Background & Aims: Antigen-presenting cells (APCs) are involved in the induction of liver inflammation. We investigated the roles of specific APCs in the pathogenesis of acute liver injury in mice. Methods: We used concanavalin A (con A) or carbon tetrachloride to induce acute liver inflammation in mice and studied the roles of macrophages that express CCR9. Results: After injection of con A, we detected CCR9+CD11b+CD11c− macrophages that express tumor necrosis factor (TNF)-α in livers of mice, whereas CCR9+Siglec-H−CD11b−CD11clow plasmacytoid DCs (pDCs), which are abundant in normal livers, disappeared. The CCR9+ macrophages were also detected in the livers of RAG-2−/− mice, which lack lymphocytes and natural killer T cells, after injection of con A. Under inflammatory conditions, CCR9+ macrophages induced naïve CD4+ T cells to become interferon gamma–producing Th1 cells in vivo and in vitro. CCR9−/− mice injected with con A did not develop hepatitis unless they also received CCR9+ macrophages from mice that received con A; more CCR9+ macrophages accumulated in their inflamed livers than CCR9− pDCs, CCR9− pDCs, or CCR9− macrophages isolated from mice that had received injections of con A. Levels of CCL25 messenger RNA increased in livers after injection of con A; neutralizing antibodies against CCL25 reduced the induction of hepatitis by con A by blocking the migration of CCR9+ macrophages and their production of TNF-α. Peripheral blood samples from patients with acute hepatitis had greater numbers of TNF-α−producing CCR9+CD14+CD16high monocytes than controls. Conclusions: CCR9+ macrophages contribute to the induction of acute liver inflammation in mouse models of hepatitis.

Keywords: Immune Regulation; Hepatic Disease; Chemokine Receptor; T-Cell Activation.

Although the liver faces continuous exposure to many pathogens and commensal bacterial products, the innate and adaptive immune responses of the liver favor the induction of immunologic tolerance. Critically, however, there are many patients who experience acute hepatic failure, with a high lethal rate owing to liver inflammation. Although various immune compartments, such as T cells including CD4+CD25+Foxp3+ regulatory T cells, natural killer cells, natural killer T (NKT) cells, macrophages (Kupffer cells), conventional DCs (cDCs), and plasmacytoid DCs (pDCs), reside in the normal liver, it is unknown which types of cells positively induce inflammation.

Accumulating evidence shows that the chemokine/chemokine receptor axis instructs immune cells into the inflamed liver. Originally, CCR9 is a representative gut-homing receptor on lymphocytes that migrate into the small intestine, where CCL25 (ligand of CCR9) is abundant. Furthermore, up-regulation of CCL25 in the inflamed small intestine following accumulation of CCR9+CD4+ T cells has been reported both in Crohn’s disease and in a murine model, and clinical trials of anti-CCR9 antagonist against Crohn’s disease are ongoing. In liver immunity, however, Eksteen et al previously showed that CCL25 is up-regulated in the inflamed liver of primary sclerosing cholangitis in humans and that CCR9-expressing T cells are accumulated in the liver of such patients. Although initial studies focused on CCR9-expressing lymphocytes irrespective of the gut and liver, Hadeiba et al recently showed that immature pDCs in the spleen and lymph nodes also express CCR9 and that those CCR9-expressing pDCs have the ability to suppress immune responses. However, little is known of the function of CCR9-expressing pDCs in the liver and also of the presence and the function of liver CCR9-expressing macrophages and cDCs.

To examine the induction of liver inflammation, we compared 2 typical conditions—the steady condition versus the inflammatory condition—using an acute T cell–mediated hepatitis model, concanavalin A (con A)–induced liver inflammation in mice, focusing on antigen-presenting cells (APCs) and the chemokine/chemokine receptor axis. Intravenous injection of con A induces massive hepatocyte necrosis, with marked infl
stration of lymphocytes in the liver and elevation of serum transaminase levels. Initially, this model was believed to be CD4+ T-cell dependent; later, however, 2 groups clarified that the pathologic CD4+ T cells were CD1d-dependent NK T cells. Against this background, we here propose that liver CCR9+ macrophages act specifically as initial inflammatory cells in the development of con A–induced T-cell hepatitis.

