Viral hepatitis remains a global health challenge despite recent progress in the development of more effective therapies. Although virus-specific CD8\(^+\) and CD4\(^+\) T cell responses are essential for viral clearance, it remains largely unknown what regulates T cell–mediated viral clearance. Thus, a better understanding of the regulation of anti-viral T cell immunity would be critical for the design of more effective therapies for viral hepatitis. Using a model of adenovirus-induced hepatitis, here we showed that adenoviral infection induced recruitment of Ly6C\(^{hi}\) monocytes to the liver in a CCR2-dependent manner. These recruited Ly6C\(^{hi}\) monocytes suppressed CD8\(^+\) and CD4\(^+\) T cell responses to adenoviral infection, leading to a delay in viral clearance. In vivo depletion of Ly6C\(^{hi}\) monocytes markedly enhanced anti-viral T cell responses and promoted viral clearance. Mechanistically, we showed that induction of iNOS and the production of NO by Ly6C\(^{hi}\) monocytes are critical for the suppression of T cell responses. In addition, a contact-dependent mechanism mediated by PD-1 and PD-L1 interaction is also required for T cell suppression by Ly6C\(^{hi}\) monocytes. These findings suggest a critical role for Ly6C\(^{hi}\) monocytes in the regulation of T cell immunity in viral hepatitis and may provide new insights into development of more effective therapies for treating viral hepatitis based on targeting the immunosuppressing monocytes.
Ly6C<sup>hi</sup> monocytes regulate T cell responses in viral hepatitis

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Viral hepatitis remains a global health challenge despite recent progress in the development of more effective therapies. Although virus-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses are essential for viral clearance, it remains largely unknown what regulates T cell–mediated viral clearance. Thus, a better understanding of the regulation of anti-viral T cell immunity would be critical for the design of more effective therapies for viral hepatitis. Using a model of adenovirus-induced hepatitis, here we showed that adenoviral infection induced recruitment of Ly6C<sup>hi</sup> monocytes to the liver in a CCR2-dependent manner. These recruited Ly6C<sup>hi</sup> monocytes suppressed CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses to adenoviral infection, leading to a delay in viral clearance. In vivo depletion of Ly6C<sup>hi</sup> monocytes markedly enhanced anti-viral T cell responses and promoted viral clearance. Mechanistically, we showed that induction of iNOS and the production of NO by Ly6C<sup>hi</sup> monocytes are critical for the suppression of T cell responses. In addition, a contact-dependent mechanism mediated by PD-1 and PD-L1 interaction is also required for T cell suppression by Ly6C<sup>hi</sup> monocytes. These findings suggest a critical role for Ly6C<sup>hi</sup> monocytes in the regulation of T cell immunity in viral hepatitis and may provide new insights into development of more effective therapies for treating viral hepatitis based on targeting the immunosuppressing monocytes.
organs involved. In mouse models of infection with *Mycobacterium tuberculosis*, *Toxoplasma gondii*, and West Nile virus, Ly6C hi monocyte mobilization to sites of infection leads to resistance to the infection (13–15). In contrast, Ly6C hi monocyte recruitment suppresses T cell responses to infections with Theiler's murine encephalomyelitis virus and murine cytomegalovirus (MCMV) (16, 17).

Whether monocytes play a role in virus-induced hepatitis remains largely unknown. Using the model of adenovirus-induced hepatitis, here we showed that adenoviral infection induced recruitment of Ly6C hi monocytes to the liver that peaked at day 5 after infection, which coincided with the infiltration of CD8 + and CD4 + T cells into the liver. In vivo depletion of these Ly6C hi monocytes markedly enhanced T cell responses to viral infection, leading to an accelerated viral clearance, suggesting a critical role for Ly6C hi monocytes in regulating T cell responses to viral infection. We further demonstrated a marked reduction in Ly6C hi monocyte recruitment and an enhanced virus-specific T cell response in the liver in mice defective for CCR2 (CCR2 −/−), suggesting that the recruitment of Ly6C hi monocytes to the liver in response to adenoviral infection is dependent on CCR2. Furthermore, these intrahepatic Ly6C hi monocytes were capable of directly suppressing CD8 + and CD4 + T cell responses in vitro. Mechanistically, we found that induction of iNOS and the production of NO by Ly6C hi monocytes are required for the suppression of T cell responses. In addition, a contact-dependent mechanism mediated by PD-1 and PD-L1 interaction is also important for T cell suppression by Ly6C hi monocytes.

