CCR5 Deficiency Drives Enhanced Natural Killer Cell Trafficking to and Activation within the Liver in Murine T Cell-Mediated Hepatitis

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Natural killer (NK) cells are innate immune cells that are enriched in the liver, but the processes underlying NK cell trafficking to the liver and cellular activation within the liver of patients with T cell-mediated liver diseases remain poorly defined. Concanavalin A (Con A) hepatitis is a murine model mimicking many aspects of human T cell-mediated liver diseases. Here we demonstrate that severe hepatitis in CCR5-deficient (KO) mice is associated with increased hepatic NK cell recruitment driven by enhanced hepatic production of CCL5 acting via CCR1 and by enhanced hepatic NK cell activation relative to that observed in wild-type mice after Con A administration. Furthermore, NK cell depletion ameliorated severe hepatitis in CCR5 KO mice but did not alter hepatitis in wild-type mice after Con A treatment. We propose that in the setting of CCR5 deficiency NK cells assume a profound effector role in Con A hepatitis via enhanced CCL5-CCR1 driven hepatic recruitment in addition to augmented cytokine-driven NK cell activation to produce interferon- γ . These results highlight the potential profound impact of altered chemokine receptor expression on the innate immune response in the setting of T cell-mediated hepatitis. (Am J Pathol 2007, 170:1975–1988; DOI: 10.2353/ajpath.2007.060690)

CCR5 is a CC chemokine receptor that is expressed on various cell types including natural killer (NK) T cells, CD4⁺ T cells, macrophages, and NK cells.^{1–4} CCR5 is a G-protein coupled receptor known to regulate the immune response by interacting with any of three chemo-

kine ligands (CCL3, CCL4, and CCL5).² CCR5 gained notoriety in the mid-1990s when this receptor was identified as one of the obligate membrane co-receptors for the binding and entry of human immunodeficiency (HIV) virus into target cells² and that its ligand CCL5 suppressed HIV replication.² About 1% of the Caucasian population is homozygous for the CCR5 Δ 32 polymorphism (which renders the CCR5 receptor nonfunctional), and these individuals resist HIV infection.^{5,6} Thus, the development of CCR5 antagonists as potential therapies for HIV infection was accelerated by the pharmaceutical industry. However, recent early clinical trials of some CCR5 antagonists for the potential treatment of HIV infection were halted due to profound hepatotoxicity,^{7–9} possibly implicating CCR5 as a modulator of the hepatic inflammatory response.¹⁰ In support of a modulatory role for CCR5 in hepatic inflammation, the CCR5 Δ 32 polymorphism has been linked to the prevalence and severity of a number of T cell-mediated liver diseases including chronic hepatitis C^{11,12} (but this remains controversial¹³) and primary sclerosing cholangitis.¹⁴ However, the impact of the CCR5 Δ 32 polymorphism on the severity of acute hepatitis has not been examined.

Concanavalin A (Con A)-induced hepatitis is a well-characterized model of T cell-mediated hepatitis mimicking many aspects of human T cell-mediated liver disease, including autoimmune hepatitis.^{4,15–26} Con A-induced hepatitis is mediated by liver-infiltrating activated CD4⁺ T cells, which produce mainly interferon- γ (IFN- γ),²² and resident hepatic NKT cells, which produce mainly interleukin-4 (IL-4) (and to a lesser extent IFN- γ). Kupffer cells, which secrete tumor necrosis factor- α ,²⁷ and neutrophils²⁸ have also been directly implicated in the pathogenesis of Con A-induced hepatitis. In agreement with the clinical studies suggesting a possible increased incidence and severity of hepatitis C virus and primary

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sclerosing cholangitis liver disease in CCR5-deficient individuals, we⁴ and others²⁹ recently demonstrated that CCR5 gene-deficient mice have a profound increase in the severity of liver damage and associated incidence of subsequent liver failure following Con A administration. Two distinctive mechanisms were proposed for these observations. In the first study, we demonstrated that activated hepatic CCR5-deficient CD1d tetramer-positive NKT cells resist apoptosis, and these cells produce more IL-4 than wild-type controls after Con A administration.⁴ In addition, *in vivo* depletion of IL-4 or NKT cells ameliorated liver damage in CCR5 gene-deficient mice post-Con A treatment.⁴ In the second study, Moreno and colleagues²⁹ suggested that enhanced recruitment of CCR1-expressing CD4⁺ T and NKT cells as well as macrophages into the liver of Con A-treated CCR5 KO mice triggers severe hepatitis in these mice after Con A administration.

Increased NK cell numbers are commonly observed within the livers of patients with T cell-mediated hepatitis^{30–32}; however, their specific role in the hepatic inflammatory response in these diseases remains poorly defined. Numerous studies have demonstrated that hepatic NK cells do not contribute to liver damage during Con A-induced hepatitis because selective depletion of hepatic NK cells with the specific NK cell-neutralizing antibody asialo-GM1 does not alter liver damage.^{17,26,33,34} In the present study, we report the novel observation that CCR5 deficiency unmasks a potent proinflammatory role for hepatic NK cells during Con A-induced hepatitis. Specifically, we demonstrate that in contrast to wild-type (WT) mice, CCR5 deficiency promotes the increased recruitment of both NK cells and IFN- γ -producing NK cells into the liver of Con A-treated CCR5-deficient mice. In addition, CCR5-deficient NK cells exhibit an enhanced ability to be activated both *in vivo* and *in vitro* to produce IFN- γ compared with WT mice. Furthermore, *in vivo* depletion of IFN- γ or NK cells prevents severe hepatitis in CCR5-deficient mice post-Con A treatment.

Materials and Methods

Animals

Male B6129PF2 wild-type mice and CCR5-deficient mice (B6129PF2 background) aged 7 to 9 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and kept in a conventional animal facility at the University of Calgary. All procedures in this study were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Disease Models

Con A-induced hepatitis is widely used as an animal model of T cell-mediated hepatitis.^{4,15,17,18,22,26,35,36} CCR5 KO mice and corresponding WT mice were in-

jected intravenously with a single dose of freshly prepared Con A (13.5 mg/kg; Sigma Chemical Co., St. Louis, MO) reconstituted in sterile phosphate-buffered saline (PBS).^{4,22,36} At selected time points (ie, 90 minutes and 8 hours)⁴ after Con A administration and under halothane anesthesia, blood was collected for measurement of plasma alanine transaminase (ALT) levels (Biotron Diagnostics, Hemet, CA), and the livers were then perfused with ice-cold sterile PBS to remove blood elements. Liver sections were processed and stained with hematoxylin and eosin (H&E) according to standard protocols for histological evaluation of liver injury.

