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***Escherichia coli* Nissle 1917 protects gnotobiotic pigs against human rotavirus by modulating plasmacytoid dendritic and natural killer cell responses**

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Abstract

Gram-positive lactic acid-producing bacteria including *Lactobacillus rhamnosus* GG (LGG) are commonly used as probiotics, while fewer gram-negative probiotics including *Escherichia coli* Nissle 1917 (EcN) are characterized. A mechanistic understanding of their individual and interactive effects on human rotavirus (HRV) disease and immunity is lacking. Noncolonized, EcN, LGG and EcN+LGG-colonized neonatal gnotobiotic (Gn) pigs were challenged with HRV. EcN colonization associated with greater protection against HRV, also induced the highest frequencies of plasmacytoid dendritic cells (DC), significantly increased natural killer (NK) cell function and decreased frequencies of apoptotic and TLR4⁺ mononuclear cells (MNCs). Consistent with the highest NK cell activity, splenic CD172⁺ MNCs (DC enriched fraction) of EcN colonized pigs produced the highest levels of IL-12 (activates NK cells) *in vitro*. LGG colonization had little effect on the above parameters, and those of EcN+LGG colonized pigs were intermediate, suggesting that the probiotics modulate each other's effects. Additionally, *in vitro* EcN-treated splenic or intestinal MNCs produced a higher but balanced cytokine repertoire (IFN- α , IL-12 and IL-10), as compared to that of pigs treated with LGG. These results indicate that the EcN-mediated greater protection against HRV was associated with potent stimulation of the innate immune system and activation of the DC-IL-12-NK immune axis.

Keywords

Probiotics; *L. rhamnosus* GG; *E. coli* Nissle 1917; human rotavirus; childhood diarrhea; dendritic cells; natural killer cells

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Conflict of interest

The authors declare no conflict of interest.

Introduction

The importance of probiotic and commensal bacteria in modulating immunity, the severity of viral infections and health is evident from numerous clinical human trials and experimental studies using animal models [1]. In spite of the overwhelming evidence, there is insufficient information about the precise mechanisms of action of individual probiotics and their interactive effects on immunity. Growing evidence suggests that dendritic cells (DCs) play a key role in probiotic bacteria stimulation of the innate immunity [5, 6]. Although suggesting an overall positive impact, systematic reviews of clinical trials and cross-sectional studies have not yielded conclusive information about mode of action of individual probiotics across different populations and age groups [7]. This is in part because the probiotic strains examined were mostly evaluated in the context of the human microbiome and underlying nutritional, environmental and health conditions that may inhibit, promote or mask individual probiotic effects [4]. There is also no consensus on whether and how probiotic bacteria interact with one another and the host microbiome [4]. Thus, gnotobiotic (Gn) animal models are indispensable to fill these knowledge gaps and to dissect the mechanisms for individual and combinatorial probiotic bacteria effects. Like infants, neonatal Gn piglets are susceptible to HRV diarrhea, similar in their anatomy and physiology, and possess a functional, although immature, immune system [9]. The neonatal Gn piglet provides a highly relevant model (simplified and highly regulated) to delineate the direct beneficial effects of probiotics on enteric viral infections and virus-induced immune responses.

Rotavirus (RV) is the leading cause of viral diarrhea in infants and children associated with at least 453,000 deaths in children younger than 5 years worldwide annually [10]. Because the two currently licensed live attenuated HRV vaccines showed reduced efficacy (~50%) in impoverished countries, where HRV diarrhea is most severe [11], alternate interventions are needed. Probiotics are increasingly recognized as promising low cost treatments to moderate infectious diseases, including HRV diarrhea, and to improve intestinal homeostasis [7, 12].

Lactobacillus rhamnosus GG (LGG) immunomodulatory properties and its potential to alleviate HRV diarrhea are documented in clinical human [7, 13–16] and animal [17–19] studies. In contrast, anti-HRV effects of *Escherichia coli* strain Nissle 1917 (EcN) – one of the few gram-negative commercialized probiotics of human origin – were not evaluated. EcN is widely used to treat various inflammatory disorders in humans [21]. Confirmed mechanisms of EcN action mostly in the context of enteric bacterial infections include enhancing the intestinal barrier [22], moderating inflammatory responses [23], influencing host smooth muscle cell activity [24], increasing expression of antimicrobial and immunomodulatory factors [25], induction of beta-defensin in epithelial cells [19] and modulation of T cell proliferation [27]. However, additional experiments are needed to evaluate EcN efficacy against enteropathogenic viruses including HRV and the related regulatory mechanisms. Gram-positive (G⁺) (mostly lactobacilli and bifidobacteria) and gram-negative (G[–]) probiotics/commensals differ in their microbe-associated molecular patterns that likely to differentially affect neonatal immune maturation and susceptibility to infections. However, there is insufficient information on whether the combined use of

genetically distant G⁺ and G⁻ (EcN) probiotic bacteria is advantageous over single probiotic use.

