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Infectivity of GII.4 human norovirus does not differ between T-B-NK$^+$ severe combined immunodeficiency (SCID) and non-SCID gnotobiotic pigs, implicating the role of NK cells in mediation of human norovirus infection

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ABSTRACT

Human noroviruses (HuNoVs) are a leading cause of acute gastroenteritis worldwide. It is unclear which arm of the immune system regulates resistance to HuNoV infection. Thus, we studied the pathogenesis of human norovirus (HuNoV) in T$^-$B$^-$NK$^+$ Severe Combined Immunodeficiency (SCID) gnotobiotic pigs to investigate the role of innate (especially, natural killer (NK) cells) immunity in HuNoV infection. Forty SCID and non-SCID pigs were randomly grouped: 1) SCID + HuNoV (n = 12); 2) non-SCID + HuNoV (n = 14); 3) SCID mock-inoculated (n = 6); and 4) non-SCID mock-inoculated (n = 8). Pigs (8–14-day-old) were inoculated orally with GII.4 HuNoV strain HS292 (mean 9.1 $\log_{10}$ genomic equivalents/pig) or mock. Daily fecal consistency and fecal viral RNA shedding, and histopathology (at euthanasia) were evaluated. Frequencies of blood and ileal T, B, and NK cells were analyzed by flow cytometry, and a NK cell cytotoxicity assay was performed at post-inoculation day (PID) 8. Unlike the increased infectivity of HuNoV observed previously in T$^-$B$^-$NK$^-$ SCID pigs (Lei et al., 2016. Sci. Rep. 6, 25,222), there was no significant difference in frequency of pigs with diarrhea and diarrhea days between T$^-$B$^-$NK$^+$ SCID + HuNoV and non-SCID + HuNoV groups. Cumulative fecal HuNoV RNA shedding at PIDs 1–8, PIDs 9–27, and PIDs 1–27 also did not differ statistically. These observations coincided with the presence of NK cells and NK cell cytotoxicity in the ileum and blood of the SCID pigs. Based on our observations, innate immunity, including NK cell activity, may be critical to mediate or reduce HuNoV infection in T$^-$B$^-$NK$^+$ SCID pigs, and potentially in immunocompetent patients.

1. Introduction

Human noroviruses (HuNoV) are single-stranded RNA viruses belonging to the *Caliciviridae* family. HuNoVs are a leading cause of acute gastroenteritis in the United States and worldwide (Siebenga et al., 2009), causing an estimated 219,000 deaths per year (Bartsch et al., 2016). Estimates of economic burden indicate that the societal costs due to HuNoV are $60.3 billion per year (Bartsch et al., 2016). Further, the incidence, as well as severity of HuNoV infection, is higher in pediatric and elderly populations (Hall et al., 2012; Phillips et al., 2010). There are no approved vaccines available to prevent HuNoV infection. However, a virus-like particle candidate vaccine has been tested clinically (phase I and II completed, advancing to phase III trials), and several other candidates are also in pre-clinical stages of development (Atmar et al., 2011; Lucero et al., 2018). Recently, a B cell (Jones et al., 2014) and a complex human intestinal enteroid monolayer culture system (Ettayebi et al., 2016) that support HuNoV replication have been developed. However, the lack of a routine and relatively low-cost *in vitro* cell culture system remains a major challenge for HuNoV research.

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Although HuNoV replicates in B cells *in vitro* (Jones et al., 2014; Karst and Wobus, 2015), a high incidence of HuNoV infection was observed in B cell-deficient ($T^-B^-NK^+$) Severe Combined Immunodeficiency (SCID) patients compared with other types of B cell-positive SCID patients ($T^-B^+NK^-$) (Brown et al., 2016). The B cell-deficient SCID children contracted HuNoV infection at a similar rate (fecal shedding HuNoV RNA titers) compared with the SCID children with B cells, indicating involvement of other cells besides B cells in HuNoV replication. Notably, mean fecal shedding HuNoV RNA titers were significantly lower in $T^-B^-NK^+$ children compared with $T^-B^+NK^-$ children (Brown et al., 2016), implying a key role for NK cells in mediation of HuNoV infection. Interestingly, increased infectivity of HuNoV was observed in B cell and NK cell-deficient ($T^-B^-NK^-$) SCID pigs compared with normal gnotobiotic (Gn) pigs (Lei et al., 2016). Thus, it is unclear which arm of the immune system mediates or regulates resistance to HuNoV infection. Among the animal models available (Todd and Tripp, 2019), Gn pigs are susceptible to infection by several genotypes of HuNoV including GII.4 (Cheetham et al., 2006) and GII.12 (Jung et al., 2012; Takanashi et al., 2011). Gn piglets inoculated with HuNoV developed mild diarrhea and/or virus shedding up to 16 days post-viral challenge (Cheetham et al., 2006; Takanashi et al., 2011).

