarized in Fig. 5.2. Because TLR9 expression in spleen and TLR2 and TLR3 expression in blood of the Gn pigs were low and did not differ significantly among PID 0, 3 and 5 in any γδ T cell subset, the data were not shown.

It is clear that all the three porcine γδ T cell subsets in ileum responded to HRV infection as evidenced by the total lack of TLR2 and TLR3 expression at PID 0 and the significantly increased expression of TLR2 and TLR3 by each subset post-inoculation. Compared to PID 0, Gn pigs had significantly higher frequencies of TLR2 and TLR3 expressing CD2+CD8+, CD2+CD8- and CD2-CD8- subsets at PID 3 and PID 5 (Fig. 5.2A). HRV infection also significantly increased frequencies of the TLR9 expressing CD2+CD8- subset in ileum at PID 5 compared to PID 0 (Fig. 5.2A).

In spleen, frequencies of TLR2 and TLR3 expressing γδ T cells were higher than those in ileum at PID 0 for all three subsets (Fig. 5.2A and 2B). Higher baseline of TLR2 and TLR3 expression in all γδ T cell subsets in spleen suggests lower threshold for antigen recognition and activation of systemic γδ T cells. After HRV inoculation, frequencies of TLR2 and TLR3 expressing CD8- γδ T cell subsets in spleen and TLR9 expressing CD8- γδ T cell subsets in blood were

higher or significantly higher at PID 5 compared to PID 0, suggesting that the CD8- subsets in spleen and blood were activated by HRV inoculation, albeit two days later than the TLR2 and TLR3 responses in ileum (Fig. 5.2B).

Both CD2+CD8+ and CD2+CD8- γδ T cell subsets generated a swift IFN-γ response after HRV inoculation. The frequencies of CD2+CD8+ γδ T cell subset increased significantly in ileum after HRV inoculation as reported in our previous study [4] and this study showed that this subset produced IFN-γ (Fig. 5.3A). Significant correlations were found between IFN-γ production and TLR3 expression in ileum (Spearman correlation coefficiency r = 0.54; p = 0.0371) and TLR9 expression in blood (r = 0.58; p = 0.0225) in the CD2+CD8+ subset. Frequencies of IFN-γ producing CD2+CD8+ subset in spleen and blood and IFN-γ producing CD2+CD8- subset in ileum and spleen of HRV-inoculated Gn pigs increased significantly at PID 3 compared to PID 0 (Fig. 5.3). At PID 5, the IFN-γ responses started to decline; but were still higher or significantly higher than those of pre-inoculation (PID 0). Frequencies of the CD2-CD8- γδ T cells in ileum and blood significantly reduced at PID 5 as shown in our previous study [4] and no significant IFN-γ response in this subset was observed after HRV inoculation (Fig. 5.3).

CD2+CD8+ γδ T cell subset in ileum, spleen and blood produced TGF-β with or without HRV inoculation. The CD2+CD8+ subset in ileum had high frequencies of TGF-β production at both PID 0 and PID 5 (Fig. 5.4). The

frequencies of TGF-β production in the CD2+CD8+ subset was higher compared to the CD8- γδ T subsets in ileum, spleen and blood. After HRV inoculation, there was a trend for reduced TGF-β production in the CD2+CD8+ subset in ileum and spleen. However, there were no statistically significant differences because of the low pig numbers in each group (n = 3-4).

CD2+CD8+ γδ T cells in intestinal and systemic sites expressed FoxP3. Transcription factor forkhead box P3 (FoxP3) acts as the regulatory T cell lineage specification factor [22]. Expression of FoxP3 is the marker of regulatory T cell function. To better clarify the potential regulatory function of the γδ T cell subsets, patterns of FoxP3 expression by each γδ T cell subset in Gn pigs were investigated. Fig. 5.5A depicts the representative dot plots of FoxP3 expression in each γδ T cell subset in ileum. Frequencies of FoxP3 expressing CD2+CD8+ γδ T cells in ileum, IEL and spleen were significantly higher compared to the CD8- subsets (Fig. 5.5B) confirming that tissue-specific CD2+CD8+ subset exhibits regulatory T cell function. The two CD8- subsets in all tissues had low or no FoxP3 expression, which is consistent with the low or no TGF-β production by these two subsets with or without HRV inoculation. FoxP3 expression was low and the frequencies did not differ among the three γδ T cell subsets in blood.

Sort-purified CD2+CD8+ subset produced IL-10 and TGF-β whereas CD8- subsets produced IFN-γ after IPP plus IL-2 stimulation. To further

differentiate immune functions among γδ T cell subsets, the cytokine production profiles of each sort-purified γδ T cell subset from spleen and IEL of Gn pigs were determined by ELISA after the cells were stimulated with the γδ T cell agonist phosphoantigen IPP for 5 days in cell culture. IPP plus IL-2 is known to activate human Vδ2 T cells [23, 24].

Mock-stimulated γδ T cells did not produce any detectable level of IFN-γ or TGF-β in any subset. Substantial amount of IL-10 were produced by the CD2+CD8+ subset from spleen (~6000 pg/ml) and IEL (~2000 pg/ml) without IPP plus IL-2 stimulation (Fig. 5.6). The CD2+CD8+ subset stimulated with IPP plus IL-2 from neither spleen nor IEL produced IFN-γ. In contract, the CD8- subsets from both spleen and IEL produced IFN-γ, although the variability was high. There were no significant differences in IFN-γ concentrations among the subsets or between the IPP plus IL-2 stimulated and mock stimulated cells due to the high variability.

The CD2+CD8+ subset from spleen and IEL produced significantly higher amount of IL-10 in IPP plus IL-2 stimulated cells compared to the mock-stimulated cells. More importantly, the CD2+CD8+ subset produced significantly higher amount of IL-10 than the two CD8- subsets in spleen (14-23 fold) and IEL (3-13 fold) with or without IPP plus IL-2 stimulation. Interestingly, IPP stimulation also significantly increased IL-10 production by the CD2+CD8- subset. The CD2+CD8+ γδ T cells from spleen were the only subset producing the detectable amount of TGF-β (Fig. 5.6). IL-17 was

undetectable in the culture supernatants of any γδ T cell subsets (data not shown).

CD8- γδ T cells differentiated into CD8+ γδ T cells during in vitro culture. Our previous studies showed that frequencies of CD8- γδ T cells decreased and CD2+CD8+ γδ T cells increased significantly at PID 5 in HRV-inoculated and lactobacilli-colonized Gn pigs, thus we postulated that CD8- γδ T cells acquired CD8 marker upon activation and differentiated into CD2+CD8+ γδ T cells [4]. In this study, γδ T cell subsets were sort-purified by MACS and FACS and the purities of total γδ T cells were > 99 % for all the samples. Three γδ T cell subsets were collected by FACS using the gating method shown in Fig. 5.7A, which ensured that the purity of each subset were > 99 %. After the cells were cultured in vitro for 5 days with or without IPP plus IL-2 stimulation, the CD8- subsets were stained again for Tcr1-N4 and CD8 and were subjected to flow cytometry analysis. As shown in Fig. 5.7B, the γδ T cells that were originally CD8- at the beginning of the culture expressed CD8 in up to 47 % of the cells from IEL and up to 35 % of the cells from spleen. The observation confirmed that extra-thymus CD8- γδ T cells can differentiate into CD8+ γδ T cells. There were more CD2+CD8- γδ cells differentiated into CD8+ γδ cells than the CD2-CD8- γδ cells. There were no significant differences in the frequencies of CD8+ γδ T cells between IPP plus IL-2 stimulated and mock stimulated γδ T cell cultures, suggesting that IPP plus IL-2 is not necessary for the differentiation.

