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ABSTRACT
Despite its importance in shaping adaptive immune responses, viral clearance, and immune-based inflammation, tissue-specific innate immunity remains poorly characterized for hepatitis C virus (HCV) infection due to the lack of access to acutely infected tissues. In this study, we evaluated the impact of natural killer (NK) cells and myeloid (mDCs) and plasmacytoid (pDCs) dendritic cells on control of virus replication and virus-induced pathology caused by another, more rapidly resolving hepacivirus, GB virus B (GBV-B), in infections of common marmosets. High plasma and liver viral loads and robust hepatitis characterized acute GBV-B infection, and while viremia was generally cleared by 2 to 3 months postinfection, hepatitis and liver fibrosis persisted after clearance. Coinciding with peak viral loads and liver pathology, the levels of NK cells, mDCs, and pDCs in the liver increased up to 3-fold. Although no obvious numerical changes in peripheral innate cells occurred, circulating NK cells exhibited increased perforin and Ki67 expression levels and increased surface expression of CXCR3. These data suggested that increased NK cell arming and proliferation as well as tissue trafficking may be associated with influx into the liver during acute infection. Indeed, NK cell frequencies in the liver positively correlated with plasma (R = 0.698; P = 0.015) and liver (R = 0.567; P = 0.057) viral loads. Finally, soluble factors associated with NK cells and DCs, including gamma interferon (IFN-γ) and RANTES, were increased in acute infection and also were associated with viral loads and hepatitis. Collectively, the findings showed that mobilization of local and circulating innate immune responses was linked to acute virus-induced hepatitis, and potentially to resolution of GBV-B infection, and our results may provide insight into similar mechanisms in HCV infection.

IMPORTANCE
Hepatitis C virus (HCV) infection has created a global health crisis, and despite new effective antivirals, it is still a leading cause of liver disease and death worldwide. Recent evidence suggests that innate immunity may be a potential therapeutic target for HCV, but it may also be a correlate of increased disease. Due to a lack of access to human tissues with acute HCV infection, in this study we evaluated the role of innate immunity in resolving infection with a hepacivirus, GBV-B, in common marmosets. Collectively, our data suggest that NK cell and DC mobilization in acute hepacivirus infection can dampen virus replication but also regulate acute and chronic liver damage. How these two opposing effects on the host may be modulated in future therapeutic and vaccine approaches warrants further study.

Hepatitis C virus (HCV) infection has become a global health epidemic, with the virus infecting more than 170 million people worldwide (1) and resulting in 350,000 HCV-related liver disease deaths each year (2). HCV infection results in the following two disparate manifestations: (i) an acute, self-resolving infection and (ii) a chronic infection, which occurs in 60 to 80% of cases and can lead to liver fibrosis and cirrhosis and even, rarely, to hepatocellular carcinoma (3, 4). However, despite the global impact of chronic HCV infection, the viral and host immune factors leading to self-resolution or chronicity still remain unclear. Although limitations in access to acute or tissue samples make understanding early hepavivirus pathology and immunology challenging, other related hepaviruses recapitulate many features of HCV. The most closely phylogenetically related virus, GB virus B (GBV-B), has a nearly identical genome organization (5, 6), and it is almost always cleared by the immune system (7–9). Most GBV-B infection studies are conducted using common marmosets, and the virus has previously been demonstrated to induce hepatitis and a T cell response very similar to those with HCV (5, 10–14). Thus, GBV-B can offer insight into both acute responses and, potentially, immune-mediated clearance.

Significant evidence indicates that at least some primates correlate GBV-B and HCV control and prevention of disease progression are cellular immunology based. Hepacivirus infection induces a potent T cell response, and CD4+ T cells upregulate PD-1 acutely after GBV-B infection, which is indicative of activation; a similar PD-1+ T cell activation is observed in early HCV infection (15–18). Acute GBV-B infection is also characterized by an influx of
of CD8 T cells into the liver (9), and clearance of GBV-B has previously been associated with strong responses against the NS3 protein (14). Anti-NS3 responses may also be protective and important for clearance of HCV (19–21). Conversely, poor T cell responses in acute HCV infection have been associated with viral persistence and chronicity, and in chronic disease there is significant evidence of T cell exhaustion and dysfunction (22–26), but there is a lack of evidence for a similar phenomenon in GBV-B infection.

