M2 Macrophages Play Critical Roles in Progression of Inflammatory Liver Disease in Hepatitis C Virus Transgenic Mice

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ABSTRACT

Macrophages in liver tissue are widely defined as important inflammatory cells in chronic viral hepatitis due to their proinflammatory activity. We reported previously that interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) play significant roles in causing chronic hepatitis in hepatitis C virus (HCV) transgenic mice (S. Sekiguchi et al., PLoS One 7:e51656, 2012, http://dx.doi.org/10.1371/journal.pone.0051656). In addition, we showed that recombinant vaccinia viruses expressing an HCV non-structural protein (rVV-N25) could protect against the progression of chronic hepatitis by suppression of macrophage activation. Here, we focus on the role of macrophages in liver disease progression in HCV transgenic mice and examine characteristic features of macrophages following rVV-N25 treatment. The number of CD11b$^+$ F4/80$^+$ CD11c$^-$ CD206$^+$ (M2) macrophages in the liver of HCV transgenic mice was notably increased compared to that of age-matched control mice. These M2 macrophages in the liver produced elevated levels of IL-6 and TNF-α. rVV-N25 infection suppressed the number and activation of M2 macrophages in liver tissue. These results suggested that inflammatory cytokines produced by M2-like macrophages contribute to the induction of chronic liver inflammation in HCV transgenic mice. Moreover, the therapeutic effect of rVV-N25 might be induced by the suppression of the number and activation of hepatic macrophages.

IMPORTANCE

HCV causes persistent infections that can lead to chronic liver diseases, liver fibrosis, and hepatocellular carcinoma; the search for an HCV curative is the focus of ongoing research. Recently, effective anti-HCV drugs have been developed; however, vaccine development still is required for the prevention and therapy of infection by this virus. We demonstrate here that M2 macrophages are important for the pathogenesis of HCV-caused liver diseases and additionally show that M2 macrophages contribute to the therapeutic mechanism observed following rVV-N25 treatment.

Macrophages play a central role in innate immunity, inflammation, and host defense (1). These highly heterogeneous cells can rapidly change their function in response to local microenvironmental signals. Notably, signals can induce macrophages to undergo classical M1 activation (stimulated by lipopolysaccharide [LPS] and gamma interferon [IFN-γ]) or, alternatively, M2 activation (stimulated by interleukin-4 [IL-4]/IL-13); these paradigms mirror the Th1-Th2 polarization of T cells (2, 3). The M1 phenotype is characterized by the secretion of high levels of proinflammatory cytokines (tumor necrosis factor alpha [TNF-α], IL-6, and IL-12), elevated production of NO and reactive oxygen species, promotion of Th1 response, and strong microbicidal and tumoricidal activity (4). In contrast, M2 macrophages are thought to facilitate antiparasitic effects and the promotion of tissue remodeling, tissue repair, and tumor progression and to have immunoregulatory functions (5).

The resident liver macrophages, Kupffer cells (KCs), are phagocytic cells that account for 80% of the total population of fixed-tissue macrophages in the body (6). There still is controversy concerning the origin of these cells: although the class is known to be derived from bone marrow, some models suggest that the cells arrive in the liver as monocytes and differentiate into tissue-resident macrophages; other models suggest that macrophages are derived via local hematopoiesis in the liver (7). There is also an increasing number of reports suggesting that KCs have important roles in liver inflammation in viral hepatitis (8–10).

In patients with chronic HCV infection, KCs are activated by the expression of CD163 and CD33 (10), and IFN production by KCs has been proposed as a source of innate immune tolerance, characterized by a lack of response to IFN therapy (11). Thus, although KCs are involved in HCV-mediated liver inflammation and efficacy of IFN therapy, the specific role of macrophages in causing tissue damage remains unknown.

We previously reported the generation of immunocompetent mice that expressed multiple HCV proteins via a Cre/*loxP* switching system (12). These HCV transgenic (HCV-Tg) mice exhibited chronic hepatitis C-related pathological findings, including steatosis, liver fibrosis, and hepatocellular carcinoma following expression of HCV core, E1, E2, and NS2 proteins in the liver (13, 14). We also found that macrophages contribute to the development of liver disease, as demonstrated by the fact that the neutralization of IL-6 receptor and TNF-α (factors produced primarily by macrophages) in these mice attenuates pathological liver changes. Based on these observations, we sought to further elucidate the role of macrophages in liver inflammation, focusing on macrophages in the liver and spleen of HCV-Tg mice. In addition, we assessed the effect on the function of macrophages in the liver of a candidate therapeutic HCV vaccine (designated rVV-N25, for recombinant vaccinia virus vector N25) that corresponded to a recombinant vaccinia virus that expresses HCV nonstructural protein N25 containing NS2, NS3, NS4A, NS4B, NS5A, and NS5B regions. In previous work, we demonstrated that immunization with rVV-N25 suppresses serum inflammatory cytokine levels and alleviates the symptoms of pathological chronic hepatitis C (12).

MATERIALS AND METHODS

Ethics statement. All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care; these guidelines conform to the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. All protocols were approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (permission number 11-078). All efforts were made to minimize the suffering of the animals.

Animals. R6CN2HCV transgenic mice that possess HCVR6 (genotype 1b) cDNA (nucleotides 294 to 3435) downstream of the CAG promoter, the neomycin resistance gene (*neo*), and poly(A) signal flanked by two *loxP* sequences (12) were bred with Mx1-Cre transgenic mice (purchased from The Jackson Laboratory) to produce R6CN2HCV-MxCre transgenic mice (CN2-29$^{+/-}$/MxCre$^{+/-}$), here referred to as HCV transgenic mice. Cre expression in the livers of these mice was induced by intraperitoneal injection of poly(I · C) (GE Healthcare UK Ltd., Buckinghamshire, England); 300 μl poly(I · C) solution {1 mg/ml calcium- and magnesium-free phosphate-buffered saline [in PBS(−)]} was injected 3 times at 48-h intervals.