In this manuscript, we report what innate immune mechanisms were associated with and possibly contributed to distinct effects of LGG and EcN colonization on protection against HRV disease and infection.

Results

Diarrhea severity and HRV fecal shedding were lower in EcN±LGG colonized pigs

Diarrhea severity (diarrhea score > 1) was significantly decreased in the EcN±LGG pigs at PCD3 and in the EcN-colonized pigs at PCD5 (Fig. 1A). Additionally, the duration of diarrhea was 1 and 2 days in EcN- and EcN+LGG-colonized pigs, respectively, compared to 5–6 days of LGG-colonized or uncolonized piglets. Significantly decreased virus shedding titers were observed in EcN±LGG-colonized pigs at PCD2,5 and 6 (Fig. 1B). In addition, EcN-colonized pigs lacked peak shedding titers (at PCD2,4 or 5) characteristic for HRV infection and observed in all other groups.

EcN (±LGG) colonization increased frequencies of systemic and intestinal plasmacytoid (pDCs)

Consistent with reduced HRV shedding and diarrhea in the EcN±LGG piglets post VirHRV challenge, higher (significantly in duodenum and spleen) frequencies of total and activated (MHCII⁺) (significantly in duodenum) pDCs were evident in these piglets compared to LGG-colonized or noncolonized pigs (Fig. 2A and B). Additionally, 70–93% of intestinal pDCs were activated in all animals; however, only 8–41% of systemic pDCs displayed an activated phenotype (Fig. 2C). There were no consistent trends observed for cDC frequencies (data not shown).

LGG or EcN probiotic colonization significantly decreased TLR4 expressing MNC frequencies

In this study, TLR4⁺ MNC frequencies increased due to VirHRV challenge were consistently decreased in all tissues of colonized pigs (significantly in EcN or LGG colonized) as compared to noncolonized pigs following virus challenge (Fig. 3C).

Contrastingly, dual EcN+LGG colonized pigs had the highest frequencies of TLR2 and 9 expressing MNCs in spleen and blood reflective of increased stimulation of these receptors by bacterial components (Fig. 3A and D). Although variation existed between the different groups and tissues, EcN colonization appeared to decrease frequencies of TLR3⁺ MNCs consistent with decreased fecal HRV shedding and improved protection (Fig. 3B). In contrast to EcN, but consistent with our previous observations [17], LGG colonization significantly increased frequencies of intestinal TLR3⁺ MNCs (Fig. 3B).

EcN colonization decreased frequencies of apoptotic MNCs in all tissues, and EcN±LGG increased proliferation in duodenal MNCs

Frequencies of apoptotic MNCs were decreased (significantly in spleen and duodenum) in all tissues of EcN-colonized pigs compared with non-colonized pigs (highest), while those of LGG- and EcN+LGG-colonized pigs were intermediate (Fig. 4A). This suggests that these probiotic bacteria might have inhibited pro-apoptotic HRV effects by decreasing virus replication in the gut, by inhibiting TLR4-mediated pro-apoptotic signaling or via activating anti-apoptotic pathways. Additionally, consistent with overall stimulatory effects of EcN on the immune system, EcN colonization (±LGG) significantly increased frequencies of spontaneous proliferation among duodenal MNCs but not MNCs in spleen (Fig. 4B).

CD172⁺ splenic, ileal and duodenal MNCs of the EcN colonized pigs produced the highest levels of IL-12 cytokine following iHRV stimulation *in vitro*

Because of importance of DCs in protection against primary RV infection, we assessed the levels of IL-6, IL-10, IL-12, TNF- α and IFN- α cytokines in the supernatants of CD172⁺ splenic MNCs (DC-enriched MNC fraction) the four experimental groups after iHRV stimulation *in vitro* (Fig. 5A-E). Compared with other cytokines, IL-12 cytokine levels were the highest in the supernatants after iHRV stimulation, suggesting that IL-12 may play an important role in DC-mediated immune responses to HRV (Fig. 5E). Additionally, the CD172⁺ MNCs from spleen of the EcN colonized pigs produced the highest levels of IL-12 *in vitro* (Fig. 5E). Finally, with the exception of IL-6 (which was slightly decreased), CD172⁺ MNCs from spleen of EcN colonized Gn pigs (compared with the other groups) produced significantly higher levels of the other cytokines (IL-10, TNF- α and IFN- α) (Fig. 5A-D).