Recent burgeoning evidence also suggests that innate immunity may contribute to viral clearance or control of hepacivirus infections. Maintenance of normal dendritic cell (DC) phenotypes and functional repertoires has been shown to be critical for sustained antiviral responses and is necessary to augment antiviral therapies, including alpha interferon (IFN-α), which can block GBV-B infection in vitro (27–29). Conversely, GBV-B has evolved to inhibit IFN-α production, and HCV has similarly developed mechanisms to subvert plasmacytoid (pDCs) and myeloid (mDCs) DCs, decreasing the levels of IFN-α, interleukin-10 (IL-10), and IL-12 (30–32), all of which are necessary for initiation of T cell responses and mobilization of natural killer (NK) cells. In HCV infection, NK cell cytotoxicity in both the periphery and inflamed livers has been associated with lysis of infected hepatocytes and with viral clearance (33–35). Moreover, polyfunctional NK cells have been associated with resistance to infection in HCV-exposed health care workers (36). Although recent data from our lab and others have characterized marmoset NK cells and found them to be highly similar to those in humans (37, 38), the reciprocal effects of GBV-B infection and NK cells are unknown.

Despite advances in the HCV field, there are still limitations in the development of vaccines and immunotherapeutics, primarily due to a lack of access to acute infection and tissue samples. These knowledge gaps can be evaluated partially by studying the immune responses in other hepacivirus infections, particularly those that are resolved by the immune system, such as GBV-B infection. In this study, we aimed to describe the mobilization of innate immune factors, both cellular and soluble, which may influence GBV-B resolution and virus-induced pathology.

MATERIALS AND METHODS

Experimental animals. Twelve common marmosets (Callithrix jacchus) of either sex and between 2 and 8 years of age were housed in biosafety level 2 (BSL2) biocontainment facilities at the New England Primate Research Center, Southborough, MA. These animal studies were performed in accordance with the guidelines of the local Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals (39). A commercial New World nonhuman primate diet was supplemented with fruits, vegetables, eggs, and nuts, and water was provided ad libitum. Whole blood was collected prior to infection and every 3 days until day 14 postinfection (p.i.) for the acute study and at weekly intervals for the first 4 weeks followed by monthly collections until day 168 p.i. for the long-term study. Liver biopsies were also performed prior to infection and at day 14 p.i. in long-term study animals. Six animals were sacrificed at day 14 p.i., and six animals were sacrificed following a full infection, at day 168 p.i. On the day of sacrifice, animals were euthanized and evident postmortem pathology was recorded by the attending veterinarian and pathologist. The blood, liver, spleen, peripheral lymph nodes, and mesenteric lymph nodes (MLN) were collected at necropsy and processed using standard laboratory protocols for subsequent analyses. GBV-B-naïve animal culls provided an additional source of normal tissue for some analyses.
RNA was converted to cDNA by use of a high-capacity reverse transcription-PCR (RT-PCR) kit (Applied Biosystems). RT-PCRs were conducted in an ABI 7900HT system, and the results were analyzed by use of ABI software. Quantitative standards were generated by in vitro transcription of a plasmid vector containing an NS3 insert. Serial dilutions ranging from $10^8$ to $10^0$ copies/reaction mix were used to generate a standard curve with a sensitivity threshold of 15 copies/reaction mix. Repeated measurements indicated that nominally 100 copies or more could be quantified reliably and reproducibly in each sample, regardless of volume. NS3 copy equivalents were extrapolated from the standard curve and expressed as copies per milliliter of plasma.

