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Cytokine and Antibody Responses in Gnotobiotic Pigs after Infection with Human Norovirus Genogroup II.4 (HS66 Strain)[▽]

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A human norovirus genogroup II.4 strain HS66 (HuNoV-HS66) infects and causes mild diarrhea in gnotobiotic (Gn) pigs (S. Cheetham, M. Souza, T. Meulia, S. Grimes, M. G. Han, and L. J. Saif, J. Virol. 80:10372–10381, 2006). In this study we evaluated systemic and intestinal humoral and cellular immune responses to HuNoV-HS66 in orally inoculated pigs. Antibodies and type I interferon (IFN-I or IFN-α), proinflammatory interleukin-6 (IL-6), Th1 (IL-12 and IFN-γ), Th2 (IL-4), and Th2/regulatory T ([T_{reg}] IL-10) cytokine profiles in serum and intestinal contents (IC) of the HuNoV-HS66-inoculated pigs and controls were assessed by enzyme-linked immunosorbent assay at selected postinoculation days (0 to 28). Using an enzyme-linked immunospot assay, we evaluated immunoglobulin M (IgM), IgA, and IgG antibody-secreting cells (ASC) and cytokine-secreting cells (CSC) in intestine, spleen, and blood. In the HuNoV-inoculated pigs, antibody titers in serum and IC were generally low, and 65% seroconverted. Pigs with higher diarrhea scores were more likely to seroconvert and developed higher intestinal IgA and IgG antibody titers. The numbers of IgA and IgG ASC were higher systemically than in the gut. In serum, HuNoV induced persistently higher Th1 (low transient IFN-γ and high IL-12) than the other cytokines, but also low Th2 (IL-4) and Th2/T_{reg} (IL-10) levels; low, transient proinflammatory (IL-6) cytokines; and, notably, a delayed IFN-α response. In contrast, intestinal innate (IFN-α early and late) and Th1 (IL-12 late) cytokines were significantly elevated postinfection. HuNoV-HS66 also elicited higher numbers of Th1 (IL-12 and IFN-γ) CSC than Th2 (IL-4) and proinflammatory (IL-6) CSC, with the latter responses low in blood and intestine, reflecting low intestinal inflammation in the absence of gut lesions. These data provide insights into the kinetics of cytokine secretion in serum and IC of HuNoV-inoculated Gn pigs and new information on intestinal humoral and cellular immune responses to HuNoV that are difficult to assess in human volunteers.

Noroviruses (NoVs) are the leading cause of food-borne illnesses in the United States (21). The NoVs are classified into five genogroups (I to V) and at least 27 genotypes. However, only strains from genogroup I (GI), for which Norwalk virus (NV) is the prototype strain, GII, and GIV have been reported to infect humans (17). The GII NoVs also occur in swine, with GII.18 NoVs being genetically and antigenically similar to human strains, raising concerns that swine may serve as potential reservoirs for GII NoVs (38). Recent increased worldwide outbreaks of NoVs highlight a need for prevention and control measures including possible vaccines (13, 28). However, the lack of an animal model for human NoVs (HuNoVs) and their failure to grow in cell culture monolayers hamper research on immunity and vaccines for HuNoVs.

Immunity to human caliciviruses (including HuNoVs) is complex and not completely understood. Early studies of human volunteers showed that serotype-specific short-term immunity is conferred by NV infection (15, 29, 43) and that not all individuals are susceptible to NV infection and/or disease. We recently showed that a subset of gnotobiotic (Gn) pigs was susceptible to infection or disease after oral inoculation with the GII.4 HuNoV strain HS66 (HuNoV-HS66) (7). Currently, two genetic factors (ABH histo-blood group antigens and secretor status) are associated with susceptibility or resistance to NV infection and disease in humans (12, 19). We further demonstrated a similar association between the phenotype A^+/H^+ of Gn pigs and the development of diarrhea and higher rates of fecal viral shedding after infection with GII.4 HuNoV-HS66 than in Gn pigs with the non-A^+/H^+ phenotype (6). However, other investigators recently showed that in contrast to NV, Snow Mountain virus (SMV) (GII.2 HuNoV) infection was not influenced by histo-blood group or secretor status, suggesting that multiple factors may influence host susceptibility to the myriad of HuNoV genotypes (18).

The immunoglobulin M (IgM) and IgG antibody responses in serum and saliva of volunteers have been studied to assess immunity to HuNoV, and at least a fourfold increase in antibody titer has been considered as seroconversion (5, 15, 24). Fecal secretory IgA (sIgA) antibodies to HuNoV have also been suggested as a marker for symptomatic disease (27). Although susceptible individuals who had memory sIgA antibody responses (indicated by NV-specific IgA antibody titers in prechallenge saliva samples of Se^+ individuals) were not infected by NV, some individuals who were susceptible to NV and who did not have strong salivary sIgA responses were also not infected (19). These findings suggest that multiple factors may be involved in susceptibility or resistance to infection and in the development of immunity to HuNoVs.

In swine, studies have shown that both humoral and cellular

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immune responses are important in resolving viral infections and also reveal the existence of a Th1/Th2-type of immune regulation as in mice and in humans (48). It is known that porcine interleukin-12 (IL-12) is similar to human IL-12 and that innate cells (dendritic cells and NK cells) produce alpha interferon (IFN-α) and that both innate (NK cells) and T cells produce IFN-γ after viral exposure (33, 41). Porcine IL-6, produced by activated macrophages and other mononuclear cells (MNCs), is a proinflammatory cytokine, as in humans, and it mediates and modulates the immune response, but it can also exacerbate inflammation. IL-4 promotes development of helper T cells (Th2 cells) and induces division of B cells and their differentiation into plasma cells (25, 26), aiding in T-cell-dependent antibody production. IL-10 may also be a regulatory cytokine in pigs as in humans, functioning in the control of inflammation (10).

In human adult volunteers exposed to SMV (GII.2 NoV), a dominant Th1 response with significant increases in serum IFN-γ and IL-2 during the acute phase of infection (postinoculation day 2 [PID 2]) was observed; however, local intestinal immunity and innate cytokine responses were not assessed (18). Thus, the aim of this study was to evaluate both intestinal and systemic antibody responses and cytokine profiles in Gn pigs after infection with GII.4 HuNoVs. To our knowledge, this is the first study to provide data on the immune responses of Gn pigs after GII.4 HuNoV infection and also on intestinal immune responses to HuNoV, which are difficult to assess in human volunteers.

MATERIALS AND METHODS

Virus inoculum. A single aliquoted pool of the original human fecal sample identified as NoV/GII/4/HS66/2001/US (HS66 strain) (7) was used for oral inoculation of Gn pigs with a dose of approximately 5.4×10^6 genomic equivalents/ml. Each Gn pig received one oral dose of the HuNoV inoculum consisting of 1 ml of the original HuNoV-HS66 strain diluted 1:10 in minimal essential medium (MEM) (Gibco-Invitrogen, Carlsbad, CA), which was further processed by vortexing, centrifugation at $3,000 \times g$ for 20 min, and filtration through a 0.8-μm-pore-size filter followed by 0.2-μm-pore-size filters. The mock inoculum was MEM.