We further compared the levels of this cytokine produced by ileal and duodenal CD172⁺ MNCs (Fig. 5F and G). Unlike with most cytokines, IL-12 levels produced by intestinal CD172⁺ MNCs were higher than those produced by splenic CD172⁺ MNCs. Additionally, the IL-12 levels produced by intestinal CD172⁺ MNCs of EcN-colonized were significantly (5–8 times) higher than those of the other groups. However, for splenic CD172⁺ MNCs, although also significant this difference was only 2–3 fold, suggesting that EcN also acts as a potent inducer of intestinal immunity.

EcN significantly enhanced NK cell function of blood MNCs

In our pilot experiments, blood MNCs demonstrated the highest levels of cytotoxicity against K562 target cells and were therefore used to assess NK cell cytotoxic function. Comparison among the 4 experimental groups demonstrated that consistent with the highest levels of IL-12 (essential for NK cell activation) produced by CD172⁺ MNCs of EcN colonized Gn pigs, blood MNCs of EcN-colonized pigs had the highest NK cell cytotoxic activity (Fig. 6A), while blood MNCs of noncolonized pigs had the lowest. Interestingly, this effect was negated in the dual EcN+LGG colonized piglets (Fig. 6A) showing the potential for counteractive effects when probiotics are combined.

Further, total MNCs from blood of EcN+VirHRV piglets were used for NK cytotoxicity assay with or without the addition of anti-porcine IL-12 Ab, and CD172[−] MNCs (lacking the

DC component) were used for NK cytotoxicity assay with or without addition of porcine recombinant IL-12 or anti-porcine IL-12 Ab. The presence of IL-12 Abs in the E:T co-culture significantly decreased the NK function in 10 ratio (Fig. 6B), suggesting that IL-12 secreted by DCs and essential in NK cell activation, may also be required for their functionality. However, the presence of IL-12 in the E:T co-culture with the CD172⁻ MNCs showed no differences in their NK function (Fig. 6C). This suggests that besides IL-12, direct contact with DCs (CD172⁺ MNCs) may be necessary for the EcN-mediated NK cell activation/function.

LGG and EcN treatment *in vitro* reduced cDC frequencies in all tissues, while EcN increased total and MHCII⁺ pDC frequencies in MLNs

To better understand the EcN/LGG and HRV interactive and immunomodulatory effects observed *in vivo* we conducted a series of *in vitro* experiments. MNCs isolated from spleen, ileum and MLNs of 4-week-old uncolonized, uninfected Gn piglets and were treated *in vitro* with live LGG or EcN probiotic bacteria with or without exposure to iHRV to understand: i) whether the major effects of EcN/LGG observed *in vivo* require persistent colonization of the intestinal epithelial cells and bystander stimulation of intestinal immune cells or if they can be reproduced through direct short-term interactions between immune cells (MNCs) and the probiotic bacteria; and ii) if the probiotic immunomodulatory effects observed *in vivo* differ in the presence or absence of HRV. The following data summarizes our *in vitro* findings.

Treatment with LGG ($\pm$ iHRV) and with EcN ($\pm$ iHRV) (to a lesser extent) decreased cDC frequencies in ileal and MLN MNCs, while either EcN or LGG decreased (minimally) the frequencies of cDC in splenic MNCs compared with the mock- or the iHRV-treated MNCs (Supplemental Table 1). The effects of EcN or LGG on total or MHCII⁺ pDC frequencies in ileal or splenic MNCs, with or without exposure to iHRV, were marginal; however, EcN ($\pm$ iHRV) treatment resulted in 2-fold increase of pDC and MHCII⁺ pDC frequencies in MLN MNCs (Supplemental Table 1). Thus, although, variable among the different tissue origins, our results demonstrated that only EcN stimulated pDC activation in the presence or absence of iHRV *in vitro* and both probiotics had modest down-regulatory effects on intestinal cDCs.

EcN induced significantly higher production of IFN- α , IL-10 and IL-12 by naïve MNCs

We further evaluated the *in vitro* production of the 3 key cytokines, IL-10, IL-12 and IFN- α , that were up-regulated, and IL-6 that was down-regulated, by EcN colonization *in vivo*. In the absence of iHRV, EcN significantly increased IL-12 and IFN- α levels produced by splenic MNCs (Fig. 7A and B); while LGG did not affect splenic MNC cytokine levels in the absence of iHRV (Fig. 7A-D). Both probiotics significantly reduced IL-6 cytokine level of iHRV-exposed splenic MNC compared with MNCs exposed to iHRV alone (Fig. 7A-D). In iHRV treated MNCs EcN significantly reduced IL-6 levels (Fig. 7C); and LGG significantly reduced IL-6 and IL-12 levels (Fig. 7A and C). Thus, as we observed *in vivo* EcN increased IL-12 and IFN- α levels in mock-treated MNCs, but decreased IL-6 production in iHRV-exposed MNCs.

