Pig model mimicking chronic hepatitis E virus infection in immunocompromised patients to assess immune correlates during chronicity

Dianjun Cao, Qian M. Cao, Sakthivel Subramaniam, Danielle M. Yugo, C. Lynn Heffron, Adam J. Rogers, Scott P. Kenney, Debin Tian, Shannon R. Matzing, Christopher Overend, Nicholas Catanzaro, Tanya LeRoith, Heng Wang, Pablo Piñeyro, Nicole Lindstrom, Sherrie Clark-Deen, Lijuan Yuan, and Xiang-Jin Meng

*Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061; and Department of Large Animal Clinical Sciences, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2016.

Contributed by Xiang-Jin Meng, May 23, 2017 (sent for review April 3, 2017; reviewed by Alexander Ploss and Christopher M. Walker)

Chronic hepatitis E virus (HEV) infection is a significant clinical problem in immunocompromised individuals such as organ transplant recipients, although the mechanism remains unknown because of the lack of an animal model. We successfully developed a pig model of chronic HEV infection and examined immune correlates leading to chronicity. The conditions of immunocompromised patients were mimicked by treating pigs with an immunosuppressive regimen including cyclosporine, azathioprine, and prednisolone. Immunocompromised pigs infected with HEV progressed to chronicity, because 8/10 drug-treated HEV-infected pigs continued fecal virus shedding beyond the acute phase of infection, whereas the majority (7/10) of mock-treated HEV-infected pigs cleared fecal viral shedding at 8 wk postinfection. During chronic infection, serum levels of the liver enzyme γ-glutamyl transferase and fecal virus shedding were significantly higher in immunocompromised HEV-infected pigs. To identify potential immune correlates of chronic infection, we determined serum levels of cytokines and cell-mediated immune responses in pigs. Results showed that HEV infection of immunocompromised pigs reduced the serum levels of Th1 cytokines IL-2 and IL-12, and Th2 cytokines IL-4 and IL-10, particularly during the acute phase of infection. Furthermore IFN-γ-specific CD4+ T-cell responses were reduced in immunocompromised pigs during the acute phase of infection, but TNF-α-specific CD8+ T-cell responses increased during the chronic phase of infection. Thus, active suppression of cell-mediated immune responses under immunocompromised conditions may facilitate the establishment of chronic HEV infection. This pig model will aid in delineating the mechanisms of chronic HEV infection and in developing effective therapeutics against chronic hepatitis E.

Hepatitis E virus | chronic HEV infection | pig | immunosuppression | cell-mediated immune responses

Hepatitis E virus (HEV) infection causes an important global public health disease burden with an estimated 20 million individuals affected worldwide every year (1) resulting in 56,600 hepatitis E-related deaths (2). HEV is a single-stranded, positive-sense, RNA virus (3) belonging to the family Hepeviridae (4). HEV has been a major cause of acute viral hepatitis in many developing countries, although sporadic and cluster cases of acute hepatitis E have been reported in many industrialized countries, including the United States (5–7). Recent reports suggest that the clinical cases and disease burden associated with HEV infection in industrialized countries have been underestimated (7).

In general, HEV infection in immunocompetent individuals develops a self-limiting acute viral hepatitis. However, the majority of HEV infections in immunocompromised individuals, such as solid-organ transplant recipients and patients with HIV infection, lymphoma, or leukemia, are likely to progress to chronicity (8). Since the first report of chronic HEV infection in liver transplant patients in 2008 (9), chronic hepatitis E has become recognized as an emerging and important clinical problem in immunocompromised individuals, especially in solid-organ transplant recipients (8, 10). Chronic hepatitis E can cause significant liver damage, which may eventually lead to cirrhosis with considerable mortality. Patients with chronic hepatitis E also shed HEV in feces for a prolonged period and can transmit the virus to immunocompetent individuals (9). Broad-spectrum antivirals such as ribavirin and pegylated IFN have been used for the treatment of chronic hepatitis E with some success (11, 12), although currently there is no established HEV-specific therapeutic protocol. Also, importantly, the fundamental mechanisms leading to the progression and establishment of chronic hepatitis E in immunocompromised patients are unknown because of the lack of an animal model for chronic hepatitis E. Therefore, an animal model that can mimic chronic HEV infection in immunocompromised individuals is urgently needed to study the underlying mechanisms of chronic infection and to develop effective and specific therapeutics against chronic hepatitis E in immunocompromised individuals.

The Hepeviridae family has two genera (Orthohepevirus, and Piscihepevirus) and five species. The species Orthohepevirus A includes HEV infecting humans and several other mammalian species and consists of at least seven distinct HEV genotypes (4): genotypes 1 and 2 infect humans exclusively; genotypes 3 and 4 infect nonhuman primates; and genotypes 5 and 6 infect pigs and other mammals. Genotypes 7 and 8 infect wild and domestic ungulates, respectively.

Significance

An estimated 20 million hepatitis E virus (HEV) infections occur yearly worldwide, leading to 56,600 deaths. Chronic HEV infection has recently become a significant clinical problem in immunocompromised individuals such as organ transplant patients. The lack of an animal model greatly hinders our ability to study chronic HEV infection and develop therapeutics. Here we report the successful development a pig model of chronic HEV infection by mimicking the conditions of immunocompromised organ transplant patients. We demonstrate that active suppression of HEV-specific cell-mediated immune responses under immunocompromised conditions may facilitate the establishment of chronic HEV infection. This unique model now affords the opportunity to delineate the mechanism leading to chronicity and to test specific antivirals against chronic hepatitis E.