Materials and Methods

Experimental Protocols of con A–Induced Hepatitis

Con A (type IV) was purchased from Sigma-Aldrich (St Louis, MO). Intravenous injections of con A (20 mg/kg) were administered into the tail vein of mice 12 hours before examination under anesthesia. In experiment 1, age-matched wild-type (WT), CCR9−/−, CCR7−/−, and MCP-1−/− mice were treated with con A. In experiment 2, CCR9+ and CCR9− macrophages as well as CCR9+ and CCR9− pDCs were isolated from the liver of con A–treated Ly5.1+ mice. These cells were then adoptively transferred to CCR9−/− Ly5.2+ mice (5 × 10^5 cells/mice), which were immediately injected with con A. As a control, WT Ly5.2+ mice were injected with con A. In experiment 3, WT mice were intraperitoneally injected with neutralizing anti-CCR25 monoclonal antibody (mAb) (500 μg/mouse, clone 89818; R&D Systems, Minneapolis, MN; n = 4) or isotype control (n = 4) 2 hours before administration of con A. All mice were killed 12 hours after administration of con A.

See Supplementary Materials and Methods for more details.

Results

Abundant CCR9+CD11b−CD11clow pDCs in the Normal Liver

To clarify the mechanism by which liver inflammation is induced, we focused on liver APCs. To segmentalize the APC compartments, we stained liver and spleen mononuclear cells obtained from WT mice with mAb against CD11b and CD11c. As shown in Figure 1A, 3 APC compartments were found in the liver and spleen: cDCs, CD11b+CD11c+; pDCs, CD11b−CD11clow; and macrophages, CD11b+CD11clow. As previously reported, pDCs are more abundant in the liver than in the spleen, although the ratios of cDCs to macrophages were comparable in these organs (Figure 1B). To further characterize these compartments, we stained the cells with a third mAb (Supplementary Figure 1). Consistent with previous reports, B220, PDCA-1, CCR9, and CD8α were preferentially expressed on pDCs but not on cDCs or macrophages in liver and spleen cells. However, not all CD11b−CD11clow cells appeared to be identical to pDCs because some of the latter cells did not express the markers. Interestingly, the liver pDC population contained a higher proportion of CCR9+ cells than spleen pDCs, which suggests that liver pDCs are more immature and tolerogenic than spleen pDCs. In contrast, F4/80 was expressed not only on spleen and liver macrophages, but also on liver cDCs and some spleen cDCs. This implies that macrophages in the liver and spleen and cDCs in the liver can be classified exclusively as monocyte phagocyte system cells generated from monocytes and also that spleen cDCs are a mixture of macrophage/dendritic precursor- and monocyte-derived DCs. In this regard, macrophages and cDCs in the liver and spleen expressed Ly6C at a high level and a low to negative level, respectively. CD80, CD86, and major histocompatibility complex class II on liver and spleen cells were expressed at a high level on cDCs but at moderate to low levels on macrophages and pDCs (Supplementary Figure 1). Collectively, these data suggest that pDCs and cDCs/macrophages are distinct populations in the liver, whereas cDCs and macrophages constitute similar but different populations in terms of activation status.

Accumulation of CCR9-Expressing Macrophages Through con A–Induced Liver Inflammation

To induce liver inflammation, we intravenously administered con A to WT mice. Twelve hours after administration, the APC compartments in the liver showed marked changes; the proportion of pDCs was significantly reduced, whereas that of cDCs/macrophages, particularly macrophages, was significantly increased (Figure 2A and B). In contrast, the APC composition of spleen cells was unchanged by administration of con A (Figure 2A and B). Consistent with these findings, the absolute cell numbers of cDCs and macrophages in the liver were signifi-