In a separate set of experiments, polyinosinic-polycytidylic acid (polyI:C, 20 mg/kg; Sigma)^{37–39} was administered intraperitoneally to CCR5 KO and WT mice to directly activate NK cells within the liver. Sixteen hours after polyI:C administration^{37,38} hepatic mononuclear cells were isolated, and NK cells and IFN- γ -producing NK cells were identified by fluorescence-activated cell sorting (FACS) as described below.

Immunohistochemistry

The localization of CCL5 in paraffin-embedded liver sections obtained from WT and CCR5 KO mice 8 hours after Con A-induced hepatitis was determined using a specific anti-murine CCL5 polyclonal antibody (Ab) (AF478; R&D Systems, Minneapolis, MN) as previously described.⁴⁰

Isolation of Hepatic Mononuclear Cells and Flow Cytometry

Hepatic mononuclear cells were isolated as previously described.^{4,22,36} For staining of NK or CD4⁺ T cells, isolated hepatic mononuclear cells were preincubated with anti-mouse CD16/32 monoclonal antibody (mAb) (BD PharMingen, San Diego, CA) to block Fc γ receptors and then incubated with fluorescein isothiocyanate-labeled NK1.1 mAb (clone PK136; BD PharMingen) or PerCP-labeled CD4⁺ mAb (clone RM4-5; BD PharMingen). In some experiments, labeled NK cells were extracellularly stained with phycoerythrin-labeled FasL mAb (clone Kay-10; BD PharMingen). In other experiments, labeled NK cells were fixed and permeabilized with Cytofix/Cytoperm^{4,22,36} and then stained with phycoerythrin-labeled IFN- γ mAb (clone XMGI.2; BD PharMingen) as recently described.^{4,22,36} In addition, labeled CD4⁺ T cells were also fixed and permeabilized with Cytofix/Cytoperm^{4,22,36} and then stained with phycoerythrin-labeled CCR1 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) as we have previously described.²² For FACS analysis, the cell population of interest was gated using forward and side scatter characteristics and analyzed using CellQuest software (Becton Dickinson, Mountain View, CA).

Neutralization and Blocking Experiments

For NK cell depletion experiments, WT or CCR5 KO mice received a single intravenous injection of anti-asialo-GM1

polyclonal Ab or rabbit IgG (25 μ l/mouse; Cedarlane Laboratories, Ontario, ON, Canada) 24 hours before Con A administration, and all mice were sacrificed 8 hours post-Con A treatment. Depletion of NK cells was confirmed by flow cytometry. For cytokine/chemokine/chemokine receptor neutralization experiments, CCR5 KO mice received intravenously a single dose of one of the following antibodies or antagonist: anti-IFN- γ mAb (clone XMG1.2, 0.3 mg/mouse; BD PharMingen); anti-IL-4 mAb (clone 11B11 0.3 mg/mouse; BD PharMingen); anti-CCL5 mAb (clone 53433, 0.1 mg/mouse; R&D Systems); Met-RANTES (30 μ g/mouse; Serono Research Pharmaceutical Institute); or corresponding dose-matched controls 24 hours before Con A administration, and mice were sacrificed 90 minutes or 8 hours post-Con A treatment. The following controls were used: PBS (for Met-RANTES study), Rat IgG₁ (purchased from R&D Systems for anti-CCL5 mAb experiments), Rat IgG₁ (purchased from BD PharMingen for anti-IL-4 mAb study), Rat Ig κ (purchased from BD PharMingen for anti-IFN- γ mAb experiments). Met-RANTES is a dual CCR1/CCR5 antagonist^{22,41} that we have previously used to inhibit the recruitment of CCR1-expressing cells to the liver during Con A-induced hepatitis.²²

Splenocyte Cultures

Single-cell suspensions were prepared from the spleens of naïve WT and naïve CCR5 KO mice using RPMI 1640 medium (supplemented with 10% fetal calf serum, non-essential amino acids, L-glutamine, β -mercaptoethanol, and penicillin-streptomycin; all reagents were purchased from Invitrogen Canada). Briefly, spleens from naïve WT and naïve CCR5 KO mice were squeezed between sterile frosted slides, passed through a 100- μ m cell strainer (BD PharMingen), and then placed in ammonium chloride lysis buffer to remove red blood cells. Next, splenocytes (2×10^6 cells/well) in RPMI 1640 medium (as above) were treated *in vitro* with the following recombinant cytokines/chemokine as follows: unstimulated, murine CCL5 (50 ng/ml; Peprotech, London, UK) alone; murine IL-12 (5 ng/ml; Peprotech) + murine IL-18 (20 ng/ml; Biosource International, Montreal, QC, Canada); or mL-12 + mL-18 + mCCL5 for 16 hours with GolgiStop (BD PharMingen) added during the last 5 hours of stimulation. Following *in vitro* stimulation, splenocytes were stained extracellularly with NK1.1 mAb (for NK cells) and then intracellularly for IFN- γ as described above.

Cytokine/Chemokine Enzyme-Linked Immunosorbent Assays

IFN- γ , CCL5, and CCL3 levels in liver homogenates were determined by specific enzyme-linked immunosorbent assay as previously described.^{22,36}

Statistical Analysis

All data are shown as means $\pm$ SEM. For comparisons of means between two experimental groups, a Student's

unpaired *t*-test was used. Comparisons among three or more experimental groups were performed using a one-way analysis of variance followed by either Dunnett's multiple comparison or Student-Newman-Keuls post hoc test. A *P* value of ≤ 0.05 was considered significant.

Results

CCR5 Deficiency Increases the Severity of Con A-Induced Hepatitis

In agreement with our recent report,⁴ intravenous administration of a single dose of Con A (13.5 mg/kg) into CCR5 KO mice was associated with the development of severe hepatitis within 8 hours of Con A administration as demonstrated biochemically by a striking augmentation (> 15 -fold increase) in plasma ALT levels (WT mice, 983 ± 113 U/L, versus CCR5 gene-deficient mice, $17,237 \pm 124$ U/L; $P < 0.001$; $n = 4$ –5 per group). Interestingly, we also observed that Con A administration caused a significant increase in the number of NK cells recruited to the liver of CCR5 KO mice within 8 hours post-Con A administration (Figure 1, A and B). The increased NK cell recruitment into the liver of Con A-treated CCR5 KO mice was specifically due to CCR5 deficiency, because NK cells were not significantly recruited into the livers of WT mice after Con A treatment at a similar time point (Figure 1, A and B).