Innate immunity has been implicated in preventing persistent murine NoV infection in microbiota depleted mice (Baldridge et al., 2015). A recent study also demonstrated the importance of type I interferons, but not adaptive immunity, in clearing murine NoV infection (Nice et al., 2016). Thus elucidating the specific roles of adaptive and innate immunity in protection against HuNoV in relevant animal models such as SCID pigs is essential to understand the immunopathogenesis of HuNoV infection. In this study, to investigate the role of innate (especially NK cells) immunity in HuNoV infection, $T^-B^-NK^+$ SCID pigs, deficient in B and T cells, but possessing NK cells (Powell et al., 2016), were utilized to assess the pathogenesis of HuNoV infection, particularly, in comparison to the increased infectivity of HuNoV observed in a previous study employing $T^-B^-NK^-$ SCID Gn pigs (Lei et al., 2016). The $T^-B^-NK^+$ SCID pigs used in our study are a useful animal model to investigate the function and role of the innate immune system in controlling viral infections (Powell et al., 2017).

2. Materials and methods

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University. Sow pregnancies to obtain SCID piglets were initiated at Iowa State University (ISU) as previously described (Powell et al., 2016). Five pregnant sows for derivation of expected 50% SCID piglets were transferred from ISU to an animal facility at the Ohio Agricultural Research and Development Center, The Ohio State University. Subsequently, caesarean-derived Gn piglets (SCID and non-SCID littermates) from the near–term sows were obtained and maintained in isolator units as described previously (Meyer et al., 1964). Piglets with SCID mutations were identified as described previously (Waide et al., 2015).

Forty SCID and non-SCID piglets were randomly assigned to one of the following four groups: 1) SCID + HuNoV (n = 12), 2) non-SCID + HuNoV (n = 14), 3) SCID mock-inoculated (n = 6), and 4) Non-SCID mock-inoculated (n = 8). The histo-blood group antigen (HBGA) A/H phenotype of pigs was determined as described previously [13]: all pigs used in our study tested A-positive. At the age of 8–14 days, piglets were inoculated orally with GII.4 HuNoV strain HS292 [mean 9.1 $\log_{10}$ genomic equivalents/pig] or mock (sterile phosphate buffered saline). Piglets received ultra-temperature-pasteurized bovine milk (Parmalat®) according to the IACUC guidelines.

Rectal swab samples were collected daily to assess the severity of diarrhea and quantify fecal virus RNA shedding titers. Daily fecal consistency and fecal viral RNA shedding, and histopathology at euthanasia were evaluated as described previously (Cheetham et al., 2006; Takanashi et al., 2011). Diarrhea was assessed by scoring fecal consistency, as follows: 0 = solid; 1 = pasty; 2 = semi-liquid; 3 = liquid, with scores of 2 or more considered diarrheic. Individual pigs' cumulative virus shedding was assessed by calculating area under the curve (AUC) of the daily viral RNA shedding. Mean AUC of viral RNA shedding titer per rectal swab sample was calculated during an early to mid [post-inoculation days (PIDs) 1–8] and later (PIDs 9–27) stage of infection (including a variety of periods of time: PIDs 9–14, PIDs 15–27, and PIDs 1–27).

Isolation of mononuclear cells (MNCs) from blood and ileum (Chattha et al., 2013), flow cytometry to detect T or B cells and to determine frequencies of $CD3^-CD4^-CD8^+$ NK cells in the lymphocyte gate (Annamalai et al., 2015), and the NK cell cytotoxicity assay (Annamalai et al., 2015) were performed at PID 8. Briefly, K562 (human erythroleukemia cell line) tumor target cells were stained with carboxy fluorescein succinimidyl ester (CFSE), washed twice and used for the assay. Isolated MNCs were used as effector cells. Effector: target cells were mixed at ratios of 25:1, 12.5:1 and 6.25:1 and incubated overnight at 37 °C. Dead cells were identified based on uptake of 7-Aminoactinomycin D (7-AAD). The cells were analyzed by flow cytometry and the percentage of $CFSE^+$ $7\text{-}AAD^+$ cells were categorized as dead K562 cells. $CFSE^+$ K562 cells incubated without MNCs and stained similarly with 7-AAD were used as controls for spontaneous death of K562 cells. Blood samples were collected every other day to quantify serum interferon-α (IFN-α), interleukin-17 (IL-17), and tumor necrosis factor-α (TNF-α) cytokine levels by enzyme-linked immunosorbent assay (ELISA), as described previously (Chattha et al., 2013).

