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Dose Effects of LAB on Modulation of Rotavirus Vaccine Induced Immune Responses

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1. Introduction

Probiotics can influence both mucosal and systemic immune responses and function as adjuvants by promoting proinflammatory cytokine production, enhancing both humoral and cellular immune responses. Adjuvant effects of several probiotic lactic acid bacteria (LAB), mostly *Lactobacillus* strains, including *L. rhamnosus* GG, *L. acidophilus* NCFM, *L. acidophilus* CRL431, *L. acidophilus* La-14, *L. fermentum* CECT5716, *L. casei* DN-114 001, and *Bifidobacterium lactis* BI-04 have been reported in studies of influenza, polio, rotavirus and cholera vaccines and rotavirus and Salmonella typhi Ty21a infections (Boge et al., 2009; Davidson et al., 2011; Isolauri et al., 1995; Kaila et al., 1992; Mohamadzadeh et al., 2008; Olivares et al., 2007; Paineau et al., 2008; Winkler et al., 2005; Zhang et al., 2008b). The word adjuvant in the phrase “probiotic adjuvant” is not used in its traditional definition in which adjuvant implies a substance included in the vaccine formulation to aid the immune response to the vaccine antigen. Instead, probiotic adjuvants enhance the immunogenicity of vaccines when orally administered repeatedly around the time of vaccination and separately from the vaccine. By skewing the balance of pro- and anti-inflammatory innate immune responses and T helper (Th) 1 and regulatory T (Treg) cell adaptive immune responses in the context of vaccination, probiotic adjuvants act as “signal zero” to reduce Treg cell suppression and unleash effector T cell activation (Rowe et al., 2012).

Although the strain-specific effects of LAB in up- or down-regulating inflammatory immune responses have been well recognized, dose effects of probiotics on innate and adaptive immune responses are not clearly understood. The same *Lactobacillus* strain is oftentimes reported by different research groups to have opposite immune modulating effects. We hypothesized that the dose effect is at least one of the reasons for the conflicting reports. Understanding dose effects of probiotics has significant implications in their use as immunostimulatory (adjuvants) versus immunoregulatory agents.

In this chapter, we discuss findings from our serial studies of gnotobiotic pigs on the dose effects of the *L. acidophilus* NCFM strain (LA) on innate and adaptive immune responses induced by an oral attenuated human rotavirus (HRV) vaccine (AttHRV). We studied the effects of low dose (total 2.11×10^6 CFU) and high dose (total 2.22×10^9 CFU) LA on the intestinal and systemic (1) rotavirus-specific IFN- γ producing CD4+ and CD8+ T cell responses; (2) CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+ Treg cell responses and the regulatory cytokine TGF- β and IL-10 production; (3) rotavirus-specific antibody-secreting cell (ASC) and serum antibody responses; and (4) plasmacytoid dendritic cell (pDC) and conventional DC (cDC) frequencies, activation status, TLR expression and cytokine production profile. The protective effect of rotavirus vaccine against virus shedding and diarrhea was assessed in AttHRV-vaccinated gnotobiotic pigs fed with high, low, or no LA and challenged with the virulent HRV.

These studies clearly demonstrated differential immune modulating effects of high dose versus low dose LA on DC and T cell responses, and consequently different effects on the protection conferred by the AttHRV vaccine in gnotobiotic pigs challenged with virulent HRV. Low dose LA enhanced the protection against rotavirus diarrhea in AttHRV-vaccinated pigs whereas high dose LA had negative effects on the effectiveness of the vaccine. Thus, the same probiotic strains at different doses can exert qualitatively different modulating effects on immune responses induced by rotavirus vaccines and possibly other vaccines as well.

2. Dose effects of LA on T cell responses

Probiotics have been reported to exert adjuvant properties by inducing pro-Th1 cytokines and promote Th1 type immune responses. For example, *L. lactis* and *L. plantarum* induced production of IL-12 and IFN- γ by splenocytes when the LAB and an allergen were co-administered intranasally to mice (Repa et al., 2003). *L. fermentum* strain CECT5716 enhanced the Th1 responses induced by an influenza vaccine in addition to enhancing virus neutralizing antibody responses (Olivares et al., 2007). Eleven different probiotic strains were tested for cytokine production in human peripheral blood mononuclear cells (MNC) and each tested bacterium was shown to induce production of TNF- α and some strains also induced production of IL-12 and IFN- γ (Kekkonen et al., 2008). Previous studies of gnotobiotic pigs showed that a mixture of LA NCFM strain and *L. reuteri* strain enhanced both Th1 (IL-12, IFN- γ) and Th2 (IL-4 and IL-10) cytokine responses to virulent HRV infection (Azevedo et al., 2012). LA NCFM strain enhanced the HRV-specific IFN- γ producing CD8+ T cell response to a rotavirus vaccine in gnotobiotic pigs, indicating adjuvanticity of the LA strain (Zhang et al., 2008b).

Dose effects of probiotics on modulating T cell immune responses have not been well studied. To address this question, we examined the dose effects of LA NCFM (NCK56) strain on IFN- γ producing CD4+ and CD8+ T cell immune responses induced by an oral rotavirus vaccine in gnotobiotic pigs (Wen et al., 2012). The animal treatment groups included (1) high dose LA plus AttHRV vaccine (HiLA+AttHRV), (2) low dose LA plus

AttHRV (LoLA+AttHRV), (3) AttHRV only, (4) high dose LA only (HiLA), (5) low dose LA only (LoLA), and (6) mock-inoculated control (Mock). Gnotobiotic pigs were orally inoculated at 5 days of age with the AttHRV vaccine at 5×10^7 fluorescent focus-forming units (FFU) per dose. A booster dose was given 10 days later at the same dose and route. Subsets of the pigs were euthanized at post-inoculation day (PID) 28 to assess immune responses and the rest were challenged with the homotypic virulent Wa (G1,P1A[8]) strain HRV at a dose of 1×10^5 FFU to assess protection from post-challenge day (PCD) 1 to 7. The 50% infectious dose and 50% diarrhea dose of the virulent HRV in gnotobiotic pigs are approximately 1 FFU (Ward et al., 1996). The AttHRV inoculation causes virus shedding in about 6% pigs, but it does not cause any illness (Ward et al., 1996). Pigs in the high dose LA groups were fed daily with 10^3 to 10^9 CFU/dose of LA for 14 days with 10-fold incremental dose increases every other day from 3-16 days of age. The accumulative total LA dose was 2.22×10^9 CFU. Pigs in the low dose LA groups were fed with 10^3 , 10^4 , 10^5 , 10^6 , and 10^6 CFU/dose of LA every other day from 3-11 days of age. The accumulative total LA dose was 2.11×10^6 CFU.