NK Cell Depletion Prevents Severe Con A-Induced Hepatitis in CCR5 KO Mice

NK cells do not contribute to the pathogenesis of Con A-induced hepatitis in normal mice.^{17,26,33,34} However, we hypothesized that the influx of NK cells into the liver of CCR5 KO mice may contribute to the severe hepatitis observed in these mice after Con A administration. In agreement with our hypothesis, the selective depletion of hepatic NK cells with a specific NK cell neutralizing Ab (anti-asialo-GM1 Ab)^{33,42} almost completely prevented the development of severe hepatitis in Con A-treated CCR5 KO mice as shown by significantly lower plasma ALT levels (control Ab + CCR5 KO mice, $16,020 \pm 134$ U/L, versus anti-asialo-GM1 Ab + CCR5 KO mice, 185 ± 21 U/L; $P < 0.01$; $n = 5$ or 6 per group; Figure 1C). Furthermore, we observed a marked improvement in hepatic histology in anti-asialo-GM1 Ab-treated CCR5 KO mice 8 hours after Con A administration. Liver sections from control Ab-treated CCR5 KO mice exhibited extensive hepatocyte damage at 8 hours following Con A administration (Figure 1D). In contrast, liver sections from anti-asialo-GM1 Ab-treated CCR5 KO mice exhibited little hepatocyte damage or hepatocellular necrosis at 8 hours after Con A treatment (Figure 1D). In agreement with previous studies,^{17,26,33} NK cell depletion did not prevent or alter Con A-induced hepatitis in WT mice 8 hours after Con A administration relative to WT mice that received control Ab (Figure 1C), a finding that is in contrast to our observations in CCR5 KO mice. These results indicate that NK cells selectively contribute to the development of

Figure 1. **A:** Time course of NK cell recruitment into the liver of WT (□; $n = 5$) and CCR5 KO (■; $n = 4$) mice during Con A-induced hepatitis; ** $P < 0.01$ versus all other groups. **B:** Representative FACS histogram depicting increased NK cell influx into the liver of CCR5 KO mice compared with WT mice at 8 hours after Con A administration. **C:** The effect of anti-asialo-GM1 Ab ($n = 6$) or control Ab ($n = 5$) treatment on ALT levels in WT and CCR5 KO mice 8 hours after Con A treatment; ** $P < 0.01$ versus control Ab-treated WT mice; ** $P < 0.01$ versus anti-asialo Ab-treated CCR5 KO mice. **D:** Representative H&E staining of liver sections showing widespread/confluent hepatocellular necrosis (white arrows) and inflammatory cell infiltrates throughout the liver in Con A-treated CCR5 KO mice relative to patchy hepatocellular necrosis and mild inflammatory cell infiltrates in CCR5 KO mice pretreated with asialo-GM1 Ab at 8 hours after Con A treatment. **E:** Representative FACS histogram depicting NK cell depletion after anti-asialo-GM1 mAb treatment in naive CCR5 KO mice.

Con A-induced hepatitis only in the setting of CCR5 deficiency. A FACS histogram confirming depletion of hepatic NK cells after anti-asialo-GM1 Ab treatment is shown in Figure 1E.

Correlation of Severe Hepatitis in CCR5 KO Mice with Increased Intrahepatic IFN- γ

Profound hepatitis in CCR5 KO mice was associated with significantly increased hepatic levels of IFN- γ at 90 minutes and 8 hours after Con A administration relative to Con A-treated WT mice (Figure 2A). However, we recently reported that the number of IFN- γ -producing CD1d tetramer-positive NKT cells in CCR5-deficient mice is similar to that observed in WT mice after Con A administration.⁴ Because NK cells are known to exert effector

functions in part via IFN- γ production following activation,^{43–45} we evaluated by flow cytometry whether increased recruitment of IFN- γ -producing NK cells to the liver of CCR5 KO mice after Con A administration might be responsible for this differential increase in hepatic IFN- γ levels in these mice. Interestingly, we observed a striking increase in the number of IFN- γ -producing NK cells recruited to the liver of CCR5 KO mice at 90 minutes (threefold) and 8 hours (fourfold) after Con A treatment relative to Con A-treated WT mice (at these respective time points) or compared with naive mice (Figure 2, B and C). In addition, the number of IFN- γ -producing NK cells recruited into the liver of WT mice was significantly increased only at 8 hours post-Con A treatment relative to the following groups: naive WT, naive CCR5 KO, and Con A-treated WT 90 minutes (Figure 2B). Therefore, NK cells

Figure 2. Correlation of severe hepatitis in CCR5 KO mice with enhanced hepatic IFN- γ production. **A:** Enzyme-linked immunosorbent assay determination of hepatic levels of IFN- γ in WT ($\square$; $n = 5$) and CCR5 KO mice ($\blacksquare$; $n = 4$) during Con A-induced hepatitis. * $P < 0.05$ versus naïve WT; * $P < 0.05$ versus Con A-treated WT (90 minutes) and naïve groups; ** $P < 0.01$ versus all WT groups. **B:** Number of IFN- γ -producing NK cells in the liver of WT ($\square$; $n = 6$) and CCR5 KO ($\blacksquare$; $n = 5$) mice after Con A administration. * $P < 0.05$ versus Con A-treated WT (90 minutes) and naïve groups; * $P < 0.05$ versus Con A-treated WT (90 minutes) and naïve groups; ** $P < 0.01$ versus all groups. **C:** Representative FACS dot plot demonstrating increased NK cell intracellular IFN- γ in the liver of CCR5 KO mice after 8 hours of Con A treatment relative to WT mice. **D:** Number of IFN- γ -producing CD4⁺ T cells in the liver of WT ($\square$; $n = 5$) and CCR5 KO ($\blacksquare$; $n = 4$) mice 8 hours after Con A administration. * $P < 0.05$ versus naïve WT and naïve CCR5 KO mice; ** $P < 0.01$ versus all other groups. **E:** Representative FACS histogram demonstrating reduced CD4⁺ T cell recruitment in the liver of CCR5 KO mice after anti-asialo-GM1 Ab treatment relative to CCR5 KO given control antibody.

(Figure 2B) and CD4⁺ T cells (our previous study)²² are important intracellular sources of IFN- γ during Con A-induced hepatitis in WT mice. Next, we determined whether increased hepatic IFN- γ levels in Con A-treated CCR5 KO mice (Figure 2A) could also be attributed to an increased recruitment of IFN- γ -producing CD4⁺ T cells to the liver of these mice. Indeed, the numbers of IFN- γ -producing CD4⁺ T cells recruited to the liver of CCR5 KO mice were increased by CCR5 deficiency 8 hours after Con A treatment relative to Con A-treated WT mice (Figure 2D). NK cell activation has been reported to regulate T cell responses.^{46,47} However, it was unclear from our current data whether activated NK cells in CCR5 KO mice directly modulate subsequent CD4⁺ T cell responses following Con A administration. Interestingly, we found that NK cell depletion prevented the recruitment of both CD4⁺ T cells (Table 1 and Figure 2E) and IFN- γ -produc-

ing CD4⁺ T cells (Table 1) to the liver of CCR5 KO mice 8 hours following Con A administration.

