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Virus-Specific T-Cell Immunity Correlates with Control of GB Virus B Infection in Marmosets▽

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GB virus B (GBV-B) is a hepatotropic virus that is closely related to hepatitis C virus (HCV). GBV-B causes acute hepatitis in infected marmosets and tamarins and is therefore a useful small-animal model for the study of HCV. We investigated virus-specific T-cell responses in marmosets infected with GBV-B. Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay responses in the peripheral blood of two marmosets were assessed throughout the course of GBV-B infection. These T-cell responses were directed against the GBV-B nonstructural proteins 3 (NS3), 4A (NS4A), and 5B (NS5B), and their appearance was temporally associated with clearance of viremia. These marmosets were then rechallenged with GBV-B at least 3 months after clearance of the primary infection to determine if the animals were protected from reinfection. There was no detectable viremia following reinfection, although a sharp increase in T-cell responses against GBV-B proteins was observed. Epitope mapping of T-cell responses to GBV-B was performed with liver and blood samples from both marmosets after rechallenge with GBV-B. Three shared, immunodominant T-cell epitopes within NS3 were identified in animals with multiple common major histocompatibility complex class I alleles. IFN-γ ELISPOT responses were also detected in the livers of two marmosets that had resolved a primary GBV-B infection. These responses were high in frequency and were directed against epitopes within GBV-B NS3, NS4A, and NS5B proteins. These results indicate that virus-specific T-cell responses are detectable in the liver and blood of GBV-B-infected marmosets and that the clearance of GBV-B is associated with the appearance of these responses.

A major impediment in the development of antiviral agents and vaccines against hepatitis C virus (HCV) has been the lack of a convenient small-animal model to test their efficacy. The chimpanzee is the only animal model that supports virus replication and has been used in studies examining vaccine efficacy (12, 26), but due to the high cost involved in their enrollment and care and their endangered status, these animals are beyond the reach of most research groups. Consequently, protective or immunosuppressive regions of the HCV polyprotein that should be incorporated into or excluded from vaccine design are still unknown. A potential small-animal model for studying HCV immunity is the common marmoset (*Callithrix jacchus*). These animals are relatively inexpensive, are not endangered, and are easily kept in captivity. Furthermore, marmosets are often relatively inbred, which facilitates the study of major histocompatibility complex (MHC)-restricted T-cell immunity. Marmosets develop acute hepatitis following infection with GB virus B (GBV-B) (4, 19), but there have been reported cases of persistent infection with similar pathological and virologic changes to those seen with persistent HCV infection (21). Infection of marmosets with GBV-B produces a peak viremia ranging from 10^7 to 10^9 genomic equivalents/ml (GE/ml) for a period of 8 to 18 weeks (4, 19, 25), which can be prolonged following suppression of T-cell immunity with FK506, suggesting that cellular immune responses are involved in viral clearance (19). GBV-B is the most closely related virus to HCV in terms of both its genomic organization and sequence homology. HCV and GBV-B polyproteins share approximately 25 to 30% homology at the amino acid level (22), leading investigators to believe that antiviral or immunotherapies developed for GBV-B will be a useful guide to the development of effective therapies against HCV (4, 25). This is supported by the observation that the nonstructural protein 3 (NS3) protease of GBV-B correctly processes the HCV polyprotein (28) and that HCV/GBV-B chimeric NS3 proteins are enzymatically active (6). Furthermore, a recent study has shown that the NS3/NS4A proteases of both viruses utilize an identical mechanism to impede the activation of both interferon regulatory factor 3 and nuclear factor kappa B (NF-κB), resulting in impaired innate antiviral defenses (8). Similar to HCV infections in humans and chimpanzees, reinfection of convalescent marmosets or tamarins with GBV-B results in viremia of reduced magnitude and duration, indicating the existence of protective immunity (3, 4). However, the role of T-cell responses in this protective response is unknown.

The use of the GBV-B–marmoset model for the study of HCV immunity has been impeded by the lack of reagents and assays for measuring T-cell immune responses in these animals. In this study, we demonstrate that GBV-B-specific T-cell responses are detectable in marmoset liver and blood samples following viral clearance. Furthermore, we also show that the appearance of these responses is associated with viral clearance and protection from reinfection.

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MATERIALS AND METHODS

Animal handling and care. Marmosets (*Callithrix jacchus*) were housed at the animal care facility at Monash University (Clayton, Victoria, Australia) under guidelines approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Marmosets were experimentally infected by intrahepatic injection of the surgically exposed liver with 70 μg GBV-B RNA transcribed in vitro from pGBB (kindly provided by Jens Bukh, NIH) (5) or were inoculated by the intravenous or intraperitoneal route with GBV-B infectious serum containing 1×10^4 GE/ml, as previously described (25). Serum samples were collected at various times postinfection and analyzed for GBV-B RNA by reverse transcription-PCR (RT-PCR). Peripheral blood mononuclear cells (PBMC) and intrahepatic lymphocytes (IHL) were collected and assessed for virus-specific T-cell responses by gamma interferon (IFN-γ)-specific enzyme-linked immunospot (ELISPOT) assay.