Inoculation of experimental pigs. Near-term pigs were derived by surgery and maintained in sterile isolator units as previously described (22). Five- to seven-day-old Gn pigs were allocated into HuNoV-HS66-inoculated or mock-inoculated control groups. After receiving 8 ml of 100 mM sodium bicarbonate orally to neutralize stomach acids, pigs were inoculated as follows: one oral dose (5.4×10^6 genomic equivalents) of HuNoV-HS66 (diluted and processed as described above to a final volume of 10 ml) ($n = 35$ pigs) or equal volumes of MEM as controls ($n = 25$). Of 35 HuNoV-HS66-inoculated pigs, 3 pigs (2 euthanized at PID 2 and 1 at PID 21) did not show diarrhea or shed virus, nor did the older pig (PID 21) seroconvert to HuNoV-HS66. These three pigs were excluded from the detailed studies of the immune responses, leaving a total of 32 HuNoV-HS66-inoculated and infected pigs. Two additional pigs were inoculated with a fecal filtrate of HS66 virus inactivated with 0.01 M binary ethylenimine (inact-HS66), as previously described (7). The HuNoV-HS66-inoculated and control pigs were euthanized at PID 2 (HuNoV, $n = 8$; controls, $n = 5$), PID 6 (HuNoV, $n = 5$; controls, $n = 5$), PID 8 (HuNoV, $n = 5$; controls, $n = 5$), PID 21 (HuNoV, $n = 8$; controls, $n = 5$), and PID 28 (HuNoV, $n = 9$; controls, $n = 5$). One of the pigs inoculated with the inact-HS66 was euthanized at PID 21 and the other at PID 28. Four additional pigs that did not receive any inoculum were euthanized at 5 days of age, and the results were used as baseline for detection of cytokines in the serum, cytokine-secreting cell (CSC) and antibody-secreting cell (ASC) enzyme-linked immunospot (ELISPOT) assays.

Assessment of diarrhea. Daily rectal swabs were collected, and diarrhea scores were noted and recorded (0, normal; 1, pasty; 2, semiliquid; 3, watery) from PID 0 to 6 as described previously (7). Samples with scores of 2 and 3 were considered diarrheic. The diarrhea cumulative score of each pig represents the sum of daily rectal swab scores from PID 1 to 6, and the mean cumulative score of each group is the sum of each pig's diarrhea cumulative score divided by the number of pigs in that group.

Detection of viral shedding by RT-PCR. Viral shedding was determined using rectal swab fluids and 1:20 dilutions of intestinal contents (IC) by reverse transcription-PCR (RT-PCR), using the primer pair Mon 431/433 (31) targeting the RNA-dependent RNA polymerase region of HuNoV GII, under the same conditions as previously described (7). However, in contrast to the previously described method, samples that were inhibited in RT-PCR, as revealed by the use of an internal control (7), were retested after being reextracted using an RNeasy Mini kit (QIAGEN Inc, Valencia, CA). Negative controls (rectal swabs from mock-inoculated pigs and RNase-free water) for RNA extraction and RT-PCR were included in each assay. A microplate hybridization assay (37) was performed to confirm the product specificity using a probe specific for HuNoV-HS66 (PmonHS665′-CTTGCTAATTTTGCTGTAGAATGATGGGCCGTGGA-3′).

Detection of viral shedding by antigen ELISA. An antigen enzyme-linked immunosorbent assay (ELISA) was performed as previously described by Cheetham et al. (7). Samples were considered positive when the mean absorbance (450 nm) of the positive coating wells minus the mean absorbance of the negative coating wells was higher than the mean absorbance of the negative control wells plus three times the standard deviation.

Viremia. Sera from blood collected from pigs on PIDs 1 and 2 were analyzed by RT-PCR and microwell hybridization for detection of HuNoV-HS66 RNA or amplicon, respectively, as previously described (7). In contrast to the previously described method (7), samples that were inhibited in RT-PCR were reextracted using an RNeasy Mini kit and retested by RT-PCR.

Antibody detection. An immunocytochemistry assay was performed to detect HS66-specific antibodies in the serum and IC of Gn pigs, as previously described (44). For this assay a recombinant baculovirus expressing HS66 capsid was used to infect *Spodoptera frugiperda* (Sf9) cells as the HuNoV antigen source, and the recombinant baculovirus-infected cells or mock-infected cells were subsequently fixed using 10% formalin in phosphate-buffered saline (PBS). The antibody titer was defined as the reciprocal of the highest serum dilution at which brown-stained cells representing NoV antibody complexed to HS66 capsid antigen could be detected.

Isolation of MNC for antibody and CSC ELISPOT assays. Segments of the small intestine (jejunum and ileum), spleen, and blood were aseptically collected at euthanasia and processed for the isolation of MNC populations, as previously described (36, 45). Single MNC suspensions from each tissue and blood sample were prepared at concentrations of 5×10^6 and 5×10^5 cells/ml in complete medium prepared with RPMI 1640 medium (GIBCO) enriched with 8% fetal bovine serum, 20 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 μg of gentamicin/ml, 100 μg of ampicillin/ml, and 50 μg of 2-mercaptoethanol.

ELISPOT assay for HuNoV-HS66-specific ASCs. An ELISPOT assay for detection of isotype-specific (IgM, IgA, and IgG) ASCs was conducted using previously published methods (8, 46). Briefly, Sf9 cell plates infected with the HS66 recombinant baculovirus and noninfected Sf9 cell plates (mock plates) were prepared and fixed as described in the antibody detection section and washed with deionized water prior to use. Single MNC suspensions from each tissue sample were added to duplicate wells (5×10^5 and 5×10^4 cells/well). Plates were then incubated at 37°C for 12 h in 5% CO_2 and then washed three times with PBS buffer and incubated at 37°C for 2 h with 100 μl/well of horseradish peroxidase-labeled antibodies: goat anti-pig IgM (μ) (0.25 μg/ml; KPL), IgA (0.3 μg/ml; Serotec), or IgG (0.25 μg/ml; KPL). Plates were then washed three times in PBS buffer and developed with TMB (3,3′,5,5′-tetramethylbenzidine) (KPL) for 2 h at room temperature. The numbers of virus-specific ASCs were determined by counting blue spots in the wells, using a light microscope, and were reported as the number of virus-specific ASCs per 5×10^5 MNC after any background spots (<5), evident on the mock plates, were subtracted.

CSC ELISPOT assay. A cytokine ELISPOT assay for detection of IL-12, IFN-γ, IL-6, IL-4, and IL-10 CSCs was performed as previously described (2) with minor modifications. Before being added to Multiscreen immunoprecipitation sterile 96-well plates (Millipore, Bedford, MA) at concentrations of 5×10^5 and 5×10^4 cells/well, the cells were stimulated with 50 μg/ml of CsCl-purified HS66 virus-like particles (7) or 10 μg/ml of phytohemagglutinin (positive control) or RPMI medium (negative control). Plates were then incubated at 37°C in 5% CO_2 for 48 h. The numbers of CSCs were counted using an ImmunoSpot series 3A analyzer (Cellular Technology Ltd., Cleveland, OH) and expressed as CSCs per 5×10^5 MNCs. The HuNoV-HS66-specific CSC numbers were computed after the numbers of CSCs (<4) in the controls (RPMI medium-stimulated cells) were subtracted from the number in cells stimulated with HS66 virus-like particles.

