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C-C Chemokine Ligand 2/Monocyte Chemoattractant Protein-1 Directly Inhibits NKT Cell IL-4 Production and Is Hepatoprotective in T Cell-Mediated Hepatitis in the Mouse¹

Maureen N. Ajuebor,* Cory M. Hogaboam,[†] Tai Le,* and Mark G. Swain^{2*}

T cell-mediated liver diseases are associated with elevated serum levels of C-C chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1). However, the extent to which the actions of CCL2/MCP-1 contribute to the pathogenesis of T cell-mediated hepatitis remains incompletely understood. Con A-induced hepatitis is a liver-specific inflammation mediated by activated T cells and is driven by an up-regulation of the hepatic expression of TNF- α , IFN- γ , and IL-4. The present study examined the role of CCL2/MCP-1 in the pathogenesis of T cell-mediated hepatitis induced by Con A administration in the mouse. We demonstrate a novel hepatoprotective role for CCL2/MCP-1 during Con A-induced hepatitis, because CCL2/MCP-1 neutralization strikingly enhanced hepatic injury, both biochemically and histologically, after Con A administration. Furthermore, CCL2/MCP-1 neutralization was associated with a significant reduction in the hepatic levels of TNF- α and IFN- γ , but with a significant increase in hepatic IL-4 levels. Moreover, IL-4 production and CCR2 expression by Con A-stimulated CD3⁺NK1.1⁺ T cells was significantly reduced by rMCP-1 treatment in vitro. In summary, we propose that CCL2/MCP-1 fulfills a novel anti-inflammatory role in T cell-mediated hepatitis by inhibiting CD3⁺NK1.1⁺ T cell-derived IL-4 production through direct stimulation of its specific receptor CCR2. These findings may have direct clinical relevance to T cell-mediated hepatitis. *The Journal of Immunology*, 2003, 170: 5252–5259.

The increasing knowledge of the basic mechanisms governing T cell recruitment and activation during infectious (hepatitis B and C) and immune-mediated (autoimmune) hepatitis has allowed the development of targeted therapies for the treatment of these diseases (1–5). However, despite advances in their management and treatment, T cell-mediated liver diseases remain a very serious concern in the clinical setting and are associated with significant morbidity and mortality worldwide (1–5). Consequently, in recent years, much effort has been focused on understanding better the mechanism(s) underlying the role played by liver-infiltrating and resident activated T cells during T cell-mediated hepatitis.

Con A-induced hepatitis in the mouse is a well-characterized model of T cell-mediated liver disease. Con A-induced hepatitis is mediated mainly by activated CD4⁺ T cells recruited to the liver, but also involves activation of resident hepatic NKT cells (6–17). This model has been extensively used as an excellent model mimicking many aspects of human T cell-mediated liver disease (6–17). A single injection of Con A is sufficient for highly specific

single organ T cell-mediated injury in the liver of mice (6–17). Clinical and histological evidence of hepatitis is observed within 8–24 h following Con A injection, as shown by elevated plasma transaminase activities and the occurrence of histological evidence of hepatic lesions characterized by a massive CD4⁺ T cell accumulation and an influx of a relatively small number of CD8⁺ T cells (6, 7). Many studies have shown that immunoregulatory cytokines have a central role in the pathogenesis of Con A-induced hepatitis with recent studies pointing to a proinflammatory role for the cytokines TNF- α , IL-12, IFN- γ , and IL-4, and a protective role for IL-10 (7–10, 16). Similar findings have been documented in patients with T cell-mediated hepatitis where progressive liver injury is associated with an elevation of hepatic levels of TNF- α , IFN- γ , and IL-4, and a down-regulation of IL-10 (18–21).

Chemokines constitute a family of small (~8- to 15-kDa) structurally related proteins, which have emerged as one of the most important regulators of leukocyte trafficking and activation (22, 23). C-C chemokine ligand 2 (CCL2)³/monocyte chemoattractant protein-1 (MCP-1) is a C-C chemokine that was originally described as a monocyte chemoattractant (24, 25); however, it has also been shown to be a potent chemoattractant for T lymphocytes (26). In addition to its pivotal role in controlling the recruitment of inflammatory cells, increases in CCL2/MCP-1 expression have been linked to the progression of experimental fungal pneumonia and autoimmune diseases including rheumatoid arthritis and multiple sclerosis (27–29). Earlier studies examining the potential role of CCL2/MCP-1 in hepatitis C, a T cell-mediated liver disease, reported increased serum levels of CCL2/MCP-1 in patients with hepatitis C infection when compared with levels observed in healthy individuals (30). However, the extent to which the actions

*Liver Unit, Gastrointestinal Research Group, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada; [†]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109

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² Address correspondence and reprint requests to Dr. Mark G. Swain, Department of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. E-mail address: swain@ucalgary.ca

³ Abbreviations used in this paper: CCL2, C-C chemokine ligand 2; MCP-1, monocyte chemoattractant protein-1; ALT, alanine transaminase; NRS, normal rabbit serum; FasL, Fas ligand.

of CCL2/MCP-1 contribute to the pathogenesis of T cell-mediated hepatitis remains unclear.

Therefore, the purpose of the present study was to assess the contribution of CCL2/MCP-1 to the pathogenesis of T cell-mediated hepatitis induced by Con A administration in mice.

Materials and Methods

Mice

Specific pathogen-free male C57BL/6 mice (5–6 wk old) were purchased from Charles River Breeding Farms (St. Constant, Quebec). All procedures in this study were approved by the Animal Care Committee of the University of Calgary and conformed to the guidelines established by the Canadian Council on Animal Care.

Con A-induced hepatitis

Mice were injected i.v. with freshly prepared Con A (0.25 mg/mouse in 0.1 ml of PBS; Sigma-Aldrich, St. Louis, MO) (10). At 30 min, 90 min, 8 h, and 24 h after Con A administration and under halothane anesthesia, blood was collected for the measurement of plasma alanine transaminase (ALT) levels (commercial kit; Sigma-Aldrich). Livers were perfused with cold sterile PBS to remove blood elements, and then used for determination of hepatic CCL2/MCP-1 and CCR2 expression, and for histological evaluation of liver injury. PBS-treated mice served as controls.

Histopathology

Liver tissues of control and Con A-treated mice were fixed overnight in 10% neutral buffered formalin, dehydrated in graded concentrations of ethanol, and then embedded in paraffin and sectioned. Liver sections (5 μ m thick) were stained with H&E according to standard protocols and analyzed by light microscopy by an observer unaware of the treatments.