All three γδ T cell subsets enhanced IFN-γ production by CD4+ αβ T cells in the co-cultures. To further identify the immune modulating functions of γδ T cell subsets on the development of adaptive immune response, sort-purified splenic γδ T cells were co-cultured with autologous CD4+ or CD8+ αβ T cells and stimulated with PHA for 5 days in cell culture. The final cytokine concentrations in the culture supernatants were measured by ELISA and the CD4+ and CD8+ αβ T cell proliferation was measured by BrdU staining and flow cytometry. As shown in Fig. 5.8, CD4+ and CD8+ αβ T cells cultured alone with PHA produced low levels of IFN-γ. The CD8- subsets, but not the CD2+CD8+ subset, cultured alone with PHA also produced low levels of IFN-γ. All three γδ T cell subsets enhanced IFN-γ production by CD4+ αβ T cells in the co-culture; however, the two CD8- γδ T cell subsets increased the concentrations of IFN-γ more substantially (5 to 7-fold by CD8- subsets versus 2-fold by the CD2+CD8+ subset). Co-culture the three γδ T cell subsets with CD8+ αβ T cells did not significantly alter the IFN-γ production by CD8+ αβ T cells (Fig. 5.8).

The CD2+CD8+ subset significantly enhanced IL-10 production by both CD4+ and CD8+ αβ T cells. All three γδ T cell subsets and CD4+ and CD8+ αβ T cells produced measurable levels of IL-10 when cultured alone with PHA. The CD2+CD8+ subset cultured alone produced significantly more IL-10 than the two CD8- subsets alone, a similar trend as the IPP plus IL-2 stimulated CD2+CD8+ γδ T cell subset (Fig. 5.6), but at a lower magnitude (~10-fold less)

(Fig. 5.8. Note the scale differences in the y axis of the figures for IL-10 concentration). The CD2+CD8+ subset significantly enhanced IL-10 production by both CD4+ and CD8+ αβ T cells. The co-culture of CD8+ αβ T cells with CD2+CD8- γδ T cells also had significantly higher IL-10 concentrations than the cells cultured alone. However, the effect appeared to be additive rather than synergistic.

The CD2+CD8+ subset significantly increased TGF-β production by both CD4+ and CD8+ αβ T cells. Without γδ T cells in the culture, CD4+ or CD8+ αβ T cells did not produce any detectable TGF-β. Interestingly, CD2+CD8- subset also significantly enhanced TGF-β production by CD4+ αβ T cells. Due to low yields of sort-purified γδ and αβ T cells from IEL, co-culture studies were not conducted for IEL.

The CD2+CD8- subset significantly enhanced CD4+ αβ T cell proliferation with PHA stimulation. Consistent with the IFN-γ production profile, splenic CD4+ αβ T cells co-cultured with the CD2+CD8- γδ T cell subset had significantly higher frequencies of BrdU+CD4+ αβ T cells compared to co-cultures with the CD2+CD8+ γδ T cell subset or without γδ T cells (Fig. 5.9). The CD2-CD8- subset also increased frequencies of BrdU+CD4+ αβ T cells in the co-culture, but the increase was not statistically significant. None of the γδ T cell subsets enhanced CD8+ αβ T cell proliferation (data not shown).

The CD2+CD8+ subset increased HRV-specific CD4+ αβ T cell proliferation without the presence of APC. Previous studies indicated that a

minority of the porcine CD2+CD8+ γδ T cells could act as professional APCs and presented African swine fever virus antigens to CD4+ T cells [25, 26]. In this study, the sort-purified splenic CD4+ αβ T cells from attenuated HRV-vaccinated pigs and collected on PID 28 were cultured in vitro for 5 days. Semi-purified Wa HRV antigen was added in all the cultures. When CD4+ αβ T cells were cultured alone or co-cultured with the CD8- γδ T cell subsets, frequencies of proliferating CD4+ αβ T cells were low and did not differ significantly (Fig. 5.10). However, when CD4+ αβ T cells were co-cultured with CD2+CD8+ γδ T cells, frequencies of proliferating CD4+ αβ T cells increased substantially. This observation suggested that the CD2+CD8+ subset acted as APC and presented Wa HRV antigen to HRV-specific CD4+ αβ T cells to promote proliferation.

5.5 Discussion

In this study, we characterized immunological functions of the three porcine γδ T cell subsets. γδ T cells play important role in antigen recognition and innate immune responses and antigen recognition by γδ T cells is not restricted to the γδ TCR. γδ T cells can sense invading pathogens or self antigens via pattern recognition receptors such as TLRs, scavenger receptors, integrins and NK receptors [25]. TLR expressions by γδ T cells have been demonstrated in humans [12], mice [13] and cattle [27]. TLR expression on porcine γδ T cell subsets was examined for the first time in this study. We demonstrated that

rotavirus infection stimulated significant alternations in TLR expression in all three γδ T cell subsets, with the highest increases in the CD2+CD8- subset in ileum and spleen. TLR2 and TLR3 expression in all three γδ T cell subsets in ileum increased significantly at PID 3-5 after HRV infection, suggesting that all the three subsets were involved in rotavirus recognition and in the innate mucosal immune responses to rotavirus infection. Splenic and circulating CD8- γδ T cells expressed increased frequencies of TLRs by PID 5, two days later than the TLR responses in ileum, suggesting that the CD8- subsets in spleen and blood were activated when rotavirus entered the circulation after the initial replication in the small intestinal epithelium. Rotavirus fecal shedding peaked at PID 1 whereas viremia peaked at PID 3 in virulent HRV orally inoculated Gn pigs [28], which explains the delayed TLR responses in splenic and circulating and γδ T cells compared to ileal γδ T cells. There was no TLR2 or TLR3 expression in ileum and a higher baseline of TLR expression in spleen in all γδ T cell subsets of naïve Gn pigs (PID 0). These observations suggest that intestinal γδ T cells are non-activated at homeostasis and are quickly activated upon recognition of invading pathogens whereas systemic γδ T cells are constitutively activated at a low level and can be further activated during enteric virus infection.

Similarly to other species, we can generally divide porcine γδ T cells into two major functional subsets: the proinflammatory (CD2+CD8- and CD2-CD8-) and

the regulatory (CD2+CD8+) subsets based on their cytokine production profile, FoxP3 expression and immunomodulating effects on αβ T cells observed in this study. The CD2+CD8- γδ T cell subset had significantly increased frequencies of IFN-γ expression in ileum and spleen of Gn pigs after rotavirus infection and produced IFN-γ after stimulation with IPP plus IL-2. CD2+CD8- and CD2-CD8- γδ T cell subsets enhanced proliferation and IFN-γ production by CD4+ αβ T cells in the co-cultures, confirming that CD2+CD8- and CD2-CD8- γδ T cells are proinflammatory effectors. In contrast, CD2+CD8+ subset expressed FoxP3 in ileum, IEL and spleen and produced TGF-β in ileum, spleen and blood of Gn pigs, produced significantly higher amount of IL-10 and TGF-β after stimulation with IPP plus IL-2, and significantly enhanced IL-10 and TGF-β production by PHA-stimulated αβ T cells in the co-cultures. These are characteristics of regulatory T cells. However, in addition to the antiinflammatory/regulatory functions, CD2+CD8+ subset also had significantly increased frequencies of IFN-γ expression in spleen and blood of HRV-infected Gn pigs and enhanced production of IFN-γ by splenic CD4+ αβ T cells in the co-cultures after PHA stimulation, although it did not enhance CD4+ αβ T cell proliferation, indicating that this subset in specific microenvironment (spleen and blood) has proinflammatory function.