The authors declare no conflict of interest.

1To whom correspondence should be addressed. Email: xjmeng@vt.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1705446114/-/DCSupplemental.
immunosuppressive drugs (see Materials and Methods for details) routinely used to prevent rejection in human organ transplant recipients. In an attempt to identify the mechanism and immune correlates leading to chronic HEV infection, the magnitude and duration of viremia and fecal virus shedding, the types of immune responses developed against the virus, and the liver pathology associated with chronic HEV infection were also determined and analyzed in chronically infected pigs.

Results

Successful Establishment of a Pig Model for Chronic HEV Infection. To mimic the immunosuppressive conditions in human solid-organ transplant recipients, pigs in the immunocompromised group were orally administered a drug mixture compounded with three immunosuppressive drugs (see Materials and Methods for details). In the immunocompetent group, pigs naturally infected with HEV served as controls. At the start of the experiment, all pigs were HEV negative. Pigs were injected intramuscularly (i.m.) with a virus suspension three times weekly for 8 wk. Virus shedding was monitored in feces and serum by qRT-PCR. At the end of the 8-wk period, pigs were killed, and their livers were examined for HEV RNA and immunohistochemistry.

Fecal virus shedding was initially monitored by HEV-specific regular RT-PCR. Viral RNAs were detected in the fecal samples of both drug-treated (immunocompromised) and mock-treated (immunocompetent) HEV-infected pigs up to 8 wk postinfection (wpi). At 12 wpi, 7 of the 10 pigs in the drug-treated HEV-infected group still shed HEV in feces, whereas only 1 of the 10 pigs in the mock-treated HEV-infected group still had detectable viral RNA in feces by the HEV-specific RT-PCR. In general, during acute HEV infection in pigs, fecal virus shedding typically is cleared by 7–8 wpi (16); thus, for the purpose of data interpretation, we arbitrarily set 8 wpi as the time point separating acute and chronic infections in this study. The results showed that we successfully established chronic HEV infection in immunocompromised pigs, because virus shedding in these pigs continued beyond 8 wpi, for at least five additional 5 weeks. All animals were killed at 13 wpi after we determined that chronic HEV infection had been established, with the exception of two immunocompromised HEV-infected pigs that were kept alive for nine more weeks. These two pigs continued to receive daily immunosuppressive drugs, and viral RNAs in their feces were detected by regular RT-PCR during the additional 9 wk. At the end of the 9-wk period these two animals were killed also.

HEV RNAs were quantified in weekly fecal samples and in biweekly serum samples by qRT-PCR. The viral RNA loads in feces peaked at 3 wpi in immunocompetent HEV-infected pigs but continued to rise and reached peak level at 5 wpi in immunocompromised HEV-infected pigs (Fig. 1A). Between 5 and 13 wpi, the viral RNA loads in feces were significantly higher in immunocompromised HEV-infected pigs than in mock-treated HEV-infected pigs (P < 0.05), except at 10 and 11 wpi (Fig. 1A). At 8 wpi, 8 of the 10 mock-treated HEV-infected pigs but only 3 of the 10 of the drug-treated HEV-infected pigs stopped fecal virus shedding (Fig. 1A). At 13 wpi, 9 of the 10 drug-treated HEV-infected pigs but only 2 of the 10 mock-treated HEV-infected pigs shed HEV in feces (Fig. 1A). Therefore, the result of fecal virus shedding tested with qRT-PCR is similar to that tested with HEV-specific regular RT-PCR. During the chronic phase of infection, from 8 to 13 wpi, the average levels of viral RNA loads were consistently higher in feces of immunocompromised HEV-infected pigs than in the feces of immunocompetent HEV-infected pigs (Fig. 1A).

Fig. 1. Quantification of HEV RNA loads by qRT-PCR in fecal and serum samples from HEV-infected pigs. (A) HEV RNA load in fecal samples collected weekly postinfection. (B) HEV RNA loads in serum samples collected biweekly. The amounts of HEV RNAs were measured by qRT-PCR at the indicated time points, converted to log_{10} of copies per milliliter of 10% fecal suspension or serum, and expressed as mean ± SEM in a scatter plot. Each symbol in the scatter plot represents the value of an individual pig; symbols on the x axis are below the assay detection limit (400 copies per 1-ml sample) and were defined as negative. Circled numbers below the x axis indicate the number of negative animals at each time point. HEV, HEV infected (n = 10); IC HEV, immunocompromised and HEV infected (n = 10). *P < 0.05.
Interestingly, at 1 wpi, the viral RNA levels were significantly higher (about 0.5 log difference) in the sera of immunocompetent HEV-infected pigs than in the sera of immunocompromised HEV-infected pigs \( (P < 0.05) \) (Fig. 1B). From 1 wpi to 13 wpi the viral RNA levels in sera were comparable, at a low level, in immunocompetent and immunocompromised HEV-infected pigs, with the exception of 9 wpi, when the serum viral RNA level was significantly higher in immunocompromised HEV-infected pigs than in immunocompetent HEV-infected pigs \( (P < 0.05) \) (Fig. 1B), which is due to further clearance of HEV in immunocompetent HEV-infected pigs.