RNase protection assay

Total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada). The yield and purity of the RNA was determined spectrophotometrically at 260 and 280 nm. RNase Protection Assay was performed on isolated RNA (10 μ g) using the RiboQuant system (BD PharMingen, San Diego, CA). The mouse template sets mCK-5 and mCR-5 (BD PharMingen) were used for the detection of CCL2/MCP-1 mRNA and CCR2 mRNA expression, respectively. The relative quantity of CCL2/MCP-1 and CCR2 mRNA was estimated by densitometric analysis using Quantity One software (Bio-Rad Laboratories, Hercules, CA) and then normalized to respective GAPDH levels. Results are presented as the mean of normalized ratio \pm SD.

CCL2/MCP-1 neutralization

Mice were injected i.p. with anti-MCP-1 antiserum (0.5 ml/mouse) 16 h before Con A administration as previously described (31). Control mice received heat-inactivated normal rabbit serum (NRS; Sigma-Aldrich). Mice were sacrificed 90 min or 8 h after Con A administration for assessment of hepatic injury, hepatic CCR2 expression, and cytokine levels within the liver. In a separate set of control experiments, mice were injected i.p. with anti-MCP-1 antiserum (0.5 ml/mouse) or NRS, but no Con A. All mice were sacrificed 16 h after Ab treatment for assessment of hepatic injury and cytokine level.

Chemokine and cytokine ELISAs

CCL2/MCP-1 and cytokine (IL-4, IFN- γ , TNF- α , IL-12, and IL-10) levels were determined in liver tissue homogenates after Con A administration by specific ELISAs (31, 32). For this, individual perfused livers were homogenized in cold PBS buffer containing protease mixture inhibitor (Sigma-Aldrich) immediately after their removal as previously described (33). Tissue homogenates were centrifuged twice, and the supernatants were filtered through a 0.45- μ m filter, and stored at -80°C until used for determination of CCL2/MCP-1 and cytokine levels by ELISA, and total protein concentration using a Bio-Rad protein colorimetric assay (Bio-Rad Laboratories).

Isolation of liver-infiltrating lymphocytes

The isolation of liver-infiltrating lymphocytes was conducted as previously described (33). Briefly, mice were pretreated with anti-MCP-1 antiserum (0.5 ml/mouse) or NRS as described in *CCL2/MCP-1 neutralization*. Perfused livers were obtained from mice 8 h after Con A injection and minced in digestive medium containing 0.05% collagenase and 0.002% DNase I. Following gentle agitation at 37°C for 30 min, the digest was passed

through a nylon mesh and then washed twice with PBS. Cells were then subjected to density gradient centrifugation on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) to isolate the lymphocyte population. Hepatic lymphocytes were resuspended in PBS, and viability was determined by trypan blue exclusion.

Flow cytometry analysis

Flow cytometric immunofluorescence analysis of liver-infiltrating lymphocytes was performed as previously described (33–35). Isolated hepatic lymphocytes (1×10^6) were fixed and permeabilized (33–35). Following permeabilization, cells were incubated with a control goat IgG Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or a polyclonal Ab against the cytoplasmic tail of murine CCR2b (Santa Cruz Biotechnology) (34, 35) for 30 min at 4°C . Cells were then washed and stained in the dark with FITC-labeled anti-goat IgG (Santa Cruz Biotechnology). For FACS analysis, the lymphocyte population was gated using forward- and side-scatter characteristics and analyzed using CellQuest software (BD Biosciences, Mountain View, CA). For hepatic CD4 $^{+}$ T cell infiltrates analysis, isolated hepatic lymphocytes (1×10^6) were stained with a PE-conjugated anti-mouse CD4 mAb (Serotec, Oxford, U.K.), according to the manufacturer's instructions, and analyzed by FACS.

Enrichment of NK1.1 $^{+}$ cells by MACS and flow cytometry analysis

Spleen cells from naive male C57BL/6 mice were stained with FITC-conjugated anti-NK1.1 mAb (clone PK136; BD PharMingen) for 25 min at 4°C . Cells were then washed and incubated with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA) (14, 15) for 20 min at 4°C . NK1.1 $^{+}$ cells were enriched by double-positive MACS according to the manufacturer's protocol (Miltenyi Biotec). About 90% of the MACS-purified cells were NK1.1 positive, and $>40\%$ were CD3 $^{+}$ NK1.1 $^{+}$ T cells. Furthermore, the NK1.1 $^{+}$ MACS-depleted cell population contained $<5\%$ NK1.1 $^{+}$ cells. NK1.1 $^{+}$ MACS-enriched spleen cells (1×10^6) were stimulated with Con A (20 μ g/ml) with or without recombinant mouse MCP-1 (100 ng/ml; R&D Systems, Minneapolis, MN) for 16 h on 24-well plates. Following Con A stimulation, cell viability was $\sim 85\%$ for all groups as determined by trypan blue. Three-color FACS staining was then used to determine the percentage of CD3 $^{+}$ NK1.1 $^{+}$ MACS-enriched cells producing IL-4. For this, Con A-stimulated FITC-labeled NK1.1 $^{+}$ MACS-enriched cells were first stained with PerCP-conjugated CD3 ϵ mAb (clone 145-2C11; BD PharMingen), and then fixed and subsequently permeabilized with Cytofix/Cytoperm Plus (BD PharMingen). Following permeabilization, cells were stained with PE-conjugated IL-4 mAb (BVD4-1011; BD PharMingen) or CCR2b polyclonal Ab. The percentage of IL-4-positive and CCR2-positive CD3 $^{+}$ NK1.1 $^{+}$ MACS-enriched cells were determined by FACS and analyzed by CellQuest software. In a separate set of experiments, NK1.1 $^{+}$ MACS-enriched spleen cells were isolated. NK1.1 $^{+}$ MACS-enriched spleen cells (1×10^6) were cultured only in the presence of NRS (1/100 dilution) or anti-MCP-1 antiserum (but no Con A treatment) for 16 h. Cell viability was $\sim 85\%$ for all groups as determined by trypan blue. Three-color FACS staining was then used to determine the percentage of CD3 $^{+}$ NK1.1 $^{+}$ MACS-enriched cells producing IL-4.

Statistical analysis

All data are shown as mean \pm SD. Comparisons between two experimental groups of data were performed using the Student's unpaired *t* test. Comparison among three or more experimental groups was performed using a one-way ANOVA followed by Dunnett's multiple comparison test for comparisons between experimental groups and control. A value of $p \leq 0.05$ was considered significant.