This dual function of porcine CD2+CD8+ γδ T cells is not unexpected. Similar findings have been reported for γδ T cells in other species. The condition of microenvironment could determine the immune regulatory function

of a specific γδ T cell subset. Human V*δ*2 T cells (proinflammatory) primed *ex vivo* towards IFN-*γ* and TNF-*α* production could be driven towards IL-4 production under appropriate Th2-priming *in vitro* culture conditions [29]. TGF-β and IL-15 could stimulate human V*δ*2 T cells to express FoxP3 and acquire regulatory functions to suppress the proliferation of anti-CD3/CD28 stimulated peripheral blood mononuclear cells [30]. A model of inflammation regulation was proposed by Holderness [6] to explain the inducible proinflammatory responses of tissue-specific γδ T cells and at the same time the antiinflammatory, tissue repair responses commonly seen from the same cells during inflammation.

Interestingly, we found that co-culture with CD2+CD8- subset not only significantly enhanced IFN-γ but also TGF-β production by CD4+ αβ T cells. Porcine CD2+CD8- *γδ* thymocytes was proposed to be the precursor of all three peripheral *γδ* T cell subsets [8], suggesting that extra-thymus porcine CD8- subsets can possibly differentiate into CD8+ subset and obtain regulatory function. In deed we observed that a large percent (32-47 %) of sort-purified splenic and IEL CD8- *γδ* T cells became CD8+ after 5 days in vitro culture. Therefore the TGF-β inducing effect was likely exerted by the newly emerged CD8+ *γδ* T cells in the culture.

Although initial studies of mice showed that FoxP3 expression highly restricted to αβ T cells and there is a lack of FoxP3 expression on any other cell populations including γδ T cells [22], FoxP3+ γδ T cells have been identified in

mice and cattle [31, 32]. Our study showed that FoxP3 was expressed, albeit in low frequencies (< 0.5 %), on the CD2+CD8+ γδ T cells in ileum, IEL and spleen of Gn pigs. This observation corroborates with the regulatory cytokine production in Gn pigs (TGF-β) and after in vitro IPP stimulation (IL-10 and TGF-β) by the CD2+CD8+ subset. The CD2+CD8- subset from spleen of Gn pigs also expressed low levels of FoxP3, which is consistent with the findings from the co-culture study that this subset promoted TGF-β production by CD4+ αβ T cells and from the IPP stimulation study that this subset produced significantly higher levels of IL-10 after IPP stimulation in the cell culture.

Previous studies suggested that IPP directly activated proinflammatory γδ T cells (i.e. Vδ2Vγ9 T cells in humans) to proliferate and produce cytokines without requiring antigen presentation by APCs [33, 34]. In our study, IPP (pus IL-2) stimulated γδ T cells to produce IFN-γ (CD8- subsets) or TGF-β (CD2+CD8+ subset). Although IL-10 was produced constitutively by the CD2+CD8+ subset, IPP stimulation significantly enhanced IL-10 production. Thus, IPP can activate both proinflammatory and regulatory porcine γδ T cells to exert their respective immune effector functions. IPP stimulation did not have an effect on the differentiation of CD8- subsets into CD8+ γδ T cells. Taken together, the data suggest that IPP stimulation did not have a bias toward expanding the pro- or antiinflammatory γδ T cell function. The factors that regulate the differentiation of extra-thymus γδ T cells require further study.

γδ T cells can present antigens to other cells to induce adaptive immune

responses. Previous studies found that porcine CD2+CD8+ γδ T cells presented antigens via MHC II to CD4+ T cells [26]. Bovine WC1 γδ T cells and human Vδ2 T cells expressed MHC II and potentially presented antigen in the context of MHC II [35, 36]. Our co-culture study with the presence of HRV antigen and without APCs confirmed that porcine CD2+CD8+ γδ T cells has APC function and can present rotavirus antigens to CD4+ T cells.

In summary, porcine CD2+CD8- and CD2-CD8- γδ T cells have mainly proinflammatory function by directly secreting IFN-γ or promoting CD4+ αβ T cell proliferation and IFN-γ production, whereas CD2+CD8+ γδ T cells mainly exert regulatory T cell function by expressing FoxP3, directly secreting IL-10 and TGF-β or increasing IL-10 and TGF-β production by CD4+ and CD8+ αβ T cells. γδ T cells responded to rotavirus infection by TLR expression and IFN-γ and TGF-β production with different patterns in CD2+CD8+ subset from CD8- subsets. The CD8- subsets can differentiate into CD8+ subset by acquiring CD8 expression. This phenomenon may explain, at least in part, the apparently dual functions of CD2+CD8+ and CD2+CD8- subsets. Thus, both CD8+ and CD8- γδ T cell subsets can contribute to anti-virus infection as well as to the maintaining and restoring intestinal and systemic homeostasis in the face of enteric virus infections.

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5.7 References

1. Hahn, Y.S., et al., *Different potentials of gamma delta T cell subsets in regulating airway responsiveness: V gamma 1+ cells, but not V gamma 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation.* J Immunol, 2004. 172(5): p. 2894-902.

2. Holderness, J., et al., *Response of gammadelta T Cells to plant-derived tannins.* Crit Rev Immunol, 2008. 28(5): p. 377-402.

3. Saalmuller, A., W. Hirt, and M.J. Reddehase, *Porcine gamma/delta T lymphocyte subsets differing in their propensity to home to lymphoid tissue.* Eur J Immunol, 1990. 20(10): p. 2343-6.

4. Wen, K., et al., *Development of gammadelta T cell subset responses in gnotobiotic pigs infected with human rotaviruses and colonized with probiotic lactobacilli.* Vet Immunol Immunopathol, 2011. 141(3-4): p. 267-75.

5. Wilson, E., et al., *Bovine gamma delta T cell subsets express distinct patterns of chemokine responsiveness and adhesion molecules: a mechanism for tissue-specific gamma delta T cell subset accumulation.* J Immunol, 2002. 169(9): p. 4970-5.

6. Holderness, J., *Select procyanidins induce γδ T cell activation and prolieration*, in *Veterinary Molecular Biology*2008, Montana State University: Bozeman, MS Thesis, 30-40. p. 30-40.

7. Davis, W.C., et al., *Analysis of monoclonal antibodies that recognize gamma delta T/null cells.* Vet Immunol Immunopathol, 1998. 60(3-4): p. 305-16.

8. Sinkora, M., J. Sinkorova, and W. Holtmeier, *Development of gammadelta thymocyte subsets during prenatal and postnatal ontogeny.* Immunology, 2005. 115(4): p. 544-55.

9. Hedges, J.F., K.J. Lubick, and M.A. Jutila, *Gamma delta T cells respond directly to pathogen-associated molecular patterns.* J Immunol, 2005. 174(10): p. 6045-53.

10. Wesch, D., et al., *Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes.* J Immunol, 2006. 176(3): p. 1348-54.

11. Deetz, C.O., et al., *Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the Toll-Like receptor 2 agonist Pam3Cys.* Infect Immun, 2006. 74(8): p. 4505-11.

12. Pietschmann, K., et al., *Toll-like receptor expression and function in subsets of human gammadelta T lymphocytes.* Scand J Immunol, 2009. 70(3): p. 245-55.