During the chronic phase of HEV infection the serum levels of liver enzyme \( \gamma \)-glutamyl transferase (GGT) were significantly higher at 9 and 11 wpi in immunocompromised HEV-infected pigs \( (P < 0.05) \) (Fig. 2A). However, there was no statistically significant difference in the serum levels of sorbitol dehydrogenase (SDH) in immunocompromised HEV-infected and immunocompetent HEV-infected pigs \( (P > 0.05) \) (Fig. 2B). Similarly, we did not observe a significant increase in serum levels of other liver enzymes, including aspartate aminotransferase (AST), total bilirubin, and alkaline phosphatase, in immunocompromised HEV-infected pigs compared with immunocompetent HEV-infected pigs. At the time of necropsy (13 wpi), no gross lesions were observed in livers from all pigs, although mild microscopic lesions were found in the livers from 5 of the 10 immunocompromised HEV-infected pigs and 4 of the 10 immunocompetent HEV-infected pigs. There were no significant differences in microscopic liver lesion scores between the two groups of HEV-infected pigs, even though the majority of immunocompromised pigs continued to shed virus in feces during the chronic phase of infection.

**Reduction of Baseline Serum Levels of Th1 Cytokines (IL-2 and IL-12) and Th2 Cytokines (IL-4 and IL-10) in Immunocompromised HEV-Infected Pigs.** To understand the host immune response to HEV infection under immunosuppressive conditions, we simultaneously measured the levels of IFN-\( \gamma \), IL-2, IL-4, IL-10, and IL-12 in serum samples by Luminex multiplex assays. The average levels of serum IL-2 were significantly (about three- to sevenfold) lower in immunocompromised HEV-infected pigs than in immunocompetent HEV-infected pigs at 1 and 4 wpi \( (P < 0.05) \) (Fig. 3A). Similarly, the average levels of serum IL-12 were significantly (about 0.5- to 0.75-fold) lower in immunocompromised HEV-infected pigs than in immunocompetent HEV-infected pigs at 1 and 4 wpi \( (P < 0.01) \) (Fig. 3B). Although serum IFN-\( \gamma \) levels were lower in immunocompromised HEV-infected pigs than in immunocompetent HEV-infected pigs at 1 and 4 wpi, the observed differences were not significant (Fig. 3C).

The average serum IL-4 level in immunocompromised HEV-infected pigs at 1 wpi was the lowest observed in all treatment groups and was significantly lower than that in immunocompetent HEV-infected pigs \( (P < 0.05) \) (Fig. 3D). Similar to IL-2 and IL-12 responses in serum, the average levels of serum IL-10 were significantly (four- to eightfold) lower in immunocompromised HEV-infected pigs than in the HEV-infected pigs at 1 and 4 wpi \( (P < 0.05) \) (Fig. 3E). However, the serum levels of all aforementioned cytokines were not significantly different among immunocompromised mock-infected, immunocompetent mock-infected, and immunocompetent HEV-infected pig groups (Fig. 3). Overall, the serum levels of Th1 cytokines IL-2 and IL-12 and Th2 cytokines IL-4 and IL-10 were modestly reduced from baseline level of these cytokines in immunocompromised HEV-infected pigs, particularly during the early phase of HEV infection.

**IFN-\( \gamma \)-Specific CD4+ T-Cell Responses Decrease During the Acute Phase of HEV Infection, but TNF-\( \alpha \)-Specific CD8+ T-Cell Responses Increase During the Chronic Phase of HEV Infection in Immunocompromised Pigs.** Peripheral blood mononuclear cells (PBMCs) were prepared, stimulated in vitro with purified recombinant HEV ORF2 antigen, and subsequently stained and analyzed by flow cytometry. The frequencies of IFN-\( \gamma \)- and/or TNF-\( \alpha \)-producing T cells and IL-4-producing T cells were analyzed in three subpopulations, CD4+CD8- T cells (hereafter, “CD4+ T cells”), CD4-CD8+ T cells (hereafter, “CD8+ T cells”), and CD4+CD8+ T cells, and were expressed as the percentage of T cells (CD3+CD8+CD4+).

HEV-specific T-cell responses were not significantly induced in immunocompetent HEV-infected pigs compared with the immunocompetent control pigs (Fig. 4A-C). Within the three aforementioned T-cell subpopulations in the peripheral blood, we observed no statistically significant differences in the mean frequencies of IFN-\( \gamma \)-secreting T-cells in immunocompromised HEV-infected pigs and in immunocompetent HEV-infected pigs at 5, 8, and 13 wpi (Fig. 4A-C). These results are consistent with the absence of a significant increase in serum IFN-\( \gamma \) levels during HEV infection in immunocompromised pigs compared with immunocompetent control pigs (Fig. 3C).