Isolation of lymphocytes from blood and liver. PBMC were isolated from peripheral blood samples by use of a Ficoll-Hypaque density gradient (GE Healthcare Bio-Sciences, Uppsala, Sweden). To recover IHL, the whole marmoset liver was cut into small pieces and gently homogenized in phosphate-buffered saline containing 1% fetal calf serum (FCS), using a Wheaton Dounce tissue homogenizer (Wheaton, Millville, NJ). PBMC and IHL were cryopreserved in FCS containing 10% dimethyl sulfoxide.

Quantification of GBV-B RNA. GBV-B RNA was extracted from 70 μl of marmoset serum by using a QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. GBV-B RNA was quantified by quantitative RT-PCR using a primer-probe which hybridized to the GBV-B core gene, as described previously (3, 15, 25). The serum viral load was calculated by comparison with a standard curve generated from serial 10-fold dilutions of RNA transcribed in vitro from pGBB.

GBV-B peptides. Overlapping peptides spanning the GBV-B proteins core (amino acids [aa] 1 to 156; 15 peptides), NS3 (aa 941 to 1250; 30 peptides), NS3/NS4A (aa 1241 to 1615; 37 peptides), and NS5B (aa 2275 to 2864; 58 peptides) were kindly provided by Chris Walker (Columbus Children's Research Institute, Columbus, OH). Individual peptides were 20 aa in length, with a 10-aa overlap.

ELISPOT assay. GBV-B-specific T-cell responses were enumerated for PBMC or IHL by an ELISPOT assay using anti-human IFN-γ antibody pairs (Mabtech, Sweden). Responses were measured against the four GBV-B peptide pools described above. For epitope mapping studies, NS3 and NS4A 20-mer peptides were arranged in intersecting mapping pools to identify individual peptides that contained GBV-B epitopes. PBMC and IHL were cultured in duplicate wells at 2×10^5/well and 2.5×10^4/well, respectively, in RPMI (Invitrogen) supplemented with 10% FCS and a 1-μg/ml final concentration of GBV-B peptide pool in precoated ELISPOT plates and then incubated at 37°C for 40 h. PBMC or IHL stimulated without antigen and with phytohemagglutinin at 5 μg/ml were used as negative and positive controls, respectively. The cells were then removed, and ELISPOT plates were developed according to the manufacturer's instructions. The number of spot-forming cells (SFC) was analyzed using an AID ELISPOT plate reader and calculated by subtracting the mean number of spots in negative control duplicates from the mean number of spots in test duplicates and then normalizing the result to SFC/10^6 PBMC or IHL.

MHC class I analysis of marmosets. Sharing of MHC class I alleles in marmosets was analyzed by reference strand-mediated conformation analysis (RSCA), using a protocol adapted from similar methods to detect pigtail macaque MHC alleles (24, 30). Approximately 700 bp of MHC class I cDNA was amplified from marmoset PBMC cDNA by using the 5′RSCA and 3′RSCA primers as described previously (30). Three to five separate reference-strand MHC class I alleles isolated from rhesus macaques were PCR amplified under the same conditions, except that 5′RSCA was replaced with 5′-end-labeled Cy5-5′RSCA. Heteroduplex reactions between each reference strand and amplified marmoset MHC class I cDNA were performed under the following conditions: 95°C for 4 min, 55°C for 5 min, and 15°C for 5 min. Heteroduplex products were then run in a nondenaturing polyacrylamide gel on an ABI377 sequencer for 7 h, along with ROX-labeled external size standards (24). The migration rate of MHC alleles in the gel is determined by the conformation of the allele with the reference strand. Identical alleles shared between marmosets have the same mobility when heteroduplexed with the same reference strand.

RESULTS

Detection of GBV-B-specific T-cell responses in marmoset peripheral blood. Recovery from HCV infection is characterized by robust $CD4^+$ and $CD8^+$ T-cell responses against multiple viral epitopes (7, 9, 10, 20). To determine if GBV-B-specific T cells could be detected in the peripheral blood following infection, we performed IFN-γ ELISPOT assays on PBMC collected from a marmoset that had resolved infection. To perform this experiment, marmoset M803 was infected with GBV-B by intravenous injection of infectious serum (2×10^4 GE/inoculation). GBV-B RNA was detected at week 1 postinfection and reached a peak viral load at week 2 postinfection (4.8×10^6 GE/ml) before clearance of the infection at week 5 (Fig. 1A). PBMC were collected at week 10 postinfection and were assessed for virus-specific T-cell responses against GBV-B peptide pools (Fig. 1B) by using an IFN-γ ELISPOT assay (Fig. 1C). GBV-B peptide pools spanning core, NS3, NS4A, and NS5B were used in these assays, as numerous $CD4^+$ and $CD8^+$ T-cell epitopes have been described for the equivalent HCV proteins (32). In addition, mutations have been described for GBV-B core, NS3, NS4A, and NS5B in a tamarin that had a prolonged course of viremia, indicating that these regions may be subject to T-cell selection pressure (21). T-cell responses were detected against GBV-B NS3/NS4A only (275 SFC/10^6 PBMC) (Fig. 1C). No responses were detected against peptide pools comprising GBV-B core, NS3, or NS5B.