Isolation of mononuclear cells from tissues of infected animals. Each liver was homogenized on a cell strainer with RPMI containing 5% fetal bovine serum (FBS) (R5). The filtrate was overlaid on 60% Percoll followed by 30% Percoll for density gradient centrifugation, and the interface layer containing the hepatic mononuclear cells was harvested. The spleen and lymph nodes were homogenized on a cell strainer and washed with R5. Contaminating red blood cells (RBCs) were lysed using hypotonic ammonium chloride solution. The mononuclear cells were washed with R5, resuspended in RPMI containing 10% FBS (R10), counted, and used for staining for flow cytometry or for enzyme-linked immunosorbent spot (ELISpot) assay.

ELISpot assay. Virus-specific IFN-γ responses were assayed using an anti-human IFN-γ ELISpot kit (MAB Tech) on mononuclear cells of the liver and spleen. Responses were measured against 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)—that were kindly provided by Chris Walker (COLUMBUS Children's Research Institute, Columbus, OH). Cells were added at a concentration of 2 × 10^5 cells/well to antigens at a concentration of 1 μg/ml and phytohemagglutinin (PHA) at a concentration of 2 μg/ml and incubated at 37°C for 10 h. The cells were washed off, and the plate was developed according to the manufacturer's instructions. Spots were then counted by Zellnet Consulting Inc.

Flow cytometric staining of whole blood. To enumerate and phenotype NK cells, dendritic cells, and T cells, polychromatic flow cytometry was performed using whole blood. Antibodies used include CD3 (allophycocyanin [APC]-Cy7; clone SP34-2; BD Biosciences), NKp46 (phycoerythrin [PE]; clone BAB281; Immunotech), HLA-DR (ECD; clone Immu-357; Beckman-Coulter), CXCR3 (PE-Cy5; clone 1C6; BD Biosciences), CD56 (PE-Cy7; clone NCAM16.2; BD Biosciences), NKG2a (APC; clone Z199; Beckman-Coulter), CD16 (Alexa Fluor 700; clone 3G8; Invitrogen), CD4 (PE; clone L200; BD Biosciences), CD45RA (PE-Cy5; clone 5H8; BD Biosciences), CD95 (APC; clone DX2; BD Biosciences), CD11c (PE; clone S-HCL-3; BD Biosciences), CD20 (peridinin chlorophyll protein [PerCP]-Cy5.5; clone L27; BD Biosciences), and CD14 (PE-Cy7; clone MoP9; BD Biosciences) antibodies. The samples were incubated with antibody for 30 min at room temperature and then lysed with fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences) according to the manufacturer's recommended protocol. Cells were then fixed using 1% formaldehyde solution. Intracellular staining was performed using Fix/Perm reagents (Caltag Laboratories) and included staining for Ki67 (fluorescein isothiocyanate [FITC] clone MIB1; BD Biosciences) and perforin (Pacific Blue clone dG9; BioLegend). Flow cytometry acquisitions were performed on an LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (version 9.6.4; Tree Star).

Flow cytometric staining of tissue mononuclear cells. Single-cell suspensions were subjected to multicolor immunostaining for flow cytometric analysis. Briefly, 1 × 10^6 cells were resuspended in phosphate-buffered saline (PBS) containing 2% FBS. Cells were then stained, and flow cytometric acquisitions were performed as described above.

Multiplex cytokine analysis of plasma. Plasma samples were analyzed for marmoset cytokines and chemokines by Luminex methodological...
ogy, using established protocols for New World primates (42). Evaluation of the analytes IL-1β, IL-1Ra, tumor necrosis factor alpha (TNF-α), IL-2, RANTES, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, IL-12 (p40), IL-8, IFN-α, IL-13, IFN-γ, IL-17, TNF-β, and vascular endothelial growth factor (VEGF) was included in this assay. Only data for analytes quantifiable above the limit of detection are presented.

**Statistical analyses.** Statistical evaluations of differences between groups included the Mann-Whitney U test, the Kruskal-Wallis test followed by Dunn’s multiple-comparison posttest, and Student’s t test and were performed using GraphPad Prism 6.0 software. Differences between the mean ranks of different time points compared to the mean rank on day 0 were considered significant when the P value was <0.05.