Figure 1. Abundant CCR9+CD11b−CD11clow pDCs in the normal liver. (A) Representative CD11b and CD11c staining on whole cells isolated from the liver and spleen of WT mice. Each box indicates cDCs, CD11b+CD11c+, pDCs, CD11b−CD11clow, and macrophages, CD11b+CD11c−. (B) Mean percentage of pDCs, cDCs, and macrophages. Data show mean ± SEM (n = 5/group). *P < .01.
significantly higher in con A–treated mice, whereas those of pDCs were comparable in phosphate-buffered saline (PBS)- and con A–treated mice (Figure 2Bii). Surprisingly, we found a dramatic change in CCR9 expression in the liver, but not in the spleen, after administration of con A: down-regulation on pDCs and a corresponding up-regulation on macrophages/cDCs (Figure 2Ai and C). This change was confirmed by time course observation of CCR9 expression after administration of con A (Figure 2D). Furthermore, the expression level of CCL25 messenger RNA (mRNA) in the liver of con A–treated mice was significantly up-regulated 12 hours after injection of con A (Figure 2E).

Interestingly, not only did macrophages/cDCs in the liver of con A–treated mice show markedly up-regulated CD80 and CD86 expression compared with those in the liver of PBS-treated mice, but also the mean fluorescent intensity of CD80 and CD86 expression of CCR9+ macrophages/cDCs was significantly higher than in CCR9− macrophages/cDCs (Supplementary Figure 2A and B). In contrast, although both CCR9+ and CCR9− pDCs in the liver of PBS-injected mice were in the CD80− and CD86− population, only CCR9− pDCs in the liver of con A–treated mice showed up-regulated CD80 and CD86 expression (Supplementary Figure 2A). Notably, CCR9+ macrophages/cDCs, but not pDCs, expressed a significantly higher level of tumor necrosis factor (TNF)-α after injection of con A (Supplementary Figure 2C and D). The phenotypic difference between CCR9+ pDCs in the steady state and CCR9+ macrophages under inflammation was also confirmed in that only the CCR9+ pDC population, not CCR9+ macrophages, coexpressed the specific pDC marker Siglec-H22.23 (Supplementary Figure 2E). Furthermore, in vitro stimulation with con A slightly up-regulated CCR9 expression on CD11b+ macrophages of the spleen and liver at 12 and 24 hours after culture. Marked up-regulation was
observed only in liver cells at 72 hours after culture (Supplementary Figure 2F), although this up-regulated CCR9 expression was less than that induced after in vivo con A administration in mice (Supplementary Figure 2F).

We next sought to establish whether the increased CCR9+ macrophages were also observed in the liver of RAG-2−/− mice following administration of con A; we wished to determine whether macrophages or T/NKT cells are the primary cells in response to con A in this model. As shown in Supplementary Figure 3A, the accumulation of CD11b+CD11c− macrophages and the up-regulation of CCR9 in the macrophages subset were similarly observed in the liver of con A–treated WT and RAG-2−/− mice. By contrast, an activated CD69hiCD3+ population, including conventional T and NKT cells, was observed in con A–treated WT mice, but it was completely absent in con A–treated RAG-2−/− mice. The severity of liver inflammation was dramatically reduced in con A–treated RAG-2−/− mice from an assessment of the liver histology and serum levels of transaminase (Supplementary Figure 3B and C). These results suggest that NKT cells might be terminal effector cells, rather than initiator cells, following the activation of macrophages in a con A hepatitis model.

**CCR9+ Macrophages in the Livers From con A–Treated Mice Promote the Development of Th1 Cell**

To investigate the function of CCR9+ macrophages in the livers from con A–treated mice (CCR9+ macrophages) in addition to CCR9+ pDCs in the livers from PBS-treated mice (CCR9+ pDCs), both subsets were isolated using FACSAria (BD Biosciences, Franklin Lakes, NJ) (Figure 3A) and their morphologic characters were assessed using Giemsa staining. CCR9+ pDCs resembled plasma cells with an eccentric kidney-shaped nucleus, whereas CCR9+ macrophages displayed a horseshoe-shaped nucleus (Figure 3B). Next, CCR9+ pDCs or CCR9+ macrophages were cocultured with carboxyfluorescein succinimidyl ester–labeled naive CD4+ T cells obtained from the spleen of DO11.10 × RAG-2−/− mice in the presence of ovalbumin peptides. After 72 hours of culture, CD4+ T cells had extensively divided in the presence of CCR9+ macrophages but showed little division in the presence of CCR9+ pDCs (Figure 3C); this finding is consistent with previous reports showing the poor stimulatory function of pDCs.24,25 We further examined the expression of interferon (IFN)-γ, interleukin (IL)-17A, and Foxp3 in cultured CD4+ T cells. As depicted in Figure 3D, CD4+ T cells cocultured with CCR9+ macrophages expressed IFN-γ but not Foxp3 and IL-17A; CD4+ T cells cocultured with CCR9+ pDCs expressed Foxp3 but not IL-17A and IFN-γ.