2.1. Low dose LA, but not high dose LA, enhanced HRV-specific IFN- γ producing T cell responses

The magnitude of HRV-specific IFN- γ producing T cell responses in pigs was differentially modulated by low versus high dose LA at both prechallenge and postchallenge (PID 28 and PCD 7). AttHRV-vaccinated and low dose LA fed pigs (LoLA+AttHRV) had significantly higher frequencies of HRV-specific IFN- γ +CD8⁺ T cells in ileum (11- and 5-fold higher pre- and postchallenge, respectively), spleen (3.8- and 2.1-fold higher pre- and postchallenge, respectively) and blood (3- and 20-fold higher pre- and postchallenge, respectively) compared to the AttHRV only pigs (Table 1). 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 postchallenge) compared to the AttHRV only pigs. In contrast, high dose LA did not significantly alter the HRV-specific IFN- γ producing CD4⁺ and CD8⁺ T cell responses in the HiLA+HRV pigs compared to AttHRV only pigs.

	Frequencies of IFN- γ +CD8 ⁺ T cells among CD3 ⁺ cells					
	PID 28			PCD 7		
	Ileum	Spleen	Blood	Ileum	Spleen	Blood
HiLA+AttHRV	0.05	0.34	0.05	0.11	0.16	0.06
LoLA+AttHRV	1.21	0.46	0.24	0.56	0.46	0.98
AttHRV only	0.11	0.12	0.08	0.11	0.22	0.05

(Summarized from Wen et al., 2012)

Table 1. Effect of low dose vs. high dose LA on IFN- γ producing CD8⁺ T cell responses

2.2. High dose LA significantly increased frequencies of intestinal and systemic CD4+CD25-FoxP3+ Treg cells whereas low dose LA decreased TGF- β and IL-10 producing Treg cell responses

Frequencies and cytokine production of Treg cells in pigs were differentially modulated by low versus high dose LA at both prechallenge and postchallenge. HiLA+AttHRV pigs had significantly higher frequencies of CD4+CD25-FoxP3+ Treg cells (ranging from 6- to 86-fold higher) in all the tissues compared to LoLA+AttHRV and AttHRV only pigs pre- and postchallenge (Table 2).

Because Treg cells exert regulatory functions through mechanisms involving TGF- β and IL-10, we also compared the effects of high and low dose LA on frequencies of the Treg cell subsets that produced TGF- β or IL-10 among the AttHRV-vaccinated pigs.

	Frequencies of CD4+CD25-FoxP3+Treg cells among total MNC					
	PID 28			PCD 7		
	Ileum	Spleen	Blood	Ileum	Spleen	Blood
HiLA+AttHRV	2.96	10.34	9.00	1.56	3.78	3.65
LoLA+AttHRV	0.24	0.54	0.10	0.08	0.22	0.05
AttHRV only	0.23	0.59	0.51	0.27	0.26	0.17

(Summarized from Wen et al., 2012)

Table 2. Effect of low dose vs. high dose LA on frequencies of Treg cells

Low dose LA reduced frequencies of TGF- β producing CD4+CD25-FoxP3+ and CD4+CD25-FoxP3+ Treg cells compared to high dose LA and AttHRV only pigs in all tissues pre- and postchallenge (Table 3; data for CD25+ Treg cells are not shown). Low dose LA also reduced pre- and postchallenge frequencies of IL-10 producing CD4+CD25-FoxP3+ and CD4+CD25-FoxP3+Treg cells compared to high dose LA and AttHRV- only (except for CD4+CD25-FoxP3+ Treg cells in ileum and spleen postchallenge) (Table 3). High dose LA induced 2.6-fold and 20-fold, respectively higher frequencies of IL-10 producing CD4+CD25-FoxP3+ Treg cells in ileum and spleen postchallenge compared to AttHRV only.

	PID 28			PCD 7		
	Ileum	Spleen	Blood	Ileum	Spleen	Blood
Frequencies of TGF- β + cells among CD4+CD25-FoxP3+ Treg cells						
HiLA+AttHRV	4.51	1.13	0.92	4.87	10.05	1.39
LoLA+AttHRV	0.32	0.15	0.31	0.00	0.17	0.38
AttHRV only	4.19	1.19	1.55	4.34	0.79	1.53
Frequencies of IL-10+ cells among CD4+CD25-FoxP3+ Treg cells						
HiLA+AttHRV	4.21	2.97	0.88	10.68	17.70	2.51
LoLA+AttHRV	0.92	0.15	0.00	5.22	6.79	0.00
AttHRV only	4.47	3.01	1.77	4.08	0.90	1.44

(Summarized from Wen et al., 2012)

Table 3. Effect of low dose vs. high dose LA on CD25-FoxP3+ Treg cell cytokine production

These data clearly demonstrated that low dose LA promoted IFN- γ producing T cell and down-regulated Treg cell responses, whereas high dose LA induced a strong Treg cell response and promoted the regulatory cytokine production by tissue-residing Treg cells postchallenge in gnotobiotic pigs. Studies of other lactobacilli strains have reported similar findings. A mixture of *L. plantarum* CEC 7315 and CEC 7316 at high dose (5×10^9 CFU/day) resulted in significant increases in the percentages of activated potential T-suppressor and NK cells, while at low dose (5×10^8 CFU/day) increased activated T-helper cells, B cells and antigen presenting cells (APCs) in institutionalized seniors (Mane et al., 2011). High concentration ($\geq 1 \times 10^6$ colony forming unit [CFU]/ml) of a combination containing LA and *Bifidobacterium* or *B. infantis* attenuated mitogen-induced immune responses by inhibiting cell proliferation and arresting the cell cycle at the G0/G1 stage in both mitogen-stimulated spleen cells and peripheral blood MNC. However, low concentration ($\leq 1 \times 10^6$ CFU/ml) promoted a shift in the Th1/Th2 balance toward Th1-skewed immunity by enhancing IFN- γ and inhibiting IL-4 response (Li et al., 2011). The differences between the “low dose” and “high dose” LAB in these studies are small, yet the immunomodulatory effects are qualitatively different.

