Human Norovirus Infection and the Acute Serum Cytokine Response

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Abbreviations: NoV, norovirus; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; SMV, Snow Mountain virus; GEC, genomic equivalence copies; RT-PCR, reverse transcription polymerase chain reaction; LLOD, lower limit of detection

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Summary

Noroviruses (NoV) are the most common cause of epidemic gastroenteritis worldwide. The acute immune response to NoV in humans is poorly understood, hindering research on prevention and treatment. To elucidate the acute immune response and test for cytokine predictors of susceptibility to infection, serum samples from two human NoV challenge studies were tested for 16 cytokines. Subjects who became infected (n=26) were age-matched with subjects who remained uninfected following NoV challenge (n=26). Samples were tested from pre-challenge and days 1-4 post-challenge. Cytokine responses were compared between infected and uninfected groups. Overall, infected individuals exhibited an elevation in Th1 and Th2 cytokines, as well as chemokines IL-8 and MCP-1, compared to uninfected individuals (all $p<0.05$). Most cytokines peaked on day 2 post-challenge in infected subjects, and TNF-α, IL-8, and IL-10 remained elevated through day 3. The only cytokine significantly elevated among infected subjects through day 4 post-challenge was IL-10 ($p=0.021$). Pre-challenge cytokine concentrations were not predictive of infection status post-challenge. There were no significant changes in serum cytokines among NoV-challenged subjects who remained uninfected. These results suggest that NoV infection elicits a Th1 type response with some Th2 activation. Persistent elevation of IL-10 among infected subjects is consistent with activation of adaptive immune responses, such as B-cell expansion, as well as down-regulation of Th1 cytokines. This study presents the first comprehensive description of the acute cytokine response to GI.1 NoV in humans.

Introduction

Noroviruses (NoV) comprise seven genogroups in the positive-sense RNA virus family *Caliciviridae* [1, 2]. NoV are responsible for 18% of gastroenteritis worldwide [3]. Despite this broad impact, human NoV immunology is poorly understood [4, 5], in part because until recently, there was no small animal model or reliable cell culture system for human NoV [6, 7]. Furthermore, there are few prospective experimental studies on the acute human innate and cellular immune response to NoV.

The genetic determinants of NoV infection (e.g. secretor blood group antigens) are relatively well characterized, but many aspects of protective innate and cellular immunity have not been described [8, 9]. Histologic studies [4, 10-12] and in vitro tests of peripheral blood mono-nuclear cells [13] suggest cytotoxic and CD8+ T-cell activation with some cells sensitized to NoV challenge antigens, but little work has been done in vivo to describe the cytokine response to NoV infection. In particular, there is a gap in understanding the acute immune response to NoV. Prior studies have shown that adaptive immunity is critical for clearing the infection [14], but there is a gap in the knowledge about early pathogenesis of NoV infection. Key cytokines for consideration include acute response-related pro-inflammatory cytokines (e.g. IL-1, IL-6, and IL-12), chemokines involved in neutrophil and monocyte recruitment (e.g. IL-8 and MCP-1), and Th1- and Th2-related cytokines (e.g. IFN-γ, IL-2, and TNF-α and IL-4, IL-5, and IL-10, respectively) involved in the cellular immune response and initiation of adaptive immune mechanisms.

The goal of this study was two-fold. The first goal was to describe serum cytokine responses to NoV infection, including the temporal trends in serum cytokines during the acute phase of human NoV infection. The second goal was to use cytokine concentrations pre-

challenge to predict infection post-challenge. Human serum samples from two prior NoV challenge studies that used the same Norwalk virus (GI.1) inoculum were analyzed for a broad panel of relevant cytokines [15, 16]. The results have important implications for determining the role of specific cytokines in NoV infection.

Materials and methods

Population and samples

The samples tested for this study were collected from subjects involved in two separate NoV challenge studies, described previously [15, 16]. However, these studies could used the same inoculum preparation, were conducted at the same institution, and used very similar protocols. Key differences between these two challenge studies (i.e. inoculum dose) were accounted for in the modeling strategy of this study. Briefly, the first study enrolled healthy secretor-positive adult volunteers, who were challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site, part of the Atlanta Clinical and Translational Science Institute Clinical Interaction Network, between May 2006 and December 2006. Volunteers ingested filtered groundwater artificially seeded with 6.5×10^7 genomic equivalent copies (GEC) of NoV inoculum, which had been incubated at room temperature in the dark for different set lengths of time. Prior to challenge, serum and stool samples were collected. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The second study enrolled healthy secretor-positive adult volunteers, who were also challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site between February 2008 and September 2009. They were randomized into control and intervention groups and administered oysters seeded with 1×10^4 GEC of virus, which had been treated with high hydrostatic pressure processing for 5 minutes (intervention) or left untreated (control). Pre-challenge stool and serum samples were collected before challenge. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The Emory University Institutional Review Board approved both studies, and both were registered on Clinical Trials.gov (identifiers NCT00313404 and NCT00674336). All subjects consented to the future use of all biological specimens from the studies. All specimens were stored at -80°C.

Of the initial participants in both NoV challenge studies, a total of 26 became infected as defined by a NoV-positive stool or emesis sample tested by RT-PCR (limit of detection: 3,570 GEC/g stool) [15, 17]. These 26 were pair-matched by age to 26 of the challenge study participants who remained uninfected following NoV challenge. Some infected subjects from one challenge group were matched with uninfected subjects from a different challenge group because within the same group there were no uninfected individuals within 3 years of age of the infected individual. The total sample size for this study was 52 participants (13 from the 2006 trial, 39 from the 2008-2009 trial). Because the focus of this study is the acute immune response, only serum samples from pre-challenge and days 1-4 post-challenge were included, for a total of five longitudinal samples per subject.

Inoculum

The two NoV challenge studies included participants who ingested water or shellfish seeded with 8FIIb NoV inoculum. The water samples were seeded with identical amounts of NoV and stored at room temperature in the dark for varying lengths of time. They showed no evidence of titer attenuation over time, as measured by RT-PCR [15]. Therefore, for these subjects, the regression models did not control for study arm. The shellfish samples were seeded with identical amounts of NoV and treated under varying processing conditions. The inocula showed some evidence of attenuation for some treatments. For subjects from this study, all adjusted models controlled for study arm as a surrogate for dose.

Detection of viral shedding

NoV presence was measured by RT-PCR for all stool and emesis samples at all time points during the original challenge studies. A subject with a positive sample at any time point, even after day 4 post- exposure, as determined in the original or subsequent studies, was defined as infected.

Detection of cytokines

Serum samples from pre-challenge (pre-challenge), and days 1-4 post-challenge (five total samples) were analyzed from all study subjects. Serum was collected during the studies, processed, and stored at -80°C until testing. Samples were tested by a commercial laboratory (EMD Millipore Corporation Discovery and Development Solutions) using a Milliplex human cytokine 16-plex assay for IFN-α2, IFN-γ, IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, MCP-1, TNF-α, and TNF-β. Samples were run in duplicate with controls. Standard curves were generated using recombinant cytokines and calculated using a five parameter logistic model for each cytokine. Lower limits of detection (LLOD) were 3.2 pg/mL for IFN-γ, IL-10, IL-12p70, IL-1b, IL-2, IL-5, IL-6, IL-8, MCP-1, and TNF-α; 16.0 pg/mL for TNF-β; and 80.0 pg/mL for IFN-α2, IL-12p40, IL-1ra, IL-1a, and IL-4. Values below LLOD were assigned the value of the LLOD.

Statistical analysis

The data were analyzed using SAS 9.4 (Cary, NC, USA). When possible, age and inoculum were controlled for in adjusted analyses. Because the exact amount of infectious NoV in the inocula for study arms with attenuation was unknown, inoculum was controlled for using a categorical variable for each study and study arm. The Wilcoxon signed rank test was to test for

the significance of the unadjusted difference between pre-challenge and post-challenge serum cytokine levels. Conditional logistic regression was used to evaluate the association between pre-challenge and post-challenge fold changes in serum cytokine levels (i.e. ratio between post-challenge and pre-challenge cytokine concentrations), controlling for inoculum and age. To summarize the differences between infected and uninfected individuals responses over time while accounting for correlation between time points, a mixed linear model was used to test the association between $\log_{10}$ cytokine concentration and day post-challenge, stratified by infection status with a random effect by subject, autoregressive correlation between different time points taken for the same individual, and fixed effect for inoculum. The parameter estimates for the effect of each day on $\log_{10}$ cytokine concentration for each stratum were averaged and Student's t-test was used to compare infected and uninfected subjects cytokine responses over time. Conditional logistic regression adjusted for inoculum and age was also used to assess whether three measures of early cytokine levels or responses could be used as predictors of post-challenge infection. The measures assessed were pre-challenge $\log_{10}$ cytokine concentration, pre-challenge to day 1 post-challenge $\log_{10}$ cytokine change, and pre-challenge to day 1 post-challenge fold change. All cytokines were modeled individually. Differences between groups were considered statistically significant at $p < 0.05$.

Results

To determine the acute serum cytokine response to GI.1 NoV infection, serum cytokine responses in individuals who became infected following experimental challenge were compared to those who remained uninfected after challenge, as determined by RT-PCR detection of GI.1 NoV RNA in stool or emesis samples. Immune responses may differ with age, therefore the infected and uninfected subjects were matched for age (Table 1). The study population was relatively young, with a mean age of 26.7 years and a median age of 25 years. There was also a similar distribution of sex and race between infected and uninfected subjects selected for the study, though neither was a matching factor. Some uninfected subjects exhibited symptoms, including fever and diarrhea, which is consistent with past NoV challenge studies [16]. All infected individuals had higher cumulative shedding than the inoculum dose (data not shown), reducing the likelihood of misclassification of infection status because of detection of inoculum post-challenge in the absence of infection. Serum samples were available for pre-challenge through day 4 post-challenge (five time points) and all samples were tested for 16 serum cytokines. Two subjects did not have samples for day 3 and values for these samples were imputed using the average of neighboring observations for the same subject. A total of 10 subjects had invalid test results for one or more cytokine tested (29 total observations). Using imputation, all but one of these invalid results was estimated to be below the LLOD.

Studies of serum cytokine levels in humans often have high rates of observations below the LLOD [18]. Overall of the 16 cytokines tested, nine had greater than 50% of observations above the LLOD, and four had greater than 75% of observations above the LLOD (Table 2). In general, infected subjects had more samples above the LLOD.

To assess whether serum cytokines exhibited a change in concentration following NoV

challenge, the Wilcoxon signed rank test was used, stratifying by infection status. Among infected individuals, there were significant increases in IL-2, IL-10, MCP-1, and TNF-α in the days post-challenge (Figure 1, all $p < 0.05$). Among uninfected individuals, there were no significant changes in serum cytokine concentrations post-challenge compared to pre-challenge. Based on conditional logistic regression models controlling for age and inoculum, there were no significant differences between infected and uninfected individuals' changes in serum cytokine concentrations between pre-challenge and day 1 post-challenge (data not shown). However, infected individuals were significantly more likely to have increased IFN-γ, IL-6, IL-8, IL-12p70, MCP-1, and TNF-α between pre-challenge and day 2 post-challenge compared to uninfected individuals (Table 3). Infected individuals were also significantly more likely to have increased levels of TNF-α, IL-8, and IL-10 from pre-challenge to day 3 post-challenge (all $p<0.05$; data not shown). At day 4 post-challenge, the only cytokine significantly elevated over pre-challenge levels was IL-10, which was significantly elevated in infected individuals (OR of infection based on a two-fold increase in IL-10 from pre-challenge to day 4 post-challenge=3.92, $p=0.049$; data not shown).

Differences between infected and uninfected individuals over time

Overall trends in serum cytokine response were analyzed using mixed models to account for correlation within subject over time (Figure 2). For infected individuals compared to uninfected individuals, there were statistically significant fold changes across the four days post-challenge from pre-challenge levels in most serum cytokines measured (i.e. IFN-α2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β, Figure 2), with all but TNF-β significantly higher in infected individuals compared to uninfected individuals. All cytokines peaked at day 2 post-challenge among infected subjects, with the exception of IL-1ra, but many were elevated by

day 1 post-challenge (i.e. IL-2, IL-6, IL-8, MCP-1, and TNF-α) or remained elevated through day 4 post-challenge (i.e. IL-10) (Figure 2).