We next performed a reverse-transcription quantitative polymerase chain reaction assay of the gene expression of CCR9+ macrophages and CCR9+ pDCs. Expression of TNF-α mRNA in CCR9+ macrophages was significantly increased; conversely, that of IL-10 and transforming growth factor β mRNAs in CCR9+ macrophages was significantly decreased (Supplementary Figure 4). Consis-
tent with the findings of a previous report, the expression patterns of Toll-like receptors (TLR9) in CCR9+ macrophages and CCR9+ pDCs were distinct; CCR9+ pDCs preferentially expressed TLR7 and TLR9 mRNAs, whereas CCR9+ macrophages preferentially expressed TLR4 and TLR6 mRNAs (Supplementary Figure 4). This suggests that these 2 populations are separately generated from different precursor cells. Furthermore, the expression of CCR2 mRNA was significantly higher in CCR9+ macrophages; conversely, the expression of CX3CR1 mRNA was significantly lower in CCR9+ macrophages than in CCR9+ pDCs. The expression of CCR3, CCR6, and CCR7 mRNAs was comparable in CCR9+ macrophages and CCR9+ pDCs (Supplementary Figure 4).

**CCR9−/− Mice Were Resistant to con A–Induced Hepatitis**

To investigate the role of CCR9 during liver inflammation, we used CCR9−/− mice in a con A–induced hepatitis model. First, the proportion of pDCs in the liver of untreated CCR9−/− mice was slightly lower than that of untreated WT mice, but the difference was not significant (data not shown). No inflammatory lesions were found in the liver of CCR9−/− mice until the age of 40 weeks (data not shown). This suggests that CCR9+ pDCs are not the only cells responsible for liver tolerance and inflammation in the steady state and that other CCR9-expressing cells, such as activated CCR9+ macrophages, are needed to induce inflammation. To test this, we administered age-matched WT, CCR9−/−, CCR7−/−, and MCP-1−/− (MCP-1 [CCL2]; CCR2 ligand) mice with con A. As expected, CCR9−/− mice did not develop liver damage after treatment with con A (Figure 4B) and showed less elevation of transaminase levels than the treated WT mice (Figure 4C); con A–treated CCR7−/− and MCP-1−/− mice developed hepatitis to a similar extent to con A–treated WT mice (Figure 4B and C). This suggests that the CCR9/CCL25 axis plays an important role in the pathogenesis of this model. The proportion of macrophages in the liver of CCR9−/− mice after con A treatment was comparable with that of WT mice treated with PBS, whereas con A–treated WT, CCR7−/−, and MCP-1−/− mice showed marked increases of CCR9+ macrophages (Figure 4A and Di). This was confirmed by the absolute cell numbers of liver CD11b+ macrophages after treatment with con A (Figure 4Dii). The diseased WT, CCR7−/−, and MCP-1−/− mice showed up-regulation of CCR9 on macrophages and down-regulation on pDCs (Figure 4E). Furthermore, we confirmed that the serum levels of TNF-α, IFN-γ, and IL-6 in con A–treated CCR9−/− mice at 12 hours after administration of con A were significantly reduced compared with those in con A–treated WT mice (Figure 4F). Importantly, the activation of NKT and T-cell subsets observed in con A–treated WT mice was diminished in con A–treated CCR9−/− mice, which suggests that the reciprocal interaction between CCR9-expressing macrophages and NKT/T cells plays a key role in the development of con A–induced acute hepatic inflammation (Supplementary Figure 5). We also confirmed that CCR9−/− mice were resistant to carbon tetrachloride–induced liver inflammation, another model of acute liver injury in mice, accompanied by the up-regulation of CCR9 expression on CD11b+ macrophages in the liver of carbon tetrachloride–treated WT mice (Supplementary Figure 6).