Dose effects may explain some of the controversies that result from the same probiotic strain used by different research groups in animal studies showing 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 (So et al., 2008a; So et al., 2008b). On the contrary, another study 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 the studies of rats (So et al., 2008a; So et al., 2008b). In the studies of rats, 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 the study of mice, the amount of *L. casei* was 2×10^8 CFU/dose per mouse, twice per week for 8 weeks (Van Overtvelt et al., 2010). Thus, different dose and frequency of administration of the same LAB strains may result in totally different *in vivo* effects.

The dose effects of LA on immune responses to the AttHRV vaccine in pigs may also partly explain why the efficacies of oral rotavirus vaccines are significantly reduced in low-income 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 (O’Ryan et al., 2009). However, the protective efficacy of RotaTeq vaccine is only 39.3% against severe rotavirus gastroenteritis in sub-Saharan Africa (Armah et al., 2010) and 48.3% in developing countries in Asia (Zaman et al., 2010). Rotarix vaccine showed a similar disparity in efficacy in low-income countries in Africa (O’Ryan & Linhares, 2009). In addition to other factors that contribute to the reduction in rotavirus vaccine efficacy (e.g., higher titers of maternal antibodies, malnutrition), during the initial colonization of human infants, exposure to high doses of commensal bacteria (common in countries with lower hygiene standards) would have a suppressive effect on IFN- γ producing T cell responses and promote Treg cell responses, thus leading to the lowered protective immunity after rotavirus vaccination.

3. Dose effects of LA on antibody and B cell responses

Probiotics are known to modulate both humoral and cellular immune responses. Probiotics can induce antigen-specific and non-specific IgA antibody responses at mucosal surfaces (Perdigon et al., 2001; Wells & Mercenier, 2008) to prevent invasion by pathogenic microorganisms. Oral administration of *L. acidophilus* L-92 strain led to a significant increase of IgA production in Peyer's patches in mice (Torii et al., 2007). *L. casei* CRL 431 strain increased induction of intestinal IgA secreting cells in mice (Galdeano & Perdigon, 2006). *L. acidophilus* La1 strain and bifidobacteria enhanced specific serum IgA titers to *S. typhi* strain Ty21a and also total serum IgA in humans (Link-Amster et al., 1994). *L. rhamnosus* GG enhanced rotavirus-specific IgA ASC responses in humans and promoted recovery from rotavirus diarrhea (Kaila et al., 1992). In our earlier study of gnotobiotic pigs, a mixture of LA strain and *L. reuteri* strain did not alter virus-specific intestinal and systemic antibody and ASC responses, but they significantly enhanced total intestinal IgA secreting cell responses and total serum IgM and intestinal IgM and IgG titers in rotavirus infected pigs (Zhang et al., 2008a).

The first reported adjuvant effect of probiotic LAB in vaccination was from a human clinical trial in which *L. rhamnosus* GG was shown to enhance rotavirus-specific IgM secreting cells and rotavirus IgA seroconversion in infants receiving a live oral rhesus-human rotavirus reassortant vaccine (Isolauri et al., 1995). In recent years, an increasing number of human clinical trials have demonstrated adjuvant effects of probiotics in enhancing vaccine-induced antibody responses. In a double-blind randomized controlled trial, *L. rhamnosus* GG or *L. acidophilus* CRL 431 increased serum poliovirus neutralizing antibody titers and poliovirus-specific IgA and IgG titers 2- to 4-fold in adult human volunteers vaccinated with the live oral polio vaccine (de Vrese et al., 2005). In another human clinical trial, six out of the seven probiotic strains tested enhanced cholera-specific IgG antibody concentration in serum; for the *B. lactis* Bl-04 and *L. acidophilus* La-14 strains the increase was more significant (Paineau et al., 2008). Daily consumption of a fermented dairy drink (*L. casei* DN-114 001 and yoghurt ferments, Actimel) was shown to increase virus specific antibody responses to the intramuscular inactivated influenza vaccine in individuals of over 70 years of age (Boge et al., 2009). In a randomized, double-blind placebo-controlled pilot study, *L. rhamnosus* GG significantly improved the development of serum antibody responses to the H3N2 strain influenza virus (84% receiving *L. rhamnosus* GG versus 55% receiving placebo had a protective titer 28 days after vaccination) in healthy adults receiving the live attenuated influenza vaccine (FluMist, Medimmune Vaccines, Gaithersburg, MD, USA) (Davidson et al., 2011). Thus, specific strains of probiotics can act as adjuvants to enhance humoral immune responses following not only mucosal (oral or intranasal) but also parenteral vaccination. Yet, dose effects of probiotics on antibodies responses have not been well studied.

In our studies, we demonstrated that high dose LA did not significantly alter the HRV-specific antibody responses whereas low dose LA had negative effects on the antibody responses. The effect of high and low dose LA NCFM strain on HRV-specific serum IgA and IgG antibody levels and HRV-specific ASC and memory B cell responses in the intestinal

and systemic lymphoid tissues of gnotobiotic pigs induced by rotavirus vaccination were examined. The animal treatment groups were the same as listed above in the studies of T cell responses. High dose LA did not significantly alter the HRV-specific antibody responses in serum and ASC responses in any tissue at PID 28 and PCD 7 in the AttHRV-vaccinated pigs (Figs 1 and 2), except to reduce the IgG ASC response in ileum of the mock-vaccinated pigs postchallenge (Fig. 2c). In contrast, low dose LA significantly reduced the HRV-specific IgA antibody titers at PID 7 and 14 (Fig. 1a) and reduced or significantly reduced IgG ASC responses in blood pre- and postchallenge as well as IgA ASC responses in spleen and blood postchallenge (Fig. 2a and 2b). Low dose LA also significantly reduced the IgA and IgG ASC responses in ileum of the mock-vaccinated pigs postchallenge (Fig. 2c).