Pre-challenge predictors of infection

To determine if pre-challenge serum cytokine concentrations or early cytokine responses could predict infection status following challenge, the associations were estimated between infection status and three measures of serum cytokines: $\log_{10}$ serum cytokine concentration, $\log_{10}$ difference between pre-challenge and day 1 post-challenge serum cytokine concentrations, and pre-challenge to day 1 post-challenge fold change (i.e. ratio between pre-challenge and day 1 post-challenge concentrations). Across these measures, the only significant predictor of infection status was pre-challenge to day 1 post-challenge $\log_{10}$ change in MCP-1 concentration (data not shown). An increase of 1 $\log_{10}$ over this time period was associated with an OR of infection of 5.74 ($p=0.018$). No other measures of early cytokine response were statistically significant predictors of infection.

Discussion

This study found overall significant elevation in IFN-a2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β in infected subjects compared to uninfected subjects. Among infected individuals, these cytokines peaked on day 2 post-challenge. The results suggest that NoV infection elicits a Th1- and Th2-type response. There also was persistent elevation of IL-10 among infected subjects, suggestive of an evolving early adaptive immune response and down-regulation of Th1 cytokines. This study represents the first comprehensive description of the dynamics of serum cytokines in the acute time period following NoV challenge, including the largest number of cytokines simultaneously described for NoV infection (Table 3).

We found that before challenge, individuals who became infected following NoV challenge were similar to individuals who did not become infected with regard to all 16 cytokines tested. From these similar pre-challenge features, differences began to emerge as early as 24 hours post-challenge and persisted through day 4 post-challenge. As previously documented with human Snow Mountain strain (SMV; GII.2) infection, there was a significant Th1 response (i.e. IFN-γ, IL-2, and TNF-α) with significant elevation of one Th2-related cytokine (Table 3) [13]. In this study, some Th2 and other cytokines (e.g. IL-4, IL-5, IL-6, IL-8, and IL-10) were also significantly elevated following NoV challenge in infected individuals.

The cytokine responses also resembled results from animal studies (Table 3) but differed in the timing and magnitude of cytokine response [19, 20]. These results suggest that the immune response by gnotobiotic animals to human NoV may be substantially different from the response in humans, perhaps due to the gnotobiotic animals' immature immune systems [21] or because of the use of a different NoV inoculum.

Initial response: IL-8, MCP-1

We found that IL-8 levels increased in infected subjects by day 1 post-challenge and remained elevated through day 3 post-challenge. IL-8 elevation has been identified in prior field studies of NoV infection (Table 3) [22, 23]. IL-8 is a powerful chemoattractant for neutrophils [24, 25] and intraepithelial lymphocytes [26, 27], and a key mediator of the immune response to other gastrointestinal pathogens [28]. Histologic findings from previous human NoV challenge studies show increases in granulocyte and monocyte cells in the lamina propria of the small intestine 12-48 hours after challenge in infected subjects [29]. Based on the rise in IL-8 alongside an increase in MCP-1 and a later rise in IL-6, two monocyte chemoattractants, these observations are consistent with neutrophil recruitment acting in concert with monocyte response to NoV infection. Earlier work found that GI and GII NoV infection is associated with elevated fecal MCP-1, suggesting local monocyte activation [23], but this study is the first to show elevated serum MCP-1, suggesting a systemic response as well.

TNF-α and symptoms

In this study, the peak serum TNF-α concentration at 48 hours post-challenge corresponded with the time most NoV-infected individuals were symptomatic. TNF-α increases cellular permeability, leading to edema mucosal damage [30]. Elevated TNF-α has also been associated with NoV infection in past human and animal studies (Table 3) [19, 23]. In some gastrointestinal infections, TNF-α is associated with symptoms [31-33]. The association between TNF-α and NoV symptoms is an important area for future research.

IL-6

IL-6 is a multi-functional cytokine with strong pro-inflammatory effects. It is associated with damage to the intestinal mucosa [29] and increased frequency of diarrhea [22], suggesting a role in pathogenesis and clinical severity of illness. In this study, IL-6 was significantly elevated on day 2 post-challenge. This confirms some clinical studies' identification of the association between IL-6 and NoV infection but contrasts with the results from a SMV challenge study (Table 3) [13, 22, 23] and suggests possible strain-related differences in immune response. This study reported a median maximum serum IL-6 level that was higher than prior studies have identified [22, 34], but this discrepancy may be the result of differences in sample collection.

IL-10 persistently elevated

Though IL-10 peaked on day 2 post-challenge, it was significantly elevated through day 4 post-challenge. This suggests an ongoing role in NoV infection beyond the acute time period. IL-10 is involved in the Th2 response and B-cell development [35], so its elevation may be associated with the development of NoV-specific antibodies, which begin to be detectable around or before day 7 post-challenge [36, 37]. Elevated IL-10 in the context of NoV infection has been described in prior studies of human NoV (Table 3). IL-10 may also play an anti-inflammatory role in down-regulating Th1 cytokine production. The significant elevation of IL-10 in infected subjects following the elevation of Th1 cytokines supports the conclusion that NoV elicits a Th1-type response. Furthermore, IL-10-deficient mice exhibit mucosal inflammation and epithelial barrier dysfunction following MNV-challenge, whereas wildtype mice do not [38]. It is possible that IL-10 plays a similar protective role in NoV-challenged humans.

Strengths and Limitations

This study used rigorously collected human NoV challenge study data, which provided a detailed, longitudinal dataset. It is the largest collection of human NoV challenge subjects yet studied for serum cytokine response. It represents broadest panel of cytokines yet examined at one time for human NoV infection. Some limitations of this study are that it may have been underpowered to detect some changes in serum cytokines, the exposure history of subjects prior to NoV challenge was unknown, and there is the possibility that the duration of sample storage may have caused cytokine degradation [39]. An additional limitation of the study is that though RT-PCR is the gold-standard for NoV testing, there may have been individuals who were misclassified as uninfected because they shed virus at levels below the limit of detection.

Conclusion

Although NoV remains a major cause of morbidity worldwide, some aspects of the immune response continue to be poorly understood. To develop effective vaccines and prophylaxis, it is important to understand the human acute immune response to NoV. This study demonstrated a Th1- and Th2-type response to GI.1 NoV infection, early elevation of chemokines IL-8 and MCP-1, and ongoing elevation of IL-10. This work confirms earlier findings of the increase in MCP-1 associated with NoV infection, suggesting macrophage and dendritic cell involvement in NoV infection. These findings buttress the existing findings from MNV models and enhance the depth of knowledge regarding the human cytokine response to NoV. Differences between the results of this study and those of other human and animal work may be attributable to strain-related variability in immune response. Future work should consider the interplay between the immune response and both clinical outcomes and viral shedding to understand NoV pathogenesis and help develop clinical and public health strategies to reduce NoV transmission.

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Figure Legends

Figure 1: Comparison of pre-challenge to post-challenge serum cytokine concentrations in norovirus-challenged individuals, stratified by infection status (infected n=26, uninfected n=26). Values below the lower limit of detection (LLOD) were assigned to the value of the LLOD. Significance of change from pre-challenge value was tested using Wilcoxon test and is denoted by an asterisk above the relevant category. Interquartile ranges (IQRs) for infected individuals (gray boxes) and for uninfected individuals (white boxes) are shown. Dark lines indicate median values. Whiskers indicate most extreme value that is no more than 1.5 times the IQR away from the bound of the IQR. Circles indicate outliers. *p<0.05, **p<0.01, ***p<0.001

Figure 2. Mixed linear model results for the association between $\log_{10}$ cytokine change from pre-challenge to post-challenge by day, with fixed effects for inoculum dose and day and a random effect by individual subject. Solid line: infected individuals, dashed line: uninfected individuals. Points represent estimates of the effect of day on change in cytokine concentration. Error bars indicate one standard error. P-value indicates significance of overall elevation in cytokine concentration across days.