Immunization with rVV-HCV. At 90 days after the third injection with poly(I · C), HCV transgenic mice were immunized intradermally with either rVV-N25, which is based on strain LC16m8 (carrying HCV NS2 to NS5 genes and expressed under the ATI/p7.5 hybrid promoter within the hemagglutinin gene region) (12), or empty recombinant vaccinia virus vector (rVV-Emp; LC16m8 strain) at 1×10^8 PFU/mouse, as reported previously (12). rVV-directed expression of HCV proteins was observed in all tissues (including the liver and spleen) in HCV-Tg mice.

Macrophage depletion. The role of macrophages in the livers of HCV transgenic mice with chronic hepatitis was evaluated by the depletion of macrophages with clodronate liposomes. Clodronate liposomes were prepared as described previously (15). Mice were injected with clodronate liposomes on days −1 and 3 (i.e., 1 day before and 3 days after immunization [on nominal day 0] with rVV-N25 or rVV-Emp).

Cell isolation. To isolate splenocytes, spleen samples were prepared by passing the tissues through a 70-μm cell strainer (BD Biosciences). To isolate intrahepatic leukocytes, single-cell suspensions were prepared from liver tissue perfused with PBS via the inferior vena cava. The per-fused liver tissue samples then were digested in 10 ml RPMI 1640 (Nissui Pharmaceutical Co., Ltd.) containing 0.02% (wt/vol) collagenase IV (Invitrogen) and 0.002% (wt/vol) DNase I (Sigma-Aldrich) for 40 min at 37°C. The digested cell suspension was layered over Lympholyte-M (Cedarlane). After centrifugation for 20 min at $1,000 \times g$, intrahepatic leukocytes were isolated at the interface. After being washed with PBS, the cell suspensions were centrifuged for 5 min at $500 \times g$. The pellets were resuspended in 1 ml RPMI 1640 containing 10% fetal bovine serum (FBS).

FACS analysis. To perform intracellular cytokine staining, intrahepatic and intrasplenic leukocytes were cultured for 4 h at 37°C in 96-well round-bottom plates containing 200 μl/well RPMI medium. Human recombinant IL-2 (50 U) and 0.2 μl of BD GolgiPlug protein transport inhibitor (BD Biosciences) were added to each well. For the macrophage experiments, lipopolysaccharide (LPS; 0.5 μg/ml; Sigma) was added with BD GolgiPlug to the culture. After incubation, the cells were harvested, washed in PBS containing 1% FBS, and incubated for 10 min on ice with unlabeled anti-mouse CD16/32 antibody (BD Biosciences) to block binding to FcγRII/III. The cells then were surface stained for 20 min on ice with the indicated antibodies. The following reagents were obtained from BioLegend (except as noted): Pacific Blue-conjugated monoclonal anti-mouse CD8 antibody (BD Biosciences), CD11b antibody, allophycocyanin-Cy7 (APC-Cy7)-conjugated monoclonal anti-mouse F4/80 antibody, CD4 antibody, fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse T cell receptor beta (TCR-β) antibody, Ly6C antibody, CD11c, APC-conjugated monoclonal anti-mouse NK1.1 antibody, F4/80 antibody; phycoerythrin (PE)-conjugated Gr-1 antibody, and PE/Cy7-conjugated monoclonal anti-mouse CD11c antibody. Following staining, cells were washed to remove unbound antibody and fixed using the Cytofix/Cytoperm kit (BD Biosciences). Cells then were subjected to secondary staining with the following reagents (obtained from BioLegend, except as noted): PE-conjugated anti-mouse TNF-α (BD Biosciences), anti-IL-6 (BD Biosciences), anti-interferon gamma, and anti-IL-12. The cells were washed again and resuspended in 1 ml fluorescence-activated cell sorting (FACS) buffer for analysis with the FACSCanto II system (BD Biosciences).

Serum inflammatory cytokines and chemokines. To measure the amounts of cytokines and chemokines in samples of mouse serum, we used Bio-Plex cytokine assay kits (Bio-Rad Laboratories) according to the manufacturer's instructions. Specifically, we used the Bio-Plex mouse cytokine 23-plex panel, which detects 23 cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 [P40], IL-12 [P70], IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage CSF [GM-CSF], IFN-γ, KC, monocyte chemoattractant protein 1 [MCP-1], macrophage inflammatory protein 1α (MIP-1α), MIP-1β, RANTES, and TNF-α), and the Bio-Plex mouse cytokine 9-plex panel, which detects nine cytokines (IL-15, IL-18, fibroblast growth factor [FGF]-basic, leukemia inhibitory factor [LIF], M-CSF, monokine induced by gamma interferon (MIG), MIP-2, platelet-derived growth factor beta [PDGF-β], and vascular endothelial growth factor [VEGF]). The plates then were washed three times by vacuum filtration using 100 μl per well Bio-Plex wash buffer, 25 μl of diluted detection antibody was added to each well, and plates were incubated with shaking for 30 min at room temperature. After another three washes using the vacuum filtration system, 50 μl of streptavidin-PE solution was added to each well, and the plates were incubated with shaking for another 10 min at room temperature. After another three washes using the vacuum filtration system, beads were suspended in Bio-Plex assay buffer, and samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex suspension array system and Bio-Plex manager software (Bio-Rad Laboratories).

Histology and histological evaluation. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4-μm thickness), and stained with hematoxylin and eosin (H&E).