Virus-specific T-cell responses in peripheral blood are associated with viral clearance. The above data from marmoset M803 demonstrated that GBV-B-specific T cells in the peripheral blood can be quantified after resolution of infection. In order to analyze the temporal association between clearance of GBV-B viremia and the appearance of virus-specific T-cell responses in infected animals, two additional marmosets (F841 and F899) were infected with GBV-B, and weekly PBMC samples were collected and assessed for virus-specific T-cell responses. Marmosets F841 and F899 were infected with GBV-B infectious serum (2×10^4 GE/inoculation) via intraperitoneal injection, as this method was found to be technically less demanding for administering the virus inoculum and results in reproducible infections. In previous studies, we found no difference in the natural history of infection by using either intrahepatic, intravenous, or intraperitoneal injection (unpublished observations), and studies with tamarins have demonstrated similar patterns of viremia after intrahepatic and intravenous challenge (5, 21, 27). GBV-B RNA was first detected at week 3 postinfection in marmoset F841 and reached a peak viral titer at week 4 (1.29×10^5 GE/ml) (Fig. 2). GBV-B viremia was cleared at week 5 and rebounded transiently at week 6 postinfection. GBV-B RNA was detectable in marmoset F899 at week 1 postinfection and reached a peak viral titer at week 3 (3.6×10^6 GE/ml) (Fig. 2). The viral load was rapidly reduced at weeks 4 and 5 postinfection, was undetectable at week 6, and rebounded transiently at week 8 postinfection.

PBMC samples were collected at multiple times postinfection and were assessed for GBV-B-specific IFN-γ production. Virus-specific T-cell responses were first detected in marmoset F841 at week 7 postinfection, coincident with clearance of viremia (Fig. 2). These responses were detected against peptide pools encompassing NS3 (52 SFC/10^6 PBMC) and NS3/NS4A (105 SFC/10^6 PBMC). The responses against NS3 remained stable from weeks 8 to 21 postinfection, while the responses to NS3/NS4A rose steadily before reaching a peak level at week 15 postinfection (207 SFC/10^6 PBMC). No re-