**CCR9+ Macrophages Were Key Inflammatory Cells for con A–Induced T-Cell Hepatitis**

Given the evidence that inducible CCR9 expression on macrophages in the liver is crucially involved in the induction of liver inflammation, we further confirmed the direct involvement of CCR9+ macrophages without the possible impact of CCR9+ lymphocytes in the T cell–mediated con A model. To this end, CCR9+ and CCR9+ macrophages and CCR9− and CCR9− pDCs were isolated from the liver of identically con A–treated Ly5.1+ WT mice, and those cells were then adoptively transferred into Ly5.2+ CCR9−/− mice, which were immediately injected with con A (Figure 5A). As a control, Ly5.2+ WT mice were injected with con A (Figure 5A). Strikingly, con A–treated CCR9−/− mice transferred with CCR9+ macrophages developed more severe hepatitis than mice transferred with CCR9+ pDCs, CCR9− pDCs, or CCR9− macrophages from an assessment of the liver histology and serum levels of transaminase (Figure 5B and C); however, con A–treated CCR9−/− mice transferred with CCR9+ macrophages showed less liver inflammation than con A–treated WT mice (Figure 5B and C). Consistent with this, the ratios of CCR9+CD11b+ macrophages were markedly increased in the liver of con A–treated WT mice and CCR9−/− mice transferred with CCR9+ macrophages, although this was not observed in CCR9−/− mice transferred with CCR9+ or CCR9− pDCs or CCR9− macrophages (Figure 5D and E). This result suggests a specific role of migrating CCR9+ macrophages as key inflammatory cells in the development of con A–induced hepatitis. Additionally, we confirmed that the increased CCR9+CD11b+ macrophages in con A–treated CCR9−/− mice transferred with CCR9+ macrophages were Ly5.1+ transferred cells, whereas those in con A–treated WT mice were Ly5.2+ endogenous cells (Figure 5E, right).

We next investigated whether the transfer of CCR9+ macrophages obtained from con A–treated Ly5.1+ WT mice into CCR9−/− mice without con A administration could induce liver inflammation (Supplementary Figure 7A). As controls, we used CCR9−/− mice without the transfer or con A administration as well as CCR9−/− mice with the transfer of CCR9+ macrophages and con A administration (Supplementary Figure 7A). However, in sharp contrast to CCR9−/− mice transferred with CCR9+ macrophages and con A administration, Ly5.1+CD11b+ macrophages were not accumulated in the liver of CCR9−/− mice transferred with CCR9+ macrophages but without con A administration and in CCR9−/− mice without the transfer or con A administration (Supplementary Figure 7B). Consistent with this, CCR9−/− mice transferred with CCR9+ macrophages and without con A administration and CCR9−/− mice without the transfer or con A
Figure 4. CCR9−/− mice are resistant to con A–induced hepatitis. (A) CD11b and CD11c staining on whole cells in the liver from PBS- or con A-injected WT, CCR9−/−, CCR7−/−, and MCP-1−/− mice. Data are representative of 5 independent experiments. (B) Photomicrographs of H&E-stained sections of liver. (C) Serum ALT level. Data show mean ± SEM (n = 6/group). Blue bars, PBS; red bars, con A. **P < .01. (Di) Mean percentage of macrophage subset of the liver and (Di) absolute cell number of macrophages of the liver. Data show mean ± SEM (n = 6/group). Blue bars, PBS; red bars, con A. ***P < .001. (E) CCR9 staining on pDC and macrophage subsets of the liver. Data are representative of 5 independent experiments. (F) Serum levels of TNF-α, IFN-γ, and IL-6 from PBS- or con A–treated mice detected by CBA assay. Data show mean ± SEM (n = 4/group). Red bars, con A. *P < .05; **P < .01.
administration did not develop hepatitis (Supplementary Figure 7C and D).