Figure 1. Recruitment of Ly6C hi monocytes and T cells to the liver upon adenoviral infection. Mice were infected with recombinant adenovirus encoding LacZ (Ad) intravenously or left uninfected (Naive). (A) Five days after infection, cells from the spleen or the liver were analyzed for the presence of Ly6C hi monocytes and Ly6G + granulocytes by flow cytometry analysis. FACS plots are shown with the percentages of Ly6C hi monocytes (CD11b +Ly6C hi Ly6G−) and Ly6G + granulocytes (CD11b +Ly6C lo Ly6G +) among CD11b + cells indicated. (B) At 4 hours and days 1, 2, 3, 4, 5, 6, and 7 after infection, total mean numbers of Ly6C hi monocytes and Ly6G + granulocytes ± SD in the spleen and liver tissues of Ad-infected mice are shown (n = 3 per group). (C) At day 5 after infection, cells from the spleen or liver were analyzed for the infiltration of CD4 + and CD8 + T cells. FACS plots are shown with the percentages of CD4 + and CD8 + T cells among total splenic or intrahepatic lymphocytes indicated. (D) At 4 hours and days 1, 2, 3, 4, 5, 6, and 7 after infection, total mean numbers of CD4 + and CD8 + T cells ± SEM in the spleen and liver tissues of Ad-infected mice are shown (n = 3 per group). Results are representative of 3 independent experiments.
Results

Recruitment of Ly6C<sup>hi</sup> monocytes to the liver upon adenoviral infection. To investigate whether monocytes play a role in adenovirus-induced hepatitis, we first examined how monocytes respond to adenoviral infection in vivo. Recombinant adenovirus encoding LacZ (Ad) intravenously on days 3 and 4 of infection with Ad. At day 5 after infection, cells harvested from the spleen and liver were analyzed for the efficiency of monocyte depletion. (B) Mice were harvested at day 5 after infection. At 1 hour prior to harvest, 2 mg of BrdU in 200 μl PBS was injected intraperitoneally into mice. Spleen and liver tissues were harvested and the cells were stained with anti-CD11b, anti-Ly6C, and anti-Ly6G and analyzed for the efficacy of monocyte depletion. The mean total numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and BrdU-positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells ± SEM are shown in the spleen (top) and liver (bottom) tissues. *P < 0.05, **P < 0.01, determined by a 2-tailed Student’s t test, n = 4. Results are representative of 3 independent experiments.