Results

CCL2/MCP-1 and CCR2 expression in Con A-induced hepatitis

In agreement with previous reports (6–17), i.v. administration of a single dose of Con A (0.25 mg/mouse) resulted in significant hepatitis, as demonstrated by a marked increase in plasma ALT levels at 8 and 24 h post-Con A injection (Fig. 1). Moreover, this dose of Con A did not cause mortality of mice. We next determined hepatic CCL2/MCP-1 mRNA expression and protein levels at 30 min, 90 min, 8 h, and 24 h following the induction of acute hepatitis by Con A administration. Using an RNase protection assay, we observed that the development of Con A-induced hepatitis was

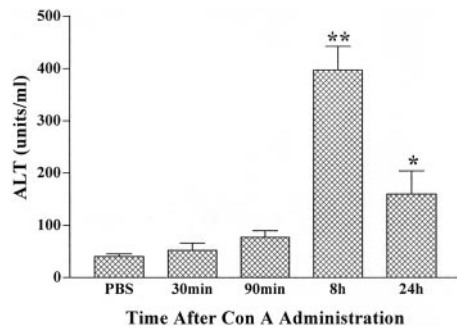


FIGURE 1. Time course of plasma ALT levels during Con A-induced hepatitis. Mice were injected with Con A (0.25 mg/mouse in 0.1 ml of PBS) or vehicle (0.1 ml in PBS), and ALT levels were measured at various times after induction of hepatitis with Con A. Results are expressed as mean \pm SD ($n = 6$ mice per group). *, $p < 0.05$; **, $p < 0.01$ vs control (PBS-treated group).

associated with increased hepatic CCL2/MCP-1 mRNA expression, with significant increases noted at 90 min and 8 h (9-fold increase above control PBS at both time points; $p < 0.01$) following Con A treatment (Fig. 2, A and B). However, by 24 h post-Con A treatment, hepatic CCL2/MCP-1 mRNA expression had returned to control levels (Fig. 2, A and B). In parallel, a serial and significant increase in hepatic levels of CCL2/MCP-1 was observed at 90 min (6-fold increase above control; $p < 0.05$) and 8 h (11-fold increase above control; $p < 0.01$) after Con A injection (Fig. 2C).

The chemokine receptor CCR2 is the sole receptor by which CCL2/MCP-1 mediates its biological effects (36). Analysis of the kinetics of hepatic CCR2 receptor mRNA expression by RNase protection assay showed significant increases at 8 h (6-fold increase above control PBS; $p < 0.01$) and 24 h (16-fold increase above control PBS; $p < 0.01$) after Con A treatment (Fig. 2, D and E).

CCL2/MCP-1 inhibition exacerbates Con A-induced hepatitis

Clinical observation (30) has suggested that CCL2/MCP-1 might be potentially beneficial during hepatitis C infection, a liver disease in which hepatic T cell recruitment and activation are of central importance (1–5). Therefore, we hypothesized that hepatic CCL2/MCP-1 produced during Con A-induced hepatitis might exhibit a hepatoprotective effect. CCL2/MCP-1 expression was highly up-regulated at 90 min and 8 h after Con A treatment (Fig. 2, A–C). Moreover, plasma ALT levels were maximal at the 8-h time point (Fig. 1). Pretreatment of mice with anti-MCP-1 antiserum resulted in enhanced hepatitis at 8 h post-Con A treatment as demonstrated biochemically by a striking elevation in plasma ALT levels (control serum, 533 ± 188 U/ml; anti-MCP-1 antiserum, 1531 ± 76 U/ml; $p < 0.001$) (Fig. 3A). Furthermore, the increased ALT level in anti-MCP-1 antiserum-treated mice was associated with marked histopathological changes in the liver (Fig. 3B). Livers of mice treated with anti-MCP-1 antiserum exhibited widespread hepatocellular necrosis (Fig. 3B). In contrast, livers from mice treated with control serum exhibited more limited hepatocellular necrosis (Fig. 3B). It is noteworthy that mice pretreated only with anti-MCP-1 antiserum (but no Con A treatment) did not exhibit any signs of hepatitis when compared with control serum-treated groups, as determined by serum ALT levels (control serum, 22 ± 12 U/ml; anti-MCP-1 antiserum, 15 ± 7 U/ml; $n = 4$ –5 per group) and histology (Fig. 3B). Thus, our observation of an anti-inflammatory role for CCL2/MCP-1 in T cell-mediated hepatic injury induced by Con A injection in mice stands in sharp contrast