In addition, we showed that the ratio of IFN-γ-expressing Th1 cells, but not IL-17A-expressing Th17 cells, was markedly increased in the liver of con A–treated CCR9−/− mice with the transfer of CCR9+ macrophages, but not in the liver of con A–treated CCR9−/− mice without the transfer (Supplementary Figure 8).

**Neutralizing Anti-CCL25 mAb Attenuated con A–Induced Liver Injury**

To directly evaluate the possible contribution of CCR9/CCL25 blockade to the clinical efficacy of the treatment of acute hepatitis (AH), we examined whether neutralizing anti-CCL25 mAb was effective in preventing con A–induced hepatitis. As expected, administration of anti-CCL25 mAb significantly ameliorated con A–induced liver injury; anti-CCL25 mAb-treated mice showed less liver damage (Figure 6A) and lower serum levels of transaminase (Figure 6B) than isotype mAb-treated con A–treated mice. The number of CCR9+ macrophages in the anti-CCL25 mAb-treated con A–treated group was significantly lower than in the isotype mAb-treated con A–treated group, whereas the number of CCR9+ pDCs was not statistically different between the isotype mAb and anti-CCL25 mAb-treated con A–treated groups (Figure 6C and D). Interestingly, the expression of TNF-α in CCR9+ macrophages in the anti-CCL25 mAb-treated con A–treated group in response to in vitro lipopolysaccharide stimulation was significantly lower than in the
Figure 6. Neutralizing anti-CCL25 mAb treatment ameliorates con A-induced liver inflammation. (A) Photomicrographs of H&E-stained sections of the liver from PBS-injected, con A/isotype mAb-injected, and con A/anti-CCL25 mAb-injected mice. Data are representative of each group (n = 4). (B) Serum ALT level. Data show mean ± SEM (n = 4/group). *P < .05. (C) CD11b and CD11c staining on liver mononuclear cells (left panel) and CCR9 staining for macrophage subsets (right panel) in the liver from 3 groups. Data are representative of 4 independent experiments (n = 4/group). (D) Absolute cell numbers of CCR9+CD11b+ cells (left panel) and CCR9+CD11c+ cells (right panel) in the liver from 3 groups. Data show mean ± SEM (n = 4/group). *P < .05. (E) Intra- and surface CCR9 expression and (F) mean percentage of TNF-α+ cells in macrophages of the liver from 3 groups. Cells were stimulated with lipopolysaccharide for 6 hours, followed by surface and intracellular staining. Data show mean ± SEM (n = 4/group). *P < .05, **P < .01. (F) Production of TNF-α, IL-6, and IL-10, in response to CCL25. CD11b+CD11c+ macrophages or CD11b−CD11clow pDCs obtained from the liver of PBS- or con A-treated mice were cultured with CCL25 (0, 100, 500 ng/mL). (Fii) Migration assay. Fold increase of migration by CCL25 compared with the baseline (without CCL25 incubation) was calculated. Data show mean ± SEM (n = 4/group). *P < .05, **P < .01.
isotype mAb-treated con A-treated group (Figure 6E); this suggests that up-regulated CCL25 under inflammation contributes to both the migration and activation of CCR9+ macrophages.

Notably, macrophages obtained from the liver of con A–treated mice—but not macrophages and pDCs from the liver of PBS-injected mice and pDCs from the liver of con A–treated mice—produced a significantly higher amount of TNF-α and IL-6 in response to CCL25 in a dose-dependent manner (Figure 6F). By contrast, pDCs from the liver of PBS-injected mice, but not macrophages and pDCs from the liver of con A–treated mice, produced significantly higher amounts of IL-10 in response to CCL25 in a dose-dependent manner (Figure 6F). We further performed an in vitro migration and cell activation experiment using macrophages and pDCs obtained from the liver of PBS- and con A–treated mice. As expected, the migratory ability of pDCs from the liver of PBS-injected mice and macrophages from the liver of con A–treated mice, both of which express CCR9, was significantly increased in the presence of CCL25 (Figure 6F).