For immunohistochemical (IHC) staining of M2 macrophages, FITC-labeled anti-mouse F4/80 antibody (clone BM8; BioLegend) and anti-mouse CD206 antibody (clone MR5D3; AbD Serotec) were used.
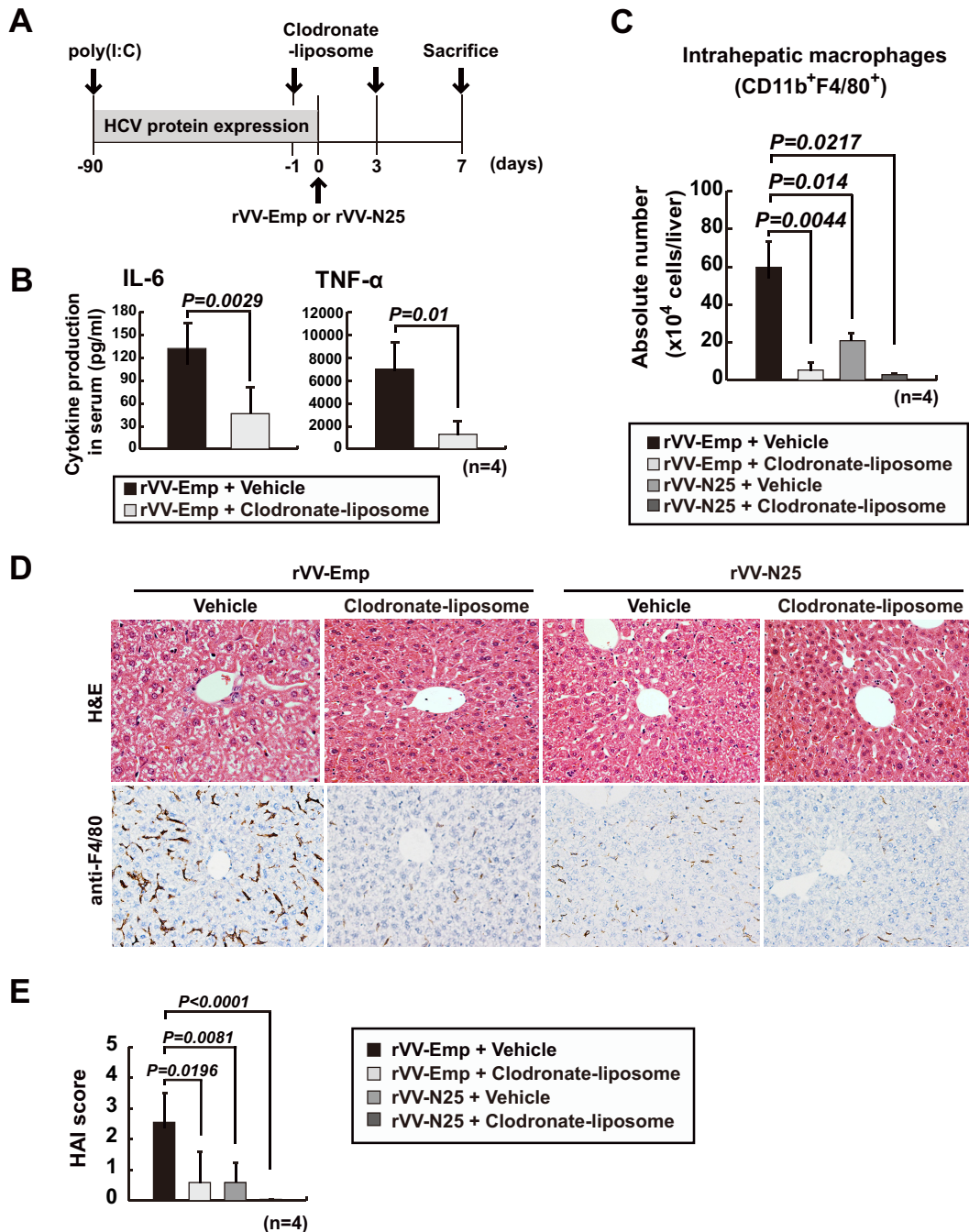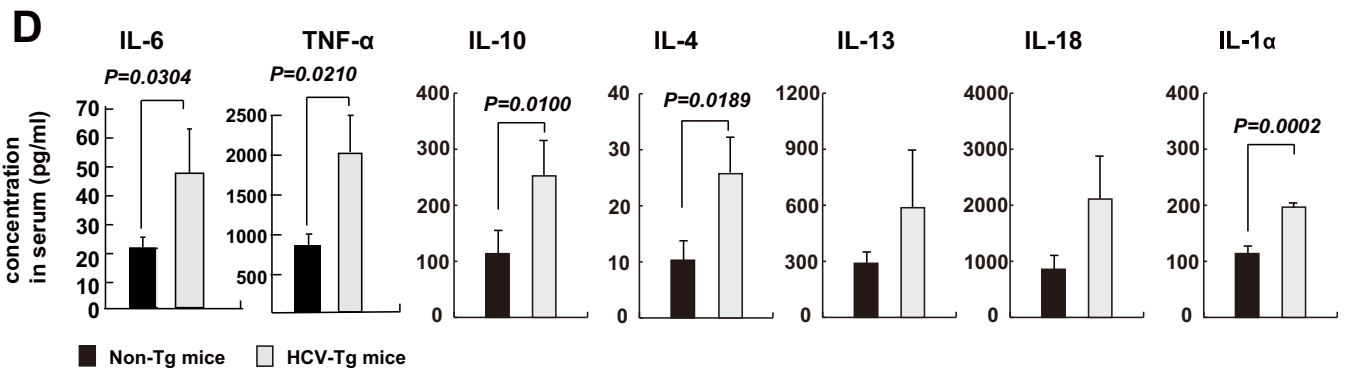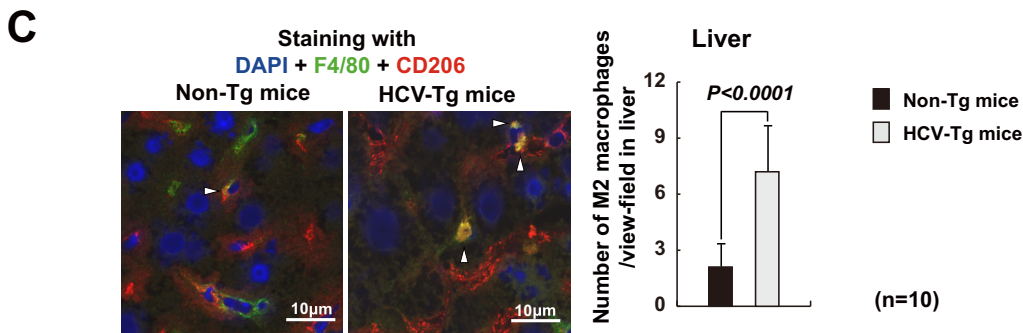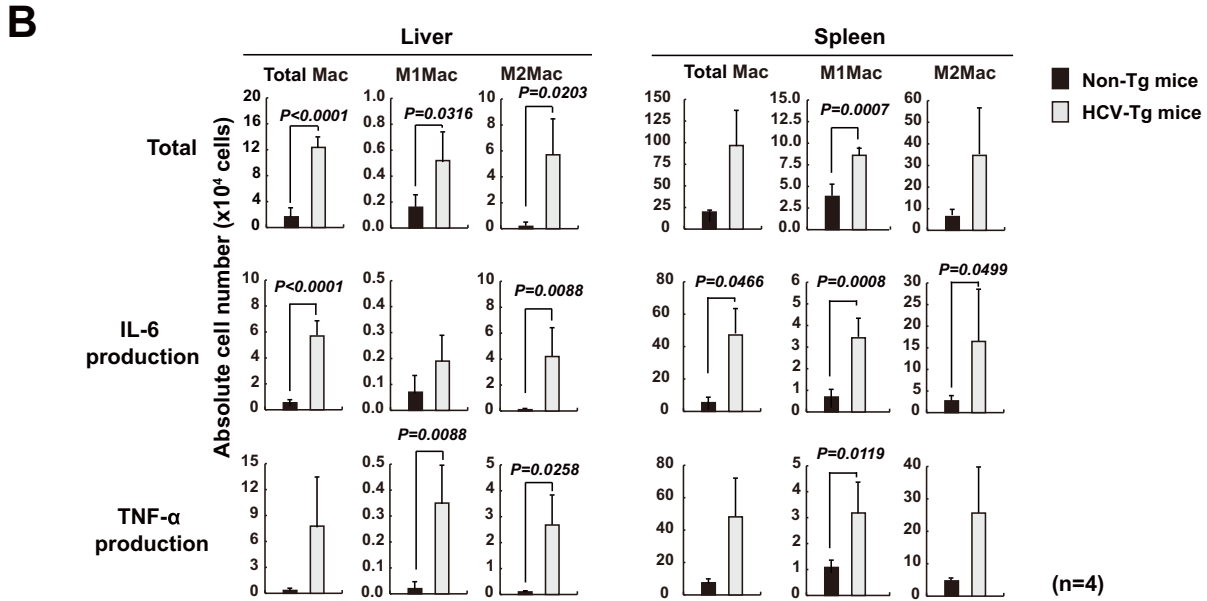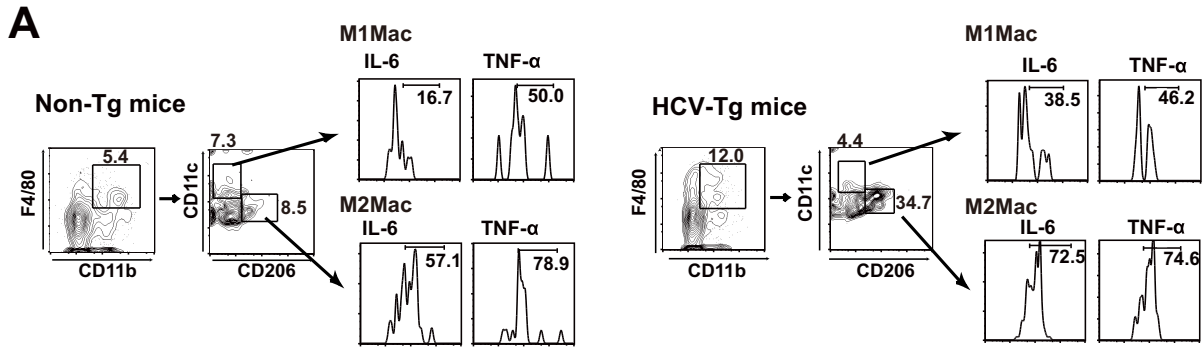
FIG 1 Macrophages are responsible for liver inflammation in HCV transgenic mice. (A) Experimental design. (B) Serum cytokine levels were measured in HCV transgenic mice that were treated with clodronate-liposome (or vehicle) prior to immunization with rVV-Emp (empty control recombinant vaccinia virus). Values are presented as means ± SD ($n = 4$). (C) Intrahepatic macrophages were isolated from the HCV transgenic mice, and the effects of clodronate-liposome on cell recruitment were analyzed. The number of CD11b+ F4/80+ cells in the liver was calculated by multiplying the total number of intrahepatic leukocytes by the frequency of this subset in the population (as determined by FACS analysis). Values are presented as means ± SD ($n = 4$). (D) Representative H&E-stained sections from livers obtained (7 days after immunization) from the four groups of HCV transgenic mice. The scale bars indicate 50 μm. (E) Histological evaluation of livers obtained (7 days after immunization) from the four groups of HCV transgenic mice. HAI scoring (see Materials and Methods) was used for the evaluation of liver inflammation. Values are presented as means ± SD ($n = 4$). P values were determined by two-tailed Student's t test; $P < 0.05$ was considered significant.