In vivo depletion of Ly6C<sup>hi</sup> monocytes enhances T cell responses and promotes viral clearance. We next examined the biological significance of hepatic recruitment of Ly6C<sup>hi</sup> monocytes in adenovirus-induced hepatitis. To address this question, we examined the effect of Ly6C<sup>hi</sup> monocyte depletion on T cell proliferation using an in vivo BrdU labeling assay. We have previously shown that administration of gemcitabine can preferentially deplete Ly6C<sup>hi</sup> monocytes in vivo (18). C57BL/6 mice were infected with Ad-LacZ intravenously at day 0 and treated with gemcitabine intraperitoneally at days 3 and 4. Consistent with our previous observation (18), gemcitabine depleted Ly6C<sup>hi</sup> monocytes, but not Ly6G<sup>+</sup> granulocytes in the spleen (Figure 2A). We further found that gemcitabine effectively depleted the infiltrating Ly6C<sup>hi</sup> monocytes in the liver (Figure 2B).
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Impaired intrahepatic recruitment of Ly6C\textsuperscript{hi} monocytes in CCR2\textsuperscript{−/−} mice enhances T cell responses to adenoviral infection and viral clearance. To further confirm that intrahepatic Ly6C\textsuperscript{hi} monocytes are responsible for the suppression of T cell responses to adenoviral infection in vivo, we used a CCR2\textsuperscript{−/−} mouse model. Previous studies have shown that the recruitment of Ly6C\textsuperscript{hi} monocytes to the site of infection in other model systems is dependent on CCR2 (11, 15). Thus, we first determined whether recruitment of Ly6C\textsuperscript{hi} monocytes to the liver during adenoviral infection was also CCR2 dependent. We found that the number of Ly6C\textsuperscript{hi} monocytes in the liver at days 3, 7, and 10 after infection was significantly (P < 0.001) lower in adenovirus-infected CCR2\textsuperscript{−/−} mice than those in infected WT controls (Figure 4A). In contrast, a significantly (P < 0.05) higher number of Ly6C\textsuperscript{hi} monocytes was detected in the bone marrow of CCR2\textsuperscript{−/−} mice than WT controls (Figure 4A). This is consistent with previous reports that in CCR2\textsuperscript{−/−} mice, monocytes are defective in migrating from the bone marrow to the sites of infection (11). Similar to the monocyte depletion experiment (Figure 3), defective recruitment of Ly6C\textsuperscript{hi} monocytes to the liver in CCR2\textsuperscript{−/−} mice promoted viral clearance in vivo (Figure 4B).

We next sought to study whether defective migration of Ly6C\textsuperscript{hi} monocytes affected intrahepatic T cell expansion and function in vivo. We used a model of OVA-specific CD8\textsuperscript{+} T cell response to Ad-OVA. A total of 2 × 10\textsuperscript{4} OT-1 OVA-specific CD8\textsuperscript{+} T cells (Thy1.1\textsuperscript{+}) were transferred into cognate WT or CCR2\textsuperscript{−/−} mice (Thy1.2\textsuperscript{+}) that were subsequently infected with 2 × 10\textsuperscript{5} PFU of Ad-OVA intravenously. Seven days after infection, cells from the liver were analyzed for the presence and function of OVA-specific CD8\textsuperscript{+} T cells.

Intrahepatic Ly6C\textsuperscript{hi} monocytes suppress CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell proliferation in vitro. To directly demonstrate that Ly6C\textsuperscript{hi} monocytes were capable of suppressing T cell responses, we utilized a previously described in vitro T cell coculture system (19). We found that addition of intrahepatic Ly6C\textsuperscript{hi} monocytes from day 5 adenovirus-infected mice to T cell cultures markedly suppressed CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell proliferation in response to stimulation with anti-CD3 and anti-CD28 Abs in a cell dose–dependent manner (Figure 5A). In contrast, no T cell suppression was observed when Ly6G\textsuperscript{hi} granulocytes were added to the culture (Figure 5A). To address whether intrahepatic Ly6C\textsuperscript{hi} monocytes were able to suppress antigen-specific T cell responses, we used influenza hemagglutinin–specific (HA-specific) CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells derived from 6.5 and clone 4 HA-TCR transgenic mice, respectively. Similarly, addition of Ly6C\textsuperscript{hi} monocytes, not Ly6G\textsuperscript{hi}...
granulocytes, greatly inhibited the proliferation of HA-specific CD4+ and CD8+ T cells in response to stimulation with their cognate peptides (Figure 5B). These results indicate that intrahepatic Ly6C+ monocytes can directly suppress antigen-specific and non–antigen-specific T cell responses.