TABLE 1. Diarrhea, fecal virus shedding, and viremia detected by RT-PCR and seroconversion detected by immunocytochemistry in Gn pigs inoculated with either HuNoV-HS66 or mock inoculum[a]

Inoculum (no. of pigs)	Virus shedding (mean no. of days [range])[b]	Diarrhea[c]			Viremia (%)[e]	Seroconversion (%)[f]	Antibodies in IC (%)[f]
		No. of positive pigs/total no. of pigs (%)	Mean no. of days (range)	Mean cumulative score (range)[d]			
HS66 (32)	4^{A} (1–6)	$27/32\ (84)^{A}$	4 (2–6)	7^{A} (2–15)	$18/32\ (56)^{A}$	$11/17\ (65)^{A}$	$9/14\ (64)^{A}$
Control (25)	0^{B}	$1/25\ (4)^{B}$	0	3^{B} (0–6)	$0/25\ (0)^{B}$	$0/10\ (0)^{B}$	$0/10\ (0)^{B}$

[a] Data are shown only for the 32 HuNoV-HS66-inoculated pigs that shed virus detected by RT-PCR; the three inoculated pigs that did not develop diarrhea or viremia or demonstrate seroconversion are not included. Values in the same column with different superscript letters (A or B) differ significantly ($P < 0.05$).
[b] Determined by RT-PCR.
[c] Diarrhea was considered to be present if fecal swab scores were ≥2 after inoculation.
[d] Represents the sum of daily rectal swab scores from PIDs 1 to 6 of each pig divided by the number of pigs in that group.
[e] Number of positive pigs/total number of pigs tested. Viremia was determined by RT-PCR on PIDs 1 and 2.
[f] Number of positive pigs/total number of pigs tested. Seroconversion and antibody titers in IC were determined by immunocytochemistry (cell-staining assay of Sf9 cells infected with HS66 recombinant baculovirus) at PID 21 and 28.

Cytokine ELISA. Blood was collected from pigs at PIDs 0, 1, 2, 4, 6, 8, 10, 12, 21, and 28, and ICs were collected at euthanasia (PIDs 0, 2, 6, 8, 21, and 28). Serum samples were processed and stored at −20°C (2). The IC samples were diluted 1:2 in MEM with a protease inhibitor cocktail to prevent cytokine degradation (1). The serum and IC were immediately frozen at −20°C until further testing. An ELISA was performed to detect IL-12, IFN-γ, IL-6, IL-4, and IL-10 as previously described (2). In addition to these cytokines, an ELISA to detect porcine IFN-α was also developed. Plates were coated with rabbit anti-porcine IFN-α (0.75 μg/ml) (PBL, Piscataway, NJ), samples were added, and then a monoclonal antibody to porcine IFN-α (1.5 μg/ml) (PBL) was added, followed by the addition of goat anti-mouse-horseradish peroxidase (5 μg/ml). Standard curves were generated using recombinant porcine IL-12 (R & D Systems), IL-6, IL-4 (Biosource, Camarillo, CA), IFN-γ, IL-10 (Biosource), and IFN-α (PBL). A computer-generated four-parameter curve fit was used to calculate the concentration of each cytokine. The detection sensitivity limits for the reactions were as follows: 7 pg/ml for IL-12, IL-6, IL-4, IL-10, and IFN-γ; 15 pg/ml for IFN-α.

Statistical analysis. The cytokine concentrations, ASC (PIDs 6, 8, 21, and 28), and CSC (PIDs 2, 6, 8, 21, and 28) numbers were compared among and within groups using a Kruskal-Wallis rank sum test (nonparametric). Spearman correlation coefficients were used to measure the correlations between diarrhea severity (cumulative scores) and the following immune responses: ASC or CSC numbers among intestinal and systemic tissues (spleen and blood) (PIDs 6, 8, 21, and 28) and HuNoV-HS66-specific convalescent-phase serum and intestinal IgA and IgG antibody titers at PIDs 21 and 28. Statistical significance was assessed at a P value of <0.05.

RESULTS

HuNoV-HS66 induces diarrhea, rectal shedding, and viremia in Gn pigs. A detailed description of clinical signs, virus shedding, and pathogenesis of HuNoV-HS66 in Gn pigs was recently published (7). In the present study, a different subset of pigs from the previous study was evaluated, and because in our previous study diarrhea was observed from PID 1 to 5, viral shedding from PID 1 to 4, and viremia from PID 1 to 2, rectal swabs were collected from only PID 1 to 6 for evaluation of diarrhea and viral shedding, and serum samples were collected from only PID 1 to 2 for detection of viremia. Thirty-two of 35 (91%) of the HuNoV-HS66-inoculated pigs shed virus, as detected by RT-PCR and microwell hybridization. Only the 32 HS66-infected pigs were evaluated further in the immunologic studies. Viral shedding was detected in the rectal swab fluids of all 32 pigs by RT-PCR, microwell hybridization, and antigen ELISA from PID 1 to 6 (data not shown), with a mean duration of shedding of 4 days (Table 1). Two of five pigs (40%) euthanized at PID 2 had viral RNA detectable in the IC (data not shown). When shedding was determined by antigen ELISA, 22/32 (69%) of the HuNoV-HS66-infected pigs tested had at least one positive rectal swab fluid sample during the acute phase of infection (PID 1 to 4), and 1 of 5 (20%) of the IC samples from pigs that were euthanized on PID 2 was positive by ELISA (data not shown). As summarized in Table 1, of the HuNoV-HS66-inoculated pigs, 27/32 (84%) had diarrhea, and 18/32 (56%) had viremia as detected by RT-PCR-positive results in the serum.

Inact-HS66 does not induce diarrhea, rectal shedding, or viremia in Gn pigs. No viral shedding, viremia, or seroconversion was detected in either of the two Gn pigs that were inoculated orally with inact-HS66. Furthermore, no HuNoV-HS66-specific ASCs were observed. The cytokine concentrations in the serum or IC and CSC responses were also low and not significantly higher than mock controls in any of the tissues at any PID in the inact-HS66 group.

HuNoV-HS66 elicits low antibody responses in Gn pigs. Antibody titers in serum and IC as determined by immunocytochemistry were generally low in the HuNoV-HS66-inoculated pigs and ranged from 20 to 160 (data not shown). The IgM antibodies were initially detected in serum at PID 4. The IgA antibodies were initially detected in serum at PID 6 and peaked at PID 28 (geometrical mean titer [GMT], 16). The IgG antibodies in serum were first detected at PID 21 and also peaked at PID 28 (GMT, 14). Sixty-five percent of the HuNoV-HS66-inoculated pigs seroconverted by PID 21 to 28 with either IgA (titers from 10 to 160) or IgG antibodies (titers from 20 to 80). In IC, IgM antibodies were initially detected and peaked on PID 6 (GMT, 10). The IgA antibody response was first detected in IC and peaked at PID 6 (GMT, 22), and IgG antibodies were initially detected at PID 21 (GMT, 7) and peaked at PID 28 (GMT, 11). The IC of 9 of 14 (64%) of HuNoV-HS66-inoculated pigs tested had IgA and/or IgG antibody titers that ranged from 10 to 160. No HuNoV-HS66-specific antibody responses were detected in the serum or IC of the control pigs.