EcN increased IL-10 levels produced by MLN, ileal or splenic (significantly) MNCs compared with mock-treated or iHRV exposed MNCs (Fig. 7D-F). In contrast, LGG decreased IL-10 levels in MLN and ileal, but not splenic MNCs compared with the mock-treated and iHRV-exposed group (Fig. 7E and F). The IL-10 levels were decreased significantly by LGG treatment in iHRV-exposed ileal MNCs (Fig. 7E).

EcN and LGG probiotics suppressed the TLR 2 and 4 expression in mock- or iHRV-exposed splenic MNCs

There were no consistent effects of EcN and LGG probiotic treatments on the expression of TLR3 (data not shown). LGG and, to a slightly lesser extent, EcN probiotics decreased the frequencies of TLR2 and TLR4 (Fig. 8) expressing splenic MNCs compared with the mock- or iHRV-exposed groups. A trend consistent with our *in vivo* findings was the TLR4 down-regulation by both probiotic bacteria.

Discussion

Here, we evaluated the immunomodulatory and protective effects of a gram-positive probiotic from the *Firmicutes* phylum (LGG), a gram-negative *Proteobacterium* (EcN), and their combined effects on HRV infection and innate immunity in neonatal Gn piglets. Greater protection against HRV infection and disease was observed in EcN colonized, compared to LGG, EcN+LGG colonized or noncolonized piglets. Consistent with the clinical parameters, the innate immune responses of the EcN+LGG group were intermediate between those of EcN- and LGG-colonized groups suggesting that probiotics modulate each other's effects.

Plasmacytoid dendritic cells were shown to contribute to RV clearance in a murine model [21]. Comparison of innate immune responses among the 4 groups demonstrated that the enhanced EcN-mediated protection against HRV was associated with increased frequencies of systemic and intestinal pDCs, augmented NK cell function and significantly increased levels of the DC-enriched fraction derived pluripotent IL-12 cytokine. The importance of the DC-IL-12-NK cell immune axis has been demonstrated experimentally in anti-infectious disease and anti-tumor immunity [36–39]. However, this is the first study to implicate it in the probiotic EcN-induced immunomodulation that confers enhanced protection against HRV. IL-12 is a proinflammatory/immunoregulatory cytokine produced by antigen presenting cells [26], neutrophils and B lymphocytes upon antigen stimulation [41, 42]. IL-12 enhances the cytotoxic activity of NK and CD8⁺ T cells and is involved in the differentiation of naive T cells into Th1 cells [40, 43]. Furthermore, while NK cell activation requires IL-12 production from DC [30], NK cells in turn interact with DCs to promote their maturation and increase IL-12 production [36, 37, 39, 45]. Consistent with these characteristics, the robust *in vitro* production of IL-12 by intestinal and splenic DCs (DC-enriched MNC fraction) of EcN colonized piglets was associated with improved protection against HRV infection and up-regulated innate immunity *in vivo*. Additionally, the increased pDC frequencies coincided with the enhanced IL-12 production and NK function of the EcN colonized pigs and may indicate that pDCs are the key DC subset mediating EcN beneficial effects. Although less robust than IL-12, DC-derived IFN- α levels were also increased in

EcN-colonized piglets, coinciding with higher pDC (potent IFN- α producers) frequencies and further supporting the hypothesis that EcN mediates its effects via pDCs. Finally, consistent with our previous observations [47], the highest frequencies of pDCs in duodenum and significantly higher proportion of ileal/duodenal pDCs that displayed an activated phenotype with upregulation of MHC class II molecules as compared to systemic pDCs. In agreement with our previous findings that intestinal but not systemic DCs predominated after HRV infection [33], this suggests that pDCs may be critical for intestinal/mucosal immunity and interactions with commensal microbiota and enteric pathogens.

Increased production of IL-6 was demonstrated previously following RV infection *in vitro* and was implied in immunopathology of infantile biliary atresia [48, 49]. Further, IL-6 levels in the study of Narvaez and colleagues [49] were decreased by CpG stimulation suggesting that the EcN-mediated decrease in HRV-induced IL-6 production observed in our study may represent another therapeutic anti-inflammatory action of probiotic treatment.