13. Schwacha, M.G. and T. Daniel, *Up-regulation of cell surface Toll-like receptors on circulating gammadelta T-cells following burn injury.* Cytokine, 2008. 44(3): p. 328-34.

14. Ward, L.A., et al., *Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs.* J Gen Virol, 1996. 77 (Pt 7): p. 1431-41.

15. Yuan, L., et al., *Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease.* J Virol, 1996. 70(5): p. 3075-83.

16. Ward, L.A., et al., *Development of mucosal and systemic lymphoproliferative responses and protective immunity to human group A rotaviruses in a gnotobiotic pig model.* Clin Diagn Lab Immunol, 1996. 3(3): p. 342-50.

17. Meyer, R.C., E.H. Bohl, and E.M. Kohler, *Procurement and Maintenance of Germfree Seine for Microbiological Investigations.* Appl Microbiol, 1964. 12: p. 295-300.

18. Zhang, W., et al., *Probiotic Lactobacillus acidophilus enhances the immunogenicity of an oral rotavirus vaccine in gnotobiotic pigs.* Vaccine, 2008. 26(29-30): p. 3655-61.

19. Wen, K., et al., *Toll-like receptor and innate cytokine responses induced by lactobacilli colonization and human rotavirus infection in gnotobiotic pigs.* Vet Immunol Immunopathol, 2009. 127: p. 304-315.

20. Yuan, L., et al., *Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are*

correlated with protection against rotavirus diarrhea in gnotobiotic pigs. Vaccine, 2008. 26(26): p. 3322-31.

21. Azevedo, M.S., et al., *Cytokine responses in gnotobiotic pigs after infection with virulent or attenuated human rotavirus.* J Virol, 2006. 80(1): p. 372-82.

22. Fontenot, J.D., et al., *Regulatory T cell lineage specification by the forkhead transcription factor foxp3.* Immunity, 2005. 22(3): p. 329-41.

23. Morita, C.T., et al., *Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells.* Immunity, 1995. 3(4): p. 495-507.

24. Sicard, H., et al., *In vivo immunomanipulation of V gamma 9V delta 2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate model.* J Immunol, 2005. 175(8): p. 5471-80.

25. Takamatsu, H.H., et al., *Porcine gammadelta T cells: possible roles on the innate and adaptive immune responses following virus infection.* Vet Immunol Immunopathol, 2006. 112(1-2): p. 49-61.

26. Takamatsu, H.H., M.S. Denyer, and T.E. Wileman, *A sub-population of circulating porcine gammadelta T cells can act as professional antigen presenting cells.* Vet Immunol Immunopathol, 2002. 87(3-4): p. 223-4.

27. Hedges, J.F., J.C. Graff, and M.A. Jutila, *Transcriptional profiling of gamma delta T cells.* J Immunol, 2003. 171(10): p. 4959-64.

28. Azevedo, M.S., et al., *Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus.* J Virol, 2005. 79(9): p. 5428-36.

29. Wesch, D., A. Glatzel, and D. Kabelitz, *Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype.* Cell Immunol, 2001. 212(2): p. 110-7.

30. Casetti, R., et al., *Cutting edge: TGF-beta1 and IL-15 Induce FOXP3+ gammadelta regulatory T cells in the presence of antigen stimulation.* J Immunol, 2009. 183(6): p. 3574-7.

31. Kang, N., et al., *Identification and characterization of Foxp3(+) gammadelta T cells in mouse and human.* Immunol Lett, 2009. 125(2): p. 105-13.

32. Gerner, W., et al., *Sensitive detection of Foxp3 expression in bovine lymphocytes by flow cytometry.* Vet Immunol Immunopathol, 2010.

33. Janeway, C.A., Jr., B. Jones, and A. Hayday, *Specificity and function of T cells bearing gamma delta receptors.* Immunol Today, 1988. 9(3): p. 73-6.

34. De Libero, G., *Sentinel function of broadly reactive human gamma delta T cells.* Immunol Today, 1997. 18(1): p. 22-6.

35. Price, S.J. and J.C. Hope, *Enhanced secretion of interferon-gamma by bovine gammadelta T cells induced by coculture with Mycobacterium bovis-infected dendritic cells: evidence for reciprocal activating signals.* Immunology, 2009. 126(2): p. 201-8.

36. Brandes, M., K. Willimann, and B. Moser, *Professional antigen-presentation function by human gammadelta T Cells.* Science, 2005. 309(5732): p. 264-8.

Fig. 5.1. Representative dot plots of TLR2 and TLR3 expressing CD2+CD8+ γδ T cell subset in ileum of Gn pigs infected with HRV at PID 5 or mock infected. Gn pigs were infected with the virulent Wa HRV. The MNCs were isolated upon euthanasia at PID 5 and freshly stained and gated for γδ T cells as described in our previous study [4]. Three defined γδ T cell subsets are shown in Fig. 5.1A with the frequencies among total γδ T cells at each corner. Fig. 5.1B to 1E show the representative dot plots for TLR2 and TLR3 expressing CD2+CD8+ γδ T cells in ileum of HRV infection and mock infected Gn pigs, respectively. The numbers above the rectangles are the frequencies of TLR2+ or TLR3+ cells among CD2+CD8+ γδ T cells.

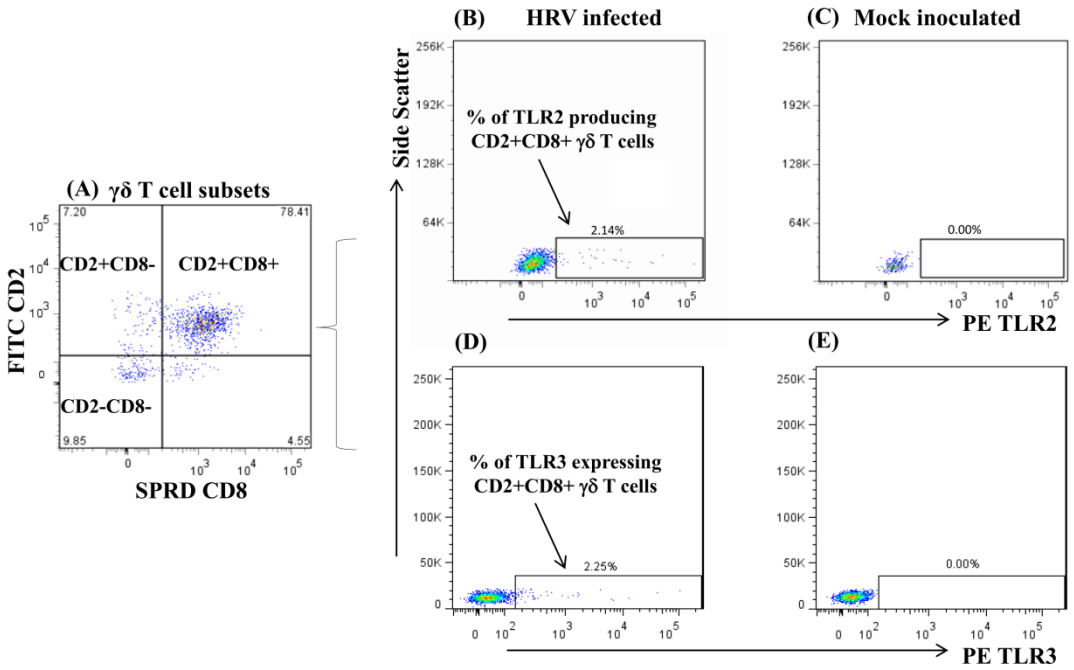

(A) γδ T cell subsets
FITC CD2
SPRD CD8
CD2+CD8-
CD2+CD8+
CD2-CD8-
7.20
78.41
9.85
4.55
(B) HRV infected
(C) Mock inoculated
Side Scatter
% of TLR2 producing
CD2+CD8+ γδ T cells
2.14%
0.00%
PE TLR2
(D)
(E)
% of TLR3 expressing
CD2+CD8+ γδ T cells
2.25%
0.00%
PE TLR3