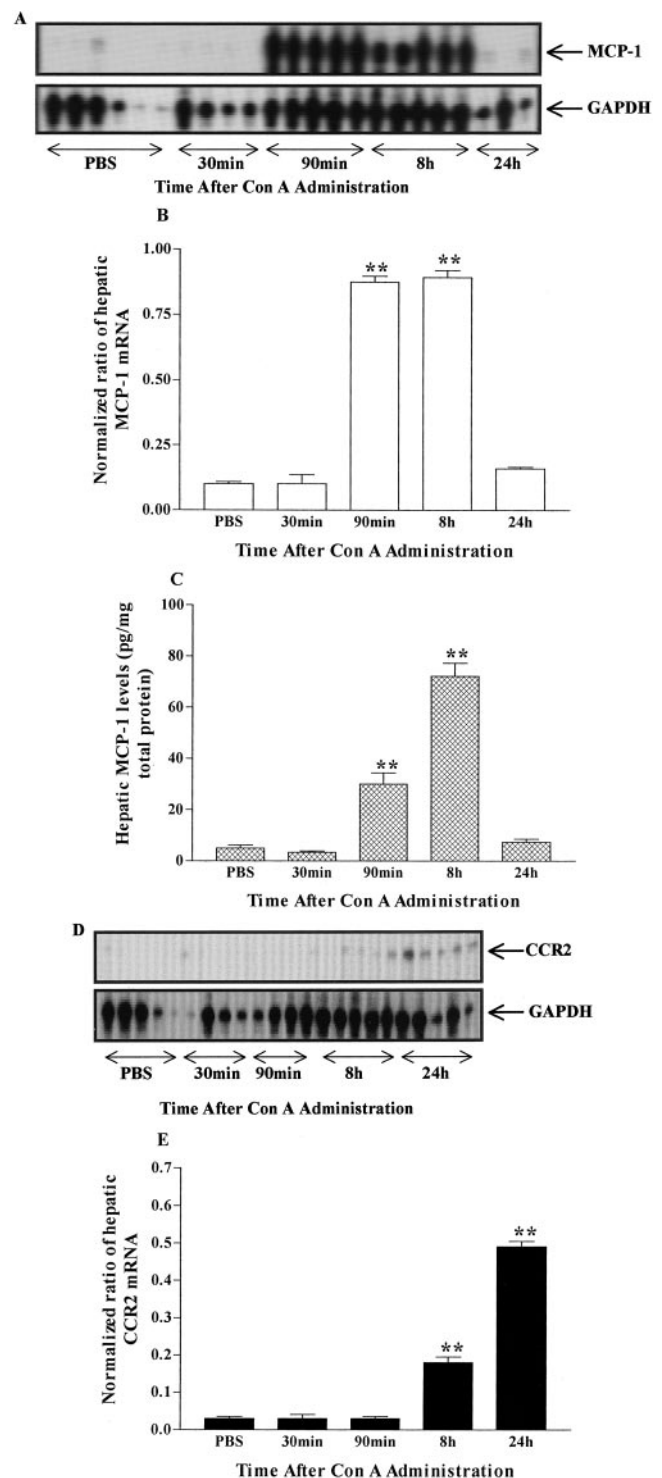
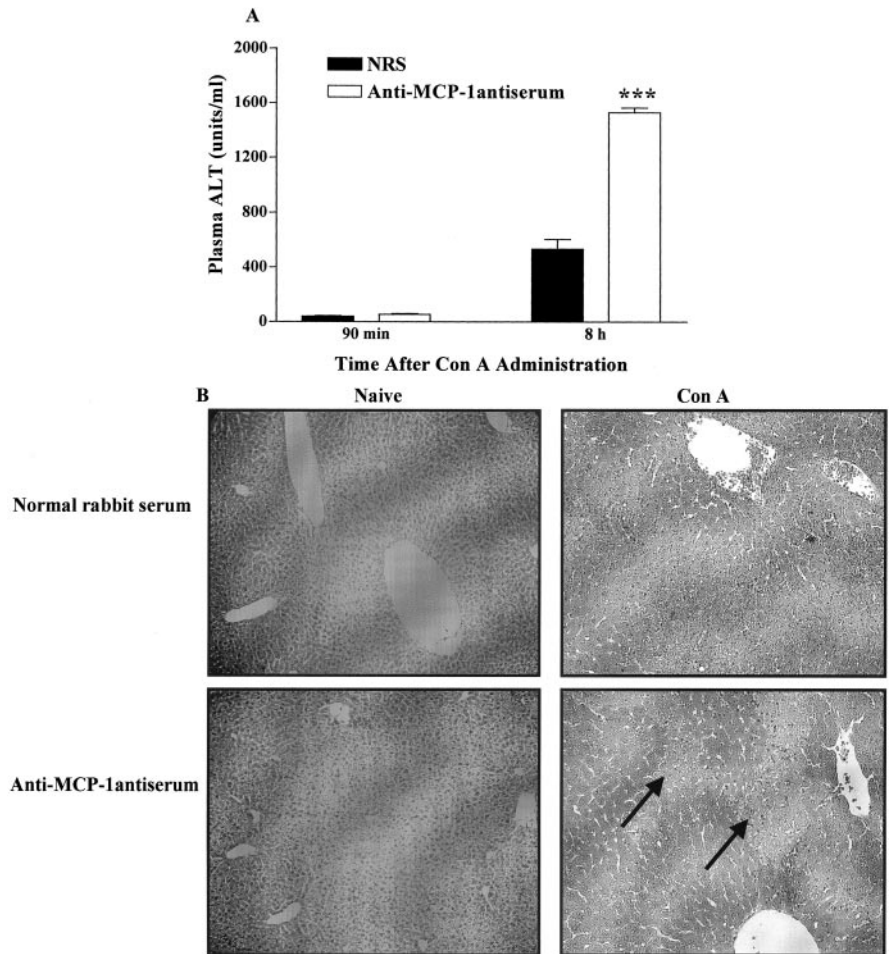


FIGURE 2. Hepatic CCL2/MCP-1 and CCR2 expression during Con A-induced hepatitis. A, Representative RNase protection assay gel for hepatic CCL2/MCP-1 mRNA expression. B, Densitometric analysis of hepatic CCL2/MCP-1 mRNA expression (as a ratio of GAPDH mRNA expression; $n = 3$ –6 per group). C, Hepatic CCL2/MCP-1 protein levels at various times (30 min to 24 h) during Con A-induced hepatitis ($n = 4$ –6 per group). D and E, RNase protection assay ($n = 3$ –4 per group) for hepatic CCR2 mRNA expression. All data are expressed as mean \pm SD. **, $p < 0.01$ vs control (PBS-treated group).

to the documented proinflammatory roles of CCL2/MCP-1 in other T cell-mediated disease models involving other organ systems including rheumatoid arthritis and multiple sclerosis (28, 29).

FIGURE 3. Neutralization of CCL2/MCP-1 exacerbates Con A-induced hepatitis. Mice were injected i.p. with anti-MCP-1 antiserum 16 h before Con A (0.25 mg/mouse) administration. Control mice received heat-inactivated NRS at the same time. Mice were sacrificed 90 min or 8 h after Con A treatment. *A*, Effect of CCL2/MCP-1 neutralization on plasma ALT levels. Results are presented as mean \pm SD ($n = 5-7$ mice per group). ***, $p < 0.001$ vs NRS. *B*, H&E staining of liver sections were obtained from mice pretreated with NRS (*top right panel*) or anti-MCP-1 antiserum (*bottom right panel*) 16 h before Con A treatment and sacrificed 8 h post-Con A administration. *Top and bottom left panels* show liver sections from mice treated with only NRS or anti-MCP-1 antiserum, respectively, but not with Con A.



CCL2/MCP-1 inhibition attenuates the recruitment of hepatic CCR2-positive lymphocytes in Con A-induced hepatitis

Con A treatment resulted in increased hepatic mRNA expression of CCR2 (CCL2/MCP-1 receptor) at both the 8- and 24-h time points (Fig. 2, *D* and *E*). Moreover, significant increases in CD4⁺ T cell infiltrates are observed in the liver at 8 h post-Con A injection (9). Therefore, we determined the recruitment of CCR2-expressing lymphocytes to the liver 8 h after Con A administration. Using RNase protection assay, we observed that mice treated with control serum showed a marked increase in hepatic CCR2 mRNA expression 8 h post-Con A (Fig. 4, *A* and *B*). However, this increase in hepatic CCR2 mRNA expression was significantly attenuated (~45% reduction; $p < 0.05$) after anti-MCP-1 antiserum treatment during Con A-induced hepatitis (Fig. 4, *A* and *B*). In contrast, the hepatic mRNA expression of other chemokine receptors (CCR1, CCR3, CCR4, and CCR5) was unaltered (data not shown). Next, we specifically determined the effect of anti-MCP-1 antiserum pretreatment on the recruitment of CCR2-expressing lymphocytes to the liver 8 h after Con A administration. By flow cytometry, we observed that the absolute number of hepatic CCR2-positive lymphocytes from mice given control serum before Con A injection was 3.51×10^5 cells/liver compared with 2.85×10^5 cells/liver in mice pretreated with anti-MCP-1 antiserum before Con A injection (Fig. 4C; $p < 0.05$). It is noteworthy that the absolute number of CD4⁺ T cells (the predominant T cell infiltrates in the liver during Con A-induced hepatitis) (6, 7, 9) recruited into hepatic tissue after Con A administration was also significantly reduced after CCL2/MCP-1 neutralization (Fig. 4D).