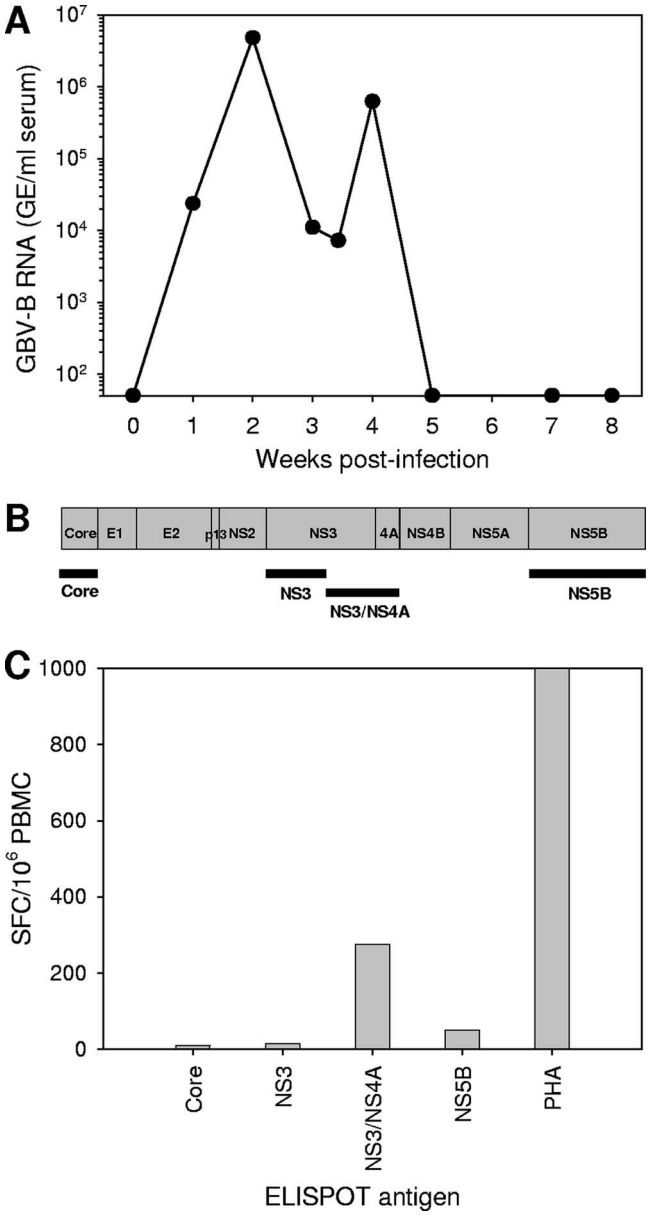

FIG. 1. GBV-B-specific T cells are detectable in the peripheral blood following recovery from GBV-B infection. (A) GBV-B viremia following intravenous injection of infectious serum. (B) Localization of GBV-B peptide pools encompassing core, NS3, NS3/NS4A, and NS5B. (C) IFN-γ ELISPOT responses in peripheral blood following clearance of infection. PBMC were isolated at week 10 postinfection and tested for IFN-γ responses against GBV-B peptide pools.

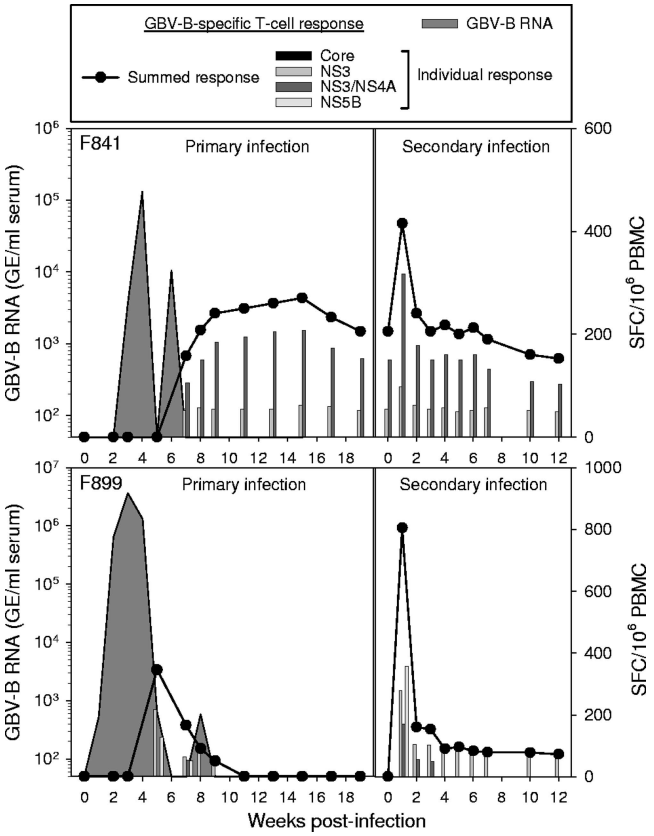

FIG. 2. The appearance of virus-specific T-cell responses is associated with viral clearance and protection from reinfection. Marmosets F841 and F899 were infected with GBV-B and allowed to recover (primary infection). The animals were then rechallenged with GBV-B 21 weeks after the primary infection (secondary infection). PBMC were collected at multiple times postinfection and assessed for IFN-γ responses by ELISPOT assay. Individual bars represent the responses to individual GBV-B peptide pools. The solid line represents the summed response to the GBV-B peptide pools. GBV-B RNA is represented by gray area plots.

sponses were detected against GBV-B core or NS5B. GBV-B-specific IFN-γ production from PBMC was first detectable in marmoset F899 at week 5 postinfection, coinciding with a 1,000-fold reduction in viral load from week 4 to week 5 (week 4, 1.3×10^6 GE/ml; week 5, 639 GE/ml) (Fig. 2). The responses were directed against NS3 (217 SFC/10^6 PBMC) and NS5B (127 SFC/10^6) and expanded to encompass NS3, NS3/NS4A, and NS5B at week 7 postinfection. Responses to NS3 only were detectable at weeks 8 and 9, and thereafter no responses were detected until reinfection.

Reinfection of marmosets with GBV-B. HCV infection studies with chimpanzees have shown that virus-specific T-cell responses are associated with viral clearance (9, 31, 33) and are able to respond rapidly to viral antigen when an animal is rechallenged (2, 14, 29). The duration and magnitude of viremia in the secondary infection are reduced in comparison to those of the primary infection and are associated with the rapid induction of memory $CD4^+$ and $CD8^+$ T cells (29). To determine if marmosets were protected from reinfection with GBV-B and if virus-specific memory T cells were associated with this protection, we reinfected marmosets F841 and F899 with GBV-B and assessed virus-specific T-cell responses in PBMC collected throughout the course of infection. Marmosets F841 and F899 were rechallenged with the same dose and by the same route of administration used in the primary infection. The marmosets were rechallenged 21 weeks after the primary infection and at least 12 weeks after clearance of virus (Fig. 2). GBV-B viremia was not detected in either of the marmosets at any time after reinfection, whereas virus-specific T-cell responses were increased significantly. For marmoset F841, total IFN-γ responses against NS3 and NS3/NS4A pep-