Anti-mouse CD206 antibody was labeled with the Alexa Fluor 555 microscale protein labeling kit (Molecular Probes). Frozen liver sections were fixed in acetone for 10 min at −20°C and then incubated with blocking buffer [1% bovine serum albumin–0.5 mM EDTA in PBS(−)] for 60 min at room temperature. Subsequently, the sections were incubated with FITC-labeled anti-mouse F4/80 antibody (1 μg/ml) and Alexa Fluor 555-labeled anti-mouse CD206 antibody (1 μg/ml) for 1 h at room temperature. After being washed with PBS(−), the sections were encapsulated with SlowFade gold antifade reagent (Molecular Probes). The nuclei were stained with 4′,6-diamidino-2-phe-

nylindole (DAPI; Molecular Probes). Fluorescence was observed under a confocal laser microscope (LSM780; Carl Zeiss).

The histological grade of hepatitis was determined using the histology activity index (HAI) scoring system. The HAI score in each group was expressed as the mean scores from five visual fields. The following histological grades of steatosis were determined: no visible fat, score 0; <5% of the liver infiltrated by fat, score 1; 5 to 25% fat, score 2; 25 to 50% fat, score 3; >50% fat, score 4.

Statistical analysis. Data are shown as means ± standard deviations (SD). P values were determined by two-tailed Student's t test. P values of less than 0.05 were considered statistically significant.

RESULTS

Depletion of macrophages reduces pathological changes in the liver of HCV transgenic mice. We previously demonstrated that inflammatory cytokines TNF-α and IL-6 play critical roles in the development of liver disease in HCV transgenic mice with chronic hepatitis. We also showed that these cytokines are produced primarily by macrophages in the liver (12). To further evaluate the role of macrophages in liver inflammation, we performed macrophage depletion by administering clodronate liposome, a treatment known to deplete macrophages in the liver. These mice were treated by two injections with clodronate liposome. One day after the first injection, the mice were immunized with rVV-N25 (vaccine) or rVV-Emp (control vector). The mice were sacrificed on day 7 (i.e., 7 days after immunization) (Fig. 1A). First, to determine whether the depletion of macrophages played a role in inflammatory response, we measured serum inflammatory cytokines and chemokines in blood samples using the Bio-Plex system. As shown in Fig. 1B, serum IL-6 and TNF-α levels were decreased in clodronate liposome-treated mice.

To evaluate the effect of clodronate liposome on inflammatory cells in the same livers, we counted the absolute number of intrahepatic leukocytes and intrasplenic leukocytes, and we calculated the number of cells belonging to each cell surface marker subset by FACS analysis. As shown in Fig. 1C, clodronate liposome treatment significantly reduced the number of macrophages (CD11b$^+$ F4/80$^+$) in the liver (about 91% reduction). Consistent with previous results, we confirmed that rVV-N25 immunization significantly decreased the number of macrophages and monocytes in the liver compared to that of the control.

To assess the histological changes in the livers after clodronate liposome treatment with rVV-N25 or rVV-Emp immunization, we observed liver tissues by H&E staining. We evaluated the grading and staging of liver tissues by an HAI score. We found that clodronate liposome-treated mice were largely attenuated for HCV-induced liver damage (Fig. 1D and E), indicating that macrophages contributed to liver inflammation in HCV transgenic mice with chronic hepatitis. Furthermore, consistent with the results of FACS analysis, IHC analysis revealed that F4/80-positive

macrophages were depleted after injection of clodronate liposome (Fig. 1D).

M1 and M2 macrophage numbers increase in the liver and spleen of HCV transgenic mice with chronic hepatitis. To determine the role of macrophages in liver inflammation, we analyzed the characteristics of macrophages in HCV transgenic mice. First, to examine whether HCV protein expression affects polarization in macrophages, we analyzed the number, phenotype, and cytokine production of macrophages in the liver and spleen of HCV transgenic mice and control mice at 3 months following poly(I·C) injection. As shown in Fig. 2A for representative data, we isolated lymphatic mononuclear cells from the two tissues, sorted the CD11b$^+$ F4/80$^+$ macrophages into M1 (CD11b$^+$ F4/80$^+$ CD11c$^+$) and M2 (CD11b$^+$ F4/80$^+$ CD206$^+$) populations, and then analyzed IL-6 and TNF-α expression following *ex vivo* LPS stimulation. As shown in Fig. 2B, macrophage numbers increased approximately 3-fold in the liver of HCV transgenic mice; interestingly, the number of M1 and M2 macrophages increased in the livers of the mice with chronic hepatitis, although the number of M2 macrophage was much higher than that for M1 macrophage. Furthermore, IL-6 and TNF-α production was increased significantly in both the M1 and M2 macrophages derived from HCV transgenic mice, although the literature (4) suggests that both cytokines are produced primarily by M1 macrophages. The number and cytokine production of macrophages in liver exhibited a pattern similar to that in spleen.

In addition, IHC analysis suggested that HCV transgenic mice had increased numbers of F4/80$^+$ CD206$^+$ cells compared to those of age-matched control mice (Fig. 2C), as quantified on the right in Fig. 2C. Serum levels of cytokines were measured, and significant increases of IL-6, TNF-α, IL-10, IL-4, and IL-1α in HCV transgenic mice were observed (Fig. 2D).

Collectively, these results showed that HCV protein expression induced the accumulation of macrophages, mainly M2 macrophages, in the liver, and that these cells might be involved in the development of liver inflammation due to the production of TNF-α and IL-6.

rVV-N25 immunization decreases the number and cytokine production of M2 macrophages in the liver. As shown in Fig. 1, we demonstrated that rVV-N25 reduces the number of macrophages in the liver and attenuates histopathological changes otherwise seen in liver tissue of HCV transgenic mice. To evaluate the effect of rVV-N25 on M2 macrophages, we analyzed the cell number and cytokine production of macrophages in the liver and spleen 7 days after rVV-N25 or rVV-Emp immunization (Fig. 3A). Serum inflammatory cytokine IL-6 levels were reduced significantly following rVV-N25 immunization (Fig. 3B). As shown in Fig. 3C, we confirmed that rVV-N25 immunization reduced the number and cytokine production of total and M2 macrophages in