Effect of CCL2/MCP-1 inhibition on hepatic cytokine levels during Con A-induced hepatitis

Various cytokines have been implicated in the pathogenesis of Con A-induced hepatitis. Specifically, hepatic TNF- α (derived from activated resident hepatic macrophages) (8, 9, 13), hepatic IL-4 (derived from activated resident hepatic NKT cells) (11, 12, 15), hepatic IFN- γ (produced by recruited CD4⁺ T cells) (9, 10), and hepatic IL-12 (16) have all been shown to exhibit proinflammatory effects during Con A-induced hepatitis. Conversely, hepatic IL-10 is known to have an anti-inflammatory effect in Con A-treated mice (10). Therefore, we next determined the effect of CCL2/MCP-1 neutralization on hepatic TNF- α , IL-4, IFN- γ , IL-12, and IL-10 levels after Con A treatment. Treatment of mice with anti-MCP-1 antiserum resulted in a significant ($p < 0.05$) decrease in hepatic TNF- α and IFN- γ levels at 90 min and 8 h, respectively, post-Con A administration when compared with mice pretreated with control serum (Fig. 5, *A* and *B*). However, CCL2/MCP-1 neutralization did not alter hepatic IL-12 levels or IL-10 after Con A injection in comparison with control mice (Fig. 5, *C* and *D*). In contrast, treatment with anti-MCP-1 antiserum resulted in a significant increase in hepatic IL-4 levels (+139%; $p < 0.05$) at 8 h post-Con A treatment when compared with mice given control serum (Fig. 5E). It is very unlikely that the increased hepatic IL-4 levels we observed in Con A-treated mice after CCL2/MCP-1 neutralization are due to nonspecific toxic effects of anti-MCP-1 antiserum. Specifically, in a separate set of experiments, mice given only anti-MCP-1 antiserum (i.e., no Con A treatment) had similar hepatic IL-4 levels as mice treated with NRS (NRS, 3.6 ± 1.1

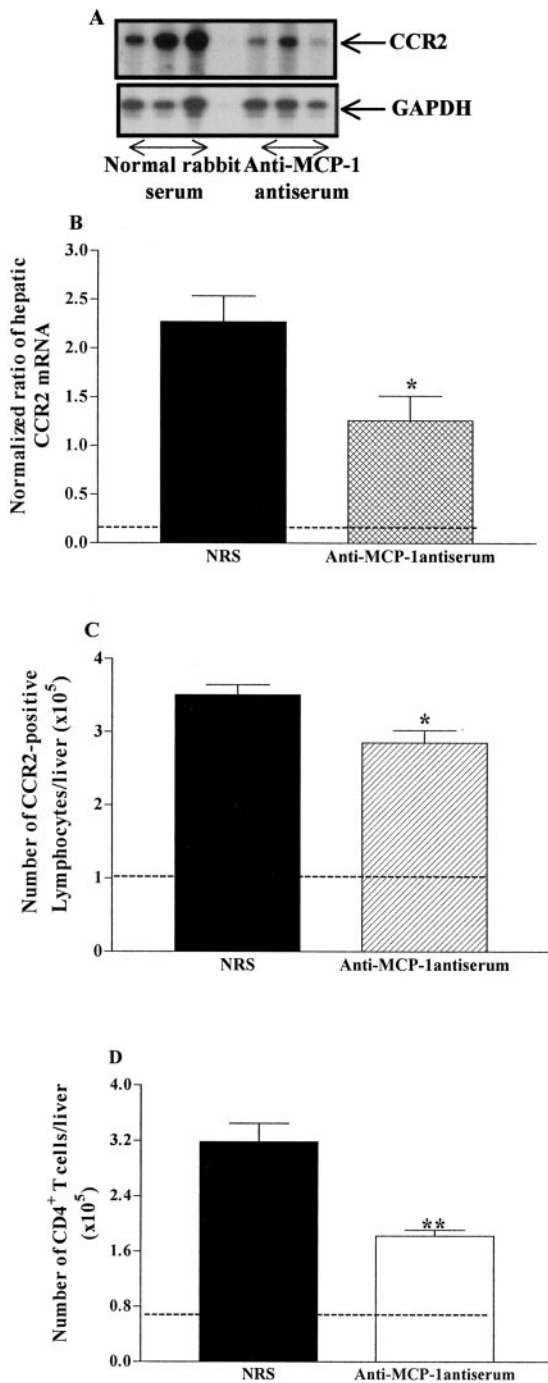


FIGURE 4. CCL2/MCP-1 inhibition decreases the influx of CCR2-positive lymphocytes into the liver during Con A-induced hepatitis. **A** and **B**, RNase protection assay ($n = 3$ per group) for hepatic CCR2 mRNA expression of mice given anti-MCP-1 antiserum or NRS 16 h before Con A (0.25 mg/mouse) administration, and sacrificed 8 h after Con A treatment. Results are expressed as mean \pm SD. *, $p < 0.05$ vs NRS. **C**, CCR2-expressing lymphocytes isolated from liver of mice pretreated with anti-MCP-1 antiserum or NRS 16 h before Con A (0.25 mg/mouse) administration, sacrificed 8 h after Con A treatment, and analyzed by flow cytometry. Dotted line (1.12×10^5 cells \pm 0.1; $n = 3$) denotes the absolute number of CCR2-expressing lymphocytes in livers of naive (no Con A) mice. **D**, CD4⁺ lymphocytes isolated from liver of mice pretreated with anti-MCP-1 antiserum or NRS 16 h before Con A (0.25 mg/mouse) administration, sacrificed 8 h after Con A treatment, and analyzed by flow cytometry. All results are expressed as mean \pm SD. *, $p < 0.05$; **, $p < 0.01$ vs NRS. Data were confirmed in two more independent experiments. Dotted line (0.8×10^5 cells \pm 0.1; $n = 3$) represents the absolute number of CD4⁺ lymphocytes in livers of naive (no Con A) mice.

pg/mg total protein; anti-MCP-1 antiserum, 2.9 ± 0.2 pg/mg total protein; $n = 3$ per group). In addition, anti-MCP-1 antiserum had no effect on CD3⁺NK1.1⁺ (NKT) T cell viability or on IL-4 production. Specifically, in CD3⁺NK1.1⁺ MACS-enriched cells incubated for 16 h with anti-MCP-1 antiserum alone (i.e., no Con A treatment), IL-4 expression was similar to that observed in NRS-treated cells (NRS, $24 \pm 6\%$; anti-MCP-1 antiserum, $27 \pm 8\%$; $n = 3$ per group). Moreover, cell viability in both groups was $\sim 85\%$.