Fisher's exact test and one-way ANOVA using GraphPad Prism software were used to determine significant differences in frequency of pigs with diarrhea and mean diarrhea duration days, respectively, between SCID + HuNoV and non-SCID + HuNoV groups, or among the three groups. Mann-Whitney test using GraphPad Prism software were used to determine significant differences in kinetics of daily fecal viral RNA shedding and mean AUC of fecal viral RNA shedding (cumulative fecal virus shedding) at PIDs 1–8, PIDs 9–14, PIDs 15–27, and PIDs 9–27 and PIDs 1–27 between SCID + HuNoV and non-SCID + HuNoV groups. NK cell activity, NK cell frequencies and cytokine levels were also analyzed by one-way ANOVA using GraphPad Prism software.

3. Results and discussion

We hypothesized that the $T^-B^-NK^+$ SCID piglets lacking adaptive immunity (B and T cells), but not NK cells, would also have increased diarrhea and fecal viral RNA shedding as compared with non-SCID piglets, as increased infectivity of HuNoV was previously observed in another type of SCID ($T^-B^-NK^-$) pigs (Lei et al., 2016). In the previous study, $T^-B^-NK^-$ SCID pigs (6 to 7-day-old) were inoculated orally with the low dose (2.74×10^4 viral RNA copies/pig) of a GII.4 HuNoV (strain 092895), which is the same genotype of HuNoV, but a different strain compared with the virus used in our study. The inoculated $T^-B^-NK^-$ SCID pigs exhibited prolonged (11 more days) and increased fecal viral RNA shedding compared with the inoculated wild-type pigs, despite no difference in the incidence of diarrhea between the two groups. In our current study, there were no significant differences in frequency of pigs with diarrhea and mean diarrhea duration days among the three groups, or between SCID + HuNoV and non-SCID + HuNoV groups (data not shown). Similarly, mean AUC of fecal HuNoV RNA shedding (cumulative fecal shedding) at PIDs 1–8, PIDs 9–27, PIDs 9–14, PIDs 15–27, and PIDs 1–27 were statistically comparable between SCID + HuNoV and non-SCID + HuNoV pigs (Fig. 1a and b). Mean onset of fecal HuNoV RNA shedding, mean days of fecal HuNoV RNA shedding, and mean HuNoV RNA peak titers were also similar between SCID + HuNoV and non-SCID + HuNoV pigs (Table 1). In addition, when percent of pigs positive for fecal virus shedding, *i.e.*

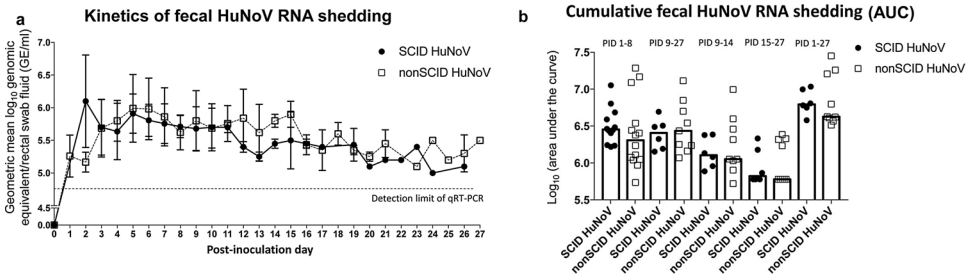

Fig. 1. (a) Kinetics of fecal HuNoV RNA shedding in SCID + HuNoV and non-SCID + HuNoV pigs expressed as geometric mean titers and standard deviations. There was no significant difference by Mann-Whitney test between SCID + HuNoV and non-SCID + HuNoV groups; (b) Cumulative virus shedding of all individual pigs was presented as $\log_{10}$ area under the curve (AUC) of daily HuNoV RNA shedding in SCID + nHuNoV and non-SCID + HuNoV pigs at post-inoculation days (PIDs) 1–8, 9–27, 9–14, 15–27, and 1–27. The box represents the median. There was no significant difference by Mann-Whitney test in cumulative viral RNA shedding between the SCID + HuNoV and non-SCID + HuNoV pigs. PIDs 1–8, SCID + HuNoV (n = 12) and non-SCID + HuNoV (n = 14); and PIDs 9–27, 9–14, 15–27, and 1–27, SCID + HuNoV (n = 6) and non-SCID + HuNoV (n = 9). Note no fecal HuNoV shedding detected in mock-inoculated pigs.