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FIG 2 M2 macrophage numbers are elevated in the liver of HCV-Tg mice. (A) Intrahepatic macrophage subpopulations were examined with or without HCV protein expression. These cells were stained with anti-CD11b, anti-F4/80, anti-CD206, and anti-CD11c monoclonal antibodies (MAbs). Representative results from three independent experiments are shown. (B) Macrophages from the liver and the spleen were isolated from non-Tg and HCV-Tg mice, and the effects of HCV protein production on cell recruitment were analyzed. The number of each subset of cells in the liver and spleen was calculated by multiplying the total number of intrahepatic or intrasplenic leukocytes by the frequency of each subset in the cell population (as determined by FACS analysis). Values are presented as means ± SD (n = 4). (C) Immunohistochemical (IHC) analysis. Liver sections were stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse F4/80 antibody and Alexa Fluor 555-labeled anti-mouse CD206 antibody. The nuclei were stained with DAPI. Fluorescence was observed under a confocal laser microscope. Values are presented as means ± SD (n = 10). P values were determined by two-tailed Student's t test; P < 0.05 was considered significant. (D) Serum levels of cytokines in HCV-Tg (gray bars) and non-Tg mice are indicated. Values are presented as means ± SD (n = 10). P values were determined by two-tailed Student's t test; P < 0.05 was considered significant.
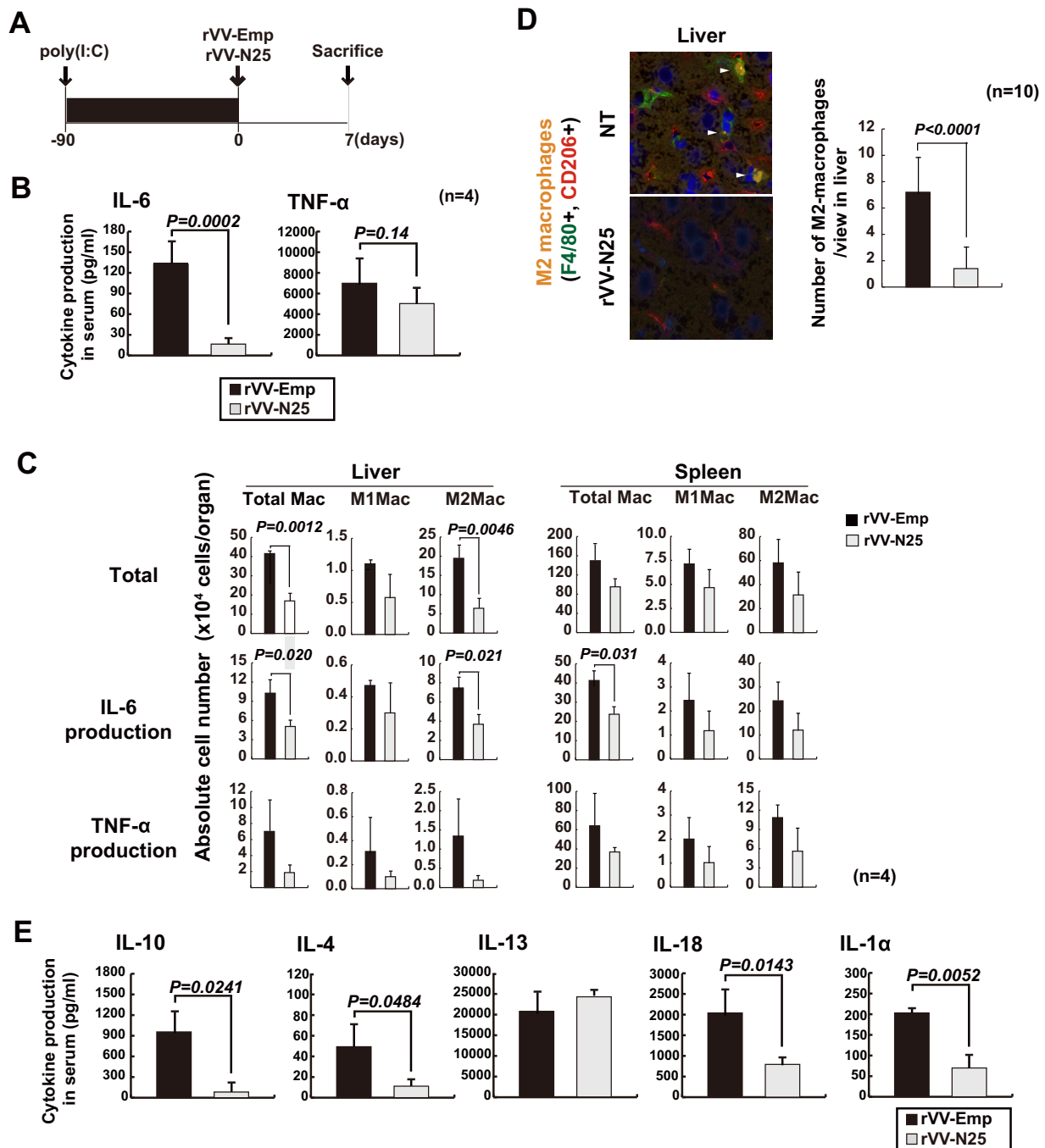
FIG 3 Immunization with recombinant vaccinia virus (rVV-N25) decreases the number and cytokine production of M2 macrophages in the liver. (A) Schematic diagram of experimental design of rVV-N25 immunization of HCV transgenic mice. (B) Serum cytokine levels were measured in HCV transgenic mice that were immunized with rVV-Emp or rVV-N25. Values are presented as means ± SD ($n = 4$). (C) Intrahepatic and intrasplenic macrophage subpopulations were examined following immunization with rVV-N25 or rVV-Emp (empty control recombinant vaccinia virus). The corresponding cells were isolated from immunized mice, and the effects of rVV-N25 on cell recruitment were analyzed. Cells were stained with anti-CD11b, anti-F4/80, anti-CD11c (for M1), and anti-CD206 (for M2). Cells also were stained with anti-IL-6 and anti-TNF-α monoclonal antibodies (MAbs). The number of each subset of cells in the liver and spleen was calculated by multiplying the total number of intrahepatic and intrasplenic leukocytes by the frequency of each subset in the cell population (as determined by FACS analysis). Values are presented as means ± SD ($n = 4$). P values were determined by two-tailed Student's t test; $P < 0.05$ was considered significant. (D) IHC analysis. Liver sections were stained with FITC-labeled anti-mouse F4/80 antibody and Alexa Fluor 555-labeled anti-mouse CD206 antibody following rVV-N25 treatment. The nuclei were stained with DAPI. Fluorescence was observed under a confocal laser microscope. White arrowheads represent M2 macrophages. The cells with positive staining in the liver were counted in 10 randomly selected microscopic fields of liver sections obtained from individual animals. (E) Serum levels of cytokines in a HCV-Tg mouse (black bars) are indicated following rVV-N25 treatment (gray bars). Values are presented as means ± SD ($n = 10$). P values were determined by two-tailed Student's t test; $P < 0.05$ was considered significant.