Fig. 5.1

Fig. 5.2. Frequencies of TLR expressing γδ T cell subsets in ileum (A), spleen (B) and blood (C) of Gn pigs infected with HRV or mock infected. The panels A, B and C depict the mean frequencies of TLR expressing cells among each γδ T cell subset in ileum (TLR2 TLR3 and TLR9), spleen (TLR3 and TLR3) and blood (TLR9) of Gn pigs infected with HRV (PID 3 and PID 5) or mock infection (PID 0). Data are presented as mean frequency ± standard error of the mean (n = 4-7). Different capital letters indicate significant differences in frequencies among the time points for the same γδ T cell subset and tissue (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

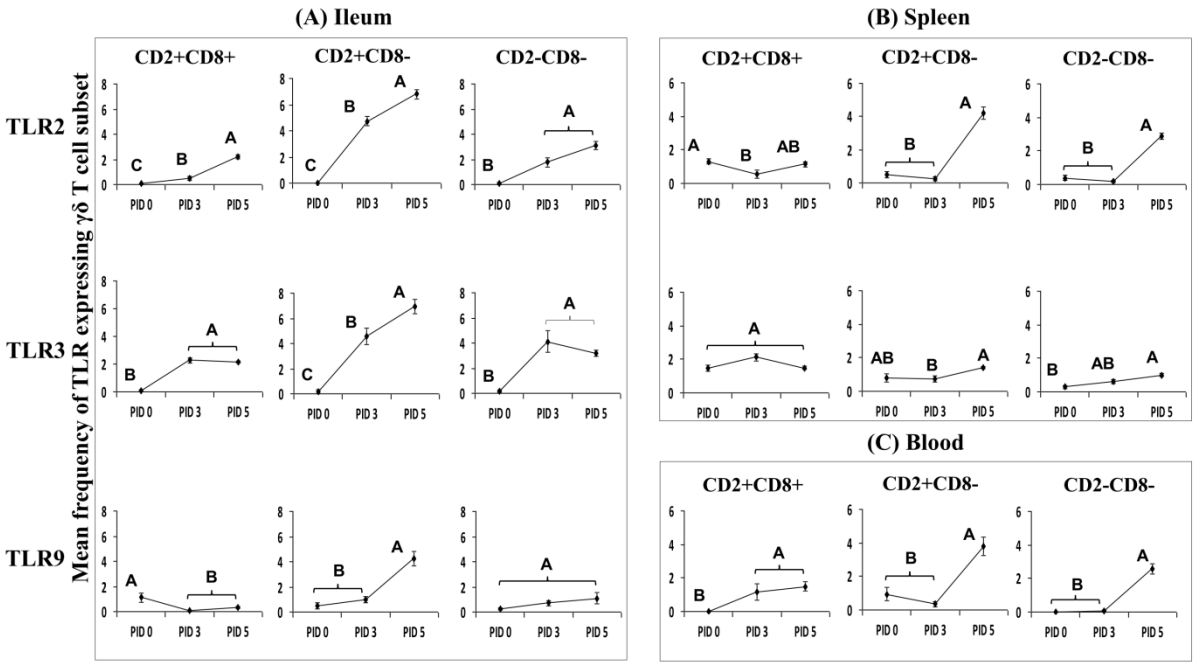

(A) Ileum
(B) Spleen
(C) Blood
CD2+CD8+
CD2+CD8-
CD2-CD8-
TLR2
TLR3
TLR9
Mean frequency of TLR expressing γδ T cell subset
PID 0
PID 3
PID 5

Fig. 5.2

Fig. 5.3. Representative dot plots (A) and mean frequencies (B) of IFN-γ producing γδ T cell subsets in ileum, spleen and blood of Gn pigs inoculated with HRV. MNCs were cultured in vitro for 17 hrs without antigen stimulation before staining for flow cytometry analysis. The right two dot plots in panel (A) show the representatives of IFN-γ producing CD2+CD8+ subset in ileum of Gn pigs infected with HRV at PID 3 or mock infected. Figures in panel (B) compare the mean frequencies of IFN-γ producing γδ T cells among the three subsets in ileum, spleen and blood of Gn pigs infected with HRV (PID 3 and PID 5) or mock infected (PID 0). Data are presented as mean frequency ± standard error of the mean (n = 4-7). Different capital letters on top of bars indicate significant differences in frequencies among the time points for the same γδ T cell subset and tissue (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

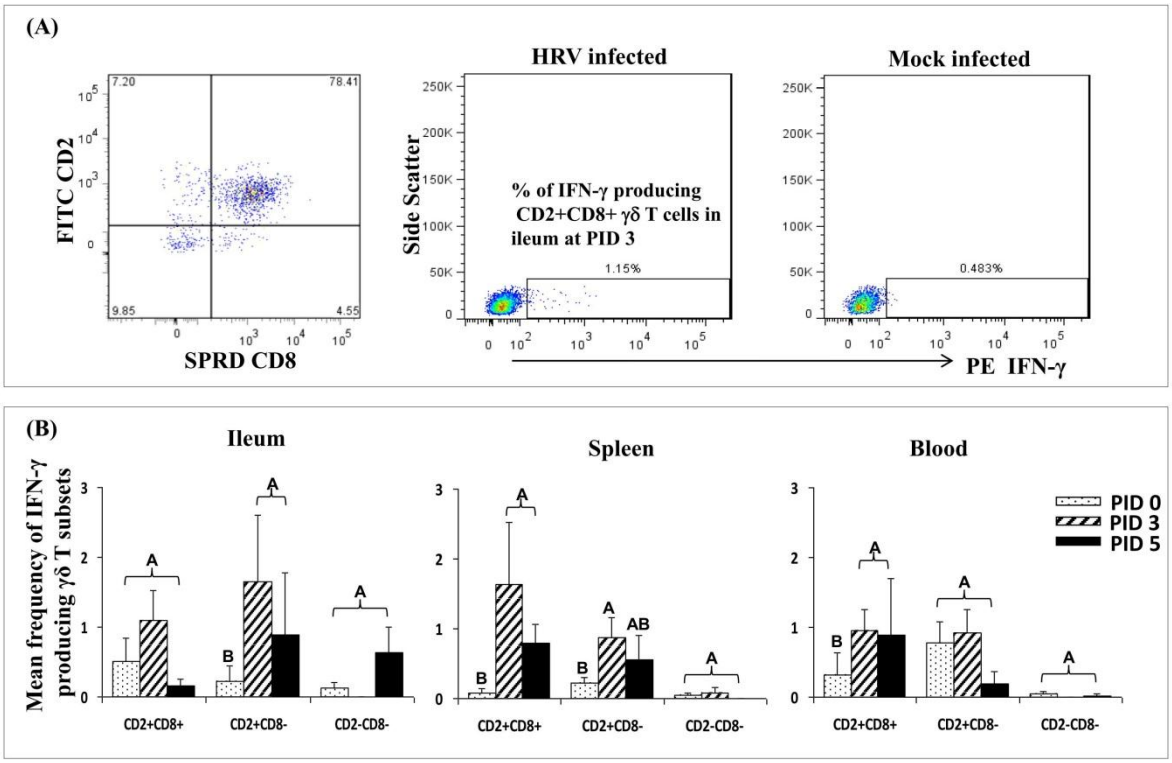

(A)
HRV infected
Mock infected
FITC CD2
SPRD CD8
Side Scatter
% of IFN-γ producing CD2+CD8+ γδ T cells in ileum at PID 3
1.15%
0.483%
PE IFN-γ
(B)
Ileum
Spleen
Blood
Mean frequency of IFN-γ producing γδ T subsets
PID 0
PID 3
PID 5
CD2+CD8+
CD2+CD8-
CD2-CD8-