**Discussion**

We here identify CCR9+ macrophages as key inflammatory cells of con A–induced AH in mice. Figure 7F gives a schematic view of the current study. Our results clearly show that CCR9+ macrophages played a key role in promoting proliferation of CD4+ T cells and generation of IFN-γ–producing Th1 cells as well as NKT cell activation in con A–induced T-cell hepatitis.

To investigate the mechanism of the breakdown of liver tolerance in the inflammatory state, we initially focused on the breakdown of liver tolerance in the inflammatory state, we finally attempted to apply the present results in mice to human AH, such as viral hepatitis and autoimmune hepatitis. To this end, we assessed the phenotypes of the peripheral blood (PB) in patients with AH, patients with chronic hepatitis (CH), and normal controls (NCs). The precise characteristics of each group are summarized in Supplementary Table 1. In particular, we focused on CD14+CD16+ cells in PB samples, because it has been reported that the subpopulation of CD14+CD16+ monocytes produces a large amount of TNF-α and other proinflammatory cytokines to participate in inflammation.28 First, the proportion of CD14+CD16high monocytes, but not CD14+CD16low or CD14+CD16high monocytes, was significantly increased in the PB of patients with AH as compared with that of NCs and patients with CH (Figure 7A, left, and B). Furthermore, the positive percentage of CCR9+ cells in CD14+CD16high monocytes was significantly higher in the PB of patients with AH than in that of NCs or patients with CH (Figure 7A, right, C, and D). Importantly, TNF-α was preferentially expressed on CD16highCCR9+ but not on CD16highCCR9− subpopulations in the PB of patients with AH (Figure 7E).

Finally, we attempted to apply the present results in mice to human AH, such as viral hepatitis and autoimmune hepatitis. To this end, we assessed the phenotypes of the peripheral blood (PB) in patients with AH, patients with chronic hepatitis (CH), and normal controls (NCs). The precise characteristics of each group are summarized in Supplementary Table 1. In particular, we focused on CD14+CD16+ cells in PB samples, because it has been reported that the subpopulation of CD14+CD16+ monocytes produces a large amount of TNF-α and other proinflammatory cytokines to participate in inflammation.28 First, the proportion of CD14+CD16high monocytes, but not CD14+CD16low or CD14+CD16high monocytes, was significantly increased in the PB of patients with AH as compared with that of NCs and patients with CH (Figure 7A, left, and B). Furthermore, the positive percentage of CCR9+ cells in CD14+CD16high monocytes was significantly higher in the PB of patients with AH than in that of NCs or patients with CH (Figure 7A, right, C, and D). Importantly, TNF-α was preferentially expressed on CD16highCCR9+ but not on CD16highCCR9− subpopulations in the PB of patients with AH (Figure 7E).
on resident CCR9-expressing pDCs in the liver because emerging evidence has shown that immature pDCs function as anti-inflammatory cells.\(^\text{13}\) We found a higher proportion of CCR9\(^+\) pDCs in the liver, which preferentially express Siglec-H, B220, PDCA-1, and CD8\(\alpha\), but which show lower expression of major histocompatibility complex class II and CD80 and CD86 than macrophages/cDCs. Moreover, liver CCR9\(^+\) pDCs showed a tolerogenic function; they suppressed the proliferation of naive CD4 T cells and instructed these cells to become Foxp3\(^+\) regulatory T cells. It is likely that pDCs induce regulatory T cells in the inflamed liver rather than in the lymph nodes, because pDCs are generally recognized to be resident cells and do not migrate to the lymph nodes.\(^\text{29}\)

These findings suggest that liver pDCs in the steady state are key players in maintaining liver tolerance. However, after administration of con A, the change in the actual number of pDCs in the liver and the phenotypic characteristics of CCR9\(^+\) pDCs was low. Furthermore, con A-treated CCR9\(^{-/-}\) mice did not develop hepatitis. To solve the discrepancy, we reevaluated CCR9 expression on other APCs in the con A-treated liver and noticed a dramatic change in CCR9 expression between normal and inflammatory livers; in con A-treated mice, pDCs down-regulate CCR9 expression, whereas macrophages/cDCs increased and up-regulate CCR9 expression. This was surprising in light of previous reports indicating that CCR9 molecules are expressed on lymphocytes and pDCs but not macrophages/cDCs.\(^\text{8,12,13}\); however, one paper recently reported CCR9-expressing macrophages in patients with rheumatoid arthritis.\(^\text{30}\) Therefore, we reconsidered that the up-regulation of CCR9 molecules in liver macrophages during the development of con A-induced hepatitis is closely associated with the gain of inflammatory responses.