The proinflammatory effects of IFN- γ during Con A-induced hepatitis are well documented.^{48,49} To characterize further the role of hepatic IFN- γ in the development of severe hepatitis in CCR5 KO mice following Con A administration, CCR5 KO mice were pretreated with a specific anti-mouse IFN- γ mAb before Con A administration. Anti-IFN- γ mAb treatment caused a significant reduction in Con A-mediated liver damage as demonstrated biochemically by lower ALT levels (control Ab + CCR5 KO mice, $12,876 \pm 1136$ U/L, versus anti-mouse IFN- γ Ab + CCR5 KO mice, 129 ± 4 U/L; $P < 0.01$; $n = 4$ or 5 per group; Figure 3A) and improved hepatic histology (Figure 3B). Specifically, liver sections from anti-IFN- γ mAb-treated CCR5 KO mice exhibited minimal hepatocyte damage and hepatocellular necrosis, whereas H&E-

Table 1. Effects of NK Cell Depletion and Cytokine Neutralization on Hepatic CD4⁺ T Cell Recruitment and Activation

Treatment groups	CD4 ⁺ T cells × 10 ⁴	IFN-γ producing CD4 ⁺ T cells × 10 ⁴
Controls	14.71 ± 1.94	8.47 ± 1.12
NK cell depletion	6.91 ± 0.47*	1.97 ± 0.21*
IFN-γ neutralization	6.45 ± 0.24*	4.87 ± 0.57*
IL-4 neutralization	7.34 ± 0.47*	3.91 ± 0.47*

CCR5-deficient mice were injected with one of the following antibodies: control antibody, NK cell depleting, anti-IFN-γ, or anti-IL-4 neutralizing antibodies as described in Materials and Methods. All CCR5-deficient mice were sacrificed at 8 hours after Con A administration. All data (*n* = 4–7) are presented as means ± SEM.

**P* < 0.05 versus controls.

stained liver sections from control Ab-treated CCR5 KO mice demonstrated extensive hepatocyte damage throughout the liver 8 hours following Con A administration (Figure 3B). It is noteworthy that anti-IFN-γ neutralizing mAb treatment of CCR5 KO mice also caused significant reductions in the recruitment of CD4⁺ T cells and IFN-γ-producing CD4⁺ T cells into the livers of these mice following Con A administration (Table 1).

IL-4 Promotes NK Cell IFN-γ Production during Severe Hepatitis in CCR5 KO Mice

IL-4, produced mainly by activated resident hepatic NKT cells, plays a critical role in the development of Con A-induced hepatitis.^{26,33,50} We recently demonstrated that CCR5 deficiency results in enhanced hepatic NKT cell activation and increased numbers of IL-4-producing NKT cells during Con A-induced hepatitis relative to WT mice.⁴ Activated NKT cells, via IFN-γ (but not IL-4) production, are known to rapidly transactivate NK cells to produce IFN-γ.^{43–45} However, a recent report demonstrated that IL-4 administration into mice increased IFN-γ production by NK cells.⁵¹ Therefore, we postulated that augmented IL-4 production by activated hepatic NKT cells during severe Con A-induced hepatitis in CCR5 KO mice might contribute to hepatic NK cell activation (ie, IFN-γ production) in these mice. In agreement with our recent observations,⁴ pretreatment of CCR5 KO mice with neutralizing anti-IL-4 mAb prevented the development of severe hepatitis 8 hours following Con A administration as depicted biochemically by ALT levels (control Ab + CCR5 KO mice, 16,200 ± 445 U/L, versus anti-mouse IL-4 mAb + CCR5 KO mice, 366 ± 55 U/L; *P* < 0.01; *n* = 4 or 5 per group). Anti-IL-4 mAb administration to CCR5 KO mice also caused a significant reduction in hepatic IFN-γ levels 8 hours following Con A administration (Figure 3C). We next determined whether reduced hepatic IFN-γ levels in Con A-treated CCR5 KO mice after anti-IL-4 mAb treatment were due to reduced recruitment of NK cells into the liver of these mice. Indeed, the number of NK cells and IFN-γ-producing NK cells recruited into the liver of CCR5 KO mice was significantly reduced by anti-IL-4 mAb treatment 8 hours post-Con A administration (Figure 3, D–F). Furthermore, anti-IL-4 mAb treatment also caused a significant reduction in NK cell activation in CCR5 KO mice 90 minutes after Con A administration, a time point before significant hepatic NK cell recruitment to the liver has occurred (Figure 1A), as

shown by a decrease in the number of hepatic IFN-γ-producing NK cells in CCR5 KO mice given anti-IL-4 mAb relative to CCR5 KO mice given control Ab at 90 minutes after Con A administration (Figure 3G). Interestingly, anti-IL-4 mAb treatment of CCR5 KO mice also caused significant reductions in the recruitment of CD4⁺ T cells and IFN-γ-producing CD4⁺ T cells in the liver of these mice following Con A administration (Table 1).

The number of IFN-γ-producing NKT cells within the liver of CCR5-deficient mice during Con A-induced hepatitis are similar to WT mice but are significantly higher than that seen in naïve WT or naïve CCR5 KO mice.⁴ Because it is well established that IFN-γ released by activated NKT cells promotes NK cell recruitment and activation,^{43–45} we also examined the effect of anti-IFN-γ mAb treatment on hepatic NK cell recruitment and activation in Con A-treated CCR5 KO mice. Anti-IFN-γ mAb treatment significantly reduced the number of both NK cells and IFN-γ-producing NK cells recruited into the liver of CCR5 KO mice relative to control Ab-treated CCR5 KO mice 8 hours after Con A administration (Figure 4, A and B). Therefore, IFN-γ and IL-4 are both important for NK cell activation and recruitment and the subsequent development of severe hepatitis in CCR5 KO mice after Con A administration. In addition to IFN-γ and IL-4, IL-12 is also a potent activator of hepatic NK cells.^{31,37,39} Therefore, we determined hepatic levels of IL-12 in WT and CCR5 KO mice to assess whether a differential increase in hepatic IL-12 levels in CCR5 KO mice (relative to WT mice) during Con A-induced hepatitis could also potentially promote enhanced hepatic NK cell activation in CCR5 KO mice. However, we observed that Con A treatment did not cause an increase in hepatic IL-12 levels in WT or CCR5 KO mice 8 hours after Con A treatment when compared with naïve WT or naïve CCR5 KO controls. In addition, IL-12 levels in the liver of CCR5 KO mice was not significantly increased relative to WT mice 8 hours after Con A treatment (WT: 5.41 ± 1.16 pg/mg total protein; WT + Con A: 5.24 ± 1.47 pg/mg total protein; CCR5 KO: 6.88 ± 2.12 pg/mg total protein; CCR5 KO + Con A: 8.25 ± 2.17 pg/mg total protein; *n* = 4 or 5 per group).