**RESULTS**

**GBV-B infection of marmosets.** To first confirm the take of infection and to evaluate the kinetics of viremia, virus loads in plasma samples and livers of all animals were quantified at multiple time points. Virus was detectable as early as 3 days p.i. in approximately 50% of infected animals, and all animals were viremic at day 14 (Fig. 1A and B). In longitudinally evaluated animals, plasma levels peaked between 10⁶ and 10⁷ copies/ml, generally by

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Time of (days) or status at sacrifice</th>
<th>H&amp;E staining</th>
<th>Hepatitis score</th>
<th>Comment(s)</th>
<th>Comment</th>
<th>Masson’s trichrome staining</th>
<th>Fibrosis score</th>
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<tbody>
<tr>
<td>04-07</td>
<td>Normal</td>
<td></td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>53-08</td>
<td>Normal</td>
<td></td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>01-09</td>
<td>14</td>
<td>Multifocal aggregates of inflammatory cells with focal necrotic hepatocytes</td>
<td>2</td>
<td></td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
</tr>
<tr>
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<td>14</td>
<td>Multifocal aggregates of inflammatory cells</td>
<td>1</td>
<td></td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
</tr>
<tr>
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<tr>
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<td>14</td>
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<td>1</td>
<td>No significant findings</td>
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<tr>
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<td>14</td>
<td>No significant findings</td>
<td>1</td>
<td></td>
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<td>14</td>
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<td></td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
</tr>
<tr>
<td>228-07</td>
<td>168</td>
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<td>1</td>
<td>Bridging fibrosis</td>
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<tr>
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<td>168</td>
<td>No significant findings</td>
<td>2</td>
<td></td>
<td>No significant findings</td>
<td>1</td>
<td>Scattered fibrosis between hepatocytes</td>
</tr>
<tr>
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<td></td>
<td>No significant findings</td>
<td>1</td>
<td>Scattered fibrosis between hepatocytes and in necrotic foci</td>
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<tr>
<td>123-10</td>
<td>168</td>
<td>Multifocal aggregates of inflammatory cells; focal hepatocellular necrotic structures</td>
<td>3</td>
<td></td>
<td>No significant findings</td>
<td>1</td>
<td>Focal areas of extensive fibrosis in the portal triad region</td>
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<tr>
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<td>168</td>
<td>Granulated hepatocytes</td>
<td>1</td>
<td></td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
</tr>
<tr>
<td>27-09</td>
<td>168</td>
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<td>1</td>
<td></td>
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<td>1</td>
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we evaluated both acute and chronic measures of liver pathology and fibrosis in GBV-B-infected animals. Histopathology analysis by H&E staining indicated multifocal lymphocyte aggregation, mild to moderate inflammation, and focal necrosis of hepatocytes in acutely infected animals compared to healthy controls (Fig. 3A; Table 1). Not surprisingly, hepatitis was more advanced in animals sacrificed at day 168, even though the virus had already been cleared. Trichrome staining revealed little to no indication of fibrosis for acute infection, but extensive fibrosis around the portal triad regions was found in animals sacrificed at day 168 (Fig. 3B; Table 1). Collectively, these data indicate that significant hepatitis occurs acutely but that advanced hepatitis and fibrosis occur later and persist even after viral clearance, and thus may continue to have impacts on liver homeostasis.

Elevated serum liver enzymes are often used as surrogate indicators of liver damage, but their association with actual hepatitis is not always consistent. Similarly, although alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) levels became elevated in some animals by day 14 or 28 p.i., and continued to stay above baseline values (Fig. 4), these changes were not significant and did not correlate with viral load (data not shown). Similar to findings for HCV-infected humans, these serum chemistry measurements may not always be effective surrogate indicators of liver damage (43, 44).