Besides TLR3, HRV up-regulated the expression of TLR2, 4, 7 and 8 in peripheral blood MNCs of pediatric patients [36]. Previous studies demonstrated that probiotic bacteria down-regulate TLR4 expression associated with pro-inflammatory and pro-apoptotic signaling [12, 37]. Toll-like receptor-4 expressed by epithelial and immune cells plays an important role in the mucosal host defense against invading pathogens. Because mucosal surfaces are colonized by the commensal microflora, TLR4 activation by microbial ligands or danger associated molecular patterns must be tightly regulated to avoid excessive stimulation and mucosal inflammation. Reportedly, extended or aberrant TLR4 activation is associated with pro-apoptotic signaling and multiple mucosal inflammatory disorders [50–53]. Consistent with previous observations [17, 32, 54], we demonstrated that HRV-induced TLR4 expression and apoptosis frequencies among MNCs were reduced by LGG and EcN probiotic colonization. In spite of the presence of TLR4 ligand (LPS), EcN colonization resulted in a more substantial decrease of apoptotic and TLR4 expressing MNCs than LGG, emphasizing the complexity of the TLR signaling pathways. Virus-triggered apoptosis is a conserved innate immune defense strategy of eliminating virus infected cells; however, it may also aid the pathogen by facilitating its spread [55]. Therefore, the EcN and LGG-mediated counteraction of the HRV-induced apoptosis that we observed in this study could have limited the HRV replication.

In this and our previous study [17], LGG colonized Gn pigs had increased intestinal TLR3⁺ MNCs that were negatively associated with HRV shedding. Contrastingly, in this study, EcN-induced protection, unlike that of LGG, is TLR3-independent.

Finally, we demonstrated that being a potent immunostimulant and increasing frequencies and function of innate immune cells, EcN also possesses immunoregulatory properties, through inducing IL-10 production. DCs of EcN colonized piglets produced higher amounts of IL-10 than noncolonized, co-colonized or LGG-colonized piglets. Additionally, splenic/ileum/MLN MNCs from naïve piglets stimulated with EcN *in vitro* produced higher levels of IL-10 than those stimulated with LGG. Our current results support the previous

hypothesis that a balanced EcN-mediated IL-10/IL-12 response can serve as a reliable predictive parameter of clinical outcome after probiotic treatment [42].

Several parallels observed in this study between the effects of the *in vivo* and *in vitro* exposure to EcN include the increase in pDC frequencies/function, increase of IL-12/IL-10/IFN- α levels and down-regulation of TLR4 expression. Our data suggest that some EcN effects observed *in vivo* could be induced by direct interactions between the probiotics and mucosal immune cells, and not only by their bystander activation via microbiota-epithelial cell interactions. This suggests that the tested probiotic bacteria may not require colonization to exert their immunomodulatory effects and could be tested further as therapeutic supplements in a host with an established intestinal microbiota.

In summary, a comparison of the anti-infectious disease (HRV) and immunomodulatory properties of LGG and EcN probiotics demonstrated that their effects in ameliorating HRV infection/disease vary greatly in magnitude and are associated with different mechanisms. We conclude that the EcN probiotic conferred greater protection against HRV by stimulating pDCs, activating the IL-12-NK cell immune axis and promoting strong, but balanced immunoregulatory/immunostimulatory responses. Finally, our results suggest that not only individual probiotic strains, but their combinations should be carefully evaluated as they may influence and even negate each other's effects by differential and simultaneous activation of stimulatory and inhibitory signaling pathways.

Materials and Methods

Virus

The virulent VirHRV Wa G1P[8] strain was used for inoculation at a dose of 1×10^6 fluorescent-forming units (FFU). The 50% infectious dose (ID_{50}) of Wa VirHRV in pigs was determined as approximately 1 FFU [43]. The Gn pig-adapted (passage 23) VirHRV Wa strain [43] was used to prepare the inactivated virus for *in vitro* treatment by using binary ethylenimine as described previously [58]. The protein concentration of inactivated HRV Wa (iHRV) was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.

Probiotic bacteria

LGG and EcN (kindly supplied by Dr. Ulrich Sonnenborn, Department of Biological Research, Ardeypharm GmbH, Herdecke, Germany) inoculums were prepared as previously described [13, 45].

Experimental design

All animal experiments were approved by the Institutional Animal Care and Use Committee at Ohio State University (protocol #2010A00000088). All the pigs were maintained, sampled, and euthanized to minimize suffering of animals. The euthanasia was performed by electrocution following anesthesia. Oral inoculation of neonatal pigs with Wa RVA caused transient followed by a spontaneous recovery within a few days. For this study, we used the lowest number of pigs previously shown to permit detection of statistical significances