Another primary function of NK cells is their capacity to exert cytotoxic effects via the FasL pathway.^{52–54} However, we found that the number of FasL-expressing NK cells in the liver of CCR5 KO mice were similar to that observed in WT mice 8 hours after Con A administration (WT + Con A: 6.61 ± 2.70 × 10³; CCR5 KO + Con A:

Figure 3. A: Plasma ALT levels 8 hours after Con A administration in CCR5 KO mice pretreated with anti-IFN- γ mAb (■; $n = 4$) relative to CCR5 KO mice given control Ab (□; $n = 4$). *** $P < 0.001$ versus control Ab-treated CCR5 KO mice. B: Representative H&E staining of liver sections depicting widespread/confluent hepatocellular necrosis (arrows) and inflammatory cell infiltrates throughout the liver in Con A-treated CCR5 KO mice (arrows) relative to patchy hepatocellular necrosis and mild inflammatory cell infiltrates in CCR5 KO mice pretreated with anti-IFN- γ mAb all at 8 hours after Con A treatment. C: Effect of anti-IL-4 mAb treatment on hepatic IFN- γ levels in CCR5 KO 8 hours after Con A administration (■; $n = 4$) relative to CCR5 KO mice given control Ab (□; $n = 4$). * $P < 0.05$ versus control Ab-treated CCR5 KO mice. D: Number of NK cells in the liver 8 hours after Con A administration in CCR5 KO mice pretreated with anti-IL-4 mAb (■; $n = 4$) relative to CCR5 KO mice given control Ab (□; $n = 4$). * $P < 0.05$ versus control Ab-treated CCR5 KO mice. E: Representative FACS histogram depicting reduced NK cell recruitment in the liver of Con A-treated CCR5 KO mice at the 8 hour time point following pretreatment with anti-IL-4 mAb relative to Con A-treated CCR5 KO mice given control Ab. F: Number of IFN- γ -producing NK cells in the liver 8 hours after Con A administration in CCR5 KO mice pretreated with anti-IL-4 mAb (■; $n = 4$) relative to CCR5 KO mice given control Ab (□; $n = 4$). * $P < 0.05$ versus control Ab-treated CCR5 KO mice. G: Number of IFN- γ -producing NK cells in the liver 90 minutes after Con A administration in CCR5 KO mice pretreated with anti-IL-4 mAb (■; $n = 4$) relative to CCR5 KO mice given control Ab (□; $n = 4$). * $P < 0.05$ versus control Ab-treated CCR5 KO.

Figure 4. **A:** Number of NK cells in the liver 8 hours after Con A administration in CCR5 KO mice pretreated with anti-IFN-γ mAb (■; *n* = 4) relative to CCR5 KO mice given control Ab (□; *n* = 4). **P* < 0.05 versus control Ab-treated CCR5 KO mice. **B:** Number of IFN-γ-producing NK cells in the liver 8 hours after Con A administration in CCR5 KO mice pretreated with anti-IFN-γ mAb (■; *n* = 4) relative to CCR5 KO mice given control Ab (□; *n* = 4). **P* < 0.05 versus control Ab-treated CCR5 KO mice.

$7.27 \pm 2.17 \times 10^3$; *n* = 3 per group), potentially excluding the FasL pathway as one of the mechanisms by which NK cells exert effector functions in CCR5 KO mice during Con A-induced hepatitis.

CCL5 Promotes NK Cell Recruitment during Con A-Induced Hepatitis in CCR5 KO Mice

We next examined possible mechanism(s) underlying increased hepatic NK cell recruitment in CCR5 KO mice during Con A-induced hepatitis. CCR5 is known to regulate immune responses by interacting with chemokine ligands, including CCL3 and CCL5.^{1,2} A previous study suggested that CCL5 can promote the chemoattraction and activation of NK cells.⁵⁵ Furthermore, CCR5 deficiency in humans, as well as in experimental animal models of inflammation and infection, is associated with significant increases in tissue levels of CCL5.^{2,56} In this study, we observed that hepatic levels of CCL5 were significantly increased in CCR5-deficient mice, but not WT mice, 8 hours following Con A administration (Figure 5A). However, this effect of CCR5 deficiency on hepatic CCL5 levels was not generalized to other CCR5 ligands,

as Con A-treated CCR5 KO mice did not demonstrate increased hepatic CCL3 levels relative to Con A-treated WT mice (Figure 5B). Our observation is in agreement with a recent report²⁹ that documented enhanced hepatic CCL5 mRNA expression in CCR5 KO mice relative to WT mice following Con A administration. In addition, we have observed by immunohistochemistry (Figure 5C) a marked increase of the expression of CCL5 in the livers of Con A-treated CCR5 KO (relative to Con A-treated WT mice). Specifically, strong CCL5 expression was documented in hepatocytes in CCR5 KO mice (restricted mostly to damaged areas of the liver and around the central veins), but only weak CCL5 expression was noted in WT mice (in a distribution similar to that observed in CCR5 KO mice) 8 hours after Con A administration (Figure 5C). In light of these findings, we next addressed the potential role of CCL5 in the recruitment of NK cells into the liver of CCR5 KO mice during Con A-induced hepatitis. Indeed, in agreement with a recent study,²⁹ treatment of Con A-treated CCR5 KO mice with anti-CCL5 neutralizing mAb significantly reduced hepatic injury (control Ab + CCR5 KO mice, 12790 ± 65 U/L, versus anti-mouse CCL5 mAb + CCR5 KO mice, 336 ± 45 U/L; *P* < 0.01; *n* = 4 or 5 per group) and was accompanied by significant reductions in the recruitment of NK cells (Figure 5D) and IFN-γ-producing NK cells (Figure 5E) to the liver of CCR5 KO mice during Con A-induced hepatitis. Furthermore, we observed that CCL5 levels in the livers of CCR5 KO mice were significantly reduced by anti-IL-4 mAb treatment 8 hours after Con A administration (control IgG + CCR5 KO: 279 ± 65 pg/mg total protein; anti-IL-4 mAb + CCR5 KO: 17 ± 3 pg/mg total protein; *P* < 0.01; *n* = 3 per group).