the liver and spleen, suggesting that M2 macrophages are targets of the therapeutic effect(s) of rVV-N25. In contrast, we did not detect a significant difference in the number of M1 macrophages in the liver. These findings were supported by IHC analysis indicating that macrophage numbers were decreased in the liver of rVV-N25-immunized HCV transgenic mice (Fig. 3D). These observations may be consistent with the significant decrease of IL-6 and TNF-α levels, although the decrease of TNF-α levels was not statistically significant.

Among cytokine levels, serum IL-4 and IL-10, which were involved in the maintenance and activation of the M2 macrophage, decreased significantly after rVV-N25 inoculation (Fig. 3E).

DISCUSSION

HCV infection-triggered liver injury and disease progression are thought to be driven by host immune responses, especially via antigen nonspecific cytotoxic T lymphocyte-induced immunopathology (16). Moreover, a decrease of T cell response specificity should make completely clearing HCV from the liver impossible (16). In addition, our previous study suggested that inflammatory liver diseases are mediated by TNF-α and IL-6 produced by macrophages in the liver (12). To clarify this distinction, we further investigated the role of macrophages in the onset of liver disease. As we report here, the number of CD11b$^+$ F4/80$^+$ macrophages was increased in the liver and spleen of HCV transgenic mice. Consistent with these results, the depletion of macrophages attenuated the pathological changes otherwise observed in the liver of HCV transgenic mice. We infer that macrophages are responsible for HCV-mediated liver inflammation. Some reports by other laboratories demonstrate that recruitment of macrophages is increased in the focal areas of inflammation, and that levels of KC-derived IL-18 and TNF-α correlate with hepatitis and liver injury in HCV-infected patients (10, 17, 18). Although HCV infection drives macrophage inflammatory responses, including the production of type 1 proinflammatory cytokines (IL-1β and IL-18) (19), few studies have thoroughly examined HCV-induced polarization of macrophages both *in vivo* and *in vitro*.

We showed that the number of M2 macrophages in the liver of HCV transgenic mice with chronic hepatitis increased about 10-fold, producing much higher levels of TNF-α and IL-6 following LPS stimulation. Although M1 macrophages in the liver also produced TNF-α and IL-6, the amounts of these cytokines were more prominent in M2 macrophages. The switch toward M2 macrophages might simply be part of a host defense mechanism used to control HCV-induced tissue damage, since the M2 population participates in the suppression of inflammation and promotion of tissue repair (2). However, it is curious that M2 macrophages produce elevated amounts of TNF-α and IL-6 compared to that of M1 macrophages in the HCV transgenic mice. Although we have not examined the production of other inflammatory cytokines (such as IL-4, IL-10, and IL-13) that are known Th2 cytokines, our results suggest that M2 macrophages have the potential to produce Th1 cytokines under some conditions. These findings imply that M2 macrophages are prominent during chronic active hepatitis, indicating that macrophage polarization depends on the period of liver disease progression. Recently, Bility et al. also demonstrated (using a humanized mouse model) that M2 macrophages are prominent during chronic active hepatitis resulting from hepatitis B virus (HBV) infection (20).

Furthermore, our HCV transgenic mice exhibited hepatic steatosis with lipid accumulation (12). Fatty acids and their cognate receptors, peroxisome proliferator-activated receptor gamma (PPARγ) and PPARδ, have been shown to induce maturation of M2 macrophages, indicating that steatosis is involved in lipid accumulation (21).

To further address the increase in the number of M2 macrophages in the liver of HCV transgenic mice, we analyzed the relative percentage of inflammatory monocytes (CD11b$^+$ Ly-6C$^+$) in bone marrow. We found that there was no significant difference for inflammatory monocytes with or without the expression of HCV protein, indicating that macrophage numbers increase by way of local proliferation in the liver.

Furthermore, we showed that the therapeutic effect of an HCV vaccine (rVV-N25) correlated with the reduction of M2 macrophage numbers in the liver. Although we have demonstrated that rVV-N25 reduces inflammatory responses in the liver (with associated decreases in the levels of inflammatory cytokines and chemokines), it remains unclear why such an inflammatory response is induced. One clue is the observation that immunization with rVV-N25 reduces macrophage numbers in liver, as assessed by FACS analysis and IHC analysis. We showed that the reduction of macrophage numbers was not due to an increase in apoptosis following rVV-N25 treatment, since we did not detect a change in the proportion of annexin V$^+$/7-aminoactinomycin D$^-$ cells among intrahepatic leukocytes. Additionally, we tested whether macrophages were infected with vaccinia virus after inoculation. Based on PCR analyses, vaccinia virus DNA was present only in the dermis and not in macrophage-infiltrated liver and spleen tissues. These results suggested that the reduction of liver macrophage numbers is not a direct reflection of vaccinia virus cytotoxicity but instead results from host immune responses following immunization. Further experiments will be required to evaluate the mechanism responsible for increasing the number of macrophages in liver.

Thus, we have demonstrated here that M2 macrophages have the potential to produce proinflammatory cytokines in the liver of HCV transgenic mice and are responsible for liver inflammation. To our knowledge, this report provides the first evidence that M2 macrophages have functions like those of M1 macrophages.

REFERENCES

1. **Akira S, Uematsu S, Takeuchi O.** 2006. Pathogen recognition and innate immunity. Cell **124:**783–801. http://dx.doi.org/10.1016/j.cell.2006.02.015.

2. **Wynn TA, Chawla A, Pollard JW.** 2013. Macrophage biology in development, homeostasis and disease. Nature **496:**445–455. http://dx.doi.org/10.1038/nature12034.