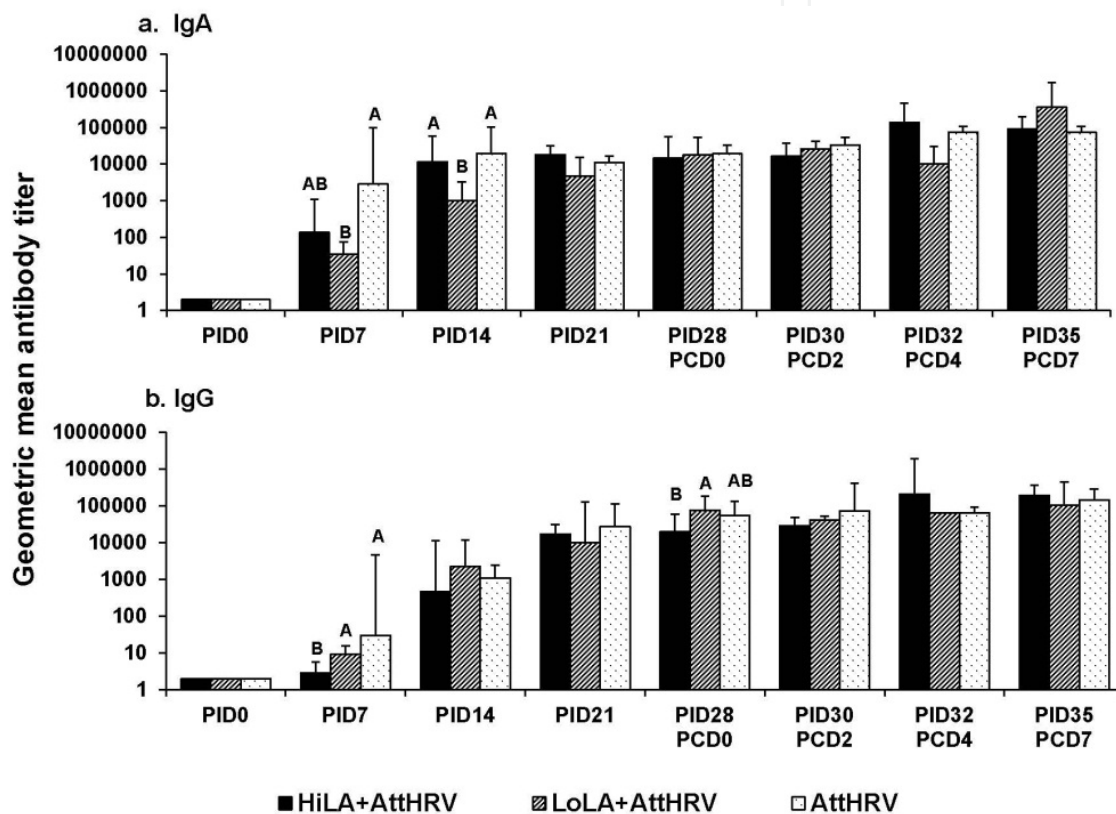


Figure 1. Rotavirus-specific serum IgA and IgG antibody responses in Gn pigs vaccinated with AttHRV with or without high or low dose LA feeding. Rotavirus-specific serum IgA (a) and IgG (b) antibody were measured by an indirect isotype-specific antibody ELISA. Error bars indicate the standard error of the mean. Different capital letters (A, B) indicate significant difference among different pig groups at the same time point (Kruskal Wallis Test, $p < 0.05$, $n = 3-27$), whereas shared letters or no letters on top indicate no significant difference.

The negative effects of low dose LA on the HRV-specific serum antibody responses and ASC responses induced by the AttHRV vaccine were undesirable for the vaccine's immunogenicity; however it is consistent with the strong pro-Th1 effect of the low dose LA. The skewed balance toward a Th1 type immune response in the low dose LA group may have resulted in the weakened antibody responses. In the subsequent studies, we evaluated the effects of a low dose and an intermediate dose of *L. rhamnosus* GG on the effector T cell,

antibody and ASC responses induced by the AttHRV vaccine and we found that *L. rhamnosus* GG enhanced the production of a balanced Th1 and Th2 immune responses to the AttHRV vaccine and significantly increased the virus-specific IFN- γ producing T cell responses, the antibody responses and the protection rate of the AttHRV vaccine (manuscripts under preparation).

