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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 become 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, and potentially to resolution of GBV-B infection; and our results may provide insight into similar mechanisms in HCV infection.
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-naive animal culls provided an additional source of normal tissue for some analyses.

![FIG 1](https://example.com/figure1.png)
formed with the forward primer CGCCGGTTGGCTCATC, the reverse
priming ammonium chloride solution. The mononuclear cells were washed
previously described (40). Liver tissues were collected on the day of sacrifice,
Virus inoculum. All marmosets were inoculated intravenously with
the reverse transcription-PCR (RT-PCR), using a modified assay as
isolated from the liver (A) and spleen (B) against overlapping pep-
Virus quantification. Virus quantification was performed by quanti-
\begin{align*}
\text{Virus inoculum. All marmosets were inoculated intravenously with } &1.0 \times 10^3 \text{ to } 4.0 \times 10^3 \text{ virus copy equivalents from one of two plasma-} \\
\text{Histopathology. Liver tissues were collected on the day of sacrifice,} \\
\text{Virus quantification. Virus quantification was performed by quanti-
\text{Histopathology. Liver tissues were collected on the day of sacrifice,} \\
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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 $10^6$ and $10^7$ 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>Fibrosis score</th>
<th>Masson’s trichrome staining</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>04-07</td>
<td>Normal</td>
<td>No significant findings</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>No significant findings</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>Multifocal aggregates of inflammatory cells</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>185-09</td>
<td>14</td>
<td>Multifocal aggregates of inflammatory cells</td>
<td>1</td>
<td>Rare small aggregates</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>370-09</td>
<td>14</td>
<td>Swollen hepatocytes with slight granularity</td>
<td>2</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>418-10</td>
<td>14</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>230-09</td>
<td>14</td>
<td>Several multifocal aggregates of inflammatory cells; focal necrotic hepatocytes</td>
<td>2</td>
<td>Several multifocal aggregates of inflammatory cells</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>249-10</td>
<td>14</td>
<td>Enlarged hepatocytes with slight granularity; focal portal triad inflammation; irregular hepatocellular vacuolization</td>
<td>2</td>
<td>Bridging fibrosis</td>
<td>2</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>228-07</td>
<td>168</td>
<td>No significant findings</td>
<td>2</td>
<td>No significant findings</td>
<td>1</td>
<td>Scattered fibrosis between hepatocytes and in necrotic foci</td>
<td>1</td>
</tr>
<tr>
<td>16-07</td>
<td>168</td>
<td>Small focus of necrotic hepatocytes with inflammatory cells and fibrosis; enlarged hepatocytes</td>
<td>2</td>
<td>Small focus of necrotic hepatocytes with inflammatory cells and fibrosis; enlarged hepatocytes</td>
<td>2</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>282-06</td>
<td>168</td>
<td>Multifocal aggregates of inflammatory cells; focal hepatocellular necrotic structures</td>
<td>3</td>
<td>Multifocal aggregates of inflammatory cells; focal hepatocellular necrotic structures</td>
<td>1</td>
<td>Focal areas of extensive fibrosis in the portal triad region</td>
<td>2</td>
</tr>
<tr>
<td>123-10</td>
<td>168</td>
<td>Granulated hepatocytes</td>
<td>1</td>
<td>Granulated hepatocytes</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>379-09</td>
<td>168</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
<tr>
<td>27-09</td>
<td>168</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
<td>No significant findings</td>
<td>1</td>
</tr>
</tbody>
</table>
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-γ 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-

**FIG 5** Acute mobilization of circulating and liver mDCs and pDCs during GBV-B infection. (A) Representative gating strategy used to delineate DC populations in marmosets. (B and C) Individual data points represent percentages of mDCs (B) and pDCs (C) following infection. (D and E) Individual data points represent percentages of mDCs (D) and pDCs (E) in tissues collected at necropsy at the indicated time points following infection. Asterisks indicate significant differences between median ranks of different time points ($P < 0.01$; Mann-Whitney U test). MLN, mesenteric lymph nodes; PLN, peripheral lymph nodes.

**FIG 6** Mobilization of circulating NK cells during GBV-B infection. (A) Representative gating strategy used to delineate NK cells and NK cell subpopulations in marmosets, as previously described (37). (B) Individual data points represent percentages of NK cells following infection. (C) Medians and ranges are shown for NK cell subpopulations. Longitudinal expression levels of intracellular perforin (D), extracellular CXCR3 (E), and intracellular Ki67 (F) are shown for circulating NK cells. Longitudinal changes were evaluated statistically by the Kruskal-Wallis test and were found to be significant for CXCR3 ($P < 0.0001$) and perforin ($P = 0.0284$). MFI, mean fluorescence intensity.
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 = 0.085) 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-

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.
involved 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-1x/H9251 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 hepatitis virus 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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