Seroconversion, serum, and intestinal convalescent antibody titers to HuNoV-HS66 are associated with diarrhea severity during the acute phase of infection. When the titers of IgA or IgG convalescent antibodies in the serum or IC (PID 21 and PID 28) were compared with the severity of diarrhea (cumulative scores) during HuNoV-HS66 infection (PID 1 to 6), moderate but significant correlations were found between diarrhea severity and convalescent-phase serum IgA and IgG antibody titers ($r = 0.5$ and $P = 0.004$; $r = 0.4$ and $P = 0.03$; respectively) and the convalescent intestinal IgA and IgG an-

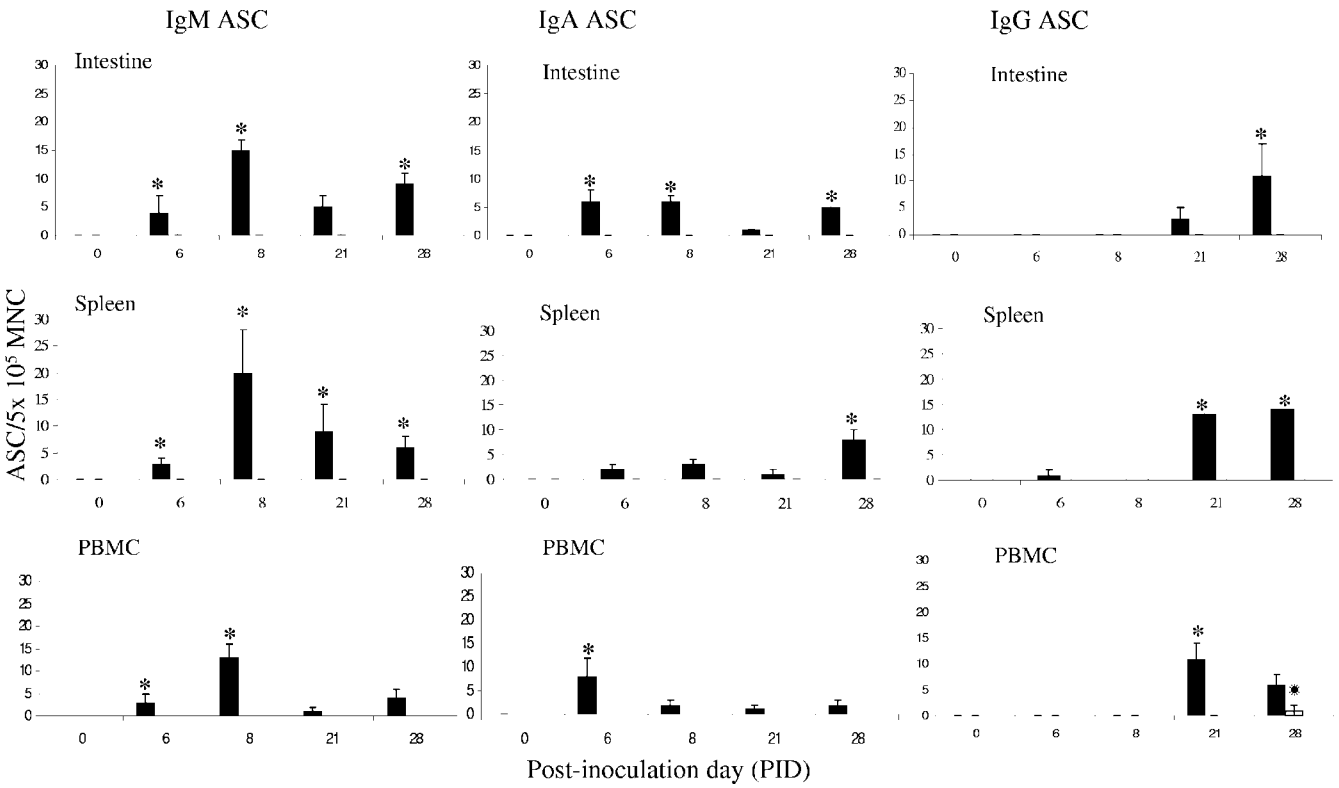

FIG. 1. Isotype-specific (IgM, IgA, and IgG) ASC responses in intestine, spleen, and PBMCs of Gn pigs inoculated with HS66 or controls. ■, HS66; □, control; ∗, significantly higher than controls ($P < 0.05$). Control bars are not visible due to lack of response by the controls to the specific antigen, except for IgG in PBMCs (indicated by filled circle with rays on the graph).

tibody titers ($r = 0.6$ and $P = 0.03$; $r = 0.7$ and $P = 0.01$; respectively).

HuNoV-HS66 induced low numbers of HS66-specific ASCs systemically and locally in Gn pigs. The results of the ELISPOT assay for detection of virus-specific ASCs in HuNoV-HS66-inoculated and control Gn pigs are depicted in Fig. 1. Overall, the number of ASCs elicited in the HuNoV-HS66-inoculated pigs was low, but most ASC responses were significantly higher than in control pigs (Fig. 1). The IgM ASCs were initially detected at PID 6 and peaked at PID 8 in both tissues and in blood peripheral blood mononuclear cells (PBMCs), with the highest numbers detected in spleen (20 ASCs per 5×10^5 MNCs). Significantly high numbers of IgA ASCs compared to controls were also first detected at PID 6 in intestine (peak at PID 6 to 8) and blood (peak at PID 6) and only later in spleen (PID 28). The IgG ASC responses peaked later, and the ASC numbers were significantly higher than in controls only at PID 28 in intestine, at PIDs 21 and 28 in spleen, and at PID 21 in blood, with similar numbers of IgG ASCs in both tissues and in the PBMCs.

Local ASC responses were strongly correlated with systemic ASC responses after HuNoV-HS66 infection. At PID 6, high correlations were observed between intestinal IgA ASCs and IgA ASCs in blood ($r = 1$; $P < 0.0001$); at PID 8 between IgM ASCs in the intestine and in spleen ($r = 0.9$; $P < 0.0001$) or in blood ($r = 1$; $P < 0.0001$) and between IgA in intestine and in spleen ($r = 1$; $P < 0.0001$); at PID21 between IgG ASCs in intestine and in spleen or in blood ($r = 0.8$ and $P = 0.0008$; $r = 0.9$ and $P < 0.0001$; respectively); and at PID 28 between IgA ASCs in intestine and in spleen ($r = 0.6$; $P = 0.02$) and IgG ASC in intestine and in spleen ($r = 0.8$; $P = 0.0005$) or in blood ($r = 0.7$; $P = 0.003$).

HuNoV-HS66 induced a balanced Th1/Th2 and a delayed IFN-I response in serum of Gn pigs. The cytokine ELISA results are summarized in Fig. 2 and Table 2. The innate cytokine IFN-I (IFN-α) was detected in the serum of all pigs at all times tested including constitutively at PID 0. A low peak of IFN-α, although not significantly higher than controls, was detected in the serum of the HuNoV-HS66-inoculated pigs at PID 1. The IFN-α concentrations in the serum of the HuNoV-HS66-inoculated pigs, which were quantitatively the highest for any cytokine, were significantly higher than controls only later at PID 10 and PID 12 (2.2- and 1.6-fold increases over controls, respectively). The proinflammatory cytokine IL-6 was detected acutely in the serum of HuNoV-HS66-inoculated pigs and was significantly higher than in control pigs at PID 2 (2.5-fold) and 4 (6.8-fold), coincident with both the diarrhea (PID 2 to 6) and viremia (PID 1 to 2) periods, and remained only slightly elevated until PID 10.