Fig. 5.3

Fig. 5.4. Frequencies of TGF-β producing γδ T cell subsets in ileum, spleen and blood of Gn pigs inoculated with HRV. MNCs were cultured in vitro for 48 hrs without antigen stimulation and Brefeldin A was added at the last 5 hrs before staining for flow cytometry analysis. Data are presented as mean frequency ± standard error of the mean (n = 3-4). The figures depict the mean frequencies of TGF-β producing cells among each γδ T cell subset in Gn pigs infected with HRV at PID 5 or mock infected. There were no statistically significant differences in frequencies of TGF-β producing cells between the time points for the same γδ T cell subset and tissue.

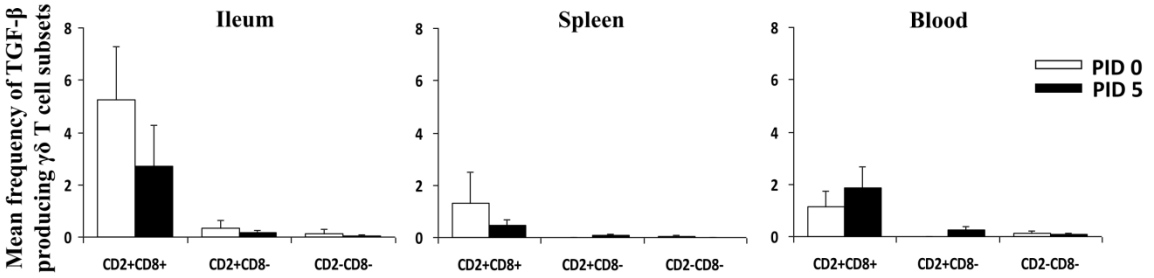

Mean frequency of TGF-β producing γδ T cell subsets
Ileum
Spleen
Blood
PID 0
PID 5
0
2
4
6
8
CD2+CD8+
CD2+CD8-
CD2-CD8-

Fig. 5.4

Fig. 5.5. Representative dot plots (A) and mean frequencies (B) of FoxP3 expressing γδ T cell subsets in intestinal and systemic lymphoid tissues of Gn pigs challenged with virulent HRV at PCD 7. MNCs were cultured in vitro with Brefeldin A for 5 hrs before staining for flow cytometry analysis. Panel (A) shows the representative dot plots of FoxP3 expressing γδ T cells in ileum of Gn pigs. Figures in panel (B) depict the mean frequencies of FoxP3 expressing γδ T cell subsets among total γδ T cells in ileum, IEL, spleen and blood. Data are presented as mean frequency ± standard error of the mean (n = 3). Different capital letters on top of bars indicate significant differences in frequencies among the three γδ T cell subsets from the same tissue (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

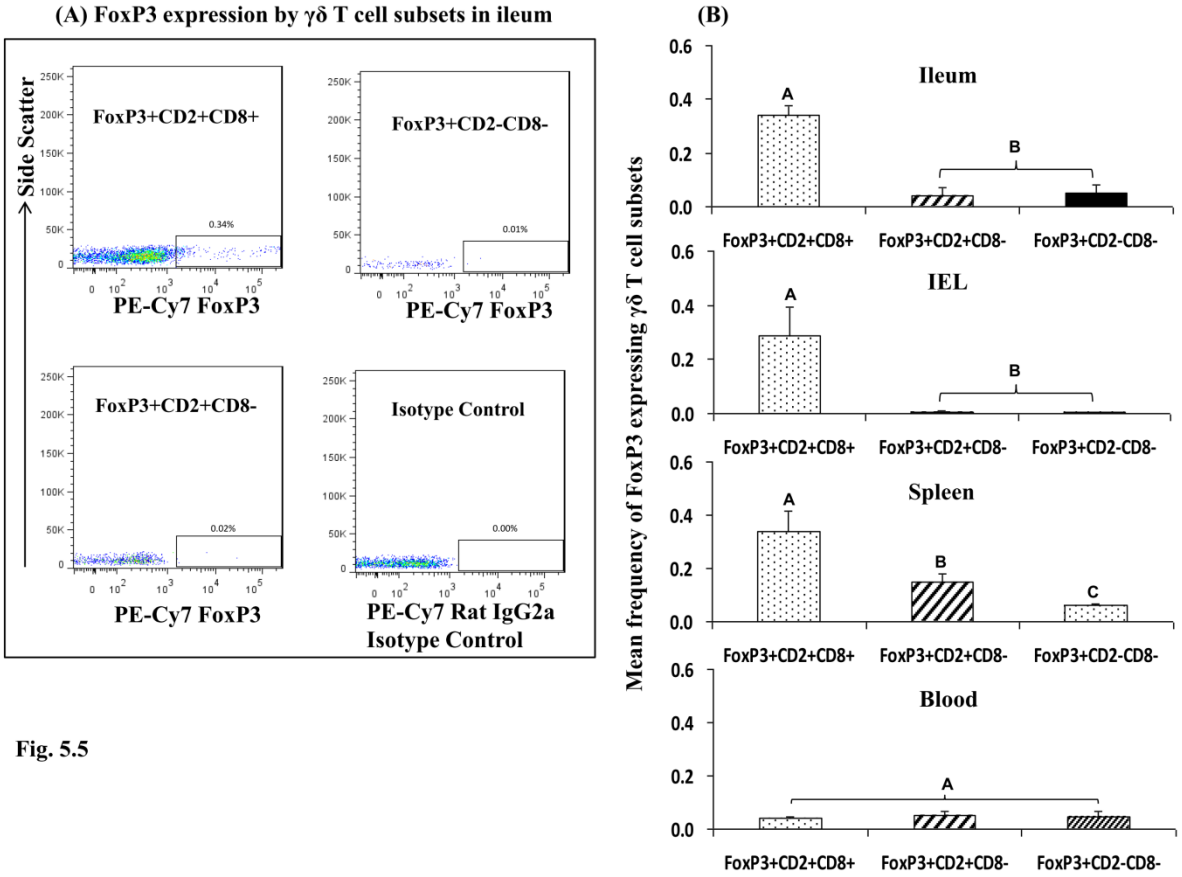

(A) FoxP3 expression by γδ T cell subsets in ileum
Side Scatter
FoxP3+CD2+CD8+
0.34%
PE-Cy7 FoxP3
FoxP3+CD2-CD8-
0.01%
PE-Cy7 FoxP3
FoxP3+CD2+CD8-
0.02%
PE-Cy7 FoxP3
Isotype Control
0.00%
PE-Cy7 Rat IgG2a
Isotype Control
(B)
Mean frequency of FoxP3 expressing γδ T cell subsets
Ileum
IEL
Spleen
Blood
A
B
C
FoxP3+CD2+CD8+
FoxP3+CD2+CD8-
FoxP3+CD2-CD8-

Fig. 5.5

Fig. 5.6. Cytokine production by splenic and IEL γδ T cell subsets in cultures with or without IPP plus IL-2 stimulation. MNCs isolated from spleen and IEL of Gn pigs infected with human norovirus GII.4 were sorted into three γδ T cell subsets by using MACS and FACS technologies. Each γδ T cell subset was cultured in vitro for 5 days with the stimulation of IPP plus IL-2 or mock stimulated. Concentrations of IFN-γ, IL-10 and TGF-β in the culture supernatants were measured by ELISA. Data are presented as mean concentrations ± standard error of the mean (n = 6). Different capital letters on top of bars indicate significant differences in cytokine concentrations between IPP plus IL-2 stimulated and mock stimulated cells for the same γδ T cell subset and tissue (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference. Different numbers of * indicates significant difference in cytokine concentrations among the three γδ T cell subsets under the same stimulation condition for the same tissue (Kruskal-Wallis test, $p < 0.05$), while same numbers of * indicate no significant difference.