tide pools increased from 205 SFC/10^6 PBMC at the time of reinfection to 415 SFC/10^6 PBMC 1 week later (Fig. 2). The T-cell responses against the NS3 and NS3/NS4A peptide pools were then reduced almost twofold 2 weeks after reinfection and remained stable up until week 12. For marmoset F899, total IFN-γ responses increased from an undetectable level at the time of reinfection to 805 SFC/10^6 PBMC 1 week after reinfection (Fig. 2). The responses 1 week after reinfection were against GBV-B peptide pools encompassing NS3, NS3/NS4A, and NS5B (277, 170, and 357 SFC/10^6 PBMC, respectively). Thereafter, total IFN-γ responses to GBV-B peptides were reduced fivefold 2 weeks after reinfection and were limited to the NS3 and NS3/NS4A peptide pools only. IFN-γ responses to the NS3 peptide pool only were detected at week 4, and these responses remained stable until week 12 postreinfection (Fig. 2).

Detection of intrahepatic virus-specific T-cell responses in marmosets following GBV-B infection. As opposed to the responses in marmoset peripheral blood, we were not able to assess intrahepatic T-cell responses in marmosets F841 and F899 throughout the course of infection, as the animals were too small for us to obtain weekly liver biopsy specimens. Consequently, intrahepatic T-cell responses were analyzed at week 12 postinfection, when the animals were sacrificed. IHL were collected from marmoset whole liver and assessed for virus-specific T-cell responses, and these responses were compared to responses measured in peripheral blood (Fig. 3). For marmoset F841, IFN-γ responses against the NS3 (355 SFC/10^6 IHL), NS3/NS4A (805 SFC/10^6 IHL), and NS5B (275 SFC/10^6 IHL) peptide pools were detected in IHL (Fig. 3A). These responses were fivefold greater than those measured in peripheral blood against the NS3 and NS3/NS4A peptide pools (72 and 157 SFC/10^6 PBMC, respectively). T-cell responses against the NS3, NS3/NS4A, and NS5B peptide pools were also detected in the liver of marmoset F899 (890, 850, and 220 SFC/10^6 IHL, respectively), while responses to NS3 only were detected in the peripheral blood (72 SFC/10^6 PBMC) (Fig. 3B).

The above data demonstrated that intrahepatic T-cell responses could be detected in marmosets F841 and F899 following secondary infection. To generate a complete picture of intrahepatic responses following clearance of infection, we also examined responses after resolution of primary infection. IHL were isolated from two marmosets (M839 and M848) that had resolved infection (15) and were assessed for virus-specific T-cell responses against GBV-B peptide pools 2 weeks after clearance of infection (Fig. 3C). Consistent with the results following secondary infection, T-cell responses against GBV-B NS3, NS3/NS4A, and NS5B were detected in both marmosets (Fig. 3C). No responses against GBV-B core were detected in either marmoset.

Epitope mapping of GBV-B-specific T-cell responses. Acute resolving HCV infections are characterized by the accumulation in the liver of HCV-specific $CD4^+$ and $CD8^+$ T cells that recognize multiple viral epitopes, whereas infections that lead to persistence are characterized by weaker T-cell responses against fewer viral epitopes (9, 14, 29, 33). To determine the specificity and breadth of the T-cell response in resolving GBV-B infections in marmosets, epitope mapping experiments were performed on liver and blood samples collected from marmosets F841 and F899 at week 12 after reinfection. In the primary and secondary infections, the NS3 and NS3/NS4A peptide pools represented the total response in marmoset F841 and 73% and 78%, respectively, of the response in marmoset F899 (Fig. 2). Consequently, epitope mapping analysis was performed on these regions, using individual GBV-B 20-mer peptides from NS3 and NS3/NS4A peptide pools arranged in intersecting mapping pools. Positive IFN-γ responses against intersecting peptide mapping pools were confirmed using the corresponding individual 20-mer peptides. For both marmosets, IFN-γ responses against three 20-mer GBV-B NS3 peptides were responsible for the total response to the NS3 and NS3/NS4A peptide pools ($NS3_{1081-1100}$ [APILCSSGHVIGM FTAARNS], $NS3_{1131-1150}$ [PTVPNEYSVQILIAPTGSGK], and