Figure 1

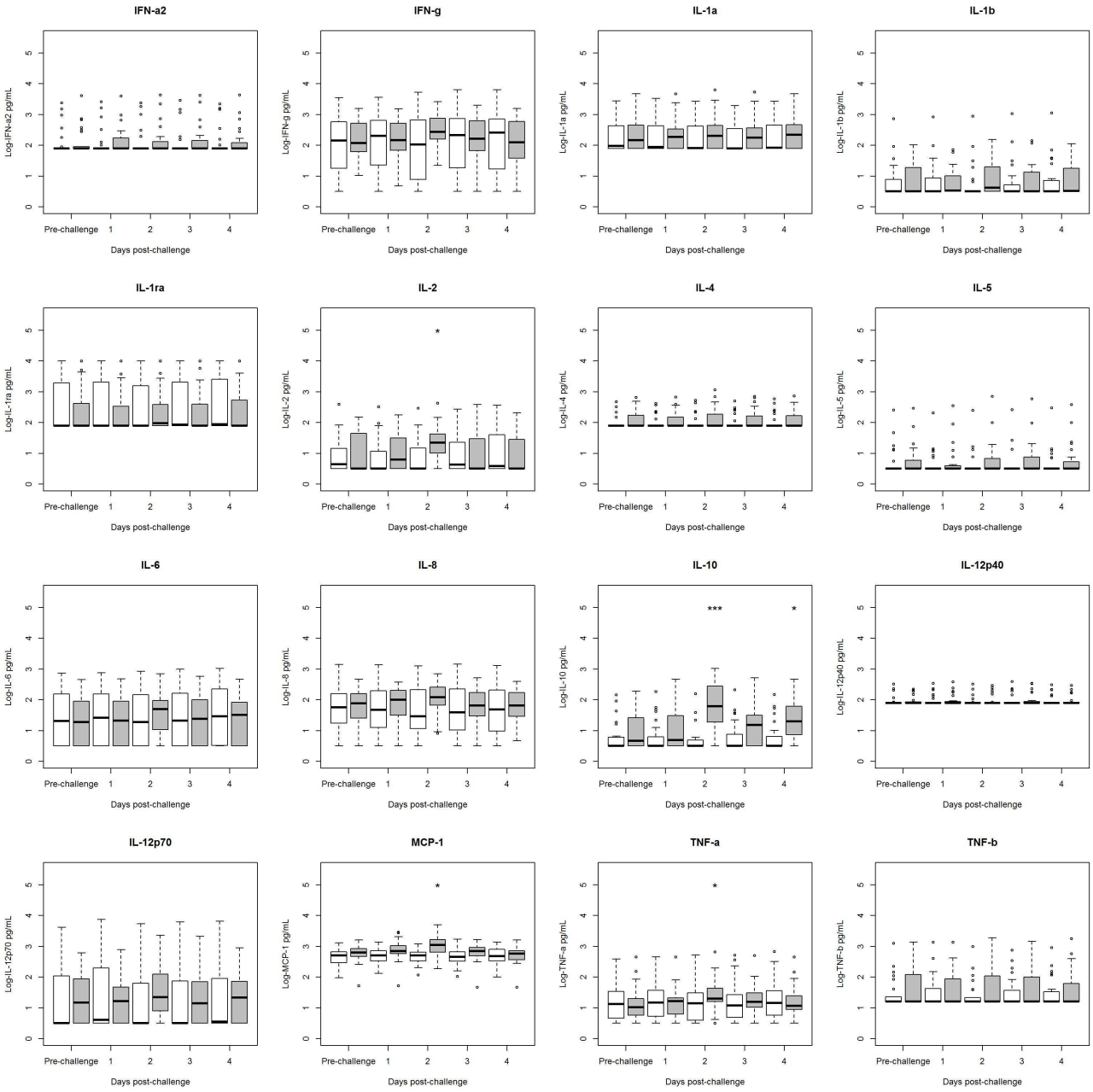

Figure 2

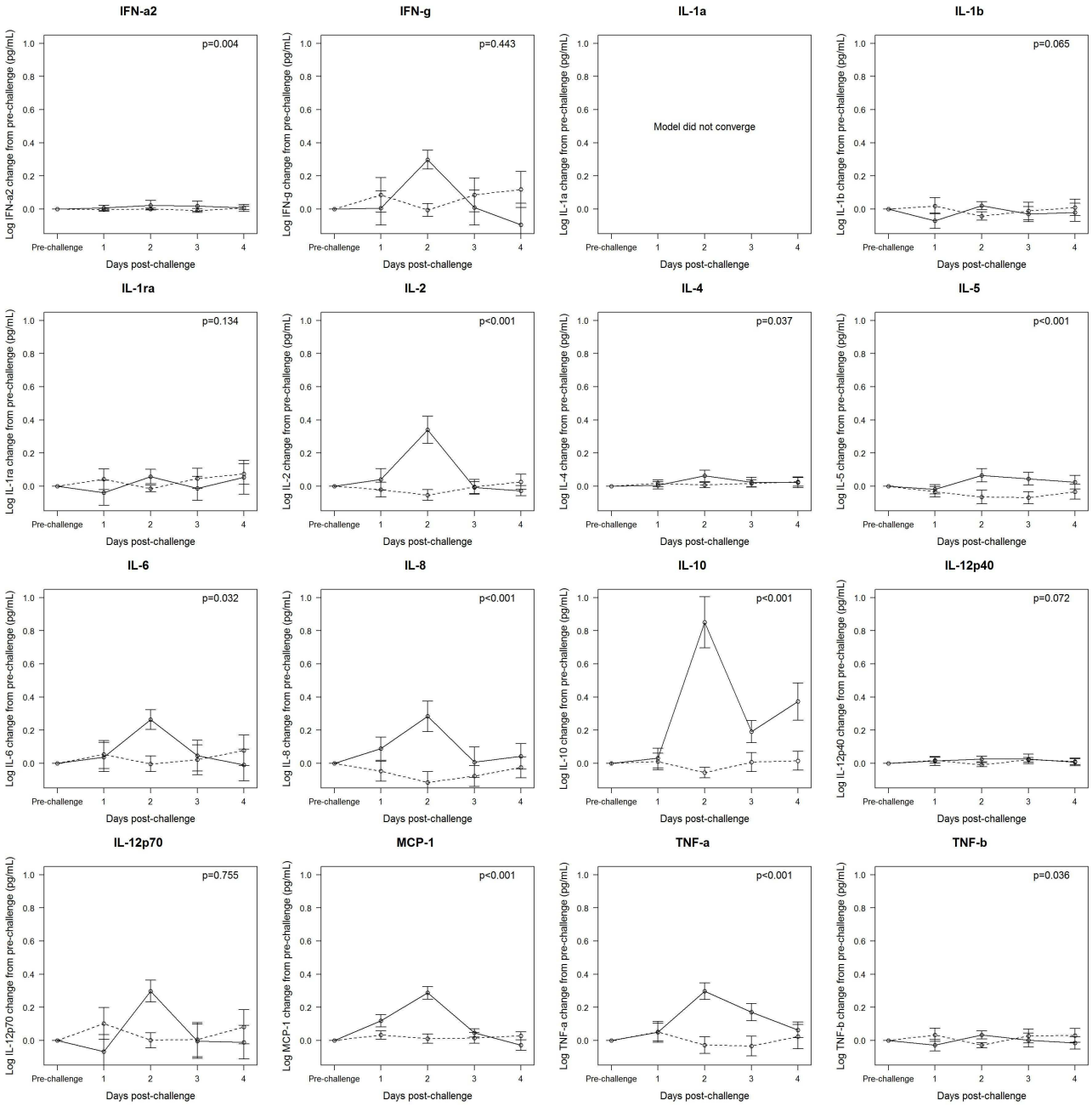

Table 1: Characteristics of norovirus-challenged individuals included in analytic sample

	Total (n=52)	Infected (n=26)	Uninfected (n=26)
Age (mean, SD)	26.7 (7.6)	26.7 (7.5)	26.7 (7.9)
Female	32 (61.5%)	17 (65.4%)	15 (57.7%)
Race			
White	25 (48.1%)	15 (57.7%)	10 (38.5%)
Black	21 (40.4%)	8 (30.8%)	13 (50.0%)
Other	6 (11.5%)	3 (11.5%)	3 (11.5%)
AGE symptoms[a]	19 (33.9%)	17 (65.4%)	2 (7.7%)
Modified Vesikari score (mean, SD)	2.6 (2.4)	4.1 (2.4)	1.1 (0.9)

Abbreviations: SD, standard deviation; AGE, acute gastroenteritis.

[a]AGE symptoms defined as diarrhea (3 or more or >= 400g loose stools in 24 hours) or emesis during days 1-4 post-challenge.

Table 2. Serum cytokine concentrations (pg/mL) of norovirus-challenged individuals pooled across days, stratified by infection status[a].

	All			Infected			Uninfected		
Cytokine	Median	IQR	% above LLOD	Median	IQR	% above LLOD	Median	IQR	% above LLOD
IFN-α2	80.0	80.0-80.0	24.8%	80.0	80.0-133.0	27.9%	80.0	80.0-80.0	21.7%
IFN-γ	186.5	40.4-656.1	90.3%	182.0	67.9-629.3	98.4%	188.3	18.2-669.5	82.2%
IL-1ra	80.0	80.0-999.3	54.3%	80.0	80.0-389.1	73.6%	80.0	80.0-1,993.9	34.9%
IL-1a	163.4	80.0-433.2	24.7%	179.8	80.0-433.2	28.5%	80.2	80.0-433.55	21.1%
IL-1b	3.2	3.2-10.3	58.9%	3.3	3.2-18.45	70.2%	3.2	3.2-7.2	48.1%
IL-2	4.6	3.2-30.1	47.8%	7.5	3.2-38.2	48.0%	3.6	3.2-17.6	47.6%
IL-4	80.0	80.0-99.9	54.7%	80.0	80.0-170.9	59.4%	80.0	80.0-80.0	50.0%
IL-5	3.2	3.2-3.9	38.4%	3.2	3.2-6.0	48.1%	3.2	3.2-3.2	28.7%
IL-6	23.9	3.2-120.2	52.5%	27.8	3.2-93.0	55.5%	21.4	3.2-161.1	49.6%
IL-8	66.5	20.7-198.1	25.4%	81.7	34.2-198.0	28.9%	45.0	11.5-202.4	21.9%
IL-10	4.4	3.2-29.5	26.8%	17.0	3.2-50.3	33.9%	3.2	3.2-6.2	19.7%
IL-12p40	80.0	80.0-80.0	70.7%	80.0	80.0-85.0	69.5%	80.0	80.0-80.0	71.9%
IL-12p70	6.1	3.2-89.8	92.2%	19.5	3.2-86.6	96.9%	3.2	3.2-96.0	87.6%
MCP-1	612.8	397.1-832.5	100.0%	693.5	510.8-944.3	100.0%	495.1	329.4-673.1	100.0%
TNF-α	14.3	6.0-27.2	85.3%	15.1	8.3-25.2	87.6%	14.0	4.9-36.2	82.9%
TNF-β	16.0	16.0-58.5	37.2%	16.0	16.0-100.5	35.7%	16.0	16.0-33.4	38.8%

Abbreviations: IQR, inter-quartile range; LLOD, lower limit of detection; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

[a]Values below LLOD included in estimates as LLOD value.

Table 3. Table comparing results from this study to prior studies of cytokine responses to human NoV infection. An 'x' indicates significant elevation compared to uninfected controls. Gray cells indicate no testing was conducted for that cytokine.

Study	This study[a]	Lindesmith 2005 [12]	Long 2011 [22]	Chen 2012 [37]	Souza 2008 [18]	Souza 2007 [19]
NoV genogroup	GI.1	GII.2	Mixed	Mixed	GII.4	GII.4
Subjects	Human	Human	Human	Human	Gn Calf	Gn Pig
Study design	Challenge	Challenge	Observational cohort	Cross-sectional	Challenge	Challenge
Cytokine						
IFN-α						x
IFN-α2	ns					
IFN-γ	x	x	ns		x[b]	x
IL-1ra	ns					
IL-1a	ns					
IL-1b	ns					
IL-2	x	x				
IL-4	ns	ns	ns		x[b]	x
IL-5	ns	x	x			
IL-6	x		ns	x		x
IL-8	x		x	x		
IL-10	x	ns	ns		x[b]	x
IL-12					x[b]	x
IL-12p40	ns					
IL-12p70	x					
MCP-1	x		x			
TNF-α	x	ns	ns		x[b]	
TNF-β	ns					

Abbreviations: ns, not significant; NoV, norovirus; Gn, gnotobiotic.

[a]Modeled using conditional logistic regression conditional on inoculum dose and adjusted for age. [b]Cytokine concentrations were elevated compared to controls but no statistical testing was conducted.

Human Norovirus Infection and the Acute Serum Cytokine Response

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Abbreviations: NoV, norovirus; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; SMV, Snow Mountain virus; GEC, genomic equivalence copies; RT-PCR, reverse transcription polymerase chain reaction; LLOD, lower limit of detection

Keywords: Caliciviruses, innate immunity, adaptive immunity

Summary

Noroviruses (NoV) are the most common cause of epidemic gastroenteritis worldwide. The acute immune response to NoV in humans is poorly understood, hindering research on prevention and treatment. To elucidate the acute immune response and test for cytokine predictors of susceptibility to infection, serum samples from two human NoV challenge studies were tested for 16 cytokines. Subjects who became infected (n=26) were age-matched with subjects who remained uninfected following NoV challenge (n=26). Samples were tested from pre-challenge and days 1-4 post-challenge. Cytokine responses were compared between infected and uninfected groups. Overall, infected individuals exhibited an elevation in Th1 and Th2 cytokines, as well as chemokines IL-8 and MCP-1, compared to uninfected individuals (all $p<0.05$). Most cytokines peaked on day 2 post-challenge in infected subjects, and TNF-α, IL-8, and IL-10 remained elevated through day 3. The only cytokine significantly elevated among infected subjects through day 4 post-challenge was IL-10 ($p=0.021$). Pre-challenge cytokine concentrations were not predictive of infection status post-challenge. There were no significant changes in serum cytokines among NoV-challenged subjects who remained uninfected. These results suggest that NoV infection elicits a Th1 type response with some Th2 activation. Persistent elevation of IL-10 among infected subjects is consistent with activation of adaptive immune responses, such as B-cell expansion, as well as down-regulation of Th1 cytokines. This study presents the first comprehensive description of the acute cytokine response to GI.1 NoV in humans.

Introduction

Noroviruses (NoV) comprise seven genogroups in the positive-sense RNA virus family *Caliciviridae* [1, 2]. NoV are responsible for 18% of gastroenteritis worldwide [3]. Despite this broad impact, human NoV immunology is poorly understood [4, 5], in part because until recently, there was no small animal model or reliable cell culture system for human NoV [6, 7]. Furthermore, there are few prospective experimental studies on the acute human innate and cellular immune response to NoV.

The genetic determinants of NoV infection (e.g. secretor blood group antigens) are relatively well characterized, but many aspects of protective innate and cellular immunity have not been described [8, 9]. Histologic studies [4, 10-12] and in vitro tests of peripheral blood mono-nuclear cells [13] suggest cytotoxic and CD8+ T-cell activation with some cells sensitized to NoV challenge antigens, but little work has been done in vivo to describe the cytokine response to NoV infection. In particular, there is a gap in understanding the acute immune response to NoV. Prior studies have shown that adaptive immunity is critical for clearing the infection [14], but there is a gap in the knowledge about early pathogenesis of NoV infection. Key cytokines for consideration include acute response-related pro-inflammatory cytokines (e.g. IL-1, IL-6, and IL-12), chemokines involved in neutrophil and monocyte recruitment (e.g. IL-8 and MCP-1), and Th1- and Th2-related cytokines (e.g. IFN-γ, IL-2, and TNF-α and IL-4, IL-5, and IL-10, respectively) involved in the cellular immune response and initiation of adaptive immune mechanisms.

The goal of this study was two-fold. The first goal was to describe serum cytokine responses to NoV infection, including the temporal trends in serum cytokines during the acute phase of human NoV infection. The second goal was to use cytokine concentrations pre-

challenge to predict infection post-challenge. Human serum samples from two prior NoV challenge studies that used the same Norwalk virus (GI.1) inoculum were analyzed for a broad panel of relevant cytokines [15, 16]. The results have important implications for determining the role of specific cytokines in NoV infection.