A Dual CCR1/CCR5 Antagonist Inhibits Severe Hepatitis in Con A-Treated CCR5 KO Mice

Moreno and colleagues²⁹ recently demonstrated that severe hepatitis in CCR5-deficient mice after Con A treatment was associated with enhanced recruitment of CCR1-expressing mononuclear cells into the liver, but the role of CCR1 in the development of severe hepatitis in CCR5 KO mice after Con A treatment was not determined. Therefore, we determined the effect of CCR1 blockade on severe hepatitis in CCR5 KO mice post-Con A administration. We observed that CCR5 deficiency both augmented liver damage (as shown by higher ALT levels) and was associated with an increase in the number of CCR1-expressing NK cells recruited into the livers of these mice relative to that observed in WT controls 8 hours following Con A treatment (Table 2). In addition, treatment of CCR5 KO mice with Met-RANTES, a functional antagonist of the chemokine receptors CCR1 and CCR5,^{4,22} resulted in significant reductions in both the severity of hepatic injury (as shown by lower ALT levels) and the number of CCR1-bearing NK cells recruited into the liver of CCR5 KO mice 8 hours after Con A administration compared with Con A-treated CCR5 KO mice administered PBS (Table 2). The effectiveness of Met-RANTES (a CCR1 and CCR5 antagonist) in ameliorating

severe hepatitis in CCR5 KO mice after Con A treatment suggests that CCR1 is a key receptor mediating the effect of CCL5 in this model. Of note, it has been previously reported that CCL5 can only activate CCR5, and not CCR1, in mice.⁵⁷ However, in light of our current observations, we propose that in the specific setting of CCR5 deficiency, CCL5 is able to use CCR1 as an alternate receptor to mediate hepatic inflammation and injury in mice.

Effect of CCL5 on CCR5-Deficient NK Cell Activation *in Vitro*

Our findings that CCL5 via CCR1 promotes the recruitment of IFN- γ -producing NK cells to the liver of CCR5 KO mice after Con A administration is consistent with CCL5 being associated with Th1 type cellular responses.⁵⁸ Therefore, we performed a series of experiments to determine whether NK cells from CCR5 KO mice demonstrate an enhanced capacity to be activated to produce IFN- γ and additionally whether the activation of CCR5-deficient NK cells could be modulated by CCL5. To do this, splenic NK cells were stimulated *in vitro* with either CCL5 alone or in the presence of recombinant cytokines IL-12 + IL-18 (as described in Materials and Methods), and intracellular NK cell IFN- γ production was determined by flow cytometry. The combination of IL-12 and IL-18 is known to promote NK cell activation.^{59–61} In a pilot experiment we determined concentrations of IL-12 + IL-18 *in vitro* that produced subthreshold activation of WT splenic NK cells to produce IFN- γ and used these concentrations (see Materials and Methods) for our formal studies of NK cell activation *in vitro* for WT and CCR5-deficient NK cells (Table 3). In contrast to WT NK cells, stimulation of CCR5-deficient splenic NK cells with IL-12 + IL-18 or IL-12 + IL-18 + CCL5 (but not CCL5 alone) caused a significant increase in the number of IFN- γ -producing NK cells relative to unstimulated (or CCL5 alone stimulated) CCR5-deficient splenic NK cells (Table 3). Moreover, CCR5-deficient splenic NK cell mean fluorescence (ie, a reflection of the amount of IFN- γ produced per cell and expressed as arbitrary units) was also significantly increased by IL-12 + IL-18 treatment and was not influenced by the presence of CCL5 (unstimulated, 35.7 ± 5.8 , versus CCL5 alone, 30.2 ± 2.5 , versus IL-12 + IL-18 stimulated: 136.5 ± 7.4 versus IL-12 +

Figure 5. CCL5 promotes NK cell recruitment into the liver of CCR5 KO mice. **A:** Enzyme-linked immunosorbent assay determination of hepatic levels of CCL5 in WT ($\square$; $n = 5$) and CCR5 KO mice ($\blacksquare$; $n = 4$) 8 hours after Con A-induced hepatitis. * $P < 0.05$ versus all other groups. **B:** Hepatic levels of CCL3 in WT ($\square$; $n = 5$) and CCR5 KO mice ($\blacksquare$; $n = 4$) as determined by enzyme-linked immunosorbent assay at 8 hours after Con A-induced hepatitis. # $P < 0.05$ versus their respective naive groups. **C:** Immunohistochemical localization of CCL5 in paraffin-embedded liver sections from WT and CCR5 KO mice 8 hours after Con A administration (magnification, $\times 40$). **D:** Number of NK cells in the liver 8 hours after Con A administration in CCR5 KO mice pretreated with anti-CCL5 mAb ($\blacksquare$; $n = 4$) relative to CCR5 KO mice given control Ab ($\blacksquare$; $n = 4$). * $P < 0.05$ versus control Ab-treated CCR5 KO mice. **E:** Number of IFN- γ -producing NK cells in the liver 8 hours after Con A administration in CCR5 KO mice pretreated with anti-CCL5 mAb ($\blacksquare$; $n = 4$) relative to CCR5 KO mice given control Ab ($\blacksquare$; $n = 4$). * $P < 0.05$ versus control Ab-treated CCR5 KO mice.

Table 2. Effects of Met-RANTES Treatment on ALT Levels and Hepatic Recruitment of CCR1-Positive NK Cells in Con A-Treated CCR5 KO Mice

Treatment groups	ALT (U/L)	CCR1-positive NK cells $\times 10^5$
PBS + WT	612 $\pm$ 58.1	0.31 $\pm$ 0.05
PBS + CCR5 KO	13,020 $\pm$ 112*	0.71 $\pm$ 0.10*
Met-RANTES + CCR5 KO	454 $\pm$ 32 [#]	0.35 $\pm$ 0.05 [#]

CCR5 KO mice were injected intravenously with either PBS or Met-RANTES (30 μ g/mouse) as described in Materials and Methods. In addition, WT mice were injected with PBS. All mice were sacrificed at 8 hours after Con A administration for determination of plasma ALT levels and the number of CCR1-expressing NK cells in the liver as described in Materials and Methods. All data ($n = 4$ per group) are presented as means $\pm$ SEM.

* $P < 0.05$ versus WT mice.

[#] $P < 0.05$ versus PBS/CCR5 KO mice.