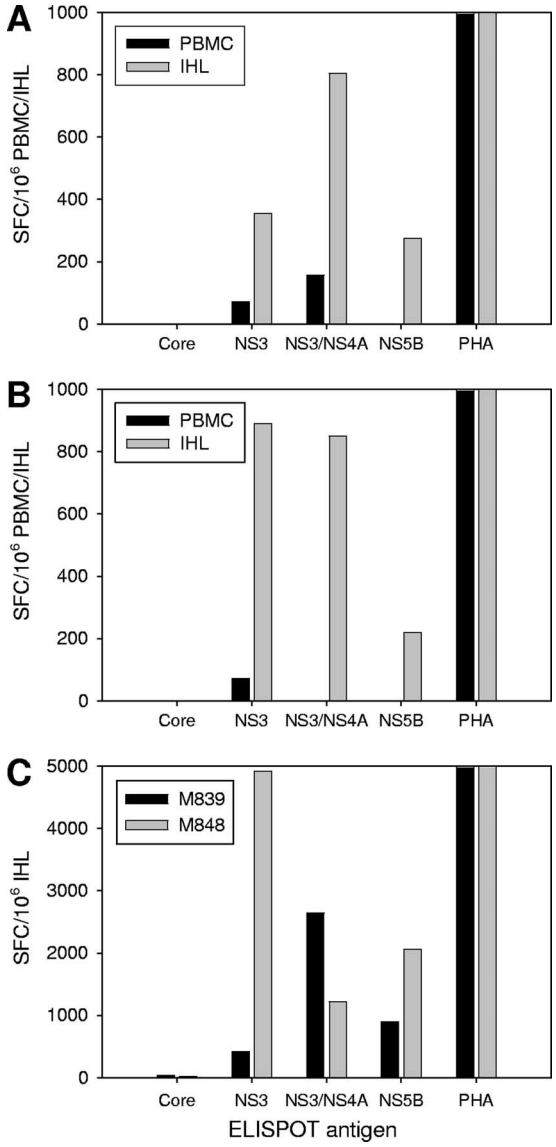

FIG. 3. Comparison of virus-specific T-cell responses in the blood and liver following clearance of infection. PBMC and IHL from marmosets F841 (A) and F899 (B) were collected 12 weeks after rechallenge with GBV-B and assessed for IFN-γ ELISPOT responses against GBV-B core, NS3, NS3/NS4A, and NS5B peptide pools. (C) IHL were also isolated from two marmosets (M839 and M848) 2 weeks after clearance of a primary GBV-B infection and assessed for IFN-γ ELISPOT responses against GBV-B peptide pools.

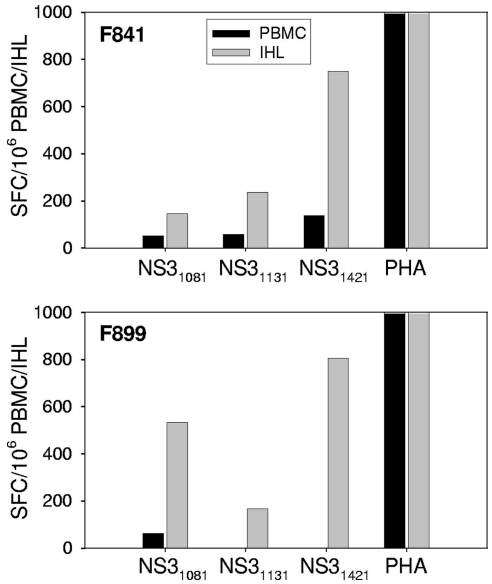

FIG. 4. The T-cell response to GBV-B infection is mediated by three immunodominant NS3 epitopes. PBMC and IHL from marmosets F841 and F899 were collected 12 weeks after rechallenge with GBV-B, and epitope mapping was performed using intersecting peptide mapping pools in IFN-γ ELISPOT assays. T-cell responses were measured against three individual GBV-B 20-mer peptides in NS3.

$NS3_{1421-1440}$ [GMVPECNIVEAFDAAKAWYG]) (Fig. 4). For marmoset F841, IFN-γ responses against all three 20-mer peptides were detected in liver and blood samples but were two- to fivefold higher in the liver than in peripheral blood (for $NS3_{1081-1100}$, 145 versus 52 SFC/10^6 IHL/PBMC; for $NS3_{1131-1150}$, 235 versus 57 SFC/10^6 IHL/PBMC; and for $NS3_{1421-1440}$, 750 versus 137 SFC/10^6 IHL/PBMC) (Fig. 4). For marmoset F899, IFN-γ responses against $NS3_{1081-1100}$, $NS3_{1131-1150}$, and $NS3_{1421-1440}$ were also detected in the liver (532, 167, and 805 SFC/10^6 IHL, respectively), whereas only $NS3_{1081-1100}$ was recognized by T cells in the peripheral blood (62 SFC/10^6 PBMC) (Fig. 4).

Responses against overlapping peptides flanking the GBV-B $NS3_{1081-1100}$, $NS3_{1131-1150}$, and $NS3_{1421-1440}$ peptides were also assessed to define the epitope more accurately. IFN-γ responses against $NS3_{1091-1110}$, which shares 10 COOH-terminal amino acids with $NS3_{1081-1100}$, were detected in both marmosets, indicating that the T-cell epitope is located in the sequence 1091-IGMFTAARNS-1100 and is likely to represent a $CD8^+$ T-cell epitope. In contrast, there were no responses to overlapping 20-mer peptides of $NS3_{1131-1150}$ or $NS3_{1421-1440}$ in either marmoset, indicating that the T-cell epitope is located in the middle of these peptides rather than at the NH_2 or COOH terminus.