among treatments. Near-term sows (Landrace × Yorkshire × Duroc cross-bred) were purchased from the Ohio State University swine center facility. Cesarean-derived Gn piglets were maintained in sterile isolators as previously described [46]. Prior to probiotic colonization, sterility was verified by aerobic and anaerobic culturing of rectal swabs to ensure no bacterial or fungal contamination. Prior to bacterial colonization, all piglets were confirmed negative for rotavirus, transmissible gastroenteritis virus, porcine epidemic diarrhea virus, calicivirus/sapovirus, astrovirus, and kobuvirus. The experiment was independently repeated three times using piglets derived from three different sows. The piglets in each of the three litters were randomly assigned to one of 4 groups (1–3/group), resulting in the following total numbers of piglets per treatment group: EcN colonized (EcN) (n=9), LGG colonized (LGG) (n=8), EcN and LGG dual-colonized (EcN+LGG) (n=6) and noncolonized (n=5). For mono-colonization of probiotics, six-day-old piglets were colonized with 10^5 colony forming units (CFUs) per pig and for dual-colonization of EcN and LGG probiotics, piglets were colonized with a 1:1 ratio of EcN and LGG, or total of 10^5 CFUs of each probiotic per pig. Probiotic inoculums were slowly instilled into the mouth using needleless syringes. Rectal swabs were collected weekly to ensure both probiotics' stable colonization. Subsequently all piglets were challenged with VirHRV Wa at post-bacterial colonization day 14. Post-VirHRV challenge, rectal swabs were collected to assess HRV shedding and record fecal scores to assess the severity of diarrhea. All piglets were euthanized at post-bacterial colonization day (PBCD) 36/post-VirHRV challenge day (PCD) 21 and blood, duodenum, ileum and spleen tissues were collected to isolate mononuclear cells (MNCs) as described previously [18].

Isolation of mononuclear cells (MNCs)

Spleen, blood, duodenum and ileum were collected the day of euthanasia and processed for isolation of MNC as described previously [60]. Flow cytometry staining was performed the same day immediately after the isolation of all tissue-derived cells.

Flow cytometry

Procedures for flow cytometry staining and analysis to assess frequencies and distribution of DC subsets, frequencies of MHCII⁺ MNCs and DCs and TLR2,3,4 and 9 expressing MNCs were performed as described previously [17]. CD172⁺/CD4⁺/CD11R1⁺-MHCII⁺/⁻ and CD172⁺/CD4⁻/CD11R1⁺/MHCII⁺ mononuclear cells were considered plasmacytoid and conventional dendritic cell-enriched fractions and were referred to as plasmacytoid (pDCs) and conventional (cDCs) dendritic cells, respectively, throughout. Frequencies and tissue distribution of apoptotic/necrotic or proliferating MNCs were assessed using Annexin V Apoptosis Detection Kit APC (eBiosciences, San Diego, CA)/Propidium Iodide Staining Solution (eBiosciences) and Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen, Grand Island, NY), respectively, as described previously [17]. The analysis and the gating strategies were as described previously [17].

Magnetic bead isolation of CD172⁺/CD172⁻ MNCs from spleen, blood, ileum or duodenum

CD172 (SWC3a) antibody (Ab) (IgG1, SouthernBiotec, Birmingham, Alabama, USA) that binds to a porcine granulocyte/monocyte was used to isolate CD172⁺/⁻ MNC fractions using magnetic beads (Miltenyi Biotec, San Diego, CA, USA) following the manufacturer's

recommendations [48]. The purity of CD172⁺ positive (DC enriched) or negative MNC fractions was confirmed by FACS analysis using CD172 Ab. CD172⁺ MNC population contained 93–97% of CD172⁺ cells, while CD172⁻ negative population contained less than 1.5% of CD172⁺ MNCs. CD172⁺/– MNCs were used for *in vitro* stimulation with HRV antigen (iHRV) or for the NK cytotoxicity assay.

Co-culture of MNCs with LGG or EcN bacteria with or without iHRV

Freshly isolated MNCs from spleen/blood of 4-week-old control Gn pigs (without previous exposure to any bacteria/viruses) were co-cultured with the live probiotic bacteria (re-suspended in PBS) LGG or EcN at a 1:10 ratio (MNC: bacteria), (n=4/treatment). Briefly, 2×10⁶ MNCs were co-cultured with 2×10⁷ CFU of EcN or LGG in enriched RPMI-1640 medium [Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlas biological, Fort Collins, CO), 1% L-glutamine, sodium pyruvate, non-essential amino acids and ampicillin/gentamicin (20µg/ml/200µg/ml) (Invitrogen)] for 24 hours under 5% CO₂ at 37°C, then exposed to iHRV (12 µg/ml) for another 24 hours under the same conditions. In our preliminary experiments, splenic MNCs produced the highest levels of most cytokines (compared to ileal, duodenal or blood MNCs); therefore, they were chosen as the major cells to assess immune function of the DC-enriched population. The CD172⁺ MNC fraction of splenic, ileal or duodenal MNCs of experimental Gn pigs was stimulated with iHRV (12 µg/ml) for 24 hours under 5% CO₂ at 37°C. Supernatants were collected and stored at –80°C until tested for cytokine levels. The MNCs were suspended in cold sterile PBS and used for flow cytometry staining immediately after the EcN/LGG and/or iHRV co-culture.