Ly6C+ monocytes suppress T cell proliferation through iNOS and NO. We next investigated how Ly6C+ monocytes suppress T cell activation. Previous studies have shown that the suppression of anti-viral T cell responses by monocytes appeared similar to that by monocytic myeloid-derived suppressor cells (m-MDSCs) in the regulation of antitumor immunity (17). Several factors have been implicated in the suppressive activity mediated by m-MDSCs including the induction of iNOS and the production of NO, the induction of arginase-1 activity, and the production of reactive oxygen species (ROS) (20). To examine the potential roles of iNOS, arginase-1, and ROS in regulating T cell activity, we added their respective inhibitors to the intrahepatic Ly6C+ monocyte/T cell coculture system. These inhibitors include L-NMMA for

Figure 4. Recruitment of Ly6C+ monocytes to the liver upon adenoviral infection is dependent on CCR2. (A and B) Wild-type (WT) or CCR2−/− mice were injected intravenously with recombinant adenovirus encoding LacZ (Ad). (A) On days 3, 7, and 10 after infection, cells from liver tissues of Ad-infected WT or CCR2−/− mice were analyzed for Ly6C+ monocytes by flow cytometry, and the total numbers of Ly6C+ monocytes in the liver and the bone marrow (BM) are indicated. Left: Mean cell numbers of Ly6C+ monocytes ± SEM in the liver. Right: Mean cell numbers of Ly6C+ monocytes ± SEM in the BM. ***P < 0.001, determined by multiple Student’s t test, n = 5 mice per group. (B) Ten days after infection, liver tissues were harvested, and cryosections were stained for LacZ expression by X-gal histochemistry. Scale bars: 100 μm. (C and D) A total of 2 × 10⁴ purified naive OT-1 CD8+ T cells (Thy1.1+) were adoptively transferred into congenic WT or CCR2−/− mice (Thy1.2+), which were subsequently infected intravenously with Ad-OVA. Seven days later, intrahepatic cells were stained with anti-CD8, anti-Thy1.1, and anti–IFN-γ intracellularly after restimulation in vitro for 6 hours with 5 μg/ml brefeldin A and 2 μg/ml of OVA peptide (257SIINFEKL264). The percentage of clonotypic OT-1 CD8+ cells among total lymphocytes are shown in C (top), and the absolute number of clonotypic cells ± SEM in the liver are indicated in D (left). The percentage of IFN-γ–producing clonotypic cells among T lymphocytes is shown in C (bottom), and the total number of IFN-γ–producing clonotypic cells ± SD in the liver are indicated in D (right). ***P < 0.001, determined by multiple Student’s t test, n = 5 mice per group. Data shown are representative of 3 independent experiments.
We found that only L-NMMA could reverse Ly6C^hi monocyte-mediated suppression of the proliferation of CD4^+ and CD8^+ T cells, whereas SOD, catalase, or nor-NOHA had no effects on Ly6C^hi monocyte-mediated suppression (Figure 6A). When added to the coculture in the absence of Ly6Chi monocytes, none of these inhibitors, including L-NMMA, could impair T cell activation (data not shown). The reversal of suppression of T cells by L-NMMA was associated with a significant (P < 0.01) reduction in NO in the culture (Figure 6B), suggesting a role for NO in mediating the suppression.

We then examined whether adenoviral infection could upregulate iNOS expression in intrahepatic Ly6Chi monocytes from the infected mice. Ad-LacZ was injected intravenously into C57BL/6 mice. Five days later, intrahepatic Ly6C^hi monocytes from naive or adenovirus-infected mice were analyzed for iNOS expression. Indeed, intrahepatic Ly6C^hi monocytes from day 5 adenovirus-infected mice produced significantly (P < 0.01) higher levels of iNOS than those from naive mice (Figure 6C). These results indicate that the Ly6C^hi monocyte–mediated suppression of T cell activity is mediated by iNOS induction and NO production.

Cell-cell contact is also required for T cell suppression by Ly6C^hi monocytes. We next examined whether cell-cell contact is also necessary for T cell suppression by Ly6C^hi monocytes utilizing the monocyte/T cell coculture system with transwells to separate monocytes from T cells. We found that the suppression of CD4^+

![Figure 5. Suppression of T cell proliferation by Ly6C^hi monocytes in vitro.](image-url)
and CD8+ T cell proliferation by Ly6C hi monocytes was abrogated in the presence of a transwell membrane, indicating that cell-cell contact is required for T cell suppression (Figure 7A). To investigate whether Ly6C hi monocytes and T cells keep close contact in situ in the liver, Ly6C hi monocytes and T cells were studied using double immunohistochemical staining with anti-CD11b (blue) and anti-Thy1.2 (brown). We observed that many CD11b+ monocytes and Thy1.2 + T cells infiltrated the liver at day 5 after adenoviral infection and that some CD11b+ cells were in close contact with Thy1.2 + T cells (Figure 7B), suggesting that cell-cell contact may also be important for monocyte-mediated suppression of T cells in vivo.