Similar to the IFN-\( \gamma \) responses in the peripheral blood, the TNF-\( \alpha \)-producing T-cell responses were not significantly increased within all three T-cell subpopulations during the acute phase of HEV infection (i.e., up to 8 wpi) (Fig. 4D and E). However, at 13 wpi the TNF-\( \alpha \)-producing CD8+ T-cell frequencies were increased significantly (two- to threefold) in both immunocompromised and immunocompetent HEV-infected pigs compared with the frequencies in the immunocompromised and immunocompetent control groups \( (P < 0.05) \) (Fig. 4F). In contrast, the TNF-\( \alpha \) responses at 13 wpi were not significantly

---

**Fig. 2.** Serum levels of the liver enzymes GGT and SDH. The serum levels of GGT (A) and SDH (B) were measured by established protocols and expressed as mean ± SEM. Control, immunocompetent noninfected \( (n = 9) \); HEV, HEV infected \( (n = 10) \); IC, immunocompromised \( (n = 10) \); IC HEV, immunocompromised and HEV infected \( (n = 10) \). *\( P < 0.05 \).
augmented in the CD4+ T-cell and CD4+CD8+ T-cell subpopulations in response to HEV in both immunocompromised and immunocompetent HEV-infected pigs compared with mock-infected pigs (Fig. 4F). When we analyzed the frequencies of single- and double-cytokine T-cell producers specific to HEV within CD3+ gated T-cell populations, the frequencies of IFN-γ+ TNF-α+ CD4+ T cells were significantly higher in immunocompetent HEV-infected pigs than in immunocompromised HEV-infected pigs (**P < 0.01) (Fig. 5A and B) and reached similar levels to those in other groups at 8 wpi and 13 wpi (Fig. 5B and C). At all time points, however, the mean frequencies of all three subpopulations of IFN-γ+ TNF-α+ T cells did not differ significantly in HEV-infected pigs (Fig. 5 D-F). Moreover, the frequencies of IFN-γ+ TNF-α+ CD8+ T cells were significantly lower in immunocompromised pigs than in immunocompetent pigs, regardless of HEV infection status at 8 wpi, and at 13 wpi the levels in immunocompromised HEV-infected pigs were increased significantly (about twofold) above the background levels observed in pigs in other treatment groups (Fig. 5F).
In summary, in immunocompromised pigs IFN-γ–specific CD4+ T-cell responses are reduced during the acute phase of HEV infection, but TNF-α–specific CD8+ T-cell responses increase moderately during the chronic phase of HEV infection.

Increased IL-4–Specific CD4+ T-Cell Responses Against HEV in Immunocompromised HEV-Infected Pigs. The mean frequencies of IL-4–secreting T cells were not increased as a result of HEV infection in all three T-cell subpopulations examined in immunocompetent HEV-infected pigs as compared with the immunocompetent control group (Fig. 6). However, in both HEV-infected and noninfected immunocompromised pigs, the mean IL-4–specific CD4+ T-cell frequencies were significantly higher than in the immunocompetent control group at 8 wpi ($P < 0.05$) (Fig. 6B). On the other hand, the mean IL-4–specific CD4+CD8+ T-cell frequencies were significantly higher in immunocompromised HEV-infected pigs than in immunocompetent HEV-infected pigs at 13 wpi ($P < 0.001$) (Fig. 6C). In contrast, the mean frequencies of IL-4+ CD8+ T cells...
did not differ significantly between treatment groups at any of the time points observed (Fig. 6). Our results suggest that the IL-4–specific CD4+ T-cell responses against HEV were greatly increased in immunocompromised HEV-infected pigs; these responses were found mainly within CD4+CD8− T cells at 8 wpi and shifted to the CD4+CD8+ T-cell compartment during the chronic phase of HEV infection.

The Immunosuppressive Regimen, Not HEV Infection, Up-Regulated the Regulatory T-Cell Immune Responses in Pigs. Using multicolor flow cytometry, we also analyzed the frequencies of Treg cells (CD4+CD25+Foxp3+ and CD4+CD25−Foxp3+ Treg cells) and intracellular regulatory cytokine production within these cells after in vitro antigen stimulation of PBMCs. No significant changes in the frequencies of either Treg cell subpopulation were observed in the peripheral blood of pigs from any of the treatment groups (Fig. 7A–C), except that at the time of necropsy (13 wpi) the frequencies of CD4+CD25+Foxp3+ Treg cells were slightly lower in immunocompromised pigs (both mock- and HEV-infected) than in immunocompetent pigs (both mock- and HEV-infected) (P < 0.05) (Fig. 7C).

When we analyzed the regulatory cytokine production in these Treg cell subpopulations at 5 wpi, the frequencies of IL-10–secreting CD4+CD25+Foxp3+ Treg cells were highest in immunocompetent HEV-infected pigs (Fig. 7D) and reached insignificant low levels at 8 wpi and 13 wpi (Fig. 7E and F); however, the difference was not statistically significant. In contrast, HEV infection exerted no apparent influence on the frequencies of IL-10–secreting CD4+CD25+Foxp3+ Treg cells in immunocompromised pigs (Fig. 7D–F). A moderate but significant increase in the frequencies of IL-10–secreting CD4+CD25+Foxp3+ Treg cells was observed in immunocompromised mock-infected pigs at 13 wpi compared with negative control pigs (P < 0.05) (Fig. 7F).