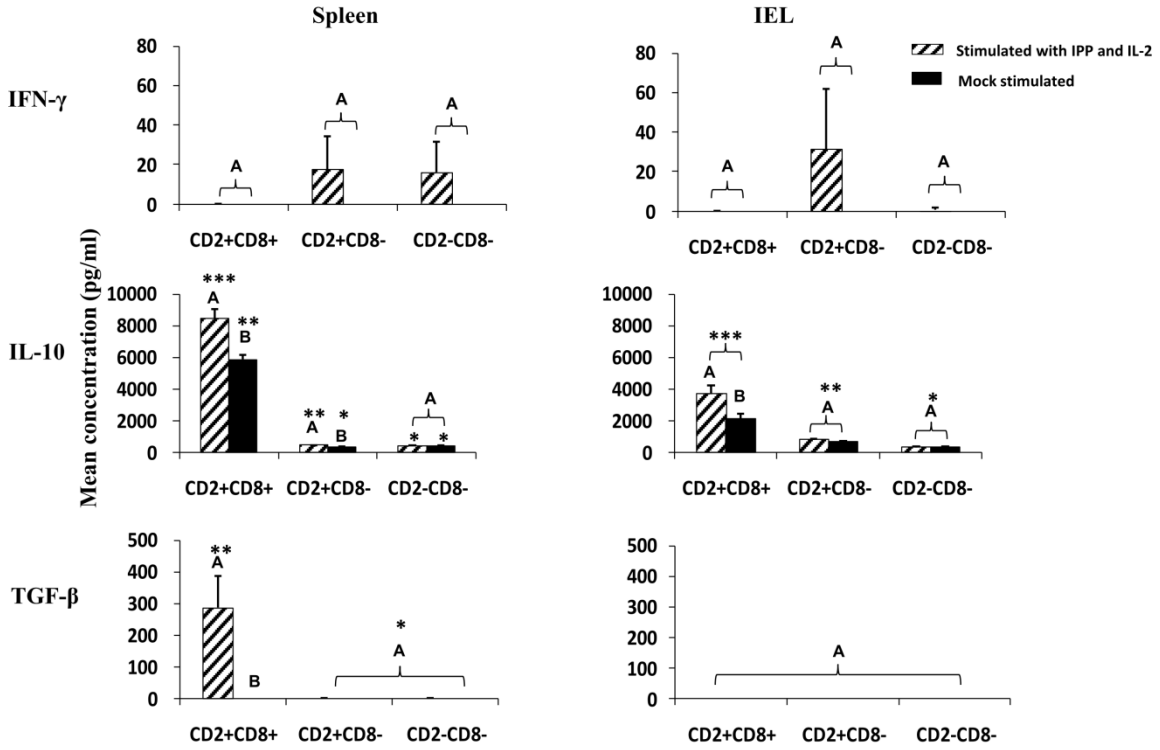
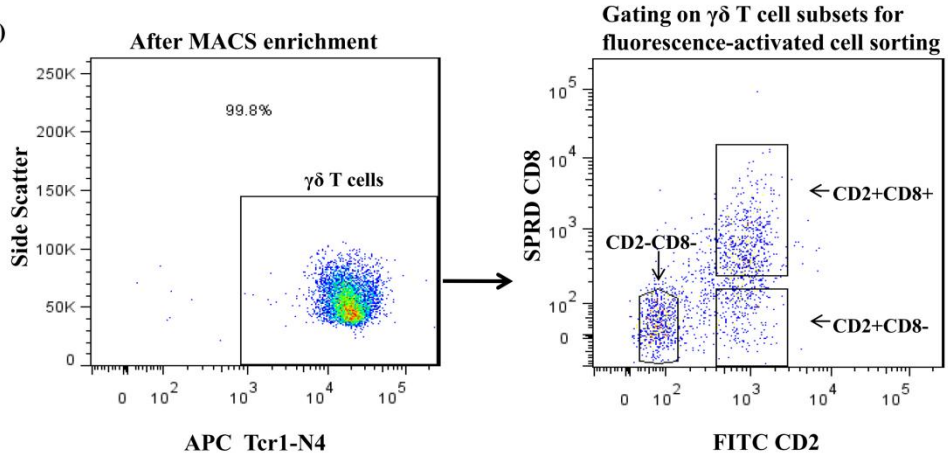

Spleen
IEL
IFN-γ
IL-10
TGF-β
Mean concentration (pg/ml)
Stimulated with IPP and IL-2
Mock stimulated
CD2+CD8+
CD2+CD8-
CD2-CD8-

Fig. 5.6

Fig. 5.7. CD8- γδ T cells differentiation into CD8+ γδ T cells during in vitro culture. MNCs isolated from spleen and IEL of Gn pigs infected with human norovirus GII.4 were sorted into three γδ T cell subsets by using MACS and FACS technologies. Each γδ T cell subset was cultured in vitro for 5 days with the stimulation of IPP plus IL-2 or mock stimulation. The culture supernatants were kept to measure IFN-γ, IL-10 and TGF-β by ELISA (See Fig. 5.6 legend) and the cells were collected for the Tcr1-N4 and CD8 staining. The left dot plot in panel (A) shows the purity of γδ T cells after enrichment by MACS and the right dot plot in panel (A) demonstrates the gating of three γδ T cell subsets to be collected by FACS. The table in panel (B) lists the mean frequencies of CD8 expression after 5 days in vitro culture of the CD2+CD8- and CD2-CD8- subsets from spleen and IEL.

(A)



(B)

	Spleen		IEL	
	Mean frequencies of CD8+ expression after 5 days in vitro culture			
γδ T cell subset	CD2+CD8-	CD2-CD8-	CD2+CD8-	CD2-CD8-
IPP+IL-2 stimulated	32	34	47	35
	(7.5)*	(6.3)	(4.7)	(0.9)
Mock-stimulated	35	34	46	37
	(6.7)	(5.6)	(7.4)	(3.9)

*Standard error of the mean (n=3).