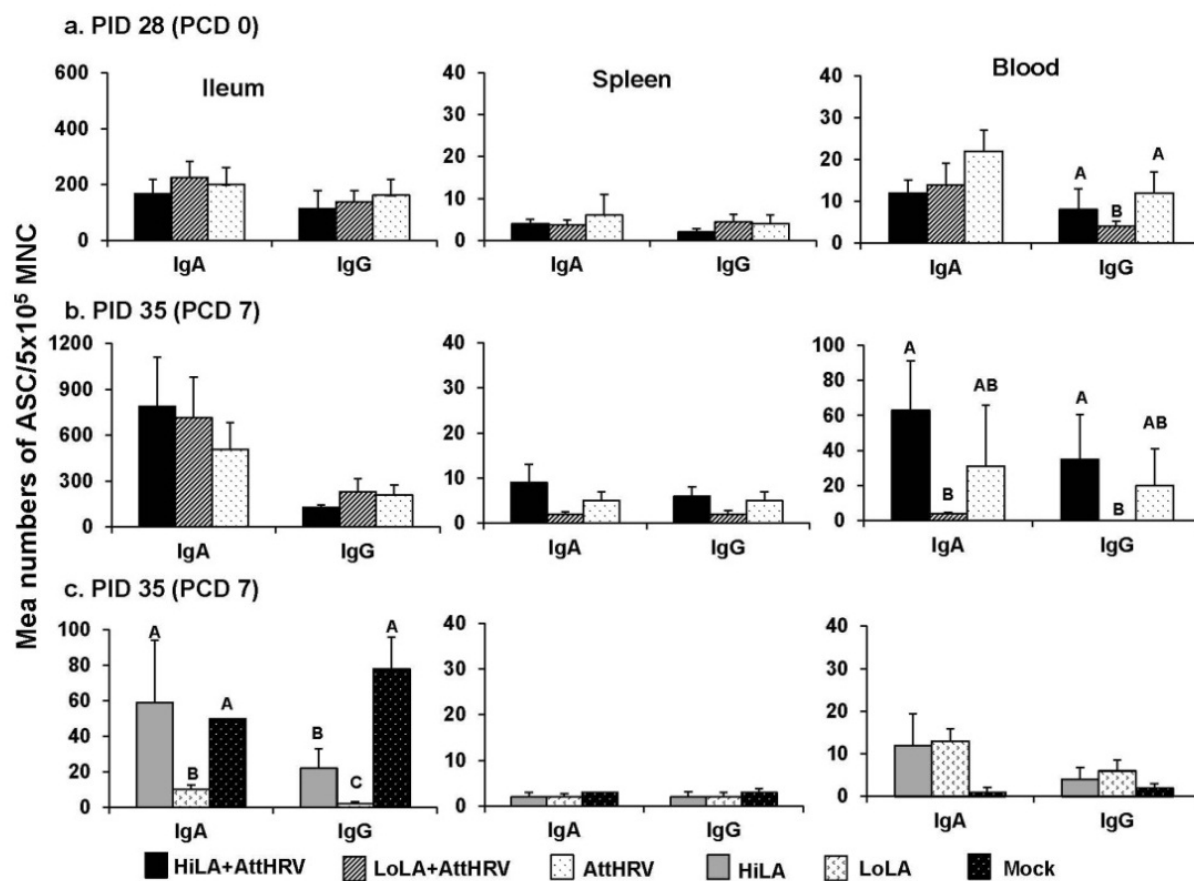


Figure 2. Rotavirus-specific IgA and IgG ASC responses in Gn pigs. Rotavirus-specific IgA and IgG ASC in the MNC isolated from ileum, spleen and blood of AttHRV-vaccinated pigs on PID 28 (PCD 0) (a) and PID 35 (PCD 7) (b) and of mock-vaccinated pigs on PID 35 (PCD 7) (c) were enumerated by using an ELISPOT assay and are presented as the mean numbers of virus-specific IgA and IgG ASC per 5×10^5 MNC. Error bars indicate the standard error of the mean. Different capital letters (A, B, C) on top of the bars indicate significant difference among the treatment groups for the same isotype in the same tissue (Kruskal Wallis Test, $p < 0.05$, $n = 3-14$), whereas shared letters or no letters on top indicate no significant difference. Note the y axis scale difference (HiLA, high dose LA; LoLA, low dose LA).

4. Dose effects of LA on DC responses

The nature and consequences of a CD4⁺ T cell response (Th1, Th2, Th17, or Treg type) largely depend on the immune functions of DCs, which are the professional antigen presenting cells that can prime and differentiate naive T cells. Both cDC and pDC are responsible for presenting microbial and dietary antigens to the adaptive immune systems, thereby influencing polarization of the adaptive immune response (Konieczna et al., 2012).

The pDC most effectively sense virus infections and are characterized by their capacity to produce large quantities of IFN- α and the pro-inflammatory cytokines IL-6 and TNF- α . These cytokines promote cDC maturation (Summerfield & McCullough, 2009). MHC II expression in professional APCs is tightly regulated. The MHC II of immature DCs are expressed at low levels at the plasma membrane, but abundantly in endocytic compartments. In the presence of inflammatory cytokines such as IFN- γ , DCs are activated; they stop capturing antigens and markedly increase MHC II expression on their plasma membrane. These MHC II are loaded with peptides derived from antigens captured at the site of inflammation. The mature DCs migrate to lymphoid tissues and up-regulate the co-stimulatory molecules (CD80/86) necessary to activate naïve T cells (Villadangos et al., 2001).

It is known that probiotics can modify the distribution, the phenotype and the function of DC subsets (Grangette, 2012). Both species-specific and strain-specific immunomodulatory effects of different LAB on DCs have been described in a large number of studies and was reviewed previously (Meijerink & Wells, 2010). Among the differential effects, several lactobacilli strains, including *L. acidophilus*, *L. gasseri*, *L. fermentum*, *L. casei*, *L. plantarum*, *L. johnsonii*, and *L. rhamnosus* have been 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 (Chiba et al., 2010; Christensen et al., 2002; Mohamadzadeh et al., 2005; Van Overtvelt et al., 2010; Vitini et al., 2000; Weiss et al., 2010; Yazdi et al., 2010). Such immune stimulating effects are characteristics of adjuvants. However, studies of dose effects of lactobacilli on DC responses are scarce, with most consisting of *in vitro* experiments, and there is a dearth of comparative studies linking *in vitro* and *in vivo* results. *L. rhamnosus* Lcr35 was shown to induce a dose-dependent immunomodulation of human DCs. Lcr35 at 10^7 CFU/ml (10 multiplicity of infection), but not 10^4 CFU/ml induced the semi-maturation of the DCs and a strong pro-inflammatory response (Evrard et al., 2011). LA NCFM induced a concentration dependent production of IL-10, and low IL-12p70 in monocyte derived DCs (Konstantinov et al., 2008). Immature DCs incubated with the LA NCFM at a bacterium to cell ratio of 1000:1 ("high dose") produced significantly higher IL-10 compared with the ratio of 10:1. In contrast, IL-12p70 was up-regulated at a lower concentration of the bacterium (10:1).

In our studies, dose effects of LA on pDC and cDC responses after rotavirus vaccination were examined in gnotobiotic pigs. The animal treatment groups were the same as listed earlier in the studies of T cell and B cell immune responses. Porcine pDC (CD172a+CD4+) and cDC (CD172a+CD11R1+) were defined as previously described (Jamin et al., 2006). The frequencies and tissue distribution, MHC II and costimulatory (CD80/86) molecular, TLR (2, 3, 9) and cytokine (IL-6, IL-10, IFN- α , TNF- α) expression by pDC and cDC in ileum, spleen and blood of gnotobiotic pigs vaccinated with the AttHRV and fed with high dose, low dose or no LA were determined using multi-color flow cytometry.