**Blocking of PD-L1/PD-1 interaction reverses T cell suppression by Ly6C hi monocytes.** The observation that cell-cell contact is important for suppression of T cell proliferation by Ly6C hi monocytes suggest that receptor-ligand interactions may be involved in T cell suppression. Galectin-9 (21), PD-1 ligand (PD-L1) (22), CTLA-4 (23), and TGF-β (24) have been implicated in the suppression of T cells in models of tumor or infection. To address whether these inhibitory molecules play a role in the suppression of T cells by Ly6C hi monocytes, blocking antibodies targeting galectin-9, PD-L1, CTLA-4, and TGF-β or a control antibody were added to the cocultures. We found that only anti-PD-L1 Ab reversed suppression of T cell proliferation by Ly6C hi monocytes (Figure 8A). We further observed that PD-L1 on intrahepatic Ly6C hi monocytes was upregulated after adenoviral infection (Figure 8B), and surface expression of PD-1 was also

Figure 6. Ly6C hi monocytes suppress T cell proliferation through iNOS. (A) CFSE-labeled splenic T cells (2 × 10^5) were cocultured without (T cell only) or with (+Monocytes) intrahepatic Ly6C hi monocytes (1 × 10^5) in the presence of anti-CD3 and -CD28 Abs. In addition, where indicated, 200 U/ml SOD, 200 U/ml catalase, 0.5 mM L-NNMA and 1 mM nor-NOHA were added to some wells at the beginning of the assay. The cultures were incubated for 72 hours and T cell proliferation was evaluated by CFSE dilution. (B) Nitric oxide (NO) was measured in culture supernatants. Mean NO ± SEM in the culture supernatant (quadruplicate per treatment) is shown. **P < 0.01, determined by multiple Student’s t test. (C) Intrahepatic Ly6C hi monocytes from naive mice or mice 5 days after infection with adeno virus (Ad) were analyzed immediately. Intracellular iNOS expression was analyzed. iNOS expression (median fluorescence intensity [MFI]) data represent the mean ± SEM on Ly6C hi monocytes. **P < 0.01, determined by a 2-tailed Student’s t test, n = 3 mice per group. Data shown are representative of 3 independent experiments.
Discussion

In this study, we showed that Ly6C<sup>hi</sup> monocytes migrated to the liver from the bone marrow in a model of adenovirus-induced hepatitis in vivo. The kinetics of Ly6C<sup>hi</sup> monocyte recruitment coincided with that of CD8<sup>+</sup> and CD4<sup>+</sup> T cell infiltration into the liver. We then showed that hepatic recruitment of Ly6C<sup>hi</sup> monocytes is dependent on CCR2, and the recruited Ly6C<sup>hi</sup> monocytes suppress CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses to adenoviral infection, leading to a delay in viral clearance. Furthermore, we demonstrated that Ly6C<sup>hi</sup> monocyte-mediated suppression of T cells was mediated by iNOS and NO, as well as a contact-dependent mechanism mediated by PD-1 and PD-L1 interaction.