There was no significant increase in the frequencies of TGF-β–secreting CD4+CD25+Foxp3+ Treg cells in the HEV-infected pigs compared with other groups at 5 wpi (Fig. 7G). We also found that the HEV infection reduced the frequencies of TGF-β–secreting CD4+CD25+Foxp3+ Treg cells in both immunocompromised and immunocompetent pigs at 8 wpi (Fig. 7H); however, the reduction was significant only in immunocompetent pigs (P < 0.05). The mean frequencies of TGF-β–secreting CD4+CD25+FoxP3+ and CD4+CD25+FoxP3+ Treg cells were at very low levels by the end of study (13 wpi) (Fig. 7I).

Discussion

Chronic hepatitis E has been increasingly recognized as an emerging and significant clinical problem in immunocompromised individuals, particularly in solid-organ transplant recipients (8, 10). The lack of an animal model for chronic HEV infection greatly hinders the development of specific antiviral...
therapies to tackle the public health burden caused by chronic hepatitis E. In this study, we successfully established a pig model for chronic hepatitis E through oral treatment of pigs with a classical immunosuppressive drug regimen similar to that used for human organ transplant recipients and experimental infection of pigs with a genotype 3 human HEV. This unique chronic HEV pig model will aid in the future study of the mechanism of HEV pathogenesis and immune responses during chronic HEV infection as well as in the development of specific antivirals for chronic hepatitis E.

The pig model presented here is ideally suited for the study of chronic hepatitis E for a number of reasons. Recently, human liver-grafted chimeric mice were proved to be a valuable small animal model for HEV study, especially for studying the intrinsic type 1 IFN signaling pathways in human hepatocytes against HEV infection (21–23). Although the chimeric mouse model can reproduce chronic HEV infection, it is not a natural host for HEV, and interaction of innate pathways of human cells with the adaptive immune pathways of mouse cells cannot be fully reproduced in chimeric mouse model. Nevertheless, this model could be improved by restoring the adaptive immune response in the chimeric mouse to provide a broader application for HEV study in the future. As a natural host of genotypes 3 and 4 HEV, the pig has been consistently proven to be an excellent animal model for HEV infection, pathogenesis, and immunity studies (24–26). Because the chronic hepatitis E cases in humans have been associated almost exclusively with genotype 3 HEV (7), and, more importantly, because the physiological characteristics and immune system of pigs closely resemble those of humans, the pig is a very attractive model system to study chronic hepatitis E by mimicking the status of immunocompromised human patients, especially solid-organ transplant recipients.

During the course of HEV infection, fecal virus shedding typically ceases at ∼3–4 wk in immunocompetent humans, although in some patients, it may last up to 7–8 wk (27). In the cases of chronic hepatitis E in humans, the fecal virus shedding continues beyond 4 wk for more than 3 mo after infection (28). In our study, fecal virus shedding ceased after 8 wpi in the great majority of pigs not treated with immunosuppressive drugs, as is consistent with the previous reports on the duration of fecal virus shedding in pigs infected with genotype 3 HEV (16). In the immunocompromised pigs, however, fecal virus shedding lasted at least 5–14 wk longer than in immunocompetent pigs, indicating that the disease course of chronic HEV infection in pigs mimics that of human organ transplant recipients undergoing the immunosuppressive regimen treatment and meets the definition of chronic HEV infection suggested by Kamar et al. (28). Our results also suggest that the serum viral RNA levels are not reliable predictors for the establishment of chronic hepatitis E, because the viral RNA levels were transiently increased in sera of immunocompromised pigs at the start of the chronic phase (9 wpi), and thereafter the levels were similar to those in immunocompetent HEV-infected pigs, remaining at a low level until the end of the study.

Because HEV infection normally causes only mild microscopic liver lesions with no gross lesions between 5 wpi and 8 wpi in pigs, it is not surprising that no pathological lesions were observed during necropsy at 13 wpi in the immunocompetent HEV-infected pigs. Moreover, the immunocompromised condition in pigs apparently did not aggravate liver damage in the chronic phase of HEV infection, as evidenced by the absence of significant differences in gross and microscopic pathological lesions in the immunocompromised HEV-infected and immunocompetent HEV-infected groups. Indeed, the higher serum levels of GGT in the immunocompromised HEV-infected pigs suggested that unapparent liver damage occurred during the chronic phase of HEV infection but did not proceed to significant pathological lesions in liver as previously reported with solid-organ transplant recipients with chronic hepatitis E (29, 30). Many factors, such as the duration of HEV infection and differences in immunological and physiological systems in pigs and humans may contribute to the observed milder pathological lesions in immunosuppressed pigs. For example, as shown by the absence of obvious adverse effects or opportunistic infections in drug-treated pigs during the course of infection, the level of immunosuppression induced in pigs is milder than that observed in human patients, and this milder immunosuppression might contribute to the observed milder pathological lesions in pigs as compared with immunosuppressed human patients. The duration of chronic HEV infection also likely played a role in the severity of immunopathogenicity. Were the chronically infected pigs kept for a longer period, it is possible that significant pathological liver lesions might develop gradually because of repeated viral-induced injury in the liver. In fact, a recent study reported more pronounced hepatic lesions in HEV-infected cynomolgus monkeys that received long-term (160-d) immunosuppressive treatment with tacrolimus (31).