Because CCR9\(^{-/-}\) mice do not show spontaneous liver inflammation, it is possible that the induction of liver inflammation requires the action of specific inflammatory CCR9\(^+\) macrophages rather than down-regulation of CCR9 expression in liver pDCs. In this regard, the finding that CCR9\(^{-/-}\) mice injected with con A and transferred with CCR9\(^+\) macrophages from con A-treated WT mice developed hepatitis, although this was not seen with CCR9\(^-\) pDCs, CCR9\(^-\) pDCs, or CCR9\(^-\) macrophages from con A-treated WT mice, clearly indicates that the liver CCR9\(^+\) macrophages are the key inflammatory cells responsible for the induction of con A-induced T-cell hepatitis. It is quite important to notice that CCR9\(^+\) macrophages play a role not only in this con A model but also in the acute carbon tetrachloride-induced hepatitis model, indicating a broad pathologic involvement of the CCR9/CCL25 axis in acute liver inflammation.

It was still unclear which cell subsets were mainly responsible for conducting acute liver injury in this model. To clarify this issue, we first showed that the accumulation of CCR9\(^+\) macrophages in the liver is not sufficient to establish hepatic injury in RAG-2\(^{-/-}\) mice, in which T cells are genetically absent. Importantly, the absence of CCR9\(^+\) macrophages resulted in milder hepatitis with less activation of CD4 T cells and NKT cells in CCR9\(^{-/-}\) mice. In addition, the adoptive transfer of CCR9\(^+\) macrophages resulted in recovery from hepatic injury at least partially by activation of CD4 T cells. These results suggest that the initial induction of CCR9\(^+\) macrophages in the liver and subsequent interaction between CCR9\(^+\) macrophages and T/NKT cells are essential in this model.

Collectively, our data show for the first time that the emergence of CCR9-expressing macrophages in the liver is centrally involved in inducing liver inflammation, resulting in the development of inflammatory liver diseases. In terms of the clinical relevance, not only the efficacy of anti-CCL25 mAb for the prevention of con A-induced hepatitis, but also the presence of human counterpart cells of murine CCR9\(^+\) macrophages, CCR9\(^+\)CD14\(^+\)CD16\(^+\) cells, in human PB of AH clearly indicate that blockade of the CCR9/CCL25 pathway would provide a feasible strategy for treating human AH. Therefore, our proposal for treating acute liver inflammation, such as fulminant liver injury by hepatitis viruses or autoimmune hepatitis, is to target pathologic CCR9\(^+\) macrophages/monocytes. The present results point to what we believe to be a novel treatment for these diseases in the near future; however, further assessments are warranted in terms of the specific sites in the liver (center for liver immunity) that produce CCL25 and the modulating factor to induce CCR9 expression in macrophages.

### Supplementary Materials

Note: To access the supplementary material accompanying this article visit the online version of Gastroenterology at www.gastrojournal.org and at doi:10.1053/j.gastro.2011.10.039.

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Received April 8, 2011. Accepted October 26, 2011.

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The authors thank K. Tsuchimoto (Kitasato Institute Hospital), R. Umeda, and S. Usui (Keio University) for providing human materials and for critical comments, as well as K. Toda (Kitasato Institute Hospital) for technical assistance.

Conflicts of interest
The authors disclose no conflicts.

Funding
Supported part by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labour and Welfare; the Japan Medical Association; Foundation for Advancement of International Science; and Keio University Medical Fund.

Writing assistance was provided by Dr Peter Hawkes (Kansai Language College) and Edanz Group Ltd and funded by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research.