Cytokine ELISA of the co-culture supernatants

The levels of porcine IFN-α, IL-6 and TNF-α, IL-12p40 (referred to as IL-12 throughout), and IL-10 cytokines in the collected supernatants were examined by ELISA using anti-swine cytokine antibodies as previously described [61].

NK cytotoxicity assay

Total blood MNCs and K562 tumor cells were used as effector and target cells, respectively. Effector: target cell ratios of 10:1, 5:1, 1:1 and 0.5:1 were used and the assay was done as described previously [50]. Additionally, to assess the effects of IL-12p40 Ab/IL-12 on NK function, the effector (total blood MNCs or CD172⁻ MNCs from EcN+VirHRV piglets) and target cells were co-cultured with or without monoclonal anti-porcine IL-12 Ab (20µg/ml, AbD Serotec) or recombinant porcine IL-12 (10µg/ml, AbD Serotec). IL-6 Ab (20µg/ml, AbD Serotec) was used as irrelevant control Ab.

Statistical analysis

One-way analysis of variance (ANOVA-general linear model), followed by Duncan's multiple range test, was used to compare mean levels of IL-6, IL-10, IL-12, TNF-α, and IFN-α cytokines and NK cell activity. The frequencies of cell populations in flow cytometry were compared among or within groups using the Kruskal-Wallis rank sum or Mann-Whitney (non-parametric) tests. Statistical significance was assessed at p = 0.05 for all

comparisons. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

RV	rotavirus
HRV	human rotavirus
VirHRV	virulent human rotavirus
iHRV	inactivated human rotavirus
Gn	gnotobiotic
MNCs	mononuclear cells
MLN(s)	mesenteric lymph node(s)
LGG	<i>Lactobacillus rhamnosus</i> strain GG
EcN	<i>Escherichia coli</i> Nissle 1917
LAB	lactic acid bacteria
LAPB	lactic acid producing bacteria
CCIF	cell-culture immunofluorescence
FFU	fluorescent-forming unit
G+	Gram-positive
G–	Gram-negative
CFU	colony-forming unit
PCD	post-challenge day
PBCD	post-bacterial colonization day
MRS	deMan, Rogosa and Sharpe
DC(s)	dendritic cell(s)
NK	natural killer

TLR	toll-like receptor
Ab	antibody
IL	interleukin
TNF	tumor necrosis factor
IFN	interferon
DAMPs	danger associated molecular patterns

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Figure 1. Diarrhea severity and HRV fecal shedding in EcN, LGG, or EcN±LGG colonized and non-colonized pigs

Diarrhea scores (A) and HRV fecal shedding (B) in different treatment groups. Different letters indicate significant differences (determined by one-way analysis of variance [ANOVA] followed by Duncan's multiple range test, $p < 0.05$) at the same time-point among treatment groups.

Figure 2. EcN ($\pm$ LGG) colonization effects on the frequencies of systemic and intestinal total (A), MHCII+ pDCs (B) and MHCII+ pDCs among total pDCs (C)

Mononuclear cells (MNCs) isolated from spleen, blood, ileum, and duodenum of the piglets from the four experimental groups at PCD21. Total and MHCII+ plasmacytoid (pDC; CD172+/CD4+/CD11R1-) frequencies among total MNCs were assessed by flow cytometry. Asterisks indicate statistically significant difference (Kruskal-Wallis test; p < 0.05). Error bars indicate SEM. Frequencies of MHCII+ (activated) pDCs (C) among total pDCs were calculated by dividing the group average of total pDCs by MHCII+ pDCs for each tissue/treatment and expressed as a % of total pDCs.

Figure 3. Frequencies of MNCs expressing TLR2, TLR3, TLR4 and TLR9 in EcN/LGG colonized piglets

Mononuclear cells (MNCs) were isolated from spleen, blood, ileum, and duodenum of the piglets from the four experimental groups at PCD21. Frequencies of TLR2,3,4 and 9 expressing MNCs among total MNCs were assessed by flow cytometry. Asterisks indicate statistically significant difference (Kruskal-Wallis test; $p < 0.05$). Error bars indicate SEM.