Because the deficiency in immune response contributes to the establishment of chronic HEV infection in humans (32, 33), it is critical to examine the nature of immune responses developed against chronic HEV infection in immunocompromised pigs to identify potential immune correlates leading to chronic HEV infection. Cytokines play important roles in the regulation of the innate, humoral, and cellular immune responses against virus infection (34). We found that HEV infection of immunocompetent pigs did not up-regulate serum levels of the cytokines participating in cell-mediated immunity. However, the baseline serum levels of Th1 cytokines (IL-2 and IL-12) were reduced when immunocompromised pigs were infected with HEV, whereas, importantly, the immunosuppressive drug treatment alone without HEV infection did not suppress these cytokines. This finding indicates that HEV infection actively suppresses Th1 immune responses in immunocompromised pigs, as has been observed in human patients chronically infected with HEV (32). Interestingly, HEV infection did not significantly increase or reduce IFN-γ cytokine levels in sera of either immunocompetent or immunocompromised pigs. However, HEV infection specifically increased the activation of CD4+CD8+ T cells to produce IFN-γ in immunocompetent pigs, and the activation was reduced significantly in pigs infected with HEV. CD4+CD8+ T cells are the critical effector memory Th cells in pigs necessary to mount an effective cell-mediated immune response against virus infections (35, 36), and these findings are consistent with previous observations in human hepatitis E patients (37, 38). Therefore, the active suppression of HEV-specific Th1 immune responses under immunocompromised conditions may be a plausible explanation for the prolonged fecal virus shedding and establishment of chronic infection observed in the immunocompromised HEV-infected pigs. Furthermore, this HEV-specific immunosuppression may be a potentiating factor for the effects of the immunosuppressive drugs in pigs, because one of the drugs, cyclosporine A, is known to down-regulate serum IL-2 and IL-12 levels (39). However, the effect of drugs alone was not strong enough to reduce these cytokine levels significantly, whereas the possible synergistic effect between drugs and HEV infection reduced these cytokine levels significantly in immunocompromised HEV-infected pigs compared with the levels in immunocompetent HEV-infected pigs.

T cells producing multiple cytokines are functionally more potent than those producing a single cytokine (40) and are reliable correlates of protective T-cell immunity. In contrast to mice and humans, there are significant CD4+CD8+ T-cell subpopulations consisting of activated Th cells, memory Th cells, and effector T cells (41) in pigs (Fig. S1). The porcine double-positive CD4+CD8+ cells are effector memory CD4+ T cells which repeatedly experienced a wide variety of antigens (pathogenic or...
derived from gut-residing microbes). They produce both Th1 and Th2 cytokines, are major producers of these cytokines when stimulated with exogenous antigen in vitro, and also have a sub-population of cytotoxic T cells that are important for antiviral immunity in pigs. Therefore, it is important to analyze the function of this T-cell subpopulation in HEV-infected pigs. When we looked into the IFN-γ and TNF-α production in CD4+CD8+ T cells during the early phase of HEV infection, we found both single (IFN-γ only) and double cytokine-producing cells in infected pigs, but, interestingly, the single (IFN-γ only) but not the double cytokine-producing CD4+CD8+ T cells were reduced in immunocompromised HEV-infected pigs. As the HEV infection progressed into the chronic phase, there was no induction of double cytokine-producing CD4+CD8+ T cells, suggesting that the protective T-cell immune responses against HEV were not induced during chronic infection. Moreover, HEV failed to induce the production of IFN-γ in the activated CD8+ T cells while maintaining or even increasing the TNF-α production in those cells, as evident from the increased levels of the single cytokine (TNF-α only)-producing CD8+ T cells in the chronic phase of HEV infection. The contribution of these cells in the progression of chronic hepatitis E warrants further investigation.

Contrary to the previous report on the study of hepatitis E pathogenesis (42), HEV infection in pigs did not increase IL-4 and IL-10 serum levels in either normal or immunocompromised pigs at 1 wpi. Even though HEV infection reduced the baseline levels of Th2 cytokines in the serum of immunocompromised pigs, it increased the levels of IL-4-producing CD4+ T cells in the peripheral blood, particularly during the chronic phase of infection. These observations suggest that, although Th1 immune responses against HEV were reduced by the treatment with immunosuppressive drugs, the Th2 immune responses against the virus were increased in immunocompromised pigs. Specifically, the Th2 immune responses were shifted from a CD4+CD8+ T-cell population to a CD4+CD8+ T-cell population as the HEV infection progressed into the chronic phase, further confirming the sustained stimulation of Th2 immunity. Because HEV in sera exists in a quasi-enveloped form, which is resistant to neutralization by antibodies (43), the enhanced levels of antibodies against HEV in immunocompromised pigs may not neutralize the virus efficiently. Overall, the imbalance in T-cell immunity (Th1 vs. Th2) against HEV infection in immunocompromised pigs might play a key role in the progression of chronic HEV infection and needs further in-depth investigation.