Materials and methods

Population and samples

The samples tested for this study were collected from subjects involved in two separate NoV challenge studies, described previously [15, 16]. However, these studies could used the same inoculum preparation, were conducted at the same institution, and used very similar protocols. Key differences between these two challenge studies (i.e. inoculum dose) were accounted for in the modeling strategy of this study. Briefly, the first study enrolled healthy secretor-positive adult volunteers, who were challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site, part of the Atlanta Clinical and Translational Science Institute Clinical Interaction Network, between May 2006 and December 2006. Volunteers ingested filtered groundwater artificially seeded with 6.5×10^7 genomic equivalent copies (GEC) of NoV inoculum, which had been incubated at room temperature in the dark for different set lengths of time. Prior to challenge, serum and stool samples were collected. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The second study enrolled healthy secretor-positive adult volunteers, who were also challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site between February 2008 and September 2009. They were randomized into control and intervention groups and administered oysters seeded with 1×10^4 GEC of virus, which had been treated with high hydrostatic pressure processing for 5 minutes (intervention) or left untreated (control). Pre-challenge stool and serum samples were collected before challenge. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The Emory University Institutional Review Board approved both studies, and both were registered on Clinical Trials.gov (identifiers NCT00313404 and NCT00674336). All subjects consented to the future use of all biological specimens from the studies. All specimens were stored at -80°C.

Of the initial participants in both NoV challenge studies, a total of 26 became infected as defined by a NoV-positive stool or emesis sample tested by RT-PCR (limit of detection: 3,570 GEC/g stool) [15, 17]. These 26 were pair-matched by age to 26 of the challenge study participants who remained uninfected following NoV challenge. Some infected subjects from one challenge group were matched with uninfected subjects from a different challenge group because within the same group there were no uninfected individuals within 3 years of age of the infected individual. The total sample size for this study was 52 participants (13 from the 2006 trial, 39 from the 2008-2009 trial). Because the focus of this study is the acute immune response, only serum samples from pre-challenge and days 1-4 post-challenge were included, for a total of five longitudinal samples per subject.

Inoculum

The two NoV challenge studies included participants who ingested water or shellfish seeded with 8FIIb NoV inoculum. The water samples were seeded with identical amounts of NoV and stored at room temperature in the dark for varying lengths of time. They showed no evidence of titer attenuation over time, as measured by RT-PCR [15]. Therefore, for these subjects, the regression models did not control for study arm. The shellfish samples were seeded with identical amounts of NoV and treated under varying processing conditions. The inocula showed some evidence of attenuation for some treatments. For subjects from this study, all adjusted models controlled for study arm as a surrogate for dose.

Detection of viral shedding

NoV presence was measured by RT-PCR for all stool and emesis samples at all time points during the original challenge studies. A subject with a positive sample at any time point, even after day 4 post- exposure, as determined in the original or subsequent studies, was defined as infected.

Detection of cytokines

Serum samples from pre-challenge (pre-challenge), and days 1-4 post-challenge (five total samples) were analyzed from all study subjects. Serum was collected during the studies, processed, and stored at -80°C until testing. Samples were tested by a commercial laboratory (EMD Millipore Corporation Discovery and Development Solutions) using a Milliplex human cytokine 16-plex assay for IFN-α2, IFN-γ, IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, MCP-1, TNF-α, and TNF-β. Samples were run in duplicate with controls. Standard curves were generated using recombinant cytokines and calculated using a five parameter logistic model for each cytokine. Lower limits of detection (LLOD) were 3.2 pg/mL for IFN-γ, IL-10, IL-12p70, IL-1b, IL-2, IL-5, IL-6, IL-8, MCP-1, and TNF-α; 16.0 pg/mL for TNF-β; and 80.0 pg/mL for IFN-α2, IL-12p40, IL-1ra, IL-1a, and IL-4. Values below LLOD were assigned the value of the LLOD.

Statistical analysis

The data were analyzed using SAS 9.4 (Cary, NC, USA). When possible, age and inoculum were controlled for in adjusted analyses. Because the exact amount of infectious NoV in the inocula for study arms with attenuation was unknown, inoculum was controlled for using a categorical variable for each study and study arm. The Wilcoxon signed rank test was to test for

the significance of the unadjusted difference between pre-challenge and post-challenge serum cytokine levels. Conditional logistic regression was used to evaluate the association between pre-challenge and post-challenge fold changes in serum cytokine levels (i.e. ratio between post-challenge and pre-challenge cytokine concentrations), controlling for inoculum and age. To summarize the differences between infected and uninfected individuals responses over time while accounting for correlation between time points, a mixed linear model was used to test the association between $\log_{10}$ cytokine concentration and day post-challenge, stratified by infection status with a random effect by subject, autoregressive correlation between different time points taken for the same individual, and fixed effect for inoculum. The parameter estimates for the effect of each day on $\log_{10}$ cytokine concentration for each stratum were averaged and Student's t-test was used to compare infected and uninfected subjects cytokine responses over time. Conditional logistic regression adjusted for inoculum and age was also used to assess whether three measures of early cytokine levels or responses could be used as predictors of post-challenge infection. The measures assessed were pre-challenge $\log_{10}$ cytokine concentration, pre-challenge to day 1 post-challenge $\log_{10}$ cytokine change, and pre-challenge to day 1 post-challenge fold change. All cytokines were modeled individually. Differences between groups were considered statistically significant at $p < 0.05$.

Results

To determine the acute serum cytokine response to GI.1 NoV infection, serum cytokine responses in individuals who became infected following experimental challenge were compared to those who remained uninfected after challenge, as determined by RT-PCR detection of GI.1 NoV RNA in stool or emesis samples. Immune responses may differ with age, therefore the infected and uninfected subjects were matched for age (Table 1). The study population was relatively young, with a mean age of 26.7 years and a median age of 25 years. There was also a similar distribution of sex and race between infected and uninfected subjects selected for the study, though neither was a matching factor. Some uninfected subjects exhibited symptoms, including fever and diarrhea, which is consistent with past NoV challenge studies [16]. All infected individuals had higher cumulative shedding than the inoculum dose (data not shown), reducing the likelihood of misclassification of infection status because of detection of inoculum post-challenge in the absence of infection. Serum samples were available for pre-challenge through day 4 post-challenge (five time points) and all samples were tested for 16 serum cytokines. Two subjects did not have samples for day 3 and values for these samples were imputed using the average of neighboring observations for the same subject. A total of 10 subjects had invalid test results for one or more cytokine tested (29 total observations). Using imputation, all but one of these invalid results was estimated to be below the LLOD.

Studies of serum cytokine levels in humans often have high rates of observations below the LLOD [18]. Overall of the 16 cytokines tested, nine had greater than 50% of observations above the LLOD, and four had greater than 75% of observations above the LLOD (Table 2). In general, infected subjects had more samples above the LLOD.

To assess whether serum cytokines exhibited a change in concentration following NoV

challenge, the Wilcoxon signed rank test was used, stratifying by infection status. Among infected individuals, there were significant increases in IL-2, IL-10, MCP-1, and TNF-α in the days post-challenge (Figure 1, all $p < 0.05$). Among uninfected individuals, there were no significant changes in serum cytokine concentrations post-challenge compared to pre-challenge. Based on conditional logistic regression models controlling for age and inoculum, there were no significant differences between infected and uninfected individuals' changes in serum cytokine concentrations between pre-challenge and day 1 post-challenge (data not shown). However, infected individuals were significantly more likely to have increased IFN-γ, IL-6, IL-8, IL-12p70, MCP-1, and TNF-α between pre-challenge and day 2 post-challenge compared to uninfected individuals (Table 3). Infected individuals were also significantly more likely to have increased levels of TNF-α, IL-8, and IL-10 from pre-challenge to day 3 post-challenge (all $p<0.05$; data not shown). At day 4 post-challenge, the only cytokine significantly elevated over pre-challenge levels was IL-10, which was significantly elevated in infected individuals (OR of infection based on a two-fold increase in IL-10 from pre-challenge to day 4 post-challenge=3.92, $p=0.049$; data not shown).

Differences between infected and uninfected individuals over time

Overall trends in serum cytokine response were analyzed using mixed models to account for correlation within subject over time (Figure 2). For infected individuals compared to uninfected individuals, there were statistically significant fold changes across the four days post-challenge from pre-challenge levels in most serum cytokines measured (i.e. IFN-α2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β, Figure 2), with all but TNF-β significantly higher in infected individuals compared to uninfected individuals. All cytokines peaked at day 2 post-challenge among infected subjects, with the exception of IL-1ra, but many were elevated by

day 1 post-challenge (i.e. IL-2, IL-6, IL-8, MCP-1, and TNF-α) or remained elevated through day 4 post-challenge (i.e. IL-10) (Figure 2).

Pre-challenge predictors of infection

To determine if pre-challenge serum cytokine concentrations or early cytokine responses could predict infection status following challenge, the associations were estimated between infection status and three measures of serum cytokines: $\log_{10}$ serum cytokine concentration, $\log_{10}$ difference between pre-challenge and day 1 post-challenge serum cytokine concentrations, and pre-challenge to day 1 post-challenge fold change (i.e. ratio between pre-challenge and day 1 post-challenge concentrations). Across these measures, the only significant predictor of infection status was pre-challenge to day 1 post-challenge $\log_{10}$ change in MCP-1 concentration (data not shown). An increase of 1 $\log_{10}$ over this time period was associated with an OR of infection of 5.74 ($p=0.018$). No other measures of early cytokine response were statistically significant predictors of infection.

Discussion

This study found overall significant elevation in IFN-a2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β in infected subjects compared to uninfected subjects. Among infected individuals, these cytokines peaked on day 2 post-challenge. The results suggest that NoV infection elicits a Th1- and Th2-type response. There also was persistent elevation of IL-10 among infected subjects, suggestive of an evolving early adaptive immune response and down-regulation of Th1 cytokines. This study represents the first comprehensive description of the dynamics of serum cytokines in the acute time period following NoV challenge, including the largest number of cytokines simultaneously described for NoV infection (Table 3).

We found that before challenge, individuals who became infected following NoV challenge were similar to individuals who did not become infected with regard to all 16 cytokines tested. From these similar pre-challenge features, differences began to emerge as early as 24 hours post-challenge and persisted through day 4 post-challenge. As previously documented with human Snow Mountain strain (SMV; GII.2) infection, there was a significant Th1 response (i.e. IFN-γ, IL-2, and TNF-α) with significant elevation of one Th2-related cytokine (Table 3) [13]. In this study, some Th2 and other cytokines (e.g. IL-4, IL-5, IL-6, IL-8, and IL-10) were also significantly elevated following NoV challenge in infected individuals.

The cytokine responses also resembled results from animal studies (Table 3) but differed in the timing and magnitude of cytokine response [19, 20]. These results suggest that the immune response by gnotobiotic animals to human NoV may be substantially different from the response in humans, perhaps due to the gnotobiotic animals' immature immune systems [21] or because of the use of a different NoV inoculum.

Initial response: IL-8, MCP-1

We found that IL-8 levels increased in infected subjects by day 1 post-challenge and remained elevated through day 3 post-challenge. IL-8 elevation has been identified in prior field studies of NoV infection (Table 3) [22, 23]. IL-8 is a powerful chemoattractant for neutrophils [24, 25] and intraepithelial lymphocytes [26, 27], and a key mediator of the immune response to other gastrointestinal pathogens [28]. Histologic findings from previous human NoV challenge studies show increases in granulocyte and monocyte cells in the lamina propria of the small intestine 12-48 hours after challenge in infected subjects [29]. Based on the rise in IL-8 alongside an increase in MCP-1 and a later rise in IL-6, two monocyte chemoattractants, these observations are consistent with neutrophil recruitment acting in concert with monocyte response to NoV infection. Earlier work found that GI and GII NoV infection is associated with elevated fecal MCP-1, suggesting local monocyte activation [23], but this study is the first to show elevated serum MCP-1, suggesting a systemic response as well.

TNF-α and symptoms

In this study, the peak serum TNF-α concentration at 48 hours post-challenge corresponded with the time most NoV-infected individuals were symptomatic. TNF-α increases cellular permeability, leading to edema mucosal damage [30]. Elevated TNF-α has also been associated with NoV infection in past human and animal studies (Table 3) [19, 23]. In some gastrointestinal infections, TNF-α is associated with symptoms [31-33]. The association between TNF-α and NoV symptoms is an important area for future research.