Intravenous administration of adenovirus is a well-established model for viral hepatitis, as the liver is the primary organ for adenoviral infection. Previous studies have shown that the adaptive immune responses mediated by CD8<sup>+</sup> and CD4<sup>+</sup> T cells are essential for the development of inflammation and the eventual clearance of viral infection (5–7, 25). Although the role of antiviral CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses in inducing viral hepatitis has been shown in a model of MCMV infection (26), to our knowledge, this study represents the first report to demonstrate a critical role for Ly6C<sup>hi</sup> monocytes in regulating T cell responses in viral hepatitis. Depending on the nature of the infection and the context, the role of Ly6C<sup>hi</sup> monocytes can be dichotomous: proinflammatory versus antiinflammatory (13–17). In our model of viral hepatitis, CCR2-dependent migration of Ly6C<sup>hi</sup> monocytes to the liver helps suppress antiviral T cell responses and delay viral clearance. This is consistent with the observations in models of infection with Theiler’s murine encephalomyelitis virus and MCMV (16, 17), but is in contrast with those in models of infection with M. tuberculosis, T. gondii, and West Nile virus, in which Ly6C<sup>hi</sup> monocyte recruitment promotes the clearance of the infection (13–15). The reason for the differential roles of Ly6C<sup>hi</sup> monocytes in different settings remains unknown, but could be related to cytokines/factors produced during the infection that may contribute to the activation and maturation of Ly6C<sup>hi</sup> monocytes. The suppressive role of Ly6C<sup>hi</sup> monocytes in T cell responses in our model of adenoviral hepatitis is also in contrast with a recent report by Huang et al. showing that intrahepatic myeloid cells may promote T cell responses in the liver (27). However, their model involves adoptive transfer of in vitro-activated T cells (by anti-CD3 and anti-CD28 antibodies in the presence of IL-12) into mice followed by further stimulation with TLR9 ligand in vivo. Although they demonstrated that the transferred T cells stimulated with TLR9 ligand in vivo enhanced clearance of subsequent viral infection, the role of myeloid cells/monocytes in promoting T cell responses was shown in upregulated on activated CD8<sup>+</sup> T cells of day 5 adenovirus-infected mice compared with naive counterparts (Figure 8C). Collectively, these data suggest that interaction of PD-L1 and PD-1, which are induced on Ly6Chi monocytes and T cells after adenoviral infection, may be involved in suppression of T cell response by Ly6C<sup>hi</sup> monocytes in vivo.
the context of TLR9 stimulation, rather than viral infection. Furthermore, in vitro–stimulated T cells were used in their study instead of endogenous naive T cells, as in our model of adenoviral infection.

Our observation that Ly6C<sup>hi</sup> monocyte–mediated suppression of T cell activation and expansion is also dependent on NO suggests that Ly6C<sup>hi</sup> monocytes function during adenoviral infection similarly to m-MDSCs in tumor immunity. This similarity suggests there is an overlap in inflammatory signals that drive the differentiation of these suppressors of T cell responses during viral infection and the status of chronic inflammation, such as the tumor microenvironment. One of these factors could be IFN-γ, which is crucial for the control of viral infection and tumor growth (28, 29). Studies have shown that IFN-γ activates iNOS in a STAT1-dependent manner, leading to NO production (30, 31), which suppresses T cell activation and proliferation by disrupting IL-2 receptor signaling (32). Thus, IFN-γ produced by activated T cells during adenoviral infection could contribute to the induction of iNOS and production of NO by Ly6C<sup>hi</sup> monocytes, leading to the control of potentially overwhelming T cell activation. Further investigations are needed to delineate the role of IFN-γ in the suppressive function of Ly6C<sup>hi</sup> monocytes in vivo.