IL-18 + CCL5: 160.8 ± 18.1 ; $n = 3$ per group; * $P < 0.001$ versus unstimulated and CCL5 alone). These results suggest that CCR5-deficient NK cells are highly sensitive to cytokine-driven activation because sub-threshold concentrations of the cytokine combination of IL-12 and IL-18 were effective in stimulating CCR5-deficient NK cells, but not WT NK cells, to produce IFN- γ . In addition, our data also suggest that this enhanced cytokine-driven production of IFN- γ by CCR5-deficient NK cells is not modulated by CCL5 acting through CCR1.

Characterization of NK Cell Activation in Vivo in CCR5 KO Mice

Our observation that hepatic NK cells in CCR5 KO but not WT mice exert proinflammatory effects after Con A treatment were unexpected. Importantly, NK cell development in CCR5 KO mice is normal relative to WT mice (Table 4). However, given our *in vitro* findings demonstrating enhanced cytokine-driven IFN- γ production in CCR5-deficient splenic NK cells versus WT controls, we performed additional experiments to determine whether direct activation of hepatic NK cells *in vivo* would also produce similar differences between CCR5-deficient and WT NK cell IFN- γ production. To examine this, polyI:C (a direct and potent activator of hepatic NK cells)^{37,38} was administered intraperitoneally to CCR5 KO and WT mice, and the number of hepatic IFN- γ -producing NK cells determined by intracellular staining. Interestingly, we found that the numbers of IFN- γ -producing NK cells within the livers of CCR5 KO mice were about twofold higher than that observed in WT mice at 16 hours after polyI:C administration (WT + polyI:C: $1.41 \pm 0.31 \times 10^4$ cells/liver versus CCR5 KO + poly I:C: $2.63 \pm 0.14 \times 10^4$ cells/liver; * $P < 0.05$; $n = 3$ per group). Therefore, hepatic NK cells are more readily activated to produce IFN- γ by direct stimulation by polyI:C in the setting of CCR5 deficiency *in vivo* compared with WT mice.

Discussion

NK cells, a unique lymphoid cell lineage that differs from T cells in that they do not rearrange the TCR receptor genes, are thymus-independent and do not require pre-immunization to exert effector functions.^{60,62,63} NK cells are abundant in the circulating blood, spleen, and liver^{60,62–64} and are well suited to mediate a rapid first line of defense against most pathogens, thus reflecting their innate nature.^{60,62,63} Recently, the contribution of NK cells to host defense has been shown to extend beyond their traditional role as killers of pathogens in that they can promote tissue injury during experimental autoimmune diseases including diabetes, because disease severity is decreased by NK cell depletion.⁶⁰ Although the liver is enriched in NK cells, the processes underlying NK cell trafficking to the liver and cellular activation within the liver during inflammation remain poorly defined.⁵⁵ Specifically, the role of NK cells in the pathogenesis of various T cell-mediated liver diseases (including hepatitis B and C and autoimmune hepatitis) is unclear, although increased NK cell infiltrates are observed in liver biopsies of patients with these liver diseases.^{30–32}

The Con A-induced hepatitis model mimics many aspects of human T cell-mediated liver diseases. It is widely accepted that NK cells do not contribute to the pathogenesis of Con A-induced hepatitis.^{17,26,33} In agreement with these previous reports,^{17,26,33} depletion of NK cells in WT mice did not influence liver injury due to Con A treatment, suggesting that NK cells are not typically effector cells in this model of hepatitis. However, in contrast to WT mice, CCR5 deficiency was associated with the increased recruitment of NK cells to the liver at the 8-hour time point post-Con A, and depletion of NK cells in CCR5 KO mice almost completely prevented liver injury. Therefore, in the specific setting of CCR5 deficiency, NK cells become key effector cells in the development of T cell-mediated hepatitis. Although a recent study²⁹ docu-

Table 3. Effects of Chemokine and Cytokine Stimulation on Intracellular NK Cell IFN- γ Production

Treatment groups	WT	CCR5 KO
Unstimulated	17.70 $\pm$ 0.37 $\times 10^2$	14.50 $\pm$ 0.43 $\times 10^2$
CCL5	20.80 $\pm$ 0.23 $\times 10^2$	11.80 $\pm$ 0.43 $\times 10^2$
IL-12 + IL-18	21.21 $\pm$ 0.23 $\times 10^2$	87.60 $\pm$ 1.65 $\times 10^{2*}$
IL-12 + IL-18 + CCL5	22.30 $\pm$ 0.76 $\times 10^2$	99.20 $\pm$ 2.17 $\times 10^{2*}$

Splenocytes from naïve WT or naïve CCR5 KO mice were stimulated for 16 hours with chemokine/cytokines (as shown above). The number of intracellular NK cell IFN- γ expression was determined by flow cytometry. All data ($n = 3$) are presented as means $\pm$ SEM.

* $P < 0.05$ versus CCL5-stimulated CCR5-deficient cells and unstimulated CCR5-deficient cells.

Table 4. Percent K Cells in Naïve WT and CCR5 KO Mice

Tissue	WT	CCR5 KO
Peripheral blood	11.10 ± 1.89	14.30 ± 1.16
Liver	24.20 ± 2.74	23.30 ± 3.03

NK cells were isolated from peripheral blood or liver of naïve WT and CCR5 KO mice and stained for NK cells as described in Materials and Methods. All data ($n = 4$ per group) are presented as means ± SEM.

mented increased recruitment of mononuclear cells (specifically CD4⁺ T and NKT cells as well as macrophages) into the liver of CCR5 KO mice following Con A administration, the authors did not address the specific role of NK cells. Therefore, our observation is unique and represents the first study to identify the central effector role for NK cells in the development of severe T cell-mediated hepatitis in the setting of CCR5 deficiency after Con A administration. Severe hepatitis in CCR5 KO mice at 8 hours after Con A treatment was also associated with increased hepatic NK cell activation (ie, increased IFN- γ production). NK cells are known to exert their effector functions at least in part via increased IFN- γ production during the inflammatory response,^{44,45,60,62} and previous reports have shown that IFN- γ exerts proinflammatory effects during Con A-induced hepatitis by direct cytotoxic effects on hepatocytes.^{48,49} Therefore, we propose that NK cells, via increased IFN- γ production, promote the development of severe hepatitis in the setting of CCR5 deficiency post-Con A, because treatment of CCR5 KO mice with anti-IFN- γ mAb strikingly reduced the severity of hepatitis at 8 hours after Con A injection. Of note, our observation of increased hepatic levels of IFN- γ in CCR5 KO relative to WT mice during Con A-induced hepatitis differs from a recent study by Moreno et al,²⁹ which suggested that the serum level of IFN- γ is not increased by CCR5 deficiency post-Con A. These different results highlight the importance of measuring tissue levels of cytokines/chemokines, because discussing mechanisms based on plasma cytokine/chemokine levels can lead potentially to misleading conclusions as documented previously in the Con A model.²⁷