IL-6

IL-6 is a multi-functional cytokine with strong pro-inflammatory effects. It is associated with damage to the intestinal mucosa [29] and increased frequency of diarrhea [22], suggesting a role in pathogenesis and clinical severity of illness. In this study, IL-6 was significantly elevated on day 2 post-challenge. This confirms some clinical studies' identification of the association between IL-6 and NoV infection but contrasts with the results from a SMV challenge study (Table 3) [13, 22, 23] and suggests possible strain-related differences in immune response. This study reported a median maximum serum IL-6 level that was higher than prior studies have identified [22, 34], but this discrepancy may be the result of differences in sample collection.

IL-10 persistently elevated

Though IL-10 peaked on day 2 post-challenge, it was significantly elevated through day 4 post-challenge. This suggests an ongoing role in NoV infection beyond the acute time period. IL-10 is involved in the Th2 response and B-cell development [35], so its elevation may be associated with the development of NoV-specific antibodies, which begin to be detectable around or before day 7 post-challenge [36, 37]. Elevated IL-10 in the context of NoV infection has been described in prior studies of human NoV (Table 3). IL-10 may also play an anti-inflammatory role in down-regulating Th1 cytokine production. The significant elevation of IL-10 in infected subjects following the elevation of Th1 cytokines supports the conclusion that NoV elicits a Th1-type response. Furthermore, IL-10-deficient mice exhibit mucosal inflammation and epithelial barrier dysfunction following MNV-challenge, whereas wildtype mice do not [38]. It is possible that IL-10 plays a similar protective role in NoV-challenged humans.

Strengths and Limitations

This study used rigorously collected human NoV challenge study data, which provided a detailed, longitudinal dataset. It is the largest collection of human NoV challenge subjects yet studied for serum cytokine response. It represents broadest panel of cytokines yet examined at one time for human NoV infection. Some limitations of this study are that it may have been underpowered to detect some changes in serum cytokines, the exposure history of subjects prior to NoV challenge was unknown, and there is the possibility that the duration of sample storage may have caused cytokine degradation [39]. An additional limitation of the study is that though RT-PCR is the gold-standard for NoV testing, there may have been individuals who were misclassified as uninfected because they shed virus at levels below the limit of detection.

Conclusion

Although NoV remains a major cause of morbidity worldwide, some aspects of the immune response continue to be poorly understood. To develop effective vaccines and prophylaxis, it is important to understand the human acute immune response to NoV. This study demonstrated a Th1- and Th2-type response to GI.1 NoV infection, early elevation of chemokines IL-8 and MCP-1, and ongoing elevation of IL-10. This work confirms earlier findings of the increase in MCP-1 associated with NoV infection, suggesting macrophage and dendritic cell involvement in NoV infection. These findings buttress the existing findings from MNV models and enhance the depth of knowledge regarding the human cytokine response to NoV. Differences between the results of this study and those of other human and animal work may be attributable to strain-related variability in immune response. Future work should consider the interplay between the immune response and both clinical outcomes and viral shedding to understand NoV pathogenesis and help develop clinical and public health strategies to reduce NoV transmission.

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Figure Legends

Figure 1: Comparison of pre-challenge to post-challenge serum cytokine concentrations in norovirus-challenged individuals, stratified by infection status (infected n=26, uninfected n=26). Values below the lower limit of detection (LLOD) were assigned to the value of the LLOD. Significance of change from pre-challenge value was tested using Wilcoxon test and is denoted by an asterisk above the relevant category. Interquartile ranges (IQRs) for infected individuals (gray boxes) and for uninfected individuals (white boxes) are shown. Dark lines indicate median values. Whiskers indicate most extreme value that is no more than 1.5 times the IQR away from the bound of the IQR. Circles indicate outliers. *p<0.05, **p<0.01, ***p<0.001

Figure 2. Mixed linear model results for the association between $\log_{10}$ cytokine change from pre-challenge to post-challenge by day, with fixed effects for inoculum dose and day and a random effect by individual subject. Solid line: infected individuals, dashed line: uninfected individuals. Points represent estimates of the effect of day on change in cytokine concentration. Error bars indicate one standard error. P-value indicates significance of overall elevation in cytokine concentration across days.

Figure 1

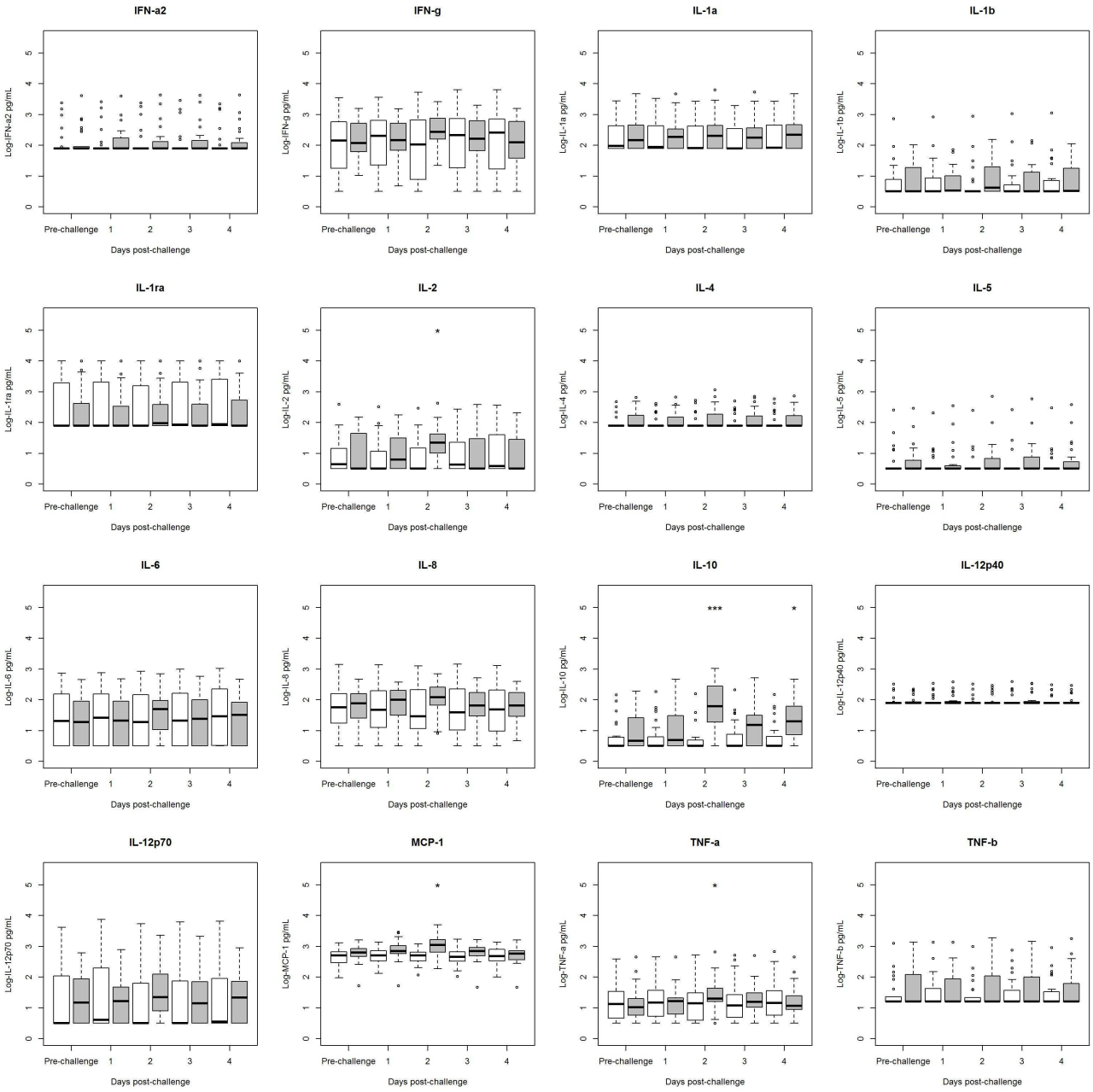

Figure 2

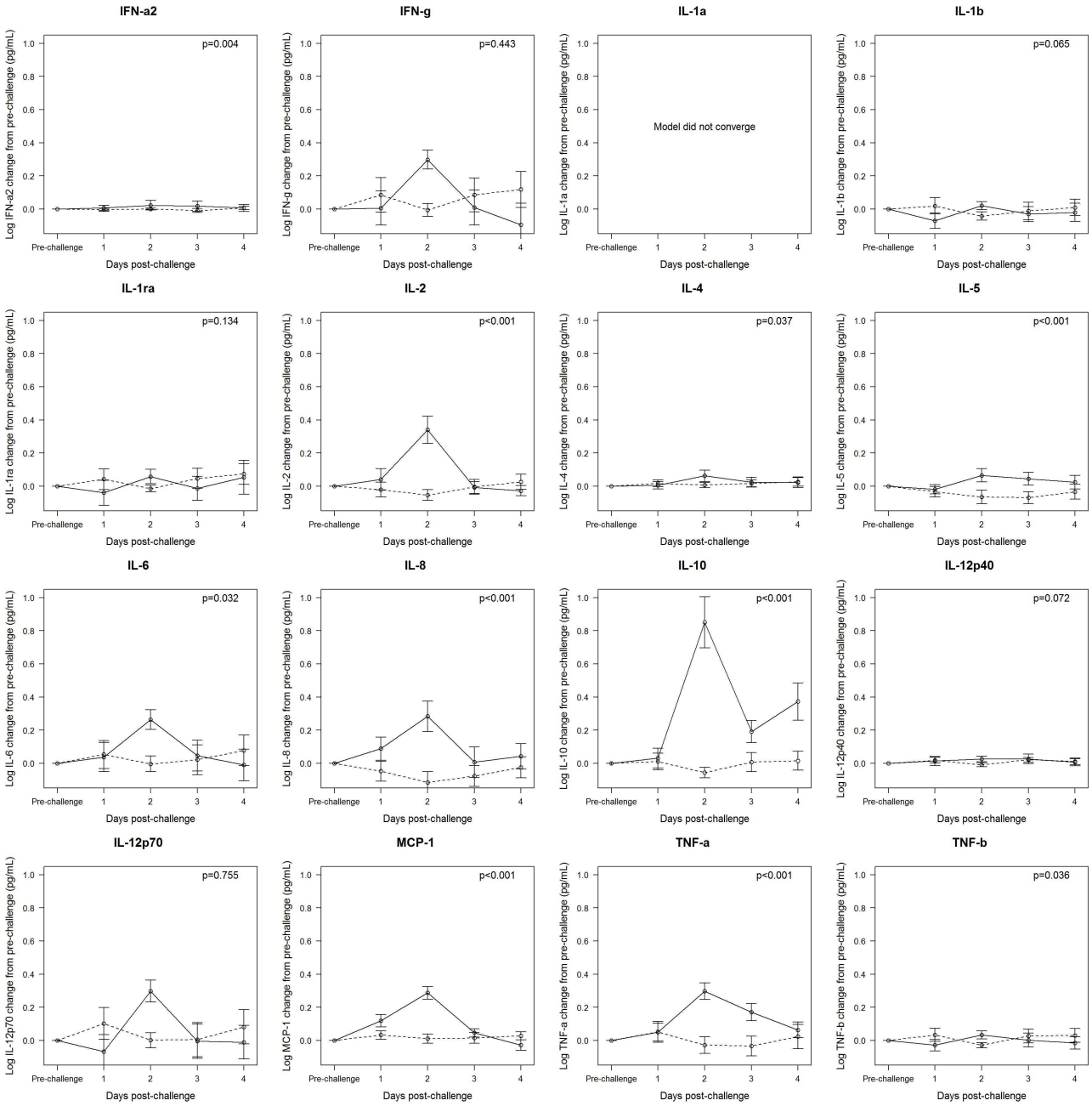

Table 1: Characteristics of norovirus-challenged individuals included in analytic sample

	Total (n=52)	Infected (n=26)	Uninfected (n=26)
Age (mean, SD)	26.7 (7.6)	26.7 (7.5)	26.7 (7.9)
Female	32 (61.5%)	17 (65.4%)	15 (57.7%)
Race			
White	25 (48.1%)	15 (57.7%)	10 (38.5%)
Black	21 (40.4%)	8 (30.8%)	13 (50.0%)
Other	6 (11.5%)	3 (11.5%)	3 (11.5%)
AGE symptoms[a]	19 (33.9%)	17 (65.4%)	2 (7.7%)
Modified Vesikari score (mean, SD)	2.6 (2.4)	4.1 (2.4)	1.1 (0.9)

Abbreviations: SD, standard deviation; AGE, acute gastroenteritis.