3. **Murray PJ, Wynn TA.** 2011. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol **11:**723–737. http://dx.doi.org/10.1038/nri3073.

4. **Sica A, Mantovani A.** 2012. Macrophage plasticity and polarization: in vivo veritas. J Clin Investig **122:**787–795. http://dx.doi.org/10.1172/JCI59643.

5. **Galli SJ, Borregaard N, Wynn TA.** 2011. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. Nat Immunol **12:**1035–1044. http://dx.doi.org/10.1038/ni.2109.

6. **Crispe IN.** 2009. The liver as a lymphoid organ. Annu Rev Immunol **27:**147–163. http://dx.doi.org/10.1146/annurev.immunol.021908.132629.

7. **Jenne CN, Kubes P.** 2013. Immune surveillance by the liver. Nat Immunol **14:**996–1006. http://dx.doi.org/10.1038/ni.2691.

8. **Sitia G, Iannacone M, Aiolfi R, Isogawa M, van Rooijen N, Scozzesi C, Bianchi ME, von Andrian UH, Chisari FV, Guidotti LG.** 2011. Kupffer cells hasten resolution of liver immunopathology in mouse models of viral hepatitis. PLoS Pathog **7:**e1002061. http://dx.doi.org/10.1371/journal.ppat.1002061.

9. **Kimura K, Kakimi K, Wieland S, Guidotti LG, Chisari FV.** 2002. Activated intrahepatic antigen-presenting cells inhibit hepatitis B virus replication in the liver of transgenic mice. J Immunol **169:**5188–5195. http://dx.doi.org/10.4049/jimmunol.169.9.5188.

10. **Dolganiuc A, Norkina O, Kodys K, Catalano D, Bakis G, Marshall C, Mandrekar P, Szabo G.** 2007. Viral and host factors induce macrophage activation and loss of toll-like receptor tolerance in chronic HCV infection. Gastroenterology **133:**1627–1636. http://dx.doi.org/10.1053/j.gastro.2007.08.003.

11. **Lau DT, Negash A, Chen J, Crochet N, Sinha M, Zhang Y, Guedj J, Holder S, Saito T, Lemon SM, Luxon BA, Perelson AS, Gale M, Jr.** 2013. Innate immune tolerance and the role of kupffer cells in differential responses to interferon therapy among patients with HCV genotype 1 infection. Gastroenterology **144:**402–413. http://dx.doi.org/10.1053/j.gastro.2012.10.044.

12. **Sekiguchi S, Kimura K, Chiyo T, Ohtsuki T, Tobita Y, Tokunaga Y, Yasui F, Tsukiyama-Kohara K, Wakita T, Tanaka T, Miyasaka M, Mizuno K, Hayashi Y, Hishima T, Matsushima K, Kohara M.** 2012. Immunization with a recombinant vaccinia virus that encodes nonstructural proteins of the hepatitis C virus suppresses viral protein levels in mouse liver. PLoS One **7:**e51656. http://dx.doi.org/10.1371/journal.pone.0051656.

13. **Kasama Y, Sekiguchi S, Saito M, Tanaka K, Satoh M, Kuwahara K, Sakaguchi N, Takeya M, Hiasa Y, Kohara M, Tsukiyama-Kohara K.** 2010. Persistent expression of the full genome of hepatitis C virus in B cells induces spontaneous development of B-cell lymphomas in vivo. Blood **116:**4926–4933. http://dx.doi.org/10.1182/blood-2010-05-283358.

14. **Machida K, Tsukiyama-Kohara K, Sekiguch S, Seike E, Tone S, Hayashi Y, Tobita Y, Kasama Y, Shimizu M, Takahashi H, Taya C, Yonekawa H, Tanaka N, Kohara M.** 2009. Hepatitis C virus and disrupted interferon signaling promote lymphoproliferation via type II CD95 and interleukins. Gastroenterology **137:**285–296. http://dx.doi.org/10.1053/j.gastro.2009.03.061.

15. **Van Rooijen N, Sanders A.** 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods **174:**83–93. http://dx.doi.org/10.1016/0022-1759(94)90012-4.

16. **Guidotti LG, Chisari FV.** 2006. Immunobiology and pathogenesis of viral hepatitis. Annu Rev Pathol **1:**23–61. http://dx.doi.org/10.1146/annurev.pathol.1.110304.100230.

17. **McGuinness PH, Painter D, Davies S, McCaughan GW.** 2000. Increases in intrahepatic CD68 positive cells, MAC387 positive cells, and proinflammatory cytokines (particularly interleukin 18) in chronic hepatitis C infection. Gut **46:**260–269. http://dx.doi.org/10.1136/gut.46.2.260.

18. **Burgio VL, Ballardini G, Artini M, Caratozzolo M, Bianchi FB, Levrero M.** 1998. Expression of co-stimulatory molecules by Kupffer cells in chronic hepatitis of hepatitis C virus etiology. Hepatology **27:**1600–1606. http://dx.doi.org/10.1002/hep.510270620.

19. **Shrivastava S, Mukherjee A, Ray R, Ray RB.** 2013. Hepatitis C virus induces IL-1beta/IL-18 in circulatory and resident liver macrophages. J Virol **87:**12284–12290. http://dx.doi.org/10.1128/JVI.01962-13.

20. **Bility MT, Cheng L, Zhang Z, Luan Y, Li F, Chi L, Zhang L, Tu Z, Gao Y, Fu Y, Niu J, Wang F, Su L.** 2014. Hepatitis B virus infection and immunopathogenesis in a humanized mouse model: induction of human-specific liver fibrosis and M2-like macrophages. PLoS Pathog **10:**e1004032. http://dx.doi.org/10.1371/journal.ppat.1004032.

21. **Odegaard JI, Ricardo-Gonzalez RR, Red Eagle A, Vats D, Morel CR, Goforth MH, Subramanian V, Mukundan L, Ferrante AW, Chawla A.** 2008. Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. Cell Metab **7:**496–507. http://dx.doi.org/10.1016/j.cmet.2008.04.003.