The HuNoV-HS66-inoculated pigs developed significantly higher Th1 (IL-12) responses than controls (1.7- to 2.5-fold higher) at all PIDs except at PIDs 0, 6, and 28. The low IFN-γ peak occurred acutely (PID 2) in serum of the HuNoV-HS66-inoculated pigs, coinciding with the transient viremia at this time (PID 1 to 2). The IFN-γ concentration at PID 2 was significantly higher (2.5-fold) than that of control pigs but was

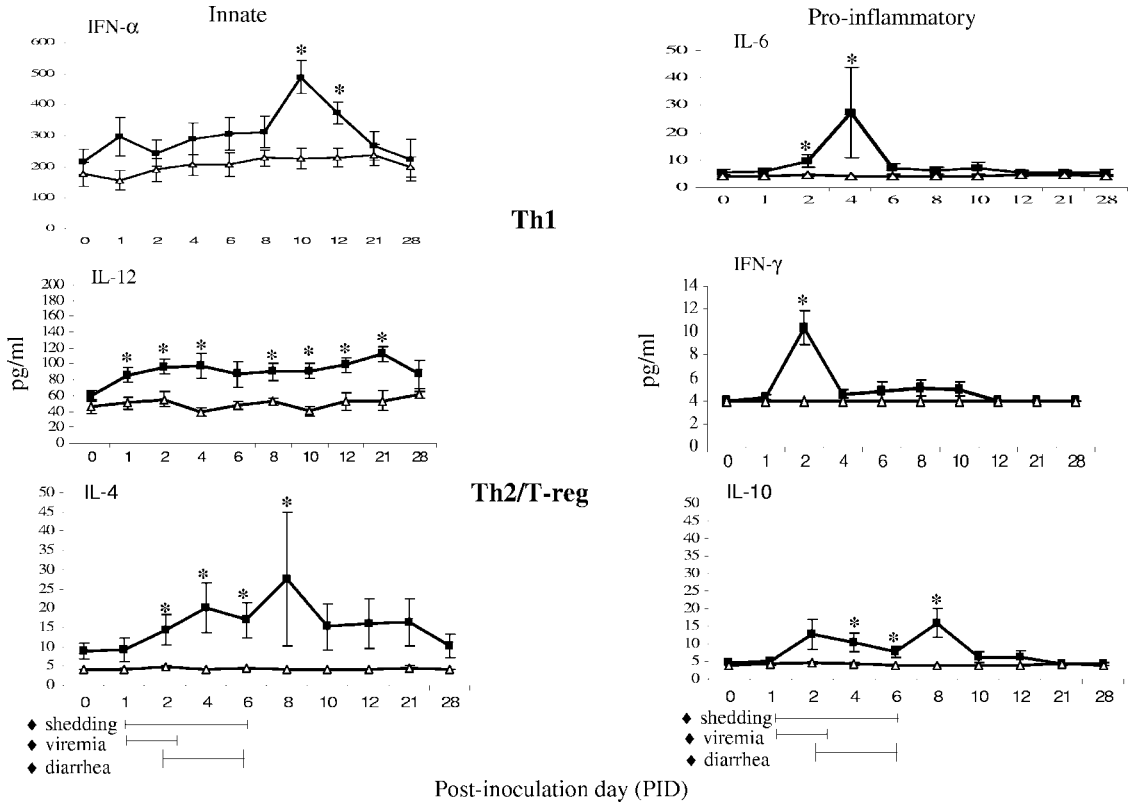

FIG. 2. Cytokine concentration in serum of Gn pigs inoculated with HuNoV-HS66 or controls. Symbols: ■, HS66; △, controls; ∗, significantly higher than control ($P < 0.05$). Due to differences in the concentrations of the cytokines, the graphs are shown using different scales. The duration period (◆) of clinical signs and stages is shown at the bottom of the graphs.

transient, remaining only slightly elevated through PID 10. Both IL-12 and IFN-α were also constitutively present at PID 0 and throughout PID 28 in the controls.

The Th2 (IL-4) and Th2/T_{reg} (IL-10) cytokines could be detected only in the serum of the HuNoV-HS66-inoculated pigs and not in the controls at each PID. The IL-4 and IL-10 concentrations were low but were significantly elevated above controls at similar PIDs (4, 6, and 8 and PID 2 for IL-4 only) with 2.5- to 7-fold increases over controls.

Only IFN-I (early and late) and IL-12 (late) cytokine concentrations were significantly elevated in the IC of the HuNoV-HS66 pigs compared to controls. The cytokine concentrations were measured in the IC of each euthanized pig and are presented as mean concentrations of each cytokine in the IC of each group for each PID that pigs were euthanized (Fig. 3). All cytokine concentrations tested peaked early (PIDs 2 and 6) in the HuNoV-HS66-infected pigs compared to controls and then peaked again at PID 21 or 28 for all but IL-10 (Fig. 3). Because of variability among pigs and possible degradation of some cytokines in IC, significant differences in cytokine concentrations in the IC of HS66-infected pigs, compared to controls, were observed only for IFN-α at PIDs 2, 8, and 21 and for IL-12 at PID 28. However, the peak concentrations of IFN-γ and IL-6 were higher in IC than in the serum (1.9- and 1.7-fold, respectively) of the HuNoV-HS66-infected pigs, whereas those for IFN-α and IL-12 were higher in serum than in IC (4.1- and 4.7-fold, respectively). In the IC, the significant peak of IFN-α at PID 2 coincided with the period of viral shedding, viremia,

TABLE 2. Cytokine responses in serum of HuNoV-HS66-infected Gn pigs

Cytokine type	Mean increase (*n*-fold) at the indicated PID[a]								
	1	2	4	6	8	10	12	21	28
Innate (IFN-α)	1.9	1.3	1.4	1.5	1.4	**2.2**	**1.6**	1.1	1.1
Proinflammatory (IL-6)	1.5	**2.5**	**6.8**	1.8	1.5	1.8	1	1	1.5
Th1									
IL-12	**1.7**	**1.7**	**2.5**	1.8	**1.7**	**2.3**	**1.9**	**2.1**	1.4
IFN-γ	1	**2.5**	1.3	1.3	1.3	1.3	1	1	1
Th2 (IL-4)	2.3	**2.8**	**5**	**4.3**	**7**	3.8	4	3.2	2.5
Th2/T_{reg} (IL-10)	1.3	2.6	**2.5**	**2**	**4**	1.5	1.5	1	1

[a] Statistically significant increases are in boldface ($P < 0.05$).