In addition to NO, we also observed that cell-cell contact is required for the suppression of T cells by intrahepatic Ly6C<sup>hi</sup> monocytes, suggesting that receptor-ligand interactions may also be involved in the suppression of T cell proliferation. Indeed, we found that adenoviral infection upregulated PD-L1 on Ly6C<sup>hi</sup> monocytes and PD-1 on activated T cells in the liver and in vitro blocking of the PD-L1/PD-1 interaction reversed the suppression of T cell proliferation by the Ly6C<sup>hi</sup> monocytes. This represents a potentially novel mechanism of T cell suppression by Ly6C<sup>hi</sup> monocytes. How does adenoviral infection upregulate the expression of PD-L1 on Ly6C<sup>hi</sup> monocytes? IFN-γ produced by tumor-infiltrating CD8<sup>+</sup> T cells can induce tumor cells to upregulate PD-L1, leading to CD8<sup>+</sup> T cell exhaustion (33), and disruption of PD-1/PD-L1 interaction leads to reversal of CD8<sup>+</sup> T cell exhaustion and restoration of antitumor activity (34). Thus, it is possible that, similar to the induction of iNOS and NO, IFN-γ produced by activated T cells during adenoviral infection could also upregulate PD-L1 on Ly6C<sup>hi</sup> monocytes. Alternatively, a recent
report indicated that PD-L1 can also be upregulated on MDSCs mediated by HIF-\(\alpha\) in response to the hypoxic tumor microenvironment (35). Such a mechanism might also be applied to PD-L1 upregulation on Ly6C\(^{hi}\) monocytes in response to viral infection. Thus, future studies are needed to define mechanisms responsible for the upregulation of PD-L1 by the suppressive monocytes.

In summary, we have provided evidence in a model of adenovirus-induced hepatitis that adenoviral infection induced recruitment of Ly6C\(^{hi}\) monocytes to the liver in a CCR2-dependent manner. These recruited Ly6C\(^{hi}\) monocytes suppress CD8\(^{+}\) and CD4\(^{+}\) T cell responses to adenoviral infection and delay viral clearance. Mechanistically, Ly6C\(^{hi}\) monocyte–mediated suppression of T cells is mediated by NO and the PD-1 pathway. These findings suggest a critical role for Ly6C\(^{hi}\) monocytes in the regulation of T cell immunity in viral hepatitis and may provide new insights into development of more effective therapies for treating viral hepatitis based on targeting the immunosuppressing monocytes.

Methods

**Mice.** C57BL/6 mice were purchased from the National Cancer Institute. The 6.5 HA-TCR transgenic mice that express a TCR recognizing an I-E\(^{d}\)-restricted HA epitope (\(^{110}\)SFGERFEIFPKE\(^{120}\)) and the clone 4 HA-TCR transgenic mice that express a TCR recognizing a K\(^{d}\)-restricted HA epitope (\(^{518}\)IYSTVASSL\(^{526}\)) have been described previously (36, 37). OT-1 transgenic mice that express a TCR recognizing a K\(^{b}\)-restricted OVA epitope (\(^{257}\)SIINFEKL\(^{264}\)) (38) and CCR2\(^{-/-}\) mice (39) were obtained from the Jackson Laboratory. All mice used were from 8 to 12 weeks of age.

**Adenoviral infection.** Recombinant adenovirus encoding LacZ (Ad-LacZ) or OVA (Ad-OVA) under the control of the cytomegalovirus promoter was generated and purified as described previously (8). For in vivo infection, mice were infected by intravenous injection of 2 \(\times\) 10\(^8\) PFU Ad-LacZ or Ad-OVA in 100 \(\mu\)l of PBS.

**Isolation of intrahepatic mononuclear cells.** Intrahepatic mononuclear cells were prepared as described (18). Briefly, the liver was homogenized and cells were isolated by density centrifugation in Ficoll reagent (Amersham Pharmacia). Cells were washed twice in 10% FBS in RPMI 1640 medium before use.

**Antibodies and flow cytometry.** Antibodies used: anti-CD11b (clone M1/70), anti-CD8 (clone 53-6.7); anti-PD-L1 (clone MIH5), anti-PD-1 (clone J43), anti-Ly6G (clone 1A8), anti-Ly6C (clone AL-21), anti-CD4 (clone RM4-5); anti-Thy1.1 (clone OKT1), anti-Ly6G (clone 1A8); anti-iNOS (clone 6/iNOS/Type II), anti-IFN-\(\gamma\) (clone XMG1.2), and anti-Ly6C (clone AL-21). These antibodies were purchased from BD Biosciences, eBioscience, AbD Serotec, or Biolegend. Flow cytometry data were collected on a BD FACS-Canto and analyzed using FACSDiva or FlowJo software.