The low dose LA group had significantly higher frequencies of pDC in ileum and spleen and cDC in spleen and blood compared to the high dose LA and AttHRV only groups (Fig. 3a). The low dose LA group had overall lower MHC II expression on pDC and cDC in all tissues and lower CD80/86 expression in blood, but significantly higher CD80/86 expression

on cDC in ileum, compared to the high dose LA and AttHRV only groups (Fig. 3b). High dose LA did not have a significant effect on DC frequencies or activation marker MHC II and CD80/86 expression, except for the significantly increased CD80/86 expression on pDC in ileum compared to the AttHRV only group (Fig. 3b).

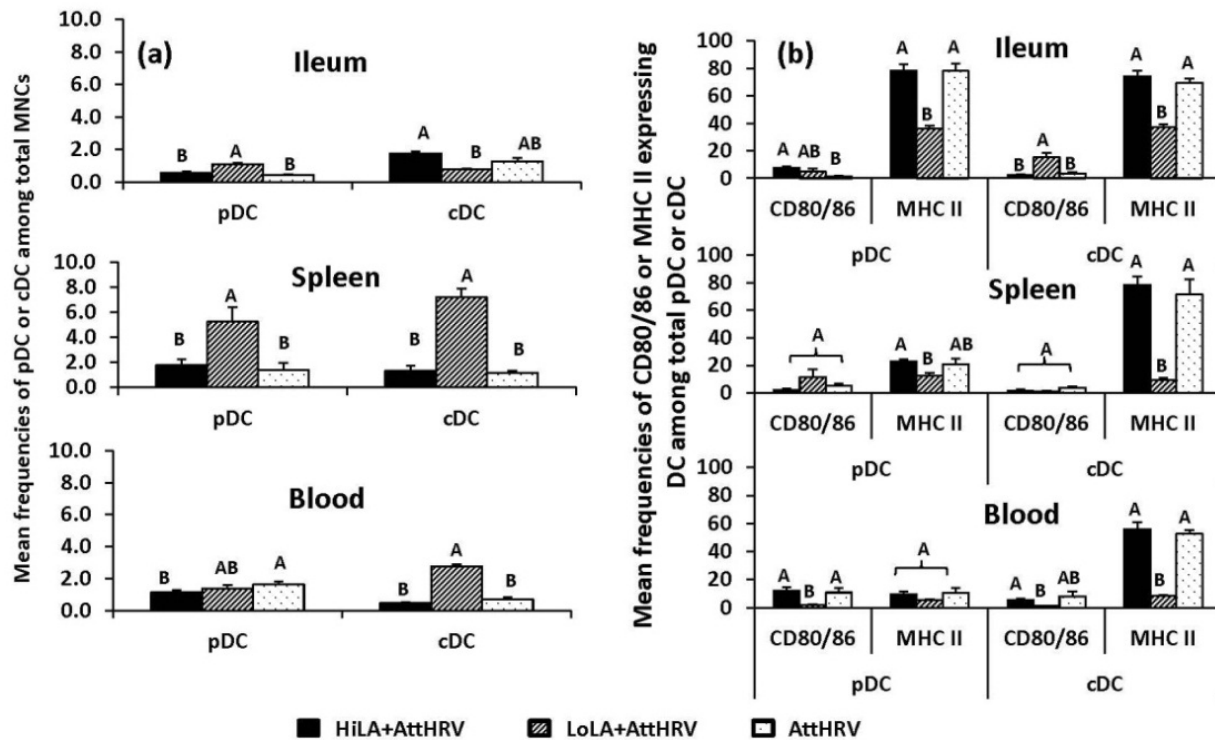


Figure 3. Frequencies of pDC and cDC (a) and the activation marker (CD80/86 and MHC II) expression (b) in intestinal and systemic lymphoid tissues of Gn pigs vaccinated with AttHRV vaccine with high dose, low dose or no LA at PID 28. MNC were stained freshly without *in vitro* stimulation before being subjected to flow cytometry analyses. Data are presented as mean frequency \pm standard error of the mean (n = 3-13). 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.

The low dose LA group had lower or significantly lower frequencies of TLR3 expression in both pDC and cDC in all tissues and significantly lower TLR2 expression on cDC in spleen compared to the high dose LA and AttHRV only groups (Fig. 4). High dose LA did not have a significant effect on TLR expression in ileum and spleen. In blood, high dose LA group had significantly lower TLR3 expression in cDC (and lower in pDC) compared to the AttHRV only group.

The most striking dose effect of LA on the cytokine production profile in DCs is the significantly increased IL-6 in the low dose LA group (Fig. 5). The low dose LA group had significantly higher frequencies of IL-6 producing pDC and cDC in all tissues compared to the high dose LA and AttHRV only groups. Interestingly, the low dose LA reduced or significantly reduced the other cytokine TNF- α , IL-10 and IFN- α production in pDC in ileum and spleen. In contrast to ileum and spleen, the low dose LA increased or significantly

increased TNF- α , IL-10 and IFN- α production in blood compared to the high dose LA or the AttHRV only group. High dose LA did not have a significant effect on IL-6, TNF- α , and IL-10 but lowered or significantly lowered IFN- α production in both pDC and cDC in all tissues compared to the AttHRV only group.