In addition to Th1 and Th2 cells, Treg cells play important roles in regulating the immune response to maintain a balance between the recognition and clearance of infectious agents and minimizing immunemediated pathology (44). Treg cells generally produce immunosuppressive cytokines such as IL-10 and TGF-β, which reflect the effector functions of the Treg cells (44–46). Our study showed that the immunosuppressive treatment of pigs increased IL-10–producing Treg cell (CD25+) levels non-specifically. In general, HEV did not modulate the immune system by inducing Treg cells (CD25+ and CD25- cells producing TGF-β or IL-10), as observed in this study. However, HEV-specific Treg responses in the CD25+ Treg compartment might be affected, and TGF-β production in these cells was reduced transiently at the start of the chronic phase of HEV infection. The relatively weak T-cell response in HEV-infected non-immunosuppressed pigs observed in this study is not surprising. The strength of antiviral immune responses is determined partly by the virulence of the virus and the level of pathogenicity. Although pigs are the natural host for genotype 3 HEV, the virus infection in pigs is typically subclinical and thus may have induced a dampened immune response. For example, at 5 wpi (the first time point tested) HEV replication and fecal virus shedding were significantly reduced, and by 8 wpi the majority of animals cleared virus infection. Thus, the weak immune response seen in nonimmunosuppressed HEV-infected pigs at these time points may be caused by a low level of virus replication or by virus clearance. Furthermore, pig blood is only a transit point for the clearance of the draining lymph nodes and spleen, and these cells may infiltrate quickly into the infected liver and reside there for a long period, until viral clearance has taken place. Therefore, it is likely that we may have missed the right time point(s) for analyzing the peak T-cell responses in peripheral blood and that a more robust immune response could have occurred earlier during the course of infection. These factors could have contributed to the observation of lower HEV-specific T-cell frequencies in pig peripheral blood. Additionally, although the major subpopulations of porcine lymphocytes are very similar to those in humans, two important differences between human and pig—the abundant surface expression of CD8α and the expression of MHC-II DR molecules on resting T lymphocytes in pigs—also might contribute to the observed differences in the course of HEV infection and immune responses in pig and human.

In conclusion, we successfully established a pig model of chronic HEV infection by oral treatment with immunosuppressive drugs routinely used for human organ transplant patients and infection with a genotype 3 human HEV. We demonstrated that this unique pig model mimicked the course of chronic HEV infection and the immune response status observed in human solid-organ transplant recipients chronically infected with HEV. We found that HEV-infected pigs at the immunocompromised stage were significantly reduced by treatment with immunosuppressive drugs and that the immune responses against HEV were skewed toward Th2 immunity through the chronic phase of HEV infection. Although we observed transient effects of CD4+CD25+ Treg immune responses against HEV infection in pigs, these effects might not have influenced the progression of chronic HEV infection. With this unique chronic HEV model, future in-depth studies can be conducted to delineate the precise roles of the different arms of immune system in the progression of chronic hepatitis E in immunosuppressed individuals and to develop effective and specific antiviral therapies for chronic hepatitis E.

Materials and Methods

Virus and Immunosuppressive Drugs. The genotype 3 HEV infectious stock (US-2 strain) (47) was prepared from the feces of a pig experimentally infected with the US-2 strain of human HEV and was used to infect pigs in this study. The viral genomic equivalent (GE) titer of this virus stock was determined by HEV-specific real-time RT-PCR. Drugs used in the immunosuppressive regimen of the study are cyclosporine (Cyclosporine oral solution USP modified, 100 mg/ml; Teva Pharmaceutical Industries Ltd.), azathioprine (50-mg tablets; Roche Laboratories, Hoffmann-La Roche Ltd.), prednisolone (prednisolone tablets; Roxane Laboratories, Boehringer Ingelheim) (48). All animal experiments were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC no. 12-16-CVM).

Treatment of Pigs with an Immunosuppressive Regimen and HEV Infection. A total of 39 HEV-negative 6-week-old pigs (purchased without consideration of sex from the Virginia Tech Tidewater Agricultural Research and Extension Center) were divided randomly into four treatment groups with 10 pigs in each group, except for the control group, which had nine pigs. In general, pigs become fully immunocompetent at the age of 7 wk (49); therefore, we purchased 6-week-old immune-maturing piglets and infected them with HEV at age 7 wk. Pigs in groups 1 and 2 were treated daily for 7 d with an oral immunosuppressive regimen similar to that used for human organ transplant recipients (cyclosporine at 10 mgkg⁻¹ d⁻¹, azathioprine at 2 mgkg⁻¹ d⁻¹, and methylprednisolone at 4 mg kg⁻¹ d⁻¹) (48). Briefly, azathioprine tablets were crushed into powder, mixed with 1 mL sterile water together with one dose of methylprednisolone and cyclosporine solutions to produce a suspension. The three-drug suspension then was mixed thoroughly with two tablets; Roxane Laboratories, Boehringer Ingelheim) (48). All animal experiments were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC no. 12-16-CVM).
negative controls, respectively. After HEV inoculation, the pigs in groups 1 and 2 were treated with the same daily immunosuppressive regimen until the end of the study. Blood, serum, and fecal samples were collected weekly until 13 wpi, when the pigs were humanely killed and necropsied. Tissue samples of liver, small intestines, and mesenteric and superficial inguinal lymph nodes were collected during necropsy for histological examination of pathological lesions. Serum, fecal, and tissue samples were stored at −80 °C until further processing and analysis.