[a]AGE symptoms defined as diarrhea (3 or more or >= 400g loose stools in 24 hours) or emesis during days 1-4 post-challenge.

Table 2. Serum cytokine concentrations (pg/mL) of norovirus-challenged individuals pooled across days, stratified by infection status[a].

	All			Infected			Uninfected		
Cytokine	Median	IQR	% above LLOD	Median	IQR	% above LLOD	Median	IQR	% above LLOD
IFN-α2	80.0	80.0-80.0	24.8%	80.0	80.0-133.0	27.9%	80.0	80.0-80.0	21.7%
IFN-γ	186.5	40.4-656.1	90.3%	182.0	67.9-629.3	98.4%	188.3	18.2-669.5	82.2%
IL-1ra	80.0	80.0-999.3	54.3%	80.0	80.0-389.1	73.6%	80.0	80.0-1,993.9	34.9%
IL-1a	163.4	80.0-433.2	24.7%	179.8	80.0-433.2	28.5%	80.2	80.0-433.55	21.1%
IL-1b	3.2	3.2-10.3	58.9%	3.3	3.2-18.45	70.2%	3.2	3.2-7.2	48.1%
IL-2	4.6	3.2-30.1	47.8%	7.5	3.2-38.2	48.0%	3.6	3.2-17.6	47.6%
IL-4	80.0	80.0-99.9	54.7%	80.0	80.0-170.9	59.4%	80.0	80.0-80.0	50.0%
IL-5	3.2	3.2-3.9	38.4%	3.2	3.2-6.0	48.1%	3.2	3.2-3.2	28.7%
IL-6	23.9	3.2-120.2	52.5%	27.8	3.2-93.0	55.5%	21.4	3.2-161.1	49.6%
IL-8	66.5	20.7-198.1	25.4%	81.7	34.2-198.0	28.9%	45.0	11.5-202.4	21.9%
IL-10	4.4	3.2-29.5	26.8%	17.0	3.2-50.3	33.9%	3.2	3.2-6.2	19.7%
IL-12p40	80.0	80.0-80.0	70.7%	80.0	80.0-85.0	69.5%	80.0	80.0-80.0	71.9%
IL-12p70	6.1	3.2-89.8	92.2%	19.5	3.2-86.6	96.9%	3.2	3.2-96.0	87.6%
MCP-1	612.8	397.1-832.5	100.0%	693.5	510.8-944.3	100.0%	495.1	329.4-673.1	100.0%
TNF-α	14.3	6.0-27.2	85.3%	15.1	8.3-25.2	87.6%	14.0	4.9-36.2	82.9%
TNF-β	16.0	16.0-58.5	37.2%	16.0	16.0-100.5	35.7%	16.0	16.0-33.4	38.8%

Abbreviations: IQR, inter-quartile range; LLOD, lower limit of detection; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

[a]Values below LLOD included in estimates as LLOD value.

Table 3. Table comparing results from this study to prior studies of cytokine responses to human NoV infection. An 'x' indicates significant elevation compared to uninfected controls. Gray cells indicate no testing was conducted for that cytokine.

Study	This study[a]	Lindesmith 2005 [12]	Long 2011 [22]	Chen 2012 [37]	Souza 2008 [18]	Souza 2007 [19]
NoV genogroup	GI.1	GII.2	Mixed	Mixed	GII.4	GII.4
Subjects	Human	Human	Human	Human	Gn Calf	Gn Pig
Study design	Challenge	Challenge	Observational cohort	Cross-sectional	Challenge	Challenge
Cytokine						
IFN-α						x
IFN-α2	ns					
IFN-γ	x	x	ns		x[b]	x
IL-1ra	ns					
IL-1a	ns					
IL-1b	ns					
IL-2	x	x				
IL-4	ns	ns	ns		x[b]	x
IL-5	ns	x	x			
IL-6	x		ns	x		x
IL-8	x		x	x		
IL-10	x	ns	ns		x[b]	x
IL-12					x[b]	x
IL-12p40	ns					
IL-12p70	x					
MCP-1	x		x			
TNF-α	x	ns	ns		x[b]	
TNF-β	ns					

Abbreviations: ns, not significant; NoV, norovirus; Gn, gnotobiotic.

[a]Modeled using conditional logistic regression conditional on inoculum dose and adjusted for age. [b]Cytokine concentrations were elevated compared to controls but no statistical testing was conducted.

Human Norovirus Infection and the Acute Serum Cytokine Response

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Running Head: Human Cytokine Response to Norovirus

Abbreviations: NoV, norovirus; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; SMV, Snow Mountain virus; GEC, genomic equivalence copies; RT-PCR, reverse transcription polymerase chain reaction; LLOD, lower limit of detection

Keywords: Caliciviruses, innate immunity, adaptive immunity

Summary

Noroviruses (NoV) are the most common cause of epidemic gastroenteritis worldwide. The acute immune response to NoV in humans is poorly understood, hindering research on prevention and treatment. To elucidate the acute immune response and test for cytokine predictors of susceptibility to infection, serum samples from two human NoV challenge studies were tested for 16 cytokines. Subjects who became infected (n=26) were age-matched with subjects who remained uninfected following NoV challenge (n=26). Samples were tested from pre-challenge and days 1-4 post-challenge. Cytokine responses were compared between infected and uninfected groups. Overall, infected individuals exhibited an elevation in Th1 and Th2 cytokines, as well as chemokines IL-8 and MCP-1, compared to uninfected individuals (all $p<0.05$). Most cytokines peaked on day 2 post-challenge in infected subjects, and TNF-α, IL-8, and IL-10 remained elevated through day 3. The only cytokine significantly elevated among infected subjects through day 4 post-challenge was IL-10 ($p=0.021$). Pre-challenge cytokine concentrations were not predictive of infection status post-challenge. There were no significant changes in serum cytokines among NoV-challenged subjects who remained uninfected. These results suggest that NoV infection elicits a Th1 type response with some Th2 activation. Persistent elevation of IL-10 among infected subjects is consistent with activation of adaptive immune responses, such as B-cell expansion, as well as down-regulation of Th1 cytokines. This study presents the first comprehensive description of the acute cytokine response to GI.1 NoV in humans.

Introduction

Noroviruses (NoV) comprise seven genogroups in the positive-sense RNA virus family *Caliciviridae* [1, 2]. NoV are responsible for 18% of gastroenteritis worldwide [3]. Despite this broad impact, human NoV immunology is poorly understood [4, 5], in part because until recently, there was no small animal model or reliable cell culture system for human NoV [6, 7]. Furthermore, there are few prospective experimental studies on the acute human innate and cellular immune response to NoV.

The genetic determinants of NoV infection (e.g. secretor blood group antigens) are relatively well characterized, but many aspects of protective innate and cellular immunity have not been described [8, 9]. Histologic studies [4, 10-12] and in vitro tests of peripheral blood mono-nuclear cells [13] suggest cytotoxic and CD8+ T-cell activation with some cells sensitized to NoV challenge antigens, but little work has been done in vivo to describe the cytokine response to NoV infection. In particular, there is a gap in understanding the acute immune response to NoV. Prior studies have shown that adaptive immunity is critical for clearing the infection [14], but there is a gap in the knowledge about early pathogenesis of NoV infection. Key cytokines for consideration include acute response-related pro-inflammatory cytokines (e.g. IL-1, IL-6, and IL-12), chemokines involved in neutrophil and monocyte recruitment (e.g. IL-8 and MCP-1), and Th1- and Th2-related cytokines (e.g. IFN-γ, IL-2, and TNF-α and IL-4, IL-5, and IL-10, respectively) involved in the cellular immune response and initiation of adaptive immune mechanisms.

The goal of this study was two-fold. The first goal was to describe serum cytokine responses to NoV infection, including the temporal trends in serum cytokines during the acute phase of human NoV infection. The second goal was to use cytokine concentrations pre-

challenge to predict infection post-challenge. Human serum samples from two prior NoV challenge studies that used the same Norwalk virus (GI.1) inoculum were analyzed for a broad panel of relevant cytokines [15, 16]. The results have important implications for determining the role of specific cytokines in NoV infection.

Materials and methods

Population and samples

The samples tested for this study were collected from subjects involved in two separate NoV challenge studies, described previously [15, 16]. However, these studies could used the same inoculum preparation, were conducted at the same institution, and used very similar protocols. Key differences between these two challenge studies (i.e. inoculum dose) were accounted for in the modeling strategy of this study. Briefly, the first study enrolled healthy secretor-positive adult volunteers, who were challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site, part of the Atlanta Clinical and Translational Science Institute Clinical Interaction Network, between May 2006 and December 2006. Volunteers ingested filtered groundwater artificially seeded with 6.5×10^7 genomic equivalent copies (GEC) of NoV inoculum, which had been incubated at room temperature in the dark for different set lengths of time. Prior to challenge, serum and stool samples were collected. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The second study enrolled healthy secretor-positive adult volunteers, who were also challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site between February 2008 and September 2009. They were randomized into control and intervention groups and administered oysters seeded with 1×10^4 GEC of virus, which had been treated with high hydrostatic pressure processing for 5 minutes (intervention) or left untreated (control). Pre-challenge stool and serum samples were collected before challenge. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The Emory University Institutional Review Board approved both studies, and both were registered on Clinical Trials.gov (identifiers NCT00313404 and NCT00674336). All subjects consented to the future use of all biological specimens from the studies. All specimens were stored at -80°C.

Of the initial participants in both NoV challenge studies, a total of 26 became infected as defined by a NoV-positive stool or emesis sample tested by RT-PCR (limit of detection: 3,570 GEC/g stool) [15, 17]. These 26 were pair-matched by age to 26 of the challenge study participants who remained uninfected following NoV challenge. Some infected subjects from one challenge group were matched with uninfected subjects from a different challenge group because within the same group there were no uninfected individuals within 3 years of age of the infected individual. The total sample size for this study was 52 participants (13 from the 2006 trial, 39 from the 2008-2009 trial). Because the focus of this study is the acute immune response, only serum samples from pre-challenge and days 1-4 post-challenge were included, for a total of five longitudinal samples per subject.

Inoculum

The two NoV challenge studies included participants who ingested water or shellfish seeded with 8FIIb NoV inoculum. The water samples were seeded with identical amounts of NoV and stored at room temperature in the dark for varying lengths of time. They showed no evidence of titer attenuation over time, as measured by RT-PCR [15]. Therefore, for these subjects, the regression models did not control for study arm. The shellfish samples were seeded with identical amounts of NoV and treated under varying processing conditions. The inocula showed some evidence of attenuation for some treatments. For subjects from this study, all adjusted models controlled for study arm as a surrogate for dose.

Detection of viral shedding

NoV presence was measured by RT-PCR for all stool and emesis samples at all time points during the original challenge studies. A subject with a positive sample at any time point, even after day 4 post- exposure, as determined in the original or subsequent studies, was defined as infected.

Detection of cytokines

Serum samples from pre-challenge (pre-challenge), and days 1-4 post-challenge (five total samples) were analyzed from all study subjects. Serum was collected during the studies, processed, and stored at -80°C until testing. Samples were tested by a commercial laboratory (EMD Millipore Corporation Discovery and Development Solutions) using a Milliplex human cytokine 16-plex assay for IFN-α2, IFN-γ, IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, MCP-1, TNF-α, and TNF-β. Samples were run in duplicate with controls. Standard curves were generated using recombinant cytokines and calculated using a five parameter logistic model for each cytokine. Lower limits of detection (LLOD) were 3.2 pg/mL for IFN-γ, IL-10, IL-12p70, IL-1b, IL-2, IL-5, IL-6, IL-8, MCP-1, and TNF-α; 16.0 pg/mL for TNF-β; and 80.0 pg/mL for IFN-α2, IL-12p40, IL-1ra, IL-1a, and IL-4. Values below LLOD were assigned the value of the LLOD.