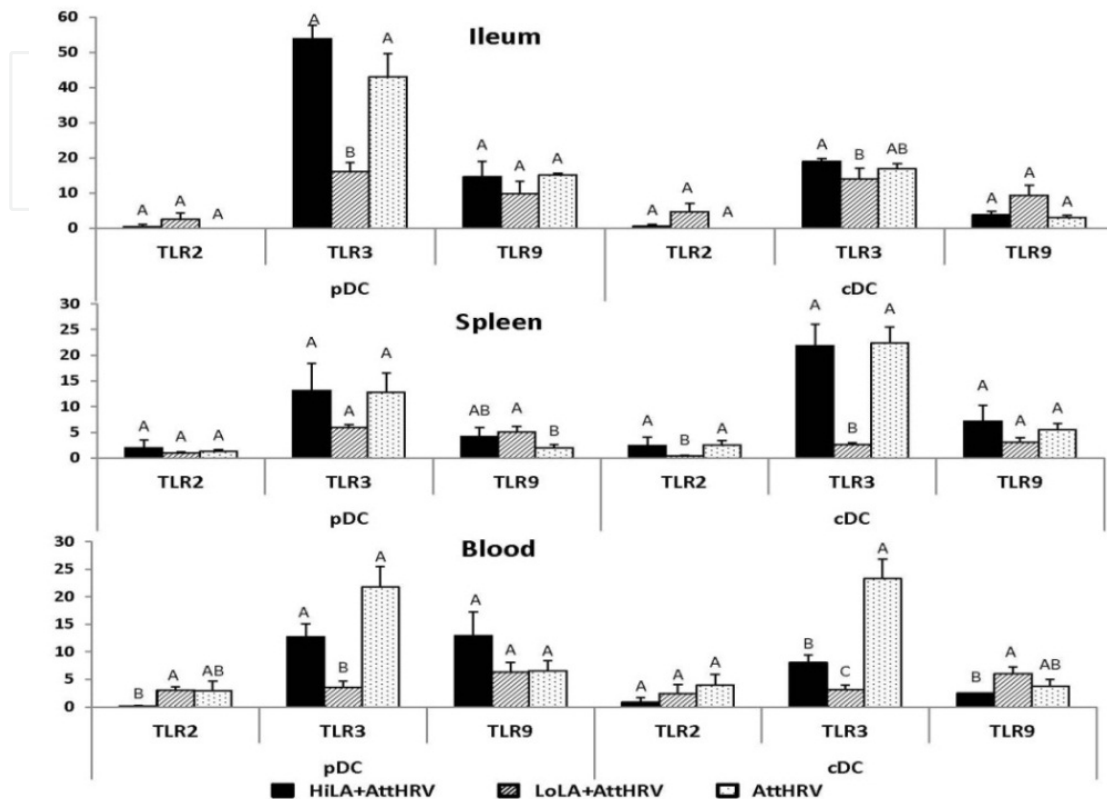


Figure 4. TLR expression patterns of pDC and cDC in intestinal and systemic lymphoid tissues of Gn pigs vaccinated with AttHRV vaccine with high dose, low dose or no LA at PID 28. MNC were stained freshly without *in vitro* stimulation before flow cytometry analyses. Data are presented as mean frequency \pm standard error of the mean ($n = 3-5$). Different letters on top of bars indicate significant differences in frequencies among groups for the same TLR in the same tissue (Kruskal–Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

Therefore, the effects of high versus low dose LA on the frequencies, maturation status and functions of DCs were strikingly different. Low dose LA significantly increased frequencies of both DC subsets, but these DCs were immature because they expressed lower frequencies of activation makers CD80/86 and MHC II and had reduced TNF- α , IL-10 and IFN- α production compared to the high dose LA and AttHRV only groups. Low dose LA promoted a strong IL-6 response in all tissues and increased all the other cytokine TNF- α , IL-10 and IFN- α production in blood for both pDC and cDC. High dose LA did not have such a significant modulating effect on the DC responses compared to the low dose (with a few exceptions). These findings are consistent with the differential effects of low dose versus high dose LA on the adaptive immune responses. The differential modulating effects of high versus low dose LA are intriguing. The biological and immunological implications of these effects and the underlying mechanisms require further investigation. From these data, it is clear that the same probiotic strain at different doses can exert qualitatively different modulating effects

on DCs and consequently on adaptive immune responses induced by rotavirus vaccines. It has been reported that the effect of low dose microbe-associated pattern molecular (MAPM), such as lipopolysaccharide, was strikingly different as compared to that of high dose on macrophage cell functions: low dose lipopolysaccharide induced a strong inflammatory response in macrophages (Maitra et al., 2011). It is plausible that a similar interaction occurs between the MAPM from LA and DCs in the gut. Future studies are needed to identify the molecular mechanisms of the dose responses of different MAPM.

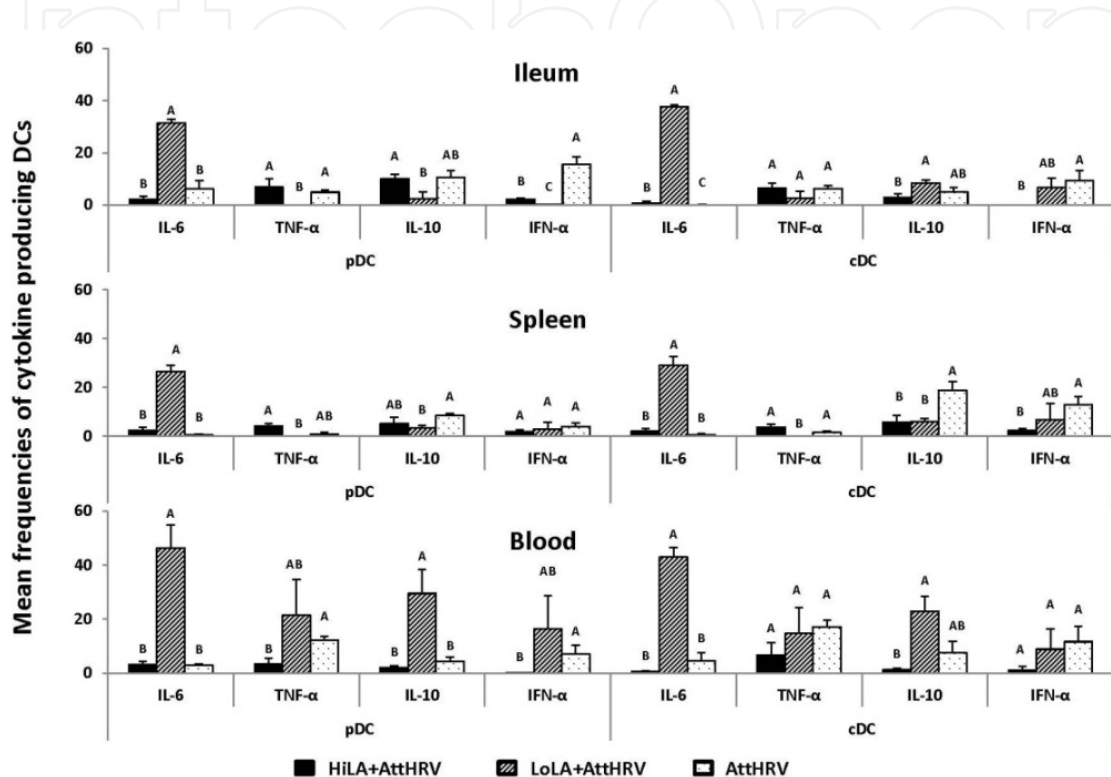


Figure 5. Cytokine production profiles of pDC and cDC in intestinal and systemic lymphoid tissues of Gn pigs vaccinated with AttHRV vaccine with high dose, low dose or no LA at PID 28. MNC were stained freshly without *in vitro* stimulation before flow cytometry analyses. Data are presented as mean frequency \pm standard error of the mean ($n = 3-8$). Different letters on top of bars indicate significant differences in frequencies among groups for the same cytokine in the same tissue (Kruskal-Wallis test, $p < 0.05$), while shared letters indicate no significant difference.