**Detection of adenoviral genomic DNA.** Total genomic DNA isolated from the liver was used to measure the amount of adenoviral DNA using primers located in the fiber gene of adenovirus by real-time quantitative PCR as described previously (8). The sequences of the forward and reverse primers for adenoviral fiber gene were

\[
5' - CCACCGATAGCAGTACCCTT - 3' \\
5' - GACCAGTTGCTACGGTCAAA - 3'
\]

**Measurement of LacZ expression.** The LacZ expression was measured in the liver homogenate by the \(\beta\)-galactosidase assay, and on frozen liver sections by X-gal histochemical staining as described previously (8, 9).

**Isolation of Ly6C\(^{hi}\) monocytes, Ly6G\(^{+}\) granulocytes, and T cells.** Ly6C\(^{hi}\) monocytes and Ly6G\(^{+}\) granulocytes were enriched from the liver and spleen from adenovirus-infected mice by positive selection with anti-CD11b microbeads (Miltenyi Biotec). Enriched cells were then stained with anti-Ly6C and anti-Ly6G. Ly6C\(^{hi}\) monocytes (CD11b\(^{+}\)Ly6C\(^{hi}\) Ly6G\(^{-}\)) and Ly6G\(^{+}\) granulocytes (CD11b\(^{+}\)Ly6C\(^{lo}\)Ly6G\(^{+}\)) were purified via FACS (purity > 90%). Both CD4\(^{+}\) and CD8\(^{+}\) T cells were enriched from splenocytes by positive selection with Thy1.2-microbeads (Miltenyi Biotec), followed by sorting with a purity of greater than 95%.

**Inhibitors and blocking antibodies.** InOS inhibitor, N\(^\text{\text{O}}\) monomethyl-L-arginine citrate (L-NMMA) was purchased from EMD Millipore; arginase 1 inhibitor, N\(^\text{\text{O}}\)-hydroxy-nor-L-arginine (nor-NOHA) from Enzo; and ROS inhibitors, SOD and catalase from Sigma-Aldrich. Anti-galectin-9 (clone R9G-35) was obtained from Biolegend; anti-PD-L1 (clone MIH1) from eBioscience; anti-CTLA-4 (clone UC10-4F10-11) from Bio X Cell, anti–TGF-\(\beta\)1, 2, 3 (clone 1D11) from R&D Systems, and control rat IgG from Jackson ImmunoResearch Laboratories.

**Double immunochemical staining.** Fresh-frozen liver sections (5 \(\mu\)m) were fixed in cold acetone for 5 minutes. The sections were washed and incubated with anti-CD11b-biotin (clone M1/70, 1:200 dilution, BD Biosciences), followed by streptavidin-conjugated alkaline phosphatase (1:500 dilution, Jackson ImmunoResearch Laboratories). Colors were developed using Vector Blue (Vector) as the alkaline phosphatase.
substrate. After blocking, the sections were then stained with anti-Thy1.2-biotin (clone 53-2.1, 1:500 dilution, BD Biosciences), followed by Vectorstain ABC solution. Color was developed using DAB (Vector) as the peroxidase substrate.

Nitrite measurement. Nitric oxide (NO) production was assessed by nitrite accumulation in 72-hour culture supernatants using the Griess reagent (Sigma-Aldrich). Cell-free culture supernatants (100 μl) were added to a microtiter plate containing 100 μl of Griess reagent and incubated for 10 minutes at room temperature. Absorbance was measured at 550 nm, and nitrite concentration calculated by comparison with a standard curve prepared with sodium nitrite.

Statistics. All statistical analyses were performed with Graph Prism 6 software. Results are expressed as the mean ± SEM. Significance between experimental groups was determined by a 2-tailed Student’s t test. A P value less than 0.05 was considered to be statistically significant for all results.

Study approval. All animal procedures were approved by the Institutional Animal Care and Use Committee at Duke University, Durham, North Carolina, USA.

Author contributions
JZ designed and performed the study, collected and analyzed data, and prepared the paper. HC performed select experiments and collected and analyzed data. XH, SJ, and YY designed research, analyzed data, and wrote the paper.

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