MHC class I analysis of marmosets F841 and F899. The finding that marmosets F841 and F899 responded to the same GBV-B epitopes, including the putative $CD8^+$ T-cell epitope located at $NS3_{1091-1100}$, suggests that they have common MHC alleles. To determine if the marmosets have common MHC class I restriction elements, we performed RSCA, using marmoset PBMC cDNA heteroduplexed separately with four reference MHC class I alleles derived from rhesus macaques (Fig. 5). This assay relies on the finding that each MHC allele, even when it differs only marginally from other MHC alleles, has a unique conformation when heteroduplexed with the fluorescent reference strand and therefore has a unique mobility profile on a nondenaturing gel. This analysis revealed that marmosets F841 and F899 shared at least four MHC class I alleles (Fig. 5). For example, cDNAs from marmosets F841 and F899 heteroduplexed with the rhesus reference allele Mamu-A*15 demonstrated four distinct heteroduplex peaks with virtually identical (<1.5 bp) mobilities. Four heteroduplex peaks shared between F841 and F899 were also observed with the other rhesus reference alleles studied. The sharing of multiple common MHC class I alleles between marmosets F841 and F899 suggests that these two marmosets are likely to respond to shared GBV-B $CD8^+$ T-cell epitopes.

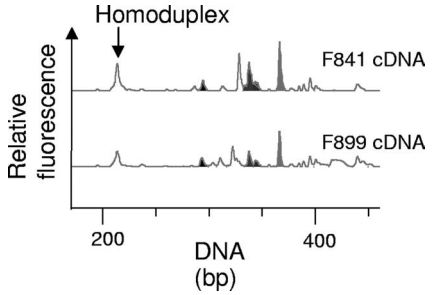

FIG. 5. Marmosets F841 and F899 share common MHC class I alleles. cDNAs from marmosets were heteroduplexed with reference strand Mamu-A15 and examined by electrophoresis on a nondenaturing gel. Each MHC allele heteroduplex has a unique mobility, with each peak representing an MHC class I allele.

DISCUSSION

Although the recent development of the JFH1-based cell culture system represents a significant advance in our understanding of virus replication in vitro, the lack of a convenient small-animal model for the study of HCV in vivo has hampered our understanding of HCV pathogenesis and persistence and impeded the development and testing of candidate vaccines. GBV-B infection of marmosets is a useful surrogate model for the study of HCV. While GBV-B infection generally results in an acute, self-limited infection, whereas HCV infection usually leads to persistence, the GBV-B/marmoset model is the most authentic small-animal model with which to study potential vaccine strategies and antiviral agents (4, 19). We have previously shown that marmosets can be infected with GBV-B chimeric viruses containing hypervariable region 1 of the E2 envelope protein of HCV (15). We have also demonstrated that GBV-B infection of marmoset primary hepatocyte cultures can be a useful in vitro cell culture system for the study of virus replication and for testing the efficacy of antiviral drugs (25). In this study, we have developed the marmoset model further by using an IFN-γ ELISPOT assay to assess GBV-B-specific T-cell responses in marmoset peripheral blood and liver samples from experimentally infected animals. Our findings on GBV-B-specific T-cell immunity in marmosets significantly expand the utility of the GBV-B/marmoset model.

Resolution of acute HCV infection in chimpanzees and humans is mediated by $CD4^+$ and $CD8^+$ T cells targeting multiple epitopes of HCV (9, 10, 20, 33). In the chimpanzee

model, depletion of $CD4^+$ or $CD8^+$ T cells prolongs infection, indicating that both T-cell subtypes are required for viral clearance (14, 29). In this study, we have shown that virus-specific T-cell responses directed against multiple GBV-B epitopes are present in the liver and blood of marmosets following clearance of viremia and that their appearance in the peripheral blood is associated with resolution of viremia. Similar to the case for HCV infections in humans and chimpanzees, responses detected in the liver were higher in frequency than those in peripheral blood. Analysis of T-cell frequencies in individuals with persistent HCV infection, using tetramers, has revealed that frequencies in the liver are at least 10-fold higher than those in peripheral blood (13, 16). In this study, using T cells isolated from marmoset whole liver without expansion, intrahepatic T-cell frequencies were higher than those in peripheral blood by a similar order of magnitude, indicating that virus-specific T cells accumulated in the liver, the site of virus replication. Although one of the epitopes ($NS3_{1091-1100}$) was mapped to a 10-mer peptide indicating a $CD8^+$ T-cell response, we were unable to definitively determine if the responses were mediated by $CD4^+$ or $CD8^+$ T cells, as antibodies specific for marmoset CD4 or CD8 that could be used in depletion experiments were not available. Serum alanine aminotransferase (ALT) levels have been used in HCV studies to assess hepatocyte destruction by $CD8^+$ cytotoxic T cells (14, 20, 29, 33). ALT levels were not elevated in marmosets M839 and M848 following infection (15), indicating that noncytolytic mechanisms may have been responsible for the control of viral replication. However, serum ALT levels have been shown to be a poor indicator of viral clearance in marmosets, and another liver enzyme (glutamate dehydrogenase) is associated with viral clearance and hepatocyte destruction (4). Therefore, it is possible that viral clearance was mediated by cytolytic $CD8^+$ T cells. We recently developed lymphocyte proliferation and cytotoxic T-cell assays with marmosets to further characterize GBV-B-specific $CD4^+$ and $CD8^+$ T-cell responses.