CCL2/MCP-1 inhibits IL-4 production and decreases CCR2 expression in Con A-stimulated CD3⁺NK1.1⁺ T cells in vitro

IL-4 has been shown to play a direct proinflammatory role in Con A-induced hepatitis (12, 15, 17). Moreover, IL-4 appears to be derived almost exclusively from activated resident hepatic NKT cells after Con A administration (15). To address the role of NKT cell-derived IL-4 in the anti-inflammatory effects of CCL2/MCP-1 during Con A-induced hepatitis, we evaluated whether recombinant mouse MCP-1 could inhibit IL-4 production by Con A-stimulated NK1.1⁺-enriched cells in vitro. We observed by flow cytometry that $29 \pm 2\%$ of unstimulated CD3⁺NK1.1⁺ MACS-enriched cells produced IL-4 (Fig. 6A), whereas $67 \pm 5\%$ of Con A-stimulated CD3⁺NK1.1⁺ MACS-enriched cells produced IL-4 (Fig. 6A). Moreover, in CCL2/MCP-1-treated, Con A-stimulated, CD3⁺NK1.1⁺ MACS-enriched cells, only $53 \pm 8\%$ of cells were IL-4 positive (Fig. 6A). CCL2/MCP-1 is known to mediate its biological effects by acting on its specific receptor, CCR2. We found that $11 \pm 1\%$ of unstimulated CD3⁺NK1.1⁺ MACS-enriched cells expressed CCR2 in vitro (Fig. 6B). However, stimulation of these cells with Con A resulted in a striking 9-fold increase in CD3⁺NK1.1⁺ cell surface expression of CCR2 (Fig. 6B). Moreover, incubation of Con A-stimulated CD3⁺NK1.1⁺ MACS-enriched cells with CCL2/MCP-1 resulted in a significant decrease in surface expression of CCR2 on these cells, consistent with internalization of the CCR2 receptor by its ligand CCL2/MCP-1 (37).

Discussion

In recent years, a number of clinical studies have provided evidence consistent with a role for a number of chemokines including CCL2/MCP-1 in the pathogenesis of T cell-mediated liver diseases (30, 38–41). However, the lack of interventional studies in T cell-mediated disease model(s) of hepatitis means that the extent to which the actions of CCL2/MCP-1 contribute to the pathogenesis of human T cell-mediated liver diseases remains largely unknown. Con A-induced hepatitis serves as a prototypic model for T cell-mediated hepatitis (6–17, 42). In our current study, we show that CCL2/MCP-1 and the expression of its specific receptor, CCR2, are elevated in the liver during Con A-induced hepatitis. We further demonstrate that CCL2/MCP-1 is beneficial during Con A-induced hepatitis because hepatic injury is exacerbated by CCL2/MCP-1 neutralization. More importantly, we provide evidence of a novel hepatoprotective role for CCL2/MCP-1 during Con A-induced hepatitis that is associated with the suppression of CD3⁺NK1.1⁺ T cell-derived IL-4 production and CCR2 expression.

One of the key defining roles of CCL2/MCP-1 is the recruitment and activation of leukocytes, including T lymphocytes, during an inflammatory response via its specific receptor, CCR2 (35, 36). Moreover, previous studies have documented a pathogenic role for activated CD4⁺ T cells recruited to the liver in Con A-mediated hepatitis (6, 7). However, in the current study, we observed that the reduction in hepatic CCR2 mRNA expression and a decrease in the recruitment of CCR2-expressing lymphocytes and CD4⁺ T cells to the liver after CCL2/MCP-1 neutralization did not diminish Con