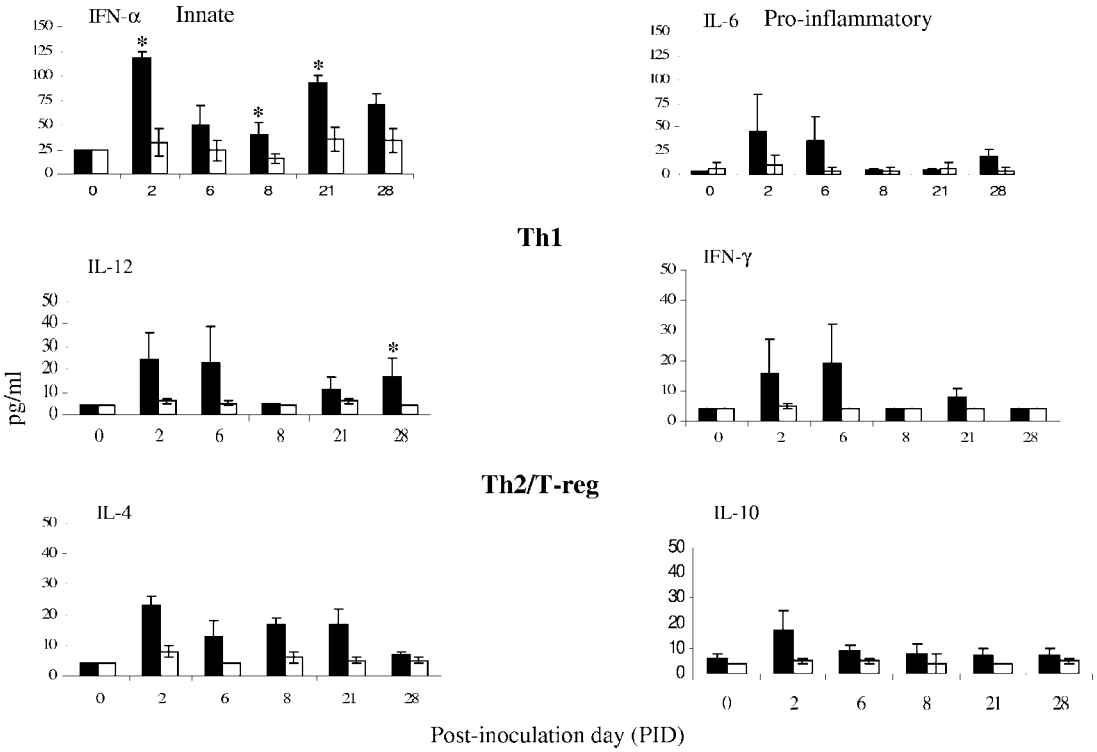

FIG. 3. Cytokine concentration in IC of Gn pigs inoculated with HuNoV-HS66 or controls. Symbols: ■, HS66; □, control; ∗, significantly higher than controls ($P < 0.05$). Results expressed are the mean value of the cytokine concentrations in the small intestinal contents of all pigs in each group.

and diarrhea, and in both serum and IC, a second and significantly elevated peak was detected at PIDs 8 and 21, respectively.

HuNoV-HS66 elicited higher numbers of Th1 (IL-12 and IFN-γ) CSCs than Th2 (IL-4) and proinflammatory (IL-6) CSCs both systemically and locally. Higher numbers of CSCs were generally detected at all times postinoculation and in all tissues and blood in the HuNoV-HS66-inoculated pigs than in controls (Fig. 4a and b). The Th1 (IFN-γ and IL-12) and Th2/T_{reg} (IL-10) CSC responses were elicited at a much higher magnitude than the proinflammatory (IL-6) and Th2 (IL-4) responses (Fig. 4a and b). For Th1 CSC responses, IL-12 showed divergent responses for both tissues and blood (Fig. 4a). IFN-γ responses in the intestine paralleled those in spleen. Significantly higher numbers of IFN-γ CSCs were detected and peaked acutely (PID 2) in intestine and spleen but peaked thereafter (PID 8) in blood and were also significantly elevated later in intestine and spleen (PIDs 21 and 28) and in blood (PID 21) compared to controls. Significantly elevated numbers of IL-12 CSCs peaked first in intestine (PID 21) and then in the spleen and blood (PID 28) and were significantly higher than controls. Compared to Th1 responses, Th2 (IL-4) CSC numbers were elicited at lower levels and were only significantly higher than controls later in infection in intestine and blood (PID 21) and spleen (PID 28) (Fig. 4b). The Th2/T_{reg} (IL-10) CSC numbers were higher overall than IL-4 CSC numbers, with the peak numbers detected later in infection in intestine (PID 21) and blood (PID 28), although none were significantly higher than controls. Peak numbers were detected earlier in spleen (PID 8) than in intestine (PID 21) and blood (PID 28).

The proinflammatory (IL-6) CSCs were elicited at a lower magnitude than all other cytokines tested (Fig. 4a and b) but were significantly increased both early (PIDs 2 and 6) and late (PID 28) in the blood and intestine of HuNoV-HS66-infected pigs compared to controls, with the significantly elevated blood CSC responses closely mimicking those in the intestine (Fig. 4a).

Systemic HuNoV-HS66 CSC responses strongly correlated with local HuNoV-HS66 CSC responses. At each PID with significant CSC numbers, we tested for correlations between intestine, spleen, and blood that might reflect trafficking of intestinal CSCs stimulated locally, by HuNoV-HS66 infection through the blood, or systemically. We found correlations of CSC numbers between proinflammatory (IL-6) CSCs in the intestine and in the spleen at PID 2 ($r = 0.9$; $P = 0.0002$) and PID 6 ($r = 1$; $P < 0.0001$) and between IL-6 CSC numbers in intestine and blood at PID 28 ($r = 0.9$; $P < 0.0001$). For Th1 (IFN-γ), correlations were found between CSCs in intestine and in spleen and blood at PID 8 ($r = 0.7$ and $P = 0.04$; $r = 1$ and $P < 0.0001$; respectively), PID 21 ($r = 0.9$ and $P < 0.0001$ for both), and PID 28 ($r = 0.9$ and $P < 0.0001$; $r = 1$ and $P < 0.0001$; respectively). For Th2 (IL-4), correlations were found between CSC numbers in intestine and spleen and blood at PID 28 ($r = 0.8$ and $P = 0.0005$; $r = 0.8$ and $P = 0.0002$; respectively) and also between CSC numbers in intestine and blood at PID 21 ($r = 0.8$ and $P = 0.003$). Generally, local and systemic HuNoV-HS66 CSC responses strongly correlated, in-