viral acquisition of individual pigs during the early stage of infection (PIDs 1–5) was analyzed by a Gehan-Breslow-Wilcox test, there was no significant difference (P = 0.18) in viral acquisition between SCID + HuNoV and non-SCID + HuNoV pigs. It is also noteworthy that the fecal HuNoV RNA shedding observed in our $T^-B^-NK^+$ SCID pigs might be the result of HuNoV infection of enterocytes, consistent with the finding of HuNoV antigen mainly in enterocytes of the HuNoV-inoculated $T^-B^-NK^-$ SCID pigs that are also B cell negative (Lei et al., 2016).

Light microscopic examination at euthanasia (PID 8 or 27) revealed that all SCID pigs exhibited depletion of lymphocytes in the lymph nodes and spleen, which completely coincided with their SCID status by DNA genotyping (Ozuna et al., 2013; Waide et al., 2015) and flow cytometry. However, none of the HuNoV-inoculated or mock-inoculated SCID and non-SCID pigs had major histological changes in the intestine at PIDs 8 and 27.

No T or B cells were detected by flow cytometry in the blood and ileal MNCs of SCID pigs, but NK cells were present. Thus, we assessed whether innate immune responses such as cytotoxicity of NK cells and innate cytokines were elicited in SCID pigs. Blood and ileal NK cell activity was significantly higher in SCID compared with non-SCID pigs tested at PID 8 (Fig. 2a and b). In the blood of SCID pigs, NK cell activity was significantly higher in uninfected pigs compared with infected pigs (Fig. 2a), although similar frequencies of NK cells were observed among the pigs (Fig. 2c), implying migration of matured NK cells from blood to the infection site such as ileum or the related lymphoid tissues, such as the Peyer's patches and mesenteric lymph nodes in the infected pigs. In the ileum of SCID pigs, concomitantly, NK cell activity was significantly higher in infected pigs compared with uninfected pigs (Fig. 2b), although similar frequencies of NK cells were observed among the pigs (Fig. 2c).

Subsequently, we assessed whether differences in frequencies of NK cells might have contributed to the observed dichotomy in NK cell activity between SCID and non-SCID piglets. However, frequencies of NK cells were statistically similar between SCID and non-SCID piglets in the blood and ileum (Fig. 2c), indicating consistent frequencies of NK cells in SCID or non-SCID pigs, regardless of the SCID status or the type of tissue. In comparison with these findings, a previous study reported significantly higher frequencies of NK cells in SCID pigs compared with non-SCID pigs (Powell et al., 2016). This might be due to experimental differences (gnotobiotic vs conventional pigs, diet, *etc.*) between the studies as well as the differences in cell surface marker phenotypes used in identifying the NK cell populations. Powell et al. identified NK cells as SWC3A $(CD172)^-$ $CD16^+$ (Powell et al., 2016). Additionally, in our study, serum IFN-α, IL-17, and TNF-α cytokine levels did not differ significantly between SCID and non-SCID piglets, regardless of infection status (Supplemental Fig. S1a-c).

We hypothesized that HuNoV infection would be more severe in $T^-B^-NK^+$ SCID Gn pigs compared with non-SCID Gn pigs. However, the extent of HuNoV infection in SCID pigs was similar to that of non-SCID pigs at the early and later stages of infection. The preserved NK cell function might be responsible for the lack of increased susceptibility to HuNoV infection in SCID pigs. In fact, an earlier study showed that NK cells of SCID piglets possessed at least similar or higher functional activity compared with NK cells of non-SCID piglets (Powell et al., 2016). In the previous and our studies, the mechanism for the higher NK cell activity in SCID compared with non-SCID piglets is unknown. One possible explanation might be that lack of regulation by regulatory T cells could result in higher NK cell activity in SCID pigs, as also observed in earlier studies (Ghiringhelli et al., 2005; Smyth et al., 2006). Furthermore, in our study, comparable serum IFN-α, IL-17, and TNF-α cytokine levels between SCID and non-SCID piglets, or between SCID + HuNoV and non-SCID + HuNoV piglets also indicated that SCID pigs were capable of producing innate immune or pro-inflammatory cytokine responses. NK cells are known to produce IL-17 (Passos et al., 2010) and TNF-α (Fauriat et al., 2010). One potential source of these cytokines might be NK cells in SCID piglets, as well as other types of myeloid cells. Thus, other myeloid cells, such as macrophages, could also be involved besides NK cells in clearance of HuNoV infection in SCID piglets, although the frequencies of the myeloid cell populations in the blood and ileum will need to be further determined in future studies.