Statistical analysis

The data were analyzed using SAS 9.4 (Cary, NC, USA). When possible, age and inoculum were controlled for in adjusted analyses. Because the exact amount of infectious NoV in the inocula for study arms with attenuation was unknown, inoculum was controlled for using a categorical variable for each study and study arm. The Wilcoxon signed rank test was to test for

the significance of the unadjusted difference between pre-challenge and post-challenge serum cytokine levels. Conditional logistic regression was used to evaluate the association between pre-challenge and post-challenge fold changes in serum cytokine levels (i.e. ratio between post-challenge and pre-challenge cytokine concentrations), controlling for inoculum and age. To summarize the differences between infected and uninfected individuals responses over time while accounting for correlation between time points, a mixed linear model was used to test the association between $\log_{10}$ cytokine concentration and day post-challenge, stratified by infection status with a random effect by subject, autoregressive correlation between different time points taken for the same individual, and fixed effect for inoculum. The parameter estimates for the effect of each day on $\log_{10}$ cytokine concentration for each stratum were averaged and Student's t-test was used to compare infected and uninfected subjects cytokine responses over time. Conditional logistic regression adjusted for inoculum and age was also used to assess whether three measures of early cytokine levels or responses could be used as predictors of post-challenge infection. The measures assessed were pre-challenge $\log_{10}$ cytokine concentration, pre-challenge to day 1 post-challenge $\log_{10}$ cytokine change, and pre-challenge to day 1 post-challenge fold change. All cytokines were modeled individually. Differences between groups were considered statistically significant at $p < 0.05$.

Results

To determine the acute serum cytokine response to GI.1 NoV infection, serum cytokine responses in individuals who became infected following experimental challenge were compared to those who remained uninfected after challenge, as determined by RT-PCR detection of GI.1 NoV RNA in stool or emesis samples. Immune responses may differ with age, therefore the infected and uninfected subjects were matched for age (Table 1). The study population was relatively young, with a mean age of 26.7 years and a median age of 25 years. There was also a similar distribution of sex and race between infected and uninfected subjects selected for the study, though neither was a matching factor. Some uninfected subjects exhibited symptoms, including fever and diarrhea, which is consistent with past NoV challenge studies [16]. All infected individuals had higher cumulative shedding than the inoculum dose (data not shown), reducing the likelihood of misclassification of infection status because of detection of inoculum post-challenge in the absence of infection. Serum samples were available for pre-challenge through day 4 post-challenge (five time points) and all samples were tested for 16 serum cytokines. Two subjects did not have samples for day 3 and values for these samples were imputed using the average of neighboring observations for the same subject. A total of 10 subjects had invalid test results for one or more cytokine tested (29 total observations). Using imputation, all but one of these invalid results was estimated to be below the LLOD.

Studies of serum cytokine levels in humans often have high rates of observations below the LLOD [18]. Overall of the 16 cytokines tested, nine had greater than 50% of observations above the LLOD, and four had greater than 75% of observations above the LLOD (Table 2). In general, infected subjects had more samples above the LLOD.

To assess whether serum cytokines exhibited a change in concentration following NoV

challenge, the Wilcoxon signed rank test was used, stratifying by infection status. Among infected individuals, there were significant increases in IL-2, IL-10, MCP-1, and TNF-α in the days post-challenge (Figure 1, all $p < 0.05$). Among uninfected individuals, there were no significant changes in serum cytokine concentrations post-challenge compared to pre-challenge. Based on conditional logistic regression models controlling for age and inoculum, there were no significant differences between infected and uninfected individuals' changes in serum cytokine concentrations between pre-challenge and day 1 post-challenge (data not shown). However, infected individuals were significantly more likely to have increased IFN-γ, IL-6, IL-8, IL-12p70, MCP-1, and TNF-α between pre-challenge and day 2 post-challenge compared to uninfected individuals (Table 3). Infected individuals were also significantly more likely to have increased levels of TNF-α, IL-8, and IL-10 from pre-challenge to day 3 post-challenge (all $p<0.05$; data not shown). At day 4 post-challenge, the only cytokine significantly elevated over pre-challenge levels was IL-10, which was significantly elevated in infected individuals (OR of infection based on a two-fold increase in IL-10 from pre-challenge to day 4 post-challenge=3.92, $p=0.049$; data not shown).

Differences between infected and uninfected individuals over time

Overall trends in serum cytokine response were analyzed using mixed models to account for correlation within subject over time (Figure 2). For infected individuals compared to uninfected individuals, there were statistically significant fold changes across the four days post-challenge from pre-challenge levels in most serum cytokines measured (i.e. IFN-α2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β, Figure 2), with all but TNF-β significantly higher in infected individuals compared to uninfected individuals. All cytokines peaked at day 2 post-challenge among infected subjects, with the exception of IL-1ra, but many were elevated by

day 1 post-challenge (i.e. IL-2, IL-6, IL-8, MCP-1, and TNF-α) or remained elevated through day 4 post-challenge (i.e. IL-10) (Figure 2).

Pre-challenge predictors of infection

To determine if pre-challenge serum cytokine concentrations or early cytokine responses could predict infection status following challenge, the associations were estimated between infection status and three measures of serum cytokines: $\log_{10}$ serum cytokine concentration, $\log_{10}$ difference between pre-challenge and day 1 post-challenge serum cytokine concentrations, and pre-challenge to day 1 post-challenge fold change (i.e. ratio between pre-challenge and day 1 post-challenge concentrations). Across these measures, the only significant predictor of infection status was pre-challenge to day 1 post-challenge $\log_{10}$ change in MCP-1 concentration (data not shown). An increase of 1 $\log_{10}$ over this time period was associated with an OR of infection of 5.74 ($p=0.018$). No other measures of early cytokine response were statistically significant predictors of infection.

Discussion

This study found overall significant elevation in IFN-a2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β in infected subjects compared to uninfected subjects. Among infected individuals, these cytokines peaked on day 2 post-challenge. The results suggest that NoV infection elicits a Th1- and Th2-type response. There also was persistent elevation of IL-10 among infected subjects, suggestive of an evolving early adaptive immune response and down-regulation of Th1 cytokines. This study represents the first comprehensive description of the dynamics of serum cytokines in the acute time period following NoV challenge, including the largest number of cytokines simultaneously described for NoV infection (Table 3).

We found that before challenge, individuals who became infected following NoV challenge were similar to individuals who did not become infected with regard to all 16 cytokines tested. From these similar pre-challenge features, differences began to emerge as early as 24 hours post-challenge and persisted through day 4 post-challenge. As previously documented with human Snow Mountain strain (SMV; GII.2) infection, there was a significant Th1 response (i.e. IFN-γ, IL-2, and TNF-α) with significant elevation of one Th2-related cytokine (Table 3) [13]. In this study, some Th2 and other cytokines (e.g. IL-4, IL-5, IL-6, IL-8, and IL-10) were also significantly elevated following NoV challenge in infected individuals.

The cytokine responses also resembled results from animal studies (Table 3) but differed in the timing and magnitude of cytokine response [19, 20]. These results suggest that the immune response by gnotobiotic animals to human NoV may be substantially different from the response in humans, perhaps due to the gnotobiotic animals' immature immune systems [21] or because of the use of a different NoV inoculum.

Initial response: IL-8, MCP-1

We found that IL-8 levels increased in infected subjects by day 1 post-challenge and remained elevated through day 3 post-challenge. IL-8 elevation has been identified in prior field studies of NoV infection (Table 3) [22, 23]. IL-8 is a powerful chemoattractant for neutrophils [24, 25] and intraepithelial lymphocytes [26, 27], and a key mediator of the immune response to other gastrointestinal pathogens [28]. Histologic findings from previous human NoV challenge studies show increases in granulocyte and monocyte cells in the lamina propria of the small intestine 12-48 hours after challenge in infected subjects [29]. Based on the rise in IL-8 alongside an increase in MCP-1 and a later rise in IL-6, two monocyte chemoattractants, these observations are consistent with neutrophil recruitment acting in concert with monocyte response to NoV infection. Earlier work found that GI and GII NoV infection is associated with elevated fecal MCP-1, suggesting local monocyte activation [23], but this study is the first to show elevated serum MCP-1, suggesting a systemic response as well.

TNF-α and symptoms

In this study, the peak serum TNF-α concentration at 48 hours post-challenge corresponded with the time most NoV-infected individuals were symptomatic. TNF-α increases cellular permeability, leading to edema mucosal damage [30]. Elevated TNF-α has also been associated with NoV infection in past human and animal studies (Table 3) [19, 23]. In some gastrointestinal infections, TNF-α is associated with symptoms [31-33]. The association between TNF-α and NoV symptoms is an important area for future research.

IL-6

IL-6 is a multi-functional cytokine with strong pro-inflammatory effects. It is associated with damage to the intestinal mucosa [29] and increased frequency of diarrhea [22], suggesting a role in pathogenesis and clinical severity of illness. In this study, IL-6 was significantly elevated on day 2 post-challenge. This confirms some clinical studies' identification of the association between IL-6 and NoV infection but contrasts with the results from a SMV challenge study (Table 3) [13, 22, 23] and suggests possible strain-related differences in immune response. This study reported a median maximum serum IL-6 level that was higher than prior studies have identified [22, 34], but this discrepancy may be the result of differences in sample collection.

IL-10 persistently elevated

Though IL-10 peaked on day 2 post-challenge, it was significantly elevated through day 4 post-challenge. This suggests an ongoing role in NoV infection beyond the acute time period. IL-10 is involved in the Th2 response and B-cell development [35], so its elevation may be associated with the development of NoV-specific antibodies, which begin to be detectable around or before day 7 post-challenge [36, 37]. Elevated IL-10 in the context of NoV infection has been described in prior studies of human NoV (Table 3). IL-10 may also play an anti-inflammatory role in down-regulating Th1 cytokine production. The significant elevation of IL-10 in infected subjects following the elevation of Th1 cytokines supports the conclusion that NoV elicits a Th1-type response. Furthermore, IL-10-deficient mice exhibit mucosal inflammation and epithelial barrier dysfunction following MNV-challenge, whereas wildtype mice do not [38]. It is possible that IL-10 plays a similar protective role in NoV-challenged humans.

Strengths and Limitations

This study used rigorously collected human NoV challenge study data, which provided a detailed, longitudinal dataset. It is the largest collection of human NoV challenge subjects yet studied for serum cytokine response. It represents broadest panel of cytokines yet examined at one time for human NoV infection. Some limitations of this study are that it may have been underpowered to detect some changes in serum cytokines, the exposure history of subjects prior to NoV challenge was unknown, and there is the possibility that the duration of sample storage may have caused cytokine degradation [39]. An additional limitation of the study is that though RT-PCR is the gold-standard for NoV testing, there may have been individuals who were misclassified as uninfected because they shed virus at levels below the limit of detection.

Conclusion

Although NoV remains a major cause of morbidity worldwide, some aspects of the immune response continue to be poorly understood. To develop effective vaccines and prophylaxis, it is important to understand the human acute immune response to NoV. This study demonstrated a Th1- and Th2-type response to GI.1 NoV infection, early elevation of chemokines IL-8 and MCP-1, and ongoing elevation of IL-10. This work confirms earlier findings of the increase in MCP-1 associated with NoV infection, suggesting macrophage and dendritic cell involvement in NoV infection. These findings buttress the existing findings from MNV models and enhance the depth of knowledge regarding the human cytokine response to NoV. Differences between the results of this study and those of other human and animal work may be attributable to strain-related variability in immune response. Future work should consider the interplay between the immune response and both clinical outcomes and viral shedding to understand NoV pathogenesis and help develop clinical and public health strategies to reduce NoV transmission.

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Conflicts of Interest

None to report.