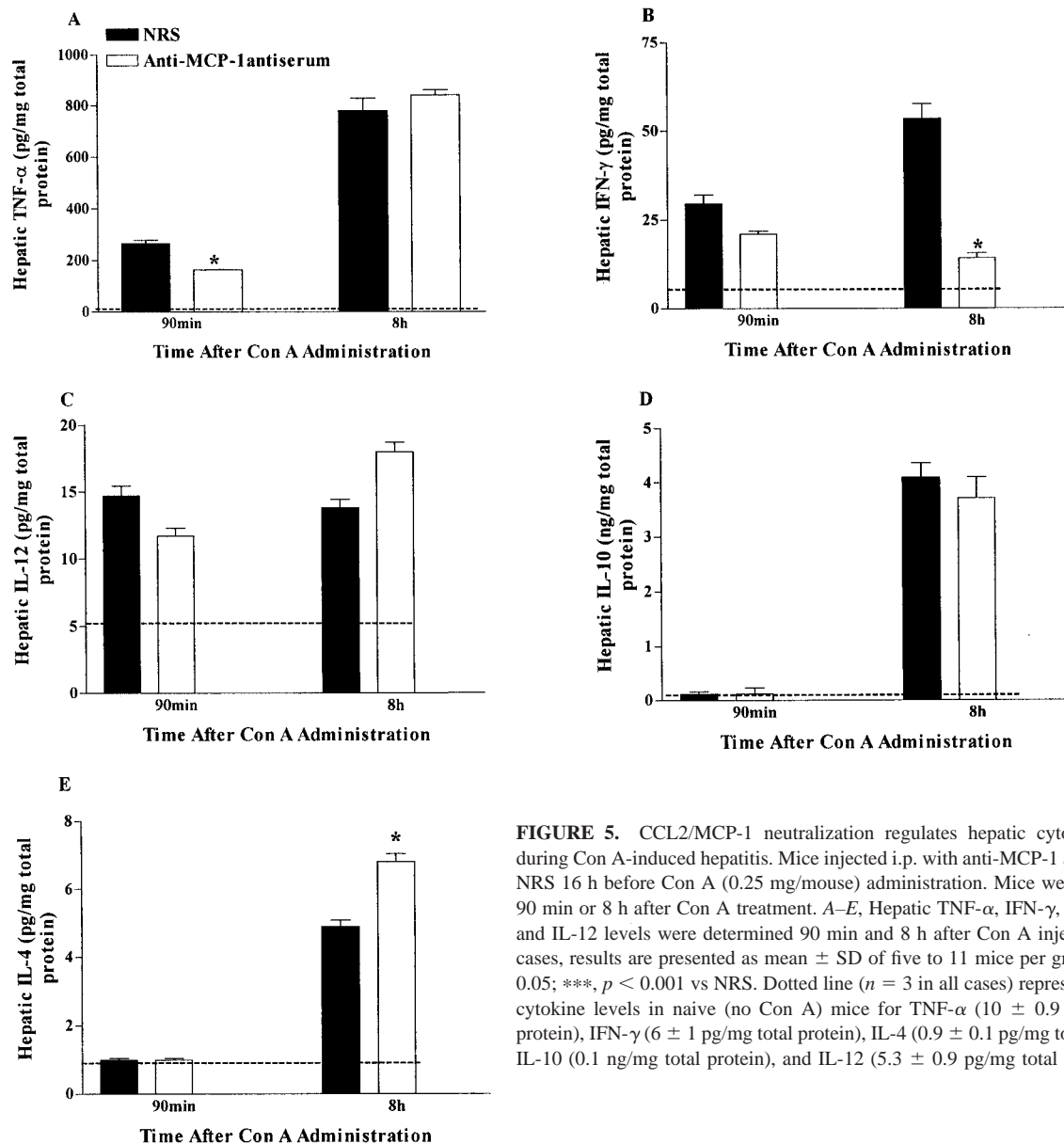


FIGURE 5. CCL2/MCP-1 neutralization regulates hepatic cytokine levels during Con A-induced hepatitis. Mice injected i.p. with anti-MCP-1 antiserum or NRS 16 h before Con A (0.25 mg/mouse) administration. Mice were sacrificed 90 min or 8 h after Con A treatment. A–E, Hepatic TNF- α , IFN- γ , IL-4, IL-10, and IL-12 levels were determined 90 min and 8 h after Con A injection. In all cases, results are presented as mean \pm SD of five to 11 mice per group. *, $p < 0.05$; ***, $p < 0.001$ vs NRS. Dotted line ($n = 3$ in all cases) represents hepatic cytokine levels in naive (no Con A) mice for TNF- α (10 ± 0.9 pg/mg total protein), IFN- γ (6 ± 1 pg/mg total protein), IL-4 (0.9 ± 0.1 pg/mg total protein), IL-10 (0.1 ng/mg total protein), and IL-12 (5.3 ± 0.9 pg/mg total protein).

A-mediated hepatic injury. In view of this finding, we propose that the hepatoprotective effects of CCL2/MCP-1 during Con A-induced liver damage may extend beyond the recruitment of activated hepatic T cells, and our findings suggest the effects of CCL2/MCP-1 on activated resident hepatic T cells (such as NKT cells) may be of central importance in the anti-inflammatory effects of CCL2/MCP-1 during Con A-induced hepatitis.

In recent years, a number of cytokines, including IFN- γ , TNF- α , and IL-12, have all been found to be proinflammatory against Con A-mediated liver damage, because mice pretreated with their respective anti-cytokine Ab or cytokine-deficient mice are resistant to Con A-mediated liver damage (7–9, 16). Conversely, IL-10 has been found to be hepatoprotective in Con A-mediated liver damage, because Ab neutralization of IL-10 exacerbates Con A-mediated hepatic injury (10). Interestingly, several lines of evidence suggest that CCL2/MCP-1 is associated with the development of a polarized Th2 response (31, 43). Therefore, in the present study, we hypothesized that the enhanced hepatic injury observed after CCL2/MCP-1 neutralization in Con A-induced hepatitis could be due to the overproduction of the Th1 cytokines TNF- α , IL-12,

and/or IFN- γ , or a decrease of the Th2 cytokine IL-10. Surprisingly, we found that the enhanced hepatic injury observed following CCL2/MCP-1 neutralization in Con A-treated mice was associated with a decrease in hepatic TNF- α and IFN- γ levels.

IFN- γ is produced mainly by activated CD4⁺ T cells recruited to the liver during Con A-mediated hepatitis (M. N. Ajuebor, unpublished observations; Refs. 10 and 15); therefore, a reduction in the absolute number of recruited CD4⁺ T cells to the liver following CCL2/MCP-1 neutralization during Con A-mediated hepatitis may underlie the reduced hepatic level of IFN- γ we documented in this study. IFN- γ is a cytokine endowed with many regulatory effects, including the induction of TNF- α secretion from tissue macrophages (44). Given the critical role of Kupffer cells in the production of TNF- α during Con A-mediated hepatitis (13), we propose that IFN- γ derived from CCR2-expressing activated lymphocytes recruited to the liver may act to augment hepatic injury by enhancing hepatic macrophage activation and TNF- α production; an effect which is attenuated by CCL2/MCP-1 neutralization. Moreover, we observed that Con A-stimulated, CD3⁺NK1.1⁺, MACS-enriched cells did not produce IFN- γ or

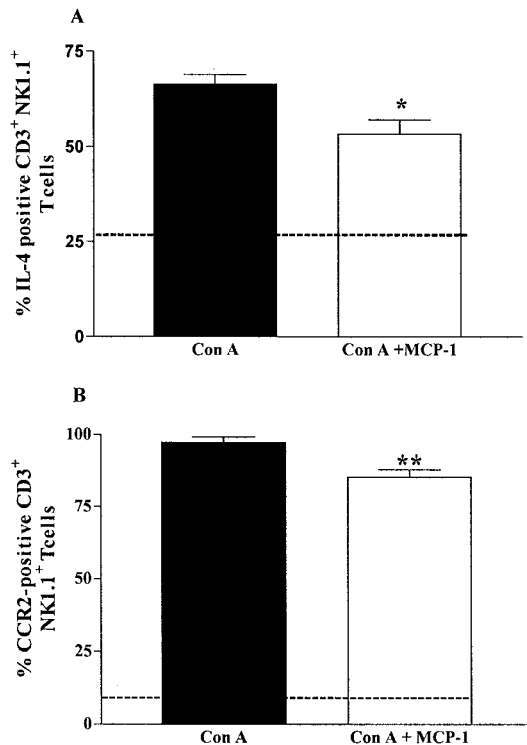


FIGURE 6. CCL2/MCP-1 decreases IL-4 production in Con A-stimulated CD3⁺NK1.1⁺ T cells in vitro. MACS-enriched spleen CD3⁺NK1.1⁺ T cells (1×10^6) were stimulated with Con A (20 μ g/ml) with or without recombinant mouse MCP-1 (100 ng/ml) for 16 h on 24-well plates, and IL-4-positive cells (A) and CCR2-positive cells (B) were determined by FACS analysis. Dotted lines in both cases represent percentages of positive cells in naive (no Con A) mice (IL-4, $29 \pm 2\%$, $n = 3$; CCR2, $11 \pm 1\%$, $n = 3$). Results are expressed as mean percentage of positive-stained cells \pm SD. *, $p < 0.05$; **, $p < 0.01$ vs Con A-stimulated group (no MCP-1 treatment). Data were confirmed in three more independent experiments.