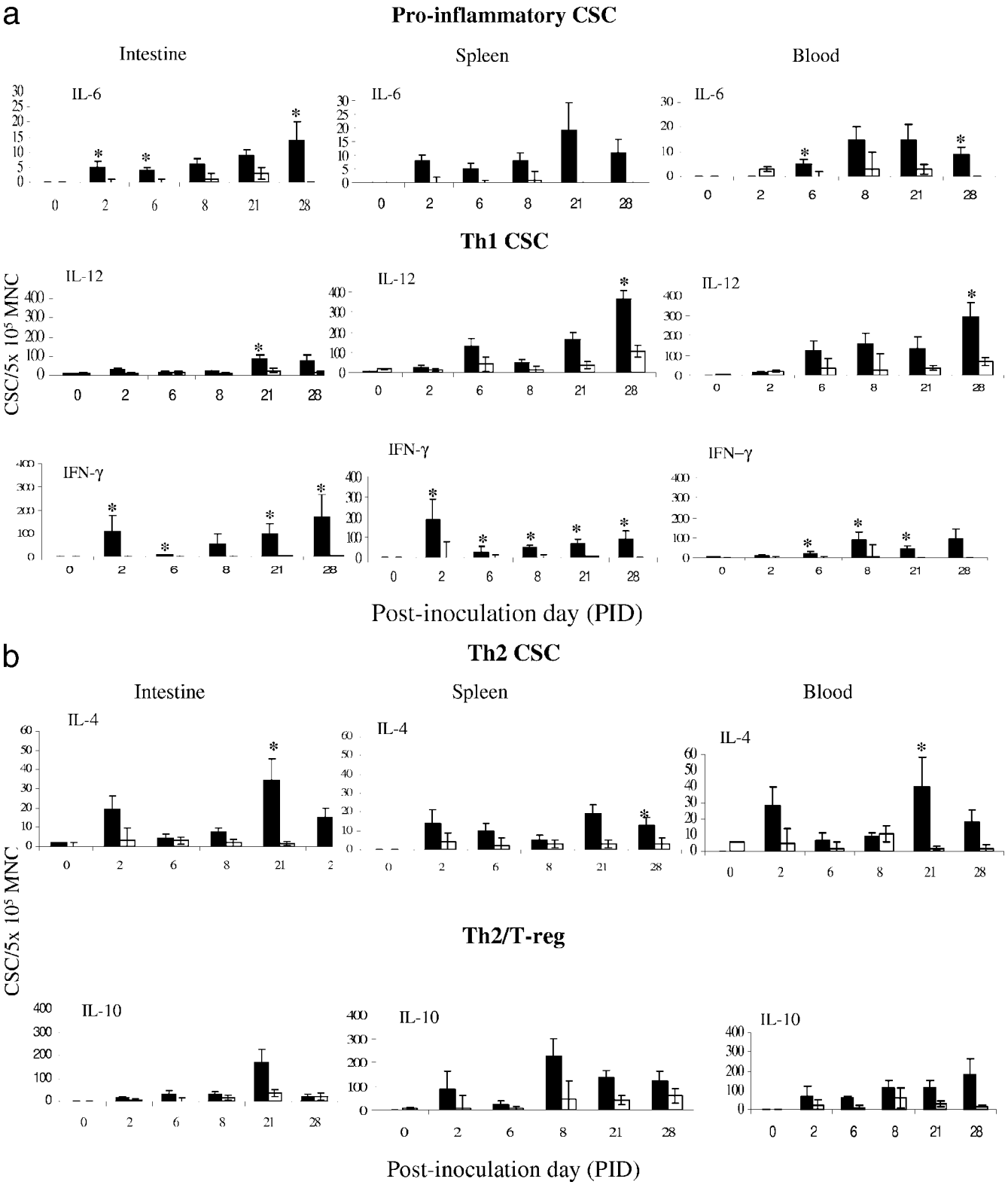

FIG. 4. (a and b) Th1 (IFN-γ and IL-12), proinflammatory (IL-6), Th2 (IL-4), and Th2/T_{reg} (IL-10) CSC responses in intestine, spleen, and PBMCs of Gn pigs inoculated with HS66 or controls. Symbols: ■, HS66; □, controls; ∗, significantly higher than controls ($P < 0.05$).

dicating that HS66 induced both local and systemic cellular immune responses.

DISCUSSION

Because studies of immunity to enteric caliciviruses in any species are limited, we evaluated B-cell (antibody and ASCs) as well as T-cell (Th1 and Th2) responses and innate (IFN-α) and proinflammatory (IL-6) cytokine responses both locally (intestine) and systemically (spleen and blood) to better understand the pathogenesis and immune responses to GII.4 HuNoV-HS66 infection in Gn pigs.

A recent study conducted in our laboratory described the detailed pathogenesis of the HuNoV-HS66 strain in Gn pigs (7). In the present study, a new subset of pigs was used, and the results were in agreement with our previous study except that we found that 32/35 (91%) of the pigs inoculated with HuNoV-HS66 had detectable viral RNA in at least one of the rectal swab fluid samples by RT-PCR and/or microwell hybridization assay in contrast to 44% of the pigs in our earlier study. This difference could be explained by the fact that all pigs in the present study, but not in our prior study, received sodium bicarbonate prior to inoculation in order to neutralize the acidic pH during viral passage through the pig's stomach, as previously described in studies with human volunteers (11, 15). Second, we reextracted and retested each sample that was inhibited in RT-PCRs, and this decreased the inhibition level of the samples and improved our detection sensitivity.

Overall, the antibody titers in serum and IC, as well as the numbers of ASCs in intestine, spleen, and blood of HuNoV HS66-infected pigs, were low. When we compared our results to Gn pigs infected with another heterologous human enteric virus, the virulent Wa human rotavirus (HRV) (46), we observed that HRV also induced an early low IgM ASC response in the small intestine that decreased by PID 21, similar to the HuNoV-infected pigs. However, HRV induced approximately 10 times more IgA and IgG ASCs in the intestine of the Gn pigs at PID 21 than HuNoV-HS66 at PID 28, but, interestingly, similar numbers of IgA and IgG ASCs were detected in spleen and blood in both HRV- and HuNoV-infected pigs at PID 21 or 28. The greater magnitude of the intestinal ASC response in the HRV-infected pigs likely reflects the longer and more pronounced intestinal viral replication and diarrhea seen, as well as the more extensive intestinal lesions noted (39). The similarities in the systemic ASC responses observed in the HRV- and HuNoV-HS66-infected Gn pigs may reflect the transient viremia evident in both.

In this study, significant positive correlations were found between intestinal and systemic ASC responses, including between IgA ASCs in intestine and spleen and between IgG ASCs in intestine and spleen and blood. Our findings are in agreement with results after virulent HRV infection of Gn pigs in which correlations were also found between IgA ASC numbers in the intestine and IgA ASCs in the blood or spleen (46).

In this study, a typical temporal distribution of ASCs indicative of primary immune responses was shown by early IgM ASC detection in local (intestine) and systemic (spleen and blood) tissues, followed by late IgG ASCs in tissues and blood. Detection of IgA ASCs early in the intestine and blood and later in spleen (memory IgA ASCs) may reflect the early trafficking of these cells between the gut lymphoid tissue and the blood and later to the systemic lymphoid tissue (9).

Data from human adult volunteer challenge studies suggested that immunity to HuNoV is variable and that the correlates of protective immunity to HuNoV are not understood (11, 15, 18, 27). Short-term immunity to homologous virus has been shown consistently; however, the seroconversion rates vary (50 to 90%) (20). In this study, 65% of the HuNoV-HS66-infected pigs that were euthanized at PID 21 or PID 28 seroconverted with either IgA, IgG, or both, and coproconversion with either IgA or IgG antibodies was detected in 9/14 (64%) of the IC from the HuNoV-HS66-infected pigs tested. Similar results were observed in studies that used primates as experimental animal models, where some animals that shed Toronto-like virus in the feces seroconverted and others did not (34).

It is important to emphasize that our animal model, the Gn pig, is a naïve animal that had not been previously exposed to any NoV or other microbe prior to HuNoV-HS66 inoculation. Therefore, the magnitude of the immune responses in the experimental animals is predictably lower than that of adult human volunteers participating in HuNoV challenge studies, who have probably been repeatedly exposed to NoVs during their lifetimes. The presence of preexisting antibodies to NoV elicited by previous infections with antigenically related viruses makes interpretation of the primary antibody responses confusing in such studies. Thus, the Gn pig model allows assessment of the primary immune responses to HuNoV in a NoV-naïve animal model.