HCV infection studies with the chimpanzee model have demonstrated that virus-specific memory $CD4^+$ and $CD8^+$ T cells are required for protection from reinfection (14, 29). In this study, marmosets were protected against reinfection several months after clearance of the primary infection. Reexposure of marmosets to GBV-B was associated with sharp increases in virus-specific T-cell responses to GBV-B nonstructural proteins. Since the nonstructural proteins of GBV-B (like those of HCV) are expressed during the intracellular infection cycle, our results suggest that these T-cell responses were boosted by an abortive infection and probably played a role in limiting viral replication. The T-cell responses measured following secondary infection were greater in magnitude and appeared more rapidly than the responses measured in the primary infection. This is consistent with the T-cell responses detected following reinfection of chimpanzees with HCV (29) and indicates that memory T cells were recalled. The fact that virus-specific T-cell responses were boosted upon reinfection suggests that viral replication occurred in the livers of these animals, triggering rapid induction of memory T cells that controlled infection. However, viral RNA was not detected at any time postinfection by an RT-PCR with a sensitivity of 10 GE/ml serum. Nevertheless, since viral RNA levels could only be assessed at weekly intervals owing to the small size of these animals and difficulties in obtaining blood, it is possible that viral RNA was present transiently before the week 1 time point. Previous studies with marmosets (4) and tamarins (3) have demonstrated reduced viral replication upon rechallenge, indicating that animals can be reinfected with GBV-B.

The role of humoral immunity in clearance of HCV and protection from reinfection is poorly understood. Previous studies with the chimpanzee model suggest that antibody to HCV envelope proteins may have a role in protection against HCV infection or may neutralize a proportion of the challenge dose (11, 17, 18). Neutralizing antibody responses to GBV-B infection have not been investigated in previous studies. In this study, neutralizing antibody responses were analyzed in marmoset M848 following in vitro infection of naïve marmoset hepatocytes as described previously (25). Although this animal had detectable antibody responses against GBV-B core following infection (15), neutralizing antibodies were not detected in this animal at the time of viral clearance or 2 weeks after clearance (unpublished data), and therefore the control of viremia in this animal was most likely due to the appearance of virus-specific T cells. Neutralizing antibody responses were not examined in marmosets F841 and F899 following GBV-B reinfection, but rechallenge of the marmosets with 4 μl of GBV-B-positive serum resulted in a boosting of the T-cell response in both animals, indicating that virus replication occurred. Thus, if neutralizing antibodies were present in these animals, the titer was insufficient to neutralize this low dose of challenge virus. Our data are consistent with previous studies demonstrating that marmosets (4) and tamarins (3) can be reinfected with GBV-B, indicating that neutralizing antibodies do not protect against reinfection.

The majority of the T-cell responses in the infected marmosets were specific for GBV-B epitopes within NS3 and NS4A. Although GBV-B peptides encompassing the entire GBV-B polyprotein were not available, all five marmosets that resolved infection showed T-cell responses against NS3 and NS4A. Epitope mapping experiments demonstrated that several peptides in NS3 were immunodominant and accounted for all of the response to the NS3 and NS3/NS4A peptide pools. Furthermore, both marmosets responded to the same three epitopes, suggesting that the animals had common MHC restriction elements. This observation was confirmed by MHC class I analysis using RSCA and revealed that marmosets F841 and F899 shared four MHC class I alleles. The larger litter size, relatively inbred nature, and limited MHC class II variability of marmosets are very useful features for the development of the GBV-B/marmoset model (1, 23).

In summary, the development of assays for measuring T-cell responses in marmosets infected with GBV-B represents a significant advance in the development of a small-animal model that can be used to understand the nature of protective immunity to members of the *Hepacivirus* genus. Ongoing work in our laboratory is focusing on various vaccine delivery systems incorporating HCV/GBV-B proteins. The development of these T-cell assays with the marmoset model will enable testing of these vaccines for efficacy in terms of immunogenicity and protection from infection and will provide important information on HCV vaccination strategies.

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