**Mobilization of innate immune responses during GBV-B infection.** Dendritic and NK cells are among the first cells to respond to viral infections, but they are poorly described for acute HCV infection, generally due to a lack of access to samples. To begin to address some of this deficit, we characterized the innate cellular response following GBV-B infection. In GBV-B-infected animals, the frequency of circulating mDCs was generally unaltered, but pDC frequencies increased following infection and then declined to normal levels by day 28 p.i. (Fig. 5A to C). Subsequent analyses of necropsy tissues found that both mDCs and pDCs were elevated in the liver during acute infection and remained after viral clearance, but they were generally unchanged in extrahepatic tissues (Fig. 5D and E). These are the first observations documenting an accumulation of DCs in livers from GBV-B-infected animals, but they are consistent with observations of other altered DC phenotypes in HCV-infected humans (30, 32, 45).

Similar to the observations for DCs, there were no overt changes in circulating NK cell frequencies or subpopulations (Fig. 6A to C). Interestingly, however, the phenotype of peripheral NK cells was altered. Intracellular perforin expression was upregulated acutely (Fig. 6D) ($P = 0.0284$; Kruskal-Wallis test), while the tissue homing marker CXCR3 (Fig. 6E) ($P < 0.0001$; Kruskal-Wallis test) and the proliferation marker Ki67 (Fig. 6F) were both upregulated early in infection and remained elevated until viral clearance. This suggested that while no net numerical change in peripheral NK cells was observed, the effects of GBV-B infection were not silent and induced increases in tissue homing, turnover, and cytotoxic arming related to ongoing virus replication. In line with these observations, the frequency of NK cells in the liver increased significantly during acute GBV-B infection (Fig. 7A), and CD16$^+$ cytotoxic NK cell, perforin, and Ki67 levels were all increased, albeit not significantly (Fig. 7B to D). IFN-$\gamma$ production by hepatic NK cells also increased significantly during acute GBV-B infection (Fig. 7E). NK cell frequencies in the liver correlated with plasma viral load ($R = 0.698; P = 0.015$) and liver viral load ($R = 0.567; P = 0.057$), indicating that
GBV-B replication likely induces NK cell recruitment. Moderate increases in NK cell frequencies were also observed in the spleen and in MLN that drain the liver, but these did not reach statistical significance.

Modulation of the inflammatory cytokine milieu during GBV-B infection. Since cellular innate immune responses were perturbed during acute GBV-B infection, we next evaluated soluble innate factors by using optimized protocols specific to detec-
tion of neotropical primate analytes by Luminex methodology. Not surprisingly, inflammatory mediators were found at low concentrations in naive animals but were selectively increased in individual animals during acute infection (Fig. 8). For example, the median serum concentration of RANTES was 11 pg/ml at baseline, but it increased to 37 and 70 pg/ml in two animals by day 14 p.i. and to 20 pg/ml in longitudinally evaluated animals by day 28 p.i. Similarly, the baseline IFN-γ level in serum was low (median, 1 pg/ml) but increased up to 9-fold by day 28 p.i. Both IFN-γ (R = 0.417; P = 0.025) and RANTES (R = 0.326; P = 0.083) levels correlated with plasma viral loads. IL-12, MIP-1β, and IFN-α levels were unchanged by day 14, but all increased longitudinally in individual animals before rapidly returning to baseline levels. Although these analyses were severely limited by the volume of plasma/serum recoverable from longitudinal samples inherent to this species, collectively these data suggest that transient increases in soluble inflammatory mediators are a characteristic of acute GBV-B infection.

**DISCUSSION**

GBV-B, a hepacivirus similar to HCV, has previously been shown to cause hepatitis in marmosets, and differences in viral immunity have been related to clearance, control, and disease progression (5, 10–13). Despite the fact that innate immunity is a well-studied correlate for both modulation of adaptive responses and indirect pathology in other disease models, its role in hepacivirus infections remains unclear. This study highlights innate immune and pathological responses to acute GBV-B infection and following virus clearance.