Two of the immunocompromised HEV-infected pigs were not necropsied at 13 wpi and were kept on the daily immunosuppressive regimen for an additional 9 wk. Weekly fecal samples from these two pigs were collected and tested by HEV-specific RT-PCR for fecal virus shedding to verify further the chronic HEV infection status.

Detection and Quantification of HEV RNA by qRT-PCR. Viral RNAs in weekly pig serum and fecal samples were quantified by qRT-PCR using HEV-specific primers. Briefly, the fecal samples were suspended in sterile PBS at 10% (vol/vol). Total RNA was extracted from 250 μL of 10% fecal suspensions or diluted serum samples with TRIzol LS Reagent (Invitrogen). HEV genomic RNAs were quantified using the SensiFAST No-ROX One-Step kit (Bioline USA, Inc.), using the forward primer (JVHEVF, 5'-GGTTGTTTCTGGGGTGAC-3') and reverse primer (JVHEVR, 5'-AGGGGTTGGTTG-AGGGTGTG-3'). A hybridization probe (JVHEVPS, 5'-TGATTCTCAGCCCTTCGC-3') following a protocol described previously (50). The qRT-PCR assays were performed in a CFX96 real-time (RT) PCR system (Bio-Rad Laboratories). In vitro-transcribed and -purified HEV genomic RNAs were used to produce a standard curve in qRT-PCR assays. The thermal cycling conditions in qRT-PCR assays were as follows: 45 °C for 10 min (reverse transcription); 95 °C for 2 min (initial denaturation); and 95 °C for 5 s followed by 60 °C for 20 s (PCR amplification) for 40 cycles. The efficiency of the qRT-PCR assay is set at 0.95 by using serial diluted copies, as reported previously (50), which is equivalent to 400 copies per 1-mL sample.

Determination of Serum Levels of Liver Enzymes in Pigs. A panel of liver enyzmes including GGT, SDH, AST, total bilirubin, and alkaline phosphatase were measured in serum samples by established protocols at the Clinical Pathology Laboratory; and 95 °C for an incubation time of 15 min for each mAb set; FITC-conjugated mouse anti-pig CD4 (IgG2b, clone 74-12-4; BD PharMingen), Spectral Red-conjugated mouse anti-pig CD8α (IgG2a, clone 76-21-2; Southern Biotech), and mouse anti-pig CD3: IgG1, clone PPT3; Southern Biotech), followed by phycoerythrin (PE)-Cy7-conjugated rat anti-mouse IgG1 (eBioscience). After staining of cell-surface markers, the PBMCs were permeabilized with BD Cytofix/Cytoperm buffer (BD Pharmingen) at 4 °C for 30 min. PBMCs were washed three times with BD Perm/Wash buffer (BD Pharmingen) and stained with R-PE-conjugated mouse anti-pig IL-2, PE-conjugated mouse anti-pig CD3 (clone PG210; eBioscience), Brilliant Violet 421-rhodamine B isoalloxazine (Rhb)-conjugated mouse anti-pig IFN-γ, Brilliant Violet 421-rhodamine B isoalloxazine (Rhb)-conjugated mouse anti-pig CD4, PE-conjugated mouse anti-pig CD8, and mouse IgG1, clone MA11 (BD Pharmingen) at 4 °C for 30 min. PBMCs stained with appropriate isotype-matched irrelevant control antibodies (BD Pharmingen, VMRD, Biolegend, or Southern Biotech) served as background staining controls. The frequencies of IFN-γ+ CD4+ CD8−, IFN-γ+ CD4− CD8+, and IFN-γ+ CD4+ CD8+ T cells were expressed as percentages of parental CD3+ cells.

In panel two, in vitro antigen-stimulated PBMCs were stained with relevant antibodies to determine the frequencies of CD4+ FoxP3+ Treg cells, the activation status (CD25), and the expression of regulatory cytokines (IL-10 and TGF-β) in these cells (54). Briefly, PBMCs (1 × 106 cells per well) were first stained at 4 °C for 15 min with FITC-conjugated mouse anti-porcine CD4, Spectral Red-conjugated mouse anti-porcine, and mouse anti-porcine CD25 (BD Pharmingen #380:2095). After 1 h of stimulation of the Treg cells, the acti-

Statistical Analysis. All data were processed with GraphPad Prism 6.01 (GraphPad Software Inc.). The differences between the mean values of two treatment groups were evaluated using a Student’s t-test or two-way ANOVA followed by Tukey multiple comparisons test. A P value of less than 0.05 was considered as statistically significant.

ACKNOWLEDGMENTS. We thank Ms. Melissa Makris for her technical assistance in flow cytometry; Dr. Ke Wen for his helpful discussion on the staining protocol of intracellular cytokines; and Karen Hall, Kimberly Allen, and Peter Jobst for their timely support in the animal study. This study was supported by NIH Grants R01AI050611 and R01AI074667.

Fig. S1. Representative dot-plots for CD4+ and CD8+ T-cell gating in pig PBMCs by flow cytometry. Stained cells were first gated for mononuclear cells based on forward scatter (FFS-A) and side scatter (SSC-A) profiles followed by live-cell gating with the LIVE/DEAD fixable dead cell stain kits. Subsequently, the CD3+ T cells were gated with CD3+ antibody. Finally, a bivariate dot plot was created to gate CD4+, CD8+, and CD4+CD8+ T-cell populations.