Very few studies have been done of local and secretory immune responses to infection with HuNoV (3, 27), and in early volunteer studies fecal sIgA antibodies to HuNoV were used as a marker for symptomatic infection (27); however, a more recent study failed to show a significant increase in convalescent fecal IgA antibody titers after oral exposure of volunteers to NV (27). In a study that used rhesus monkeys as experimental animal models, those that had clinical signs of infection and shed NV virus in the feces did not seroconvert, except for one that shed virus for a longer time (32), coinciding with our finding of positive correlations between severity of disease and IgA and IgG antibody titers in both serum and IC of HuNoV-inoculated pigs. Our results suggest that diarrhea severity may reflect the intensity of intestinal stimulation, leading to increased local and systemic antibody titers.

In our study, innate (IFN-α), proinflammatory (IL-6), Th1 (IL-12 and IFN-γ), and Th2/T_{reg} (IL-4 and IL-10) cytokine responses were elicited in the serum of HuNoV-HS66-infected pigs. As shown in previous work done in our laboratory, the intestinal cytokine concentrations are probably underestimated due to their instability to acidic pH and sensitivity to proteolysis by intestinal enzymes. However, the cytokine levels in the IC of the HuNoV-HS66-infected pigs were consistently higher than the levels in the IC of the controls.

The production of IFNs-I is usually upregulated in the early phase of viral infection (4). We speculate that the earlier increase (PID 2) of IFN-α secretion in the IC observed in this study was the result of a local, early response of the host's innate immune system (epithelial or dendritic cells or macrophages) to viral infection (30). It is possible that HuNoV could replicate in macrophages and dendritic cells, as has been described for murine noroviruses (42), and that persistent anti-

gen presentation after acute virus infection or persistence of viral genomic RNA inducing very low levels of transcription and translation of viral proteins, as suggested for vesicular stomatitis virus and influenza virus (35, 47), may also occur for NoVs, thereby stimulating the secretion of innate cytokines even after virus shedding was no longer detectable. This would explain the later peaks of IFN-α in the IC and in the serum after clearance of acute viral infection.

Low to moderate levels of proinflammatory IL-6 (6 to 28 pg/ml) cytokine in serum of HuNoV-HS66-infected pigs were similarly detected in NoV-challenged humans by Lindesmith et al. (18), although levels of IL-6 in postchallenge human sera did not vary greatly from prechallenge levels. Low numbers of IL-6 CSCs, although significantly higher than the numbers in the controls, were also detected in the intestine, spleen, and blood of HS66-infected pigs. The low IL-6 responses to viral infection could reflect the low virus load during virus replication and the limited pathology observed, evoking only low inflammation in the gut. In contrast to HuNoV infection of Gn pigs (serum IL-6 levels increased 1.5- to 6.8-fold over controls), IL-6 levels in the serum of HRV-infected Gn pigs were higher and significantly elevated at PID 1 to 5 (17- to 57-fold higher than Gn controls), reflecting the high virus load during replication in the gut and the extensive intestinal pathology induced (2).

IL-12 (Th1 inducer) was detected in serum from both infected and control pigs at each time point, with significantly higher levels both early and again later in HuNoV-HS66-infected pigs. An early transient peak (PID 2) of IFN-γ was also observed in serum of the infected Gn pigs that coincided with the early peak of IFN-γ in the serum of human volunteers inoculated with SMV (GII.2 HuNoV strain) and with the peak viremia, based on our pig study (18). The early increases of IL-12 and of IFN-γ in the serum, likely produced by macrophages and dendritic cells (or also by NK cells for IFN-γ), may represent the early innate responses to viral infection.

Significantly higher numbers of IFN-γ CSCs in the intestine, spleen, and blood also during the acute phase of infection (PIDs 2 and 6) corroborate the potential role of IFN-γ CSCs in stimulating $CD8^+$ T cells that function in viral clearance during the immune response to viral pathogens, including curtailment of viremia and fecal virus shedding (16). Significantly higher numbers of IFN-γ CSCs were also detected later (PID 21 or 28) in both tissues and blood, most likely representing the pool of antigen-specific effector memory Th1 lymphocytes at the local site of antigen encounter (gut), and also systemically.

Significantly elevated serum and IC IL-12 cytokine concentrations and CSC numbers were also detected later in infection in the HuNoV-HS66-infected pigs, first locally in intestine and later systemically in spleen and blood. They may reflect the pool of circulating antigen-presenting cells that secrete IL-12 after antigen stimulation (40) or the presence of Th1 effector memory cells secreting IL-12.

The Th2 (IL-4) and Th2/T_{reg} (IL-10) cytokines were detected in the serum of HuNoV-HS66-infected pigs at moderate to low levels at most PIDs and were significantly elevated early in the HuNoV-HS66-infected pigs. Numbers of IL-4 and IL-10 CSCs were also low, but significantly higher numbers of IL-4 CSC were detected locally in intestine and systemically in spleen later in infection. In studies of immunity to SMV in adult volunteers with preexisting antibodies to NoVs, no significant changes were detected between pre- and postchallenge concentrations of IL-10 in the serum of adult volunteers or the ability of their PBMCs to secrete IL-4 or IL-10 after in vitro stimulation with SMV (18). Furthermore, because IL-10 is also considered to be a regulatory cytokine in pigs and humans (10, 23, 26), functioning in the control of inflammation (10), the low levels of IL-10 in serum and the lack of significantly elevated numbers of IL-10 CSCs in HuNoV-HS66-infected pigs could be due to the low level of pathology (7) and inflammation induced by the GII.4 HS66 strain in pigs. This finding is also reflected by low, very transient levels of the proinflammatory cytokine IL-6 in serum and low numbers of IL-6 CSCs in intestine, spleen, and blood in the HuNoV-HS66-infected pigs.

The early detection of IL-6 CSCs (intestine and blood) and IFN-γ (intestine, spleen, and blood), followed by late IL-12 and IL-4 (intestine, spleen, and blood), shows that an early innate response to viral replication occurred, and it was followed by the later induction of memory Th1 (IL-12 and IFN-γ) and Th2 (IL-4) responses both locally and systemically. Furthermore, the positive associations found between local (intestine) and systemic (blood) IL-6, IFN-γ, and IL-4 CSC numbers suggest that these memory CSCs migrate from the original site of viral replication (intestine) transiently through the blood, as also shown for humans (14).

In conclusion, HuNoV-HS66 induced low levels of antibodies and low numbers of ASCs, both systemically and in the gut mucosa, and 65% seroconversion in pigs. In serum, HuNoV-HS66 induced Th2 responses but higher Th1 responses. Innate IFN-I responses were observed acutely in IC but were delayed in serum of HuNoV-HS66-infected pigs. The low proinflammatory (IL-6) CSC responses coincided with the lack of pronounced diarrhea and histological lesions (7) induced by HuNoV-HS66 in Gn pigs. This study provides data on the pattern and time progression of antibody and cytokine responses after GII HuNoV infection and further supports the previously described replication of HuNoV in Gn pigs (7). To our knowledge, this is the first study that comprehensively delineates the systemic and intestinal antibody, ASC, cytokine, and CSC responses after HuNoV infection in an experimental animal model.

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