In general, the acute NK cell response to GBV-B infection was characterized by increased cytotoxic arming, IFN-γ production, cell proliferation, tissue homing, and accumulation in the liver. These features persisted in the postacute period, but most functions resolved to baseline levels by the time of virus clearance. Empirical HCV studies suggest that enhanced NK cell function during acute infection can lead to viral containment (46–48), and in persons accidentally exposed to HCV, NK cells exert upregulated effector functions (36). Collectively, these data may suggest that the robust NK cell response we observed in acute GBV-B infection is related to the virus clearance normally observed (40, 49). Further, CXCR3 expression on circulating NK cells was upregulated soon after GBV-B infection, which may indicate recruitment of immunocompetent cells into the liver. CXCR3 expression on liver NK cells has previously been associated with liver cell damage and fibrosis (50, 51), and biomarkers such as increased CXCR3 or NK cell perforin may be indicative of acute hepacivirus infection and hepatitis. Indeed, the kinetics and accumulation of NK cells and other innate immune functions coincided with acute hepatitis (Fig. 3). Similarly, hepatocytes upregulate NK cell NKG2D ligands in a murine model of HBV infection, rendering the hepatocytes susceptible to increased lysis (52), and NKG2D-mediated hepatocyte killing has been described for nonalcoholic steatohepatitis (53). NK cell upregulation of TRAIL, FAS, and other granzymes and perforins has been shown to be in-

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**FIG 7** Liver NK cells in GBV-B infection. (A) Individual data points represent individual animal samples analyzed at necropsy; horizontal bars represent median percentages of NK cells. (B) Medians and ranges are shown for NK cell subpopulations at the indicated necropsy time points. Intracellular Ki67 (C) and intracellular perforin (D) expression levels are shown for bulk NK cells analyzed ex vivo at necropsy. (E) IFN-γ production in liver NK cell subpopulations following mitogen stimulation. Data are shown as means, 25th and 75th percentiles, and minimum-to-maximum whiskers (n = 6). Asterisks indicate significant differences compared to normal samples (P < 0.05; Mann-Whitney U test). D14, day 14; D168, day 168.
volved in both hepatocellular damage and HCV clearance (54–56). Taken together, our data suggest that although NK cells may be a correlate of virus clearance, they may also be a cause of liver pathology.

Similar to the observations for NK cells, GBV-B infection induced a partial accumulation of DCs in the liver. In patients with chronic HCV infection, there is a preferential migration of mDCs to areas of liver inflammation (57, 58), coinciding with a reduction in circulating DCs driven by HCV E2 protein-mediated RANTES and MIP-1α secretion (58). Subsequently, dysfunctional DCs are trapped inside the liver, unable to migrate to lymph nodes. A similar mechanism may occur in GBV-B infection, which would explain the accumulation of mDCs and pDCs in the liver without changes in the lymph nodes. Perturbation of the dendritic cell compartment may lead to altered antigen processing and skew the T cell response, both of which are well documented for HCV (41, 59), and accumulation may lead to overproduction of inflammatory mediators and subsequent liver damage.

The overaccumulation of innate immune cells in the livers of infected animals may also be an indirect source of inflammation and hepatitis mediated by soluble factors. Indeed, RANTES, MIP-1β, and IFN-γ are all produced by NK cells, mDCs, or both, and they may be associated with generalized activation. Similarly, activated pDCs and mDCs may contribute to increased IFN-α and IL-12 levels. Each of these factors has some association with virus control but also has well-described mechanisms for hepatocyte death, particularly IFN-γ (58). This may suggest that in acute hepatitis, DC- and NK cell-mediated inflammation and hepatocyte death are indirect mechanisms via cytokine production.

In summary, modeling acute hepacivirus infection in GBV-B-infected marmosets can aid in clarifying mechanisms of both virus clearance and pathology. Innate responses were robust during acute infection, whereas adaptive T cell responses, not surprisingly, were weak; previous observations suggest that humoral responses also develop much later (60). We surmise that this increased innate inflammation can have a bifurcated effect on the host: it (i) dampens virus replication but (ii) also regulates acute and chronic liver damage. The full contribution of these responses and how they can be targeted in therapeutic or vaccine modalities will need to be elucidated in further studies.

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