Table 1
No statistical difference occurred in viral RNA shedding as determined by qRT-PCR in the feces of GII.4 HuNoV (HS292 strain)-inoculated SCID compared with inoculated non-SCID pigs.

Group/ Treatment	Viral RNA shedding		
	Mean onset of virus shedding (PID) (SD)[a]	Mean days of shedding (SD)[a]	Mean peak titer ($\log_{10}$ GE/ml)[a]
SCID + HuNoV (n = 12)	3.3 (0.2)	11.1 (4.7) (n = 7)[b]	6.0 (0.1) (n = 7)[b]
Non-SCID + HuNoV (n = 14)	2.8 (0.4)	11.6 (4.9) (n = 9)[b]	6.2 (0.2) (n = 9)[b]
Mock-inoculated (n = 14)	.	.	.

PID, post-inoculation day; SD, standard deviation.
Real-time PCR was conducted on rectal swab samples collected daily.
[a] Student's t-test was used for statistical analysis. No significant differences between SCID + HuNoV and non-SCID + HuNoV groups or among the three groups.
[b] Pig numbers after early euthanasia at PID 8.

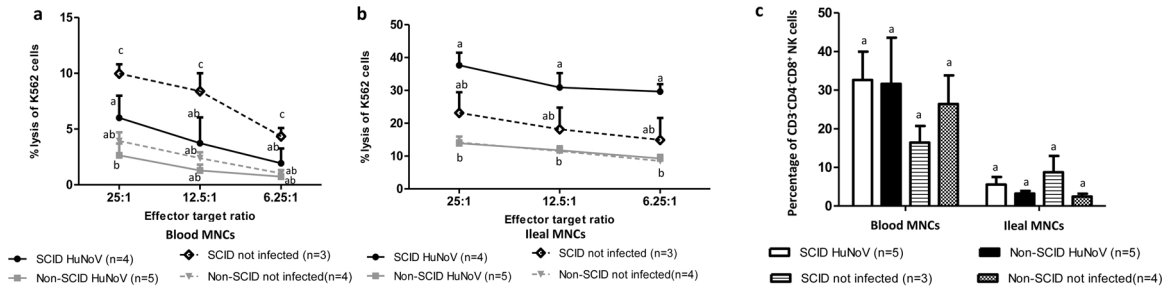

Fig. 2. Cytotoxicity and frequency of natural killer (NK) cells in SCID and non-SCID pigs post-HuNoV inoculation. NK cell activity was significantly higher in SCID vs non-SCID pigs using equal numbers of (a) blood (25:1 ratio only for infected SCID pigs) and (b) ileal MNCs (infected SCID pigs only) at post-inoculation day (PID) 8. K562 (human erythroleukemia cell line) tumor cells were used as target cells and effector: target cell ratios of 25:1, 12.5:1 and 6.25:1 were used. The line graphs labeled with different alphabetical letters differ significantly by one-way ANOVA ($P < 0.01$). (c) No significant differences were evident in frequencies of NK cell in the blood and ileum of SCID and non-SCID pigs at PID 8.

The role of innate immunity in resistance to HuNoV infection was reported in an earlier study of murine NoV (Karst et al., 2003). Mice deficient in both recombination-activating gene (RAG) (B and T cell-deficient) and signal transducer and activator of transcription 1 (STAT1) (IFN-α/β/γ response-deficient) succumbed to murine NoV infection and died, but RAG gene-deficient ($RAG^{-/-}$) mice survived murine NoV infection (Karst et al., 2003). As STAT1 functions through IFN-dependent innate immune signaling pathways, NK cell activity might be involved in reducing HuNoV infection in SCID piglets. Additionally, a previous study also showed that NK cells of SCID piglets were capable of responding to cytokines which resulted in increased NK function, including cytotoxicity against tumor cell lines (Powell et al., 2016). In a recent study, RAG2/IL2RG deficient SCID pigs that lacked B, T and NK cells had higher and prolonged HuNoV infection compared with non-SCID pigs (Lei et al., 2016). Based on these and our observations, particularly, as compared with the increased infectivity of HuNoV in the $T^-B^-NK^-$ SCID pigs, innate immunity, including NK cell activity, may be critical to mediate or reduce HuNoV infection in $T^-B^-NK^+$ SCID pigs, and potentially in immunocompetent patients.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. All authors have seen and approved the manuscript.

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Appendix A. Supplementary data

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