Fig. 5.7

Fig. 5.8. Modulation of cytokine production of sort-purified splenic CD4+ or CD8+ αβ T cells by each γδ T cell subset. Three γδ T cell subsets and CD4+ and CD8+ αβ T cells were sort-purified from splenic MNCs of Gn pigs by using MACS and FACS. γδ T cell subsets and CD4+ and CD8+ αβ T cells were cultured individually or co-cultured with PHA stimulation for 5 days. The cells from the co-cultures were collected for the BrdU staining (see Fig. 5.9 legend). Concentrations of IFN-γ, IL-10 and TGF-β in the culture supernatants were measured by ELISA. The three figures on the left panel depict mean cytokine concentrations in the co-cultures with CD4+ αβ T cells, and the three figures on the right panel with CD8+ αβ T cells. Data are presented as mean concentration ± standard error of the mean (n = 6). Different letters on top of bars indicate significant differences in cytokine concentrations among the cell cultures (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

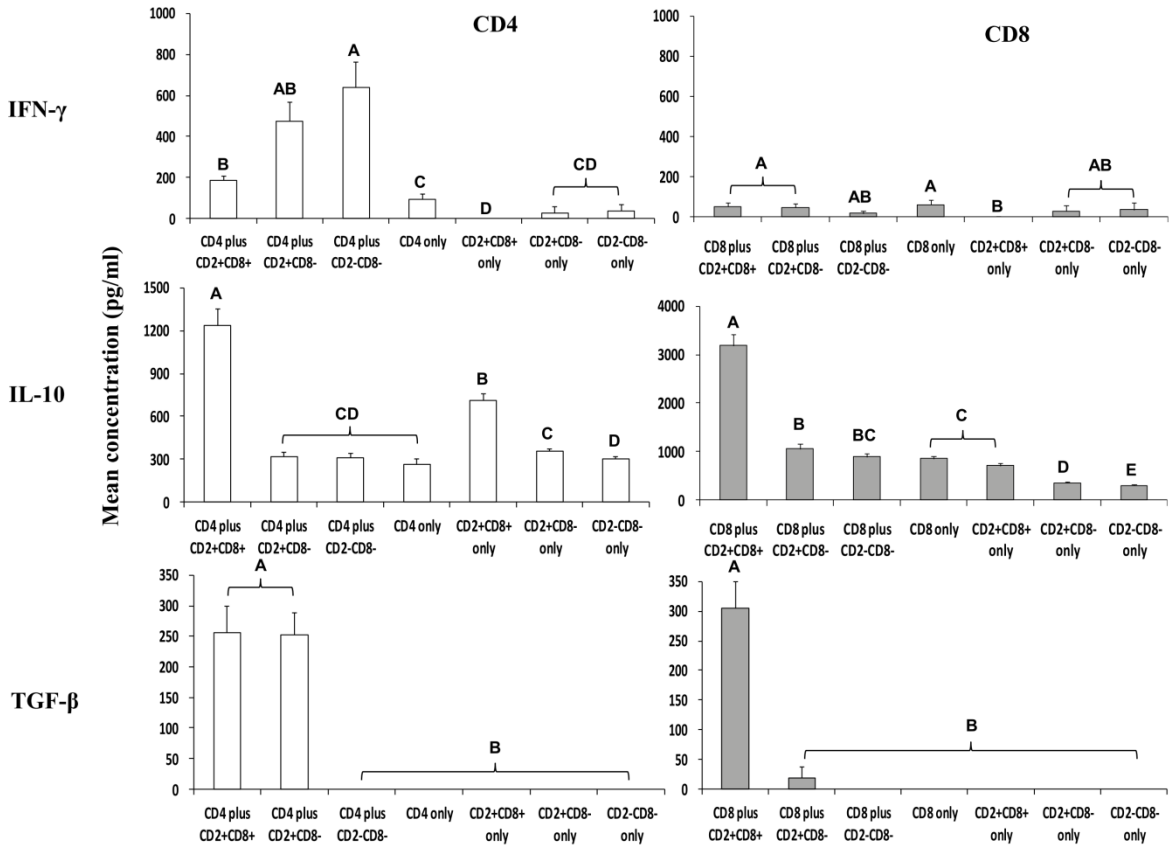

IFN-γ
IL-10
TGF-β
Mean concentration (pg/ml)
CD4
CD8
CD4 plus CD2+CD8+
CD4 plus CD2+CD8-
CD4 plus CD2-CD8-
CD4 only
CD2+CD8+ only
CD2+CD8- only
CD2-CD8- only
CD8 plus CD2+CD8+
CD8 plus CD2+CD8-
CD8 plus CD2-CD8-
CD8 only

Fig. 5.8

Fig. 5.9. Proliferation of sort-purified splenic CD4+ T cells co-cultured with each γδ T cell subset. Cells collected from the co-cultures (see Fig. 5.8 legend) were stained with antibodies to CD3, CD4, and BrdU to measure proliferation of CD4+ αβ T cells by flow cytometry. Frequencies of proliferating cells were defined as BrdU+CD4+ T cells among total CD3+ T cells. Data are presented as mean frequencies ± standard error of the mean (n = 6). Different letters on top of bars indicate significant differences in frequencies of proliferating CD4+ αβ T cells among the cell cultures (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

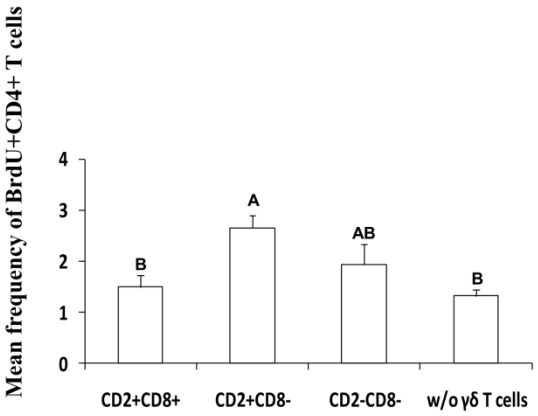

Mean frequency of BrdU+CD4+ T cells
4
3
2
1
0
B
A
AB
B
CD2+CD8+
CD2+CD8-
CD2-CD8-
w/o γδ T cells

Fig. 5.9

Fig. 5.10. Proliferation of sort-purified splenic CD4+ T cells co-cultured with each γδ T cell subset. Three γδ T cell subsets and CD4+ αβ T cells were sort-purified from MNCs isolated from spleen of Gn pigs inoculated with two oral doses of attenuated HRV and challenged with virulent HRV. The co-cultures of CD4+ αβ T cells with each γδ T cell subset or without were stimulated with Wa HRV antigen for 5 days and the cells were collected for CD3, CD4, and BrdU staining to measure proliferation of CD4+ αβ T cells. The proliferation rate was defined as the frequencies of BrdU+CD4+ T cells among CD3+ T cells. Data are presented as mean concentration ± standard error of the mean (n = 3). Different letters on top of bars indicate significant differences in frequencies of proliferating CD4+ αβ T cells among the cell cultures (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

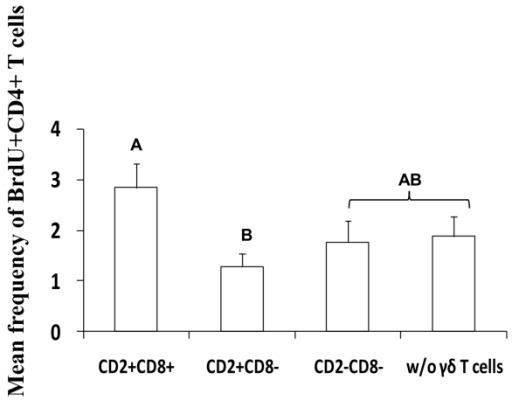

Mean frequency of BrdU+CD4+ T cells
4
3
2
1
0
A
B
AB
CD2+CD8+
CD2+CD8-
CD2-CD8-
w/o γδ T cells

Fig. 5.10