Figure 4. Frequencies of apoptotic MNCs (A) and frequency of spontaneous proliferation (B) in colonized and non-colonized piglets

Mononuclear cells were dually stained with Propidium Iodide (PI) and Annexin V-APC using Apoptosis Detection Kit APC (eBiosciences, San Diego, CA)/Propidium Iodide Staining Solution (eBiosciences). Frequencies of apoptotic MNCs (PI-/Annexin-APC-) among total MNCs isolated from spleen, blood, ileum, and duodenum of the piglets from the four experimental groups at PCD21 were evaluated by Flow Cytometry. Frequencies of proliferating MNCs (EdU+ MNCs) among total MNCs isolated from spleen and duodenum of the piglets from the four experimental groups at PCD21 were evaluated using Click-iT®

EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen, Grand Island, NY). Frequencies of apoptotic and proliferating MNCs among total MNCs were assessed by flow cytometry. Asterisks indicate statistically significant difference (Kruskal-Wallis test; $p < 0.05$). Error bars indicate SEM.

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SWC3a+ MNCs stimulated in vitro with iHRV

Figure 5. Cytokine production by splenic, ileal and duodenal CD172+ MNCs following iHRV stimulation *in vitro*

Magnetic bead isolated CD172+ MNCs from spleen, ileum and duodenum of the piglets from the 4 experimental groups were stimulated *in vitro* with iHRV. The levels of IL-6, IL-10, IL-12, IFN-α and TNF-α (A-E) in the supernatants of stimulated splenic CD172+ MNCs were evaluated using cytokine ELISA. IL-12 cytokine production was also measured in the supernatants of iHRV stimulated duodenal and ileal CD172+ MNCs (F and G). Asterisks indicate statistically significant difference (One-way ANOVA followed by Duncan's multiple range test; $p < 0.05$). Error bars indicate SEM.

Figure 6. Natural killer cell activity of blood MNCs

Blood MNCs (from the piglets from all experimental groups) and carboxyfluorescein succinimidyl ester (CFSE) stained K562 tumor cells were used as effector and target cells, respectively, and co-cultured at set ratios to assess the NK cytotoxic function (A). To assess the effects of IL-12 on the NK function, the effector (blood MNCs isolated from EcN-VirHRV pigs) and target (CFSE-K562) cells were co-cultured with or without anti-porcine IL-12 Abs (20µg/ml, AbD Serotec) (B). Additionally, to assess the effects of dendritic cells on the NK function, the magnetic bead isolated CD172⁺ blood MNCs (isolated from EcN-VirHRV pigs), lacking the DCs, (effector) and CFSE-K562 (target) cells were co-cultured with or without anti-porcine recombinant porcine IL-12 (10µg/ml, AbD Serotec) (C). IL-6 Ab (20µg/ml, AbD Serotec) was used as an irrelevant control Ab. The effector : target cell co-cultures were stained with 7-Aminoactinomycin D (7AAD) after 12 hours of incubation at 37°C, and the frequencies of CFSE-7AAD double positive cells (lysed K562 target cells) were assessed by flow cytometry. (B) and (C) Pooled data are the mean ± SEM from three independent experiments. Asterisks indicate statistically significant difference (One-way ANOVA followed by Duncan's multiple range test; $p < 0.05$). Error bars indicate SEM.

Figure 7. IL-12, IFN-α, IL-6 and IL-10 cytokine production by naïve splenic MNCs treated with LGG, EcN and iHRV in vitro

MNCs from spleen, ileum and MLNs of the negative control (without previous exposure to any microorganisms) piglets were stimulated *in vitro* with EcN and LGG with or without iHRV (n=4/treatment). The IL-12, IFN-α, IL-6 and IL-10 levels (A-D) in the supernatants of EcN and LGG (±iHRV) treated splenic MNCs were evaluated using cytokine ELISA.

Additionally, the IL-10 cytokine production was also measured in the supernatants of EcN and LGG (±iHRV) treated splenic MNCs ileal and MLN MNCs (E and F). Pooled data are the mean ± SEM from three independent experiments. Different letters indicate statistically significant difference (One-way ANOVA followed by Duncan's multiple range test; $p < 0.05$). Error bars indicate SEM.

Figure 8. EcN, LGG and iHRV exposure effects on the TLR 2 and 4 expression in by splenic MNCs

MNCs from spleen, ileum and MLNs of the negative control (without previous exposure to any microorganisms) piglets were stimulated *in vitro* with EcN and LGG with or without iHRV (n=4/treatment). Pooled data are the mean $\pm$ SEM from three independent experiments. Frequencies of TLR2 and 4 expressing MNCs among total MNCs were assessed by flow cytometry. Kruskal-Wallis test demonstrated no significant differences among EcN, LGG and/or iHRV treated MNCs. Error bars indicate SEM.