TNF- α (M. N. Ajuebor, unpublished observation). Contrary to the reduction in hepatic TNF- α and IFN- γ after anti-MCP-1 antiserum treatment in Con A-treated mice, CCL2/MCP-1 neutralization did not alter hepatic levels of IL-12 and IL-10. Therefore, our findings strongly suggest that CCL2/MCP-1 is hepatoprotective in T cell-mediated hepatitis induced by Con A administration by a pathway independent of IL-10 and IL-12, and the classical Th1/Th2 paradigm.

Until recently, Con A-induced hepatitis was considered a Th1-mediated disease model (6–10, 16). However, recently several reports have revealed that IL-4, a Th2 cytokine that is elevated in the liver following Con A injection, exhibits proinflammatory effects during Con A-induced hepatitis as demonstrated by anti-IL-4 Ab or *IL-4* gene-deficient mice studies (12, 15, 17). For this reason, we evaluated whether the exaggerated hepatic injury seen after CCL2/MCP-1 neutralization in Con A-treated mice could be attributed to overproduction of hepatic IL-4. Indeed, in our study, we observed that the enhanced hepatic injury observed after CCL2/MCP-1 neutralization of Con A-treated mice was associated with increased hepatic levels of IL-4. Interestingly, our findings are in agreement with clinical studies where successful treatment of hepatitis C-infected patients with IFN- α therapy was found to increase CCL2/MCP-1 serum levels and concomitantly suppress serum IL-4 levels (21, 30).

NKT cells, a unique group of T lymphocytes that share receptor structures with conventional T cells and NK cells (45, 46), are resident intrahepatic lymphocytes accounting for 20–30% of all intrahepatic lymphocytes (45–47). These cells exert regulatory

functions by promptly releasing significant amounts of cytokines such as IL-4 and IFN- γ following stimulation by anti-CD3 mAb, Con A, and the glycolipid ligand α -galactosylceramide (11, 15, 45–47). NKT cells recognize lipid Ags in the context of the CD1d molecule which normally associates with β_2 -microglobulin on the cell surface (11, 15, 45, 46). Thus, *CD1d* gene-deficient and β_2 -microglobulin gene-deficient mice express very few NKT cells (11, 15, 45–47), and these mice are resistant to Con A-induced hepatitis (11, 12, 15). A wealth of recent evidence demonstrates a regulatory role for resident hepatic NKT cells in augmenting hepatic damage via the secretion of IL-4 during Con A-induced hepatitis (12, 15, 17). Specifically, Kaneko et al. (15) demonstrated that IL-4 produced by Con A-activated resident hepatic NK1.1⁺ T cells acts on NK1⁺ T cells in an autocrine fashion to induce the up-regulation of Fas ligand (FasL) expression on these cells, ultimately resulting in an enhancement of NK1.1⁺ T cell-mediated cytotoxicity (12, 15, 17). Therefore, the possibility that the suppression of NKT cell-derived IL-4 may play a direct and crucial role in the anti-inflammatory effects of CCL2/MCP-1 during Con A-induced hepatitis was investigated. Our study demonstrated that Con A-stimulated CD3⁺NK1.1⁺ MACS-enriched spleen cells synthesized significant amounts of IL-4 which was inhibited by rMCP-1 treatment. The CCR2 receptor is the sole receptor by which CCL2/MCP-1 mediates its biological effects (35, 36). Although CCR2 expression has been detected on human NKT cells (47), its expression on mouse NKT cells remains undefined. Interestingly, by flow cytometry, we observed that CCR2 was basally expressed on CD3⁺NK1.1⁺ T cells. Moreover, Con A stimulation resulted in a significant increase in CCR2 expression on CD3⁺NK1.1⁺ MACS-enriched cells, and this increase in CCR2 expression was significantly suppressed by rMCP-1 treatment. An attempt to make similar comparisons by using hepatic NKT cells was unsuccessful because of the inherent difficulty in obtaining sufficient number of NKT cells from the livers of mice (M. N. Ajuebor, unpublished observations; Ref. 37). Taken together, this study provides novel evidence suggesting a direct role for CCL2/MCP-1 in suppressing CD3⁺NK1.1⁺ T cell-derived IL-4 production via a specific interaction with its receptor CCR2 as the mechanisms mediating the anti-inflammatory effects of CCL2/MCP-1 during T cell-mediated hepatitis induced by Con A injection in mice. However, we cannot exclude the possibility that CCL2/MCP-1 may also exert a hepatoprotective role during Con A-induced hepatitis by suppressing the production of other mediators of inflammation that are currently unknown.

The Fas-FasL interaction has been implicated in the pathogenic effects of IL-4 during Con A-induced hepatitis as discussed above (11, 15, 48). In support of this link between hepatic IL-4 and FasL expression, we observed that enhanced hepatic IL-4 expression seen after CCL2/MCP-1 neutralization in Con A-treated mice was also accompanied by increased hepatic FasL mRNA expression as determined by RT-PCR (M. N. Ajuebor, unpublished observations). Moreover, we found an up-regulation of FasL expression on CD3⁺NK1.1⁺ T cells stimulated with Con A in vitro (M. N. Ajuebor, unpublished observations). However, rMCP-1 treatment of Con A-stimulated CD3⁺NK1.1⁺ MACS-enriched cells in vitro did not reduce FasL expression (M. N. Ajuebor, unpublished observations) despite the suppression of IL-4 synthesis on these cells.

In summary, our findings suggest a novel hepatoprotective effect of CCL2/MCP-1 in Con A-induced hepatitis which is driven by a suppression of CD3⁺NK1.1⁺ T cell-derived IL-4 production. That CCL2/MCP-1 is anti-inflammatory in T cell-mediated hepatitis, but is proinflammatory in T cell-mediated animal disease models of multiple sclerosis and rheumatoid arthritis (28, 29, 49) can be potentially explained by the uniqueness of the liver in which

NK1.1⁺ T cells are the main resident T cell type present (45–47). Therefore, manipulation of hepatic CCL2/MCP-1 levels may be a novel potential target for the treatment of T cell-mediated liver diseases.

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