5. Dose effects of LA on protection conferred by the oral AttHRV vaccine against virulent HRV challenge

To examine the effects of low and high dose LA on improving the protection conferred by the AttHRV vaccine, subsets of gnotobiotic pigs from each treatment group were challenged with the virulent HRV Wa strain at PID 28. Clinical signs and virus shedding were monitored for 7 days postchallenge (Table 4).

After challenge, although the proportion of pigs that developed virus shedding and diarrhea did not differ significantly among the three AttHRV vaccinated pig groups, the LoLA+AttHRV group had the shortest mean durations of fecal virus shedding and diarrhea

and the lowest mean cumulative fecal consistency score among all the treatment groups. The durations of diarrhea in the LoLA+AttHRV pigs were significantly shorter compared to the AttHRV only 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 AttHRV only groups (8.4 and 9.0, respectively) were significantly lower than the control group, indicating significant protection against the severity of diarrhea. 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.

Treatments	n	Clinical signs			Fecal virus shedding (by CCIF and/or ELISA)		
		% with diarrhea ^{*, a}	Mean duration days ^{**}	Mean cumulative score ^{** , c}	% shedding virus [*]	Mean duration days ^{**}	Mean peak titer (FFU/ml) ^{** , d}
HiLA+AttHRV	13	92 ^A	4.3 (0.7 ^b) ^{AB}	12.5 (1.4) ^{AB}	31 ^B	3.8 (0.3) ^A	2.0 ^B
LoLA+AttHRV	8	88 ^A	2.4 (0.7) ^B	8.4 (1.3) ^B	36 ^B	1.0 (0.0) ^B	5.6 ^B
AttHRV only	12	67 ^A	4.6 (0.5) ^A	9.8 (1.4) ^B	50 ^B	1.3 (0.2) ^B	4.9 ^B
Mock control	9	100 ^A	5.6 (0.3) ^A	14.4 (1.0) ^A	100 ^A	4.7 (0.7) ^A	4558 ^A

^Δ The data was partially presented previously (Wen et al., 2012).

^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 Standard error of the mean.

^c Mean cumulative score calculation included all the pigs in each group.

^d FFU, fluorescent focus forming units. Geometric mean peak titers were calculated among pigs that shed virus.

^{*} Proportions in the same column with different superscript letters (A, B) differ significantly (Fisher's exact test, $p \leq 0.05$).

^{**} Means in the same column with different superscript letters (A, B, C) differ significantly (Kruskal Wallis Test, $p \leq 0.05$).

Table 4. Clinical signs and rotavirus fecal shedding in Gn pigs after virulent HRV challenge^Δ

We reported previously that protection rates against rotavirus diarrhea are correlated with virus-specific intestinal IgA ASC and IFN- γ producing T cell responses at PID 28 in Gn pigs (Yuan et al., 1996; Yuan et al., 2008). 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). Because virus-specific intestinal

IgA ASC responses probably play a more important role in rotavirus protective immunity than the IFN- γ producing CD8⁺ T cell responses (Yuan et al., 1996; Yuan et al., 2008), the effect of LA on virus-specific ASC responses also need to be taken into consideration regarding the differences in the protection conferred by the AttHRV vaccine with high or low dose LA. Although the low dose LA enhanced IFN- γ producing CD8⁺ T cell responses, it had negative effects on the serum antibody and ASC responses induced by the AttHRV vaccine. To improve the AttHRV vaccine efficacy, a different dose of LA (possible an intermediate dose) or a different probiotic strain (i.e. LGG) may be optimal to promote a balanced Th1 and Th2 response without increasing Treg cell responses.

6. Conclusion

Differential modulating effects on innate and adaptive immune responses by low dose versus high dose of the same LA NCFM strain were clearly demonstrated in gnotobiotic pigs. Low dose LA significantly enhanced the Th1 type effector T cell responses and decreased Treg cell functions in AttHRV-vaccinated pigs. Meanwhile, low dose LA resulted in a suppressed Th2 response, as evidenced by significantly reduced virus-specific ASC responses and serum antibody titers compared to the AttHRV only group. The dose effects of LA on IFN- γ producing T cell and CD4⁺CD25⁺FoxP3⁺ Treg cell immune responses were similar between the intestinal and systemic lymphoid tissues. Thus the same probiotic strain used in different doses can either increase or reduce mucosal and systemic immune responses induced by vaccines. These findings have significant implications in the use of probiotic lactobacilli as immunostimulatory versus immunoregulatory agents. Probiotic products are increasingly used to improve health, alleviate disease symptoms, and enhance vaccine efficacy. 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 not only strain but also dose selection in probiotic studies.

The gnotobiotic pig model is a valuable animal model for study of probiotic-virus-host interaction because of the many similarities between human and porcine intestinal physiology and mucosal immune system (Meurens et al., 2012). The gnotobiotic status prevents confounding factors from commensal microflora that are present in conventionally reared animals or in humans. Unlike gnotobiotic mice, gnotobiotic 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 (Butler, 2009; Yuan & Saif, 2002). Although data from studies of gnotobiotic animal models may not be generalized directly to normal animals or humans, gnotobiotic animals provide a medium in which investigating the complex interrelationships of the host and its associated microbes become possible (Coates, 1975). Our findings provide a good starting point for identification of the optimal dosage of a probiotic strain. But nonetheless, the optimal dosage needs to be confirmed in conventionalized gnotobiotic pigs and in human clinical trials in order to achieve the appropriate adjuvant effect for rotavirus and other vaccines.

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