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Figure Legends

Figure 1: Comparison of pre-challenge to post-challenge serum cytokine concentrations in norovirus-challenged individuals, stratified by infection status (infected n=26, uninfected n=26). Values below the lower limit of detection (LLOD) were assigned to the value of the LLOD. Significance of change from pre-challenge value was tested using Wilcoxon test and is denoted by an asterisk above the relevant category. Interquartile ranges (IQRs) for infected individuals (gray boxes) and for uninfected individuals (white boxes) are shown. Dark lines indicate median values. Whiskers indicate most extreme value that is no more than 1.5 times the IQR away from the bound of the IQR. Circles indicate outliers. *p<0.05, **p<0.01, ***p<0.001

Figure 2. Mixed linear model results for the association between $\log_{10}$ cytokine change from pre-challenge to post-challenge by day, with fixed effects for inoculum dose and day and a random effect by individual subject. Solid line: infected individuals, dashed line: uninfected individuals. Points represent estimates of the effect of day on change in cytokine concentration. Error bars indicate one standard error. P-value indicates significance of overall elevation in cytokine concentration across days.

Table 1: Characteristics of norovirus-challenged individuals included in analytic sample

	Total (n=52)	Infected (n=26)	Uninfected (n=26)
Age (mean, SD)	26.7 (7.6)	26.7 (7.5)	26.7 (7.9)
Female	32 (61.5%)	17 (65.4%)	15 (57.7%)
Race			
White	25 (48.1%)	15 (57.7%)	10 (38.5%)
Black	21 (40.4%)	8 (30.8%)	13 (50.0%)
Other	6 (11.5%)	3 (11.5%)	3 (11.5%)
AGE symptoms[a]	19 (33.9%)	17 (65.4%)	2 (7.7%)
Modified Vesikari score (mean, SD)	2.6 (2.4)	4.1 (2.4)	1.1 (0.9)

Abbreviations: SD, standard deviation; AGE, acute gastroenteritis.

[a]AGE symptoms defined as diarrhea (3 or more or >= 400g loose stools in 24 hours) or emesis during days 1-4 post-challenge.

Table 2. Serum cytokine concentrations (pg/mL) of norovirus-challenged individuals pooled across days, stratified by infection status[a].

	All			Infected			Uninfected		
Cytokine	Median	IQR	% above LLOD	Median	IQR	% above LLOD	Median	IQR	% above LLOD
IFN-α2	80.0	80.0-80.0	24.8%	80.0	80.0-133.0	27.9%	80.0	80.0-80.0	21.7%
IFN-γ	186.5	40.4-656.1	90.3%	182.0	67.9-629.3	98.4%	188.3	18.2-669.5	82.2%
IL-1ra	80.0	80.0-999.3	54.3%	80.0	80.0-389.1	73.6%	80.0	80.0-1,993.9	34.9%
IL-1a	163.4	80.0-433.2	24.7%	179.8	80.0-433.2	28.5%	80.2	80.0-433.55	21.1%
IL-1b	3.2	3.2-10.3	58.9%	3.3	3.2-18.45	70.2%	3.2	3.2-7.2	48.1%
IL-2	4.6	3.2-30.1	47.8%	7.5	3.2-38.2	48.0%	3.6	3.2-17.6	47.6%
IL-4	80.0	80.0-99.9	54.7%	80.0	80.0-170.9	59.4%	80.0	80.0-80.0	50.0%
IL-5	3.2	3.2-3.9	38.4%	3.2	3.2-6.0	48.1%	3.2	3.2-3.2	28.7%
IL-6	23.9	3.2-120.2	52.5%	27.8	3.2-93.0	55.5%	21.4	3.2-161.1	49.6%
IL-8	66.5	20.7-198.1	25.4%	81.7	34.2-198.0	28.9%	45.0	11.5-202.4	21.9%
IL-10	4.4	3.2-29.5	26.8%	17.0	3.2-50.3	33.9%	3.2	3.2-6.2	19.7%
IL-12p40	80.0	80.0-80.0	70.7%	80.0	80.0-85.0	69.5%	80.0	80.0-80.0	71.9%
IL-12p70	6.1	3.2-89.8	92.2%	19.5	3.2-86.6	96.9%	3.2	3.2-96.0	87.6%
MCP-1	612.8	397.1-832.5	100.0%	693.5	510.8-944.3	100.0%	495.1	329.4-673.1	100.0%
TNF-α	14.3	6.0-27.2	85.3%	15.1	8.3-25.2	87.6%	14.0	4.9-36.2	82.9%
TNF-β	16.0	16.0-58.5	37.2%	16.0	16.0-100.5	35.7%	16.0	16.0-33.4	38.8%

Abbreviations: IQR, inter-quartile range; LLOD, lower limit of detection; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

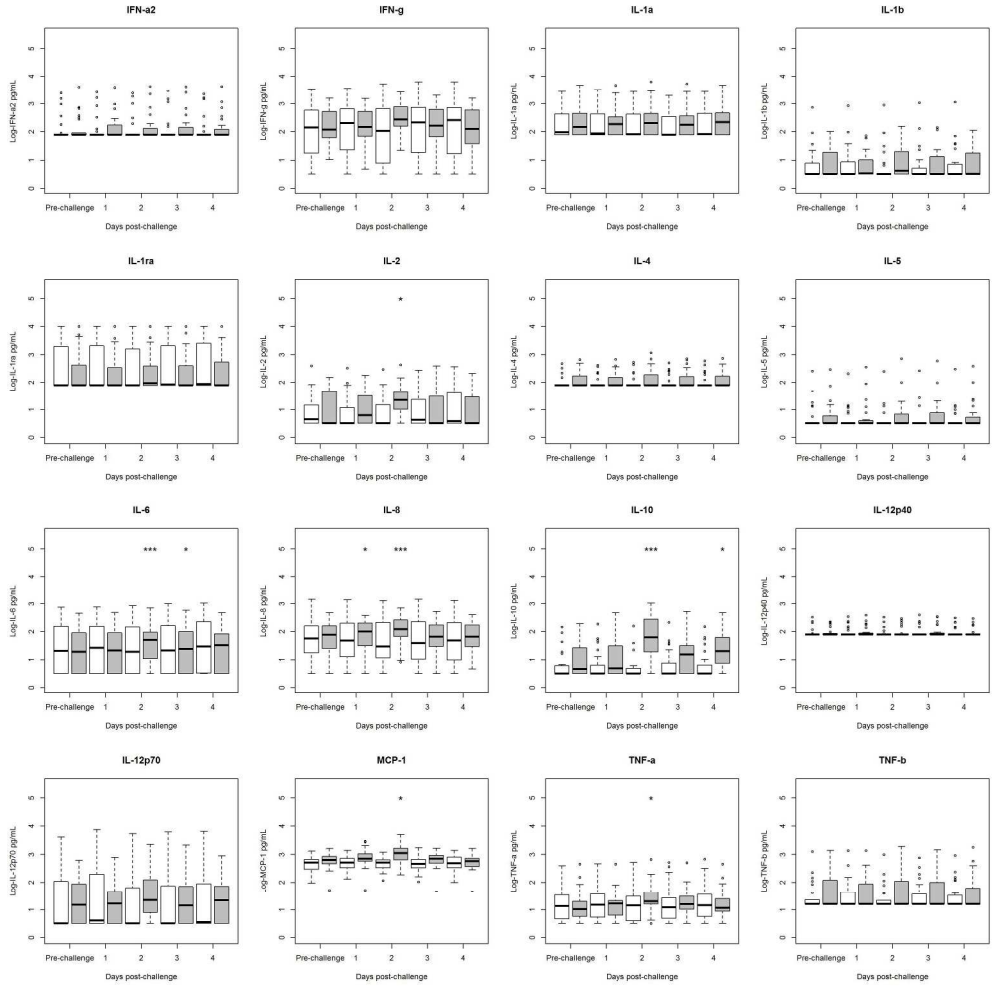
[a]Values below LLOD included in estimates as LLOD value.

Table 3. Table comparing results from this study to prior studies of cytokine responses to human NoV infection. An 'x' indicates significant elevation compared to uninfected controls. Gray cells indicate no testing was conducted for that cytokine.

Study	This study[a]	Lindesmith 2005 [12]	Long 2011 [22]	Chen 2012 [37]	Souza 2008 [18]	Souza 2007 [19]
NoV genogroup	GI.1	GII.2	Mixed	Mixed	GII.4	GII.4
Subjects	Human	Human	Human	Human	Gn Calf	Gn Pig
Study design	Challenge	Challenge	Observational cohort	Cross-sectional	Challenge	Challenge
Cytokine						
IFN-α						x
IFN-α2	ns					
IFN-γ	x	x	ns		x[b]	x
IL-1ra	ns					
IL-1a	ns					
IL-1b	ns					
IL-2	x	x				
IL-4	ns	ns	ns		x[b]	x
IL-5	ns	x	x			
IL-6	x		ns	x		x
IL-8	x		x	x		
IL-10	x	ns	ns		x[b]	x
IL-12					x[b]	x
IL-12p40	ns					
IL-12p70	x					
MCP-1	x		x			
TNF-α	x	ns	ns		x[b]	
TNF-β	ns					

Abbreviations: ns, not significant; NoV, norovirus; Gn, gnotobiotic.

[a]Modeled using conditional logistic regression conditional on inoculum dose and adjusted for age. [b]Cytokine concentrations were elevated compared to controls but no statistical testing was conducted.



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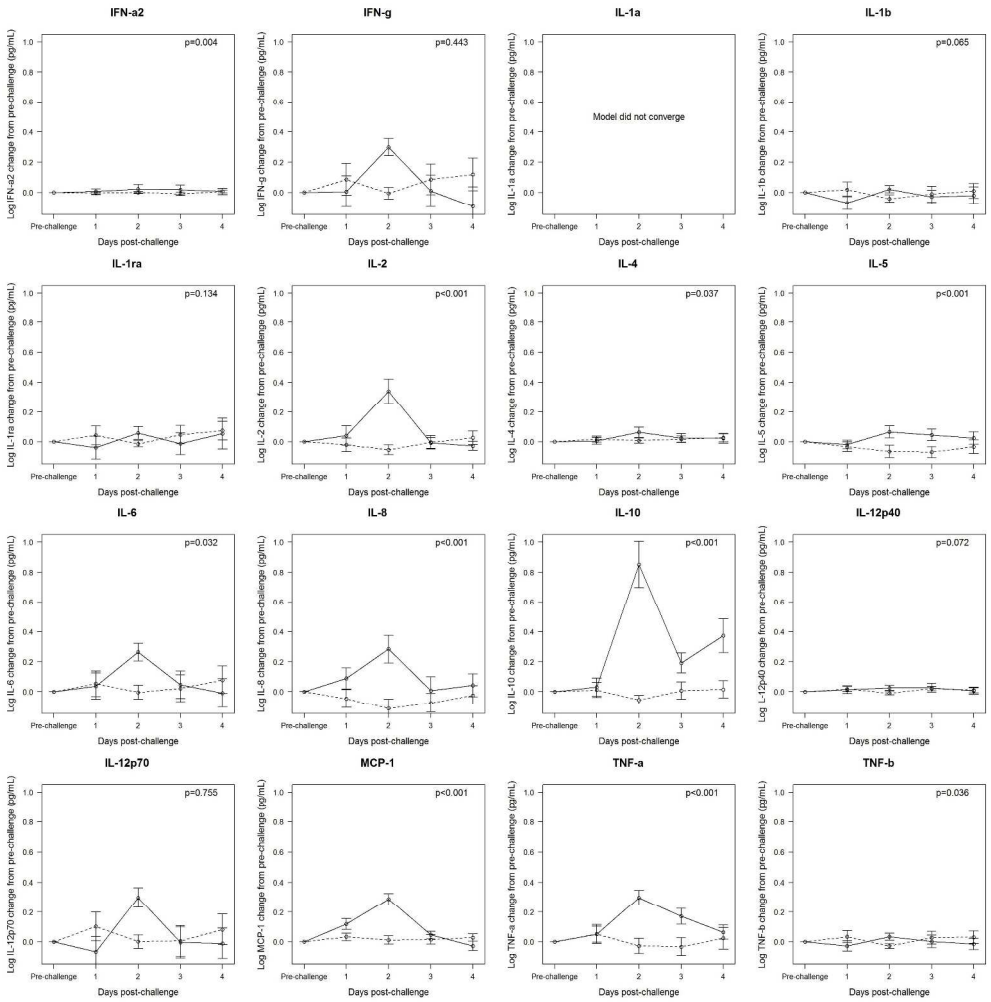

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