NKT and NK cells (via cytokine production) have been reported to influence the development of adaptive immunity by regulating CD4⁺ T cell responses during inflammation.^{44,46,47} To this effect, the present study also assessed whether cytokine(s) produced by activated hepatic NKT cells and NK cells could modulate the recruitment of CD4⁺ T cells in CCR5-deficient mice after Con A administration. Moreover, previous studies have demonstrated that CD4⁺ T cells are key effector cells in Con A-induced hepatitis, because athymic mice or mice treated with CD4⁺ T cell mAb do not develop liver injury.^{15,20,26} Along these lines, treatment of CCR5 KO mice with anti-IL-4 mAb, anti-IFN- γ mAb, or asialo-GM1 Ab (to deplete NK cells) caused significant reductions in the hepatic recruitment of CD4⁺ T cells and IFN- γ -producing CD4⁺ T cells in CCR5 KO mice 8 hours post-Con A treatment. Therefore, CCR5 deficiency is associated with the increased hepatic recruitment and activation of NK cells, which in turn drive the subsequent recruitment of

CD4⁺ T cells into the liver after Con A injection. However, it is important to emphasize that CD4⁺ T cells could be recruited into the livers of CCR5 KO mice during Con A-induced hepatitis by an NK cell-independent mechanism, because NK cell depletion did not completely prevent (~60% reduction) the hepatic recruitment of CD4⁺ T cells in Con A-treated CCR5 KO mice. In view of the fact that Met-RANTES is also effective in reducing Con A-induced hepatitis in WT mice by suppressing the recruitment of IFN- γ -producing CD4⁺ T cells (but not NK cells) as we previously demonstrated,²² we propose that both NK cell-dependent and NK cell-independent mechanisms underlie the recruitment of CD4⁺ T cells into the livers of CCR5 KO mice after Con A administration. In contrast, CD4⁺ T cells are recruited into the livers of WT mice during Con A-induced hepatitis by a mechanism independent of NK cells.

Next we investigated the potential mechanisms whereby NK cells exert their proinflammatory effector functions in CCR5 KO mice, but not in WT mice, post-Con A. A distinct feature of NK cells is that they can be stimulated swiftly in an antigen-independent manner by cytokines (including IFN- γ and possibly IL-4)^{55,60,62,63,65} to exert potent effector functions, in part via elevated IFN- γ production. In recent years it has become increasingly evident that chemokines are not just chemoattractants, but they can also modulate cell effector functions independent of leukocyte recruitment.³⁶ Specifically, we recently demonstrated an increase in the number of IL-4-producing hepatic NKT cells in CCR5 KO mice, but not WT mice, very early (ie, 90 minutes) post-Con A.⁴ In contrast, the number of IFN- γ -producing hepatic NKT cells in CCR5 KO mice were increased to levels similar to those found in WT mice at 90 minutes after Con A injection.⁴ Therefore, we postulated that CCR5-deficient hepatic NK cells, but not WT hepatic NK cells, might be more sensitive to the activating effects of IL-4 and IFN- γ released within the hepatic milieu during Con A-induced hepatitis. Indeed, we observed that NK cell activation in CCR5 KO mice was an early event occurring during Con A-induced hepatitis, as shown by increased numbers of IFN- γ -producing hepatic NK cells in CCR5 KO mice, but not WT mice, 90 minutes after Con A administration (a time point before which significant hepatic NK cell recruitment has occurred and hepatic levels of CCL5 are not increased). Furthermore, anti-IL-4 mAb treatment of CCR5 KO mice caused a significant reduction in hepatic NK cell activation (ie, number of IFN- γ -producing NK cells) 90 minutes after Con A treatment relative to CCR5 KO mice given control Ab. Support for our data suggesting that IL-4 can activate NK cells derives from two recent reports suggesting that IL-4 can directly promote NK cell IFN- γ release.^{51,65} Therefore, augmented hepatic NKT cell IL-4 production in CCR5 KO mice after Con A treatment in conjunction with NKT cell IFN- γ release may underlie the early (ie, 90 minutes) hepatic NK cell activation observed in CCR5 KO mice, but not WT mice, after Con A injection. Moreover, our findings that either neutralization of IL-4 or IFN- γ separately prevents subsequent NK cell activation and recruitment 8 hours post-Con A suggests that increased levels of both cytokines

are necessary to be present within the liver to drive subsequent NK cell activation and recruitment in the setting of CCR5 deficiency.

In addition to enhanced NK cell activation by IFN- γ and IL-4 within the livers of CCR5 KO mice, it seemed also possible that CCR5-deficient hepatic NK cells may be inherently more sensitive to direct activation compared with WT hepatic NK cells. Interestingly, we found that polyI:C administration (a specific activator for NK cells) *in vivo* increased the number of IFN- γ -producing NK cells in the livers of CCR5 KO mice relative to that observed in WT mice (without altering the number of NK cells recruited into the liver of CCR5 KO compared with WT mice). PolyI:C is known to activate hepatic NK cells independent of the actions of IL-4 and IFN- γ but requiring IL-12.³⁹ Moreover, our *in vitro* data are consistent with our polyI:C observations and strongly suggest that CCR5-deficient NK cells are inherently more sensitive to cytokine-driven activation (ie, IL-12 + IL-18 in our *in vitro* studies) to produce IFN- γ than are WT NK cells. The cause of this increased sensitivity of CCR5-deficient hepatic NK cells to cytokine-driven activation remains unclear but may be related to altered activation receptor expression on these cells. The stimulation of NK cells by polyI:C is known to be IL-12-dependent.³⁹ In addition, both IL-12 and IL-18 exert proinflammatory effects during Con A-induced hepatitis.^{66,67} In light of our *in vitro* data, we speculate that IL-12 and IL-18 produced within the liver may promote enhanced NK cell activation in CCR5 KO mice during Con A-induced hepatitis.

Our studies indicate that hepatic NK cell activation and recruitment is critical for the development of severe hepatitis in CCR5 KO mice following Con A administration. A key issue that remained to be addressed is how CCR5 deficiency promotes increased NK cell trafficking into the liver at 8 hours (but not at 90 minutes) after Con A treatment. Chemokines have traditionally been felt to act as pure chemoattractants for leukocytes. Because the CCR5 ligands (CCL5 and CCL3) are known to promote NK cell recruitment during inflammation,^{55,68} we examined the role of these ligands in NK cell recruitment into the liver in CCR5 KO mice during Con A-induced hepatitis. We observed that CCL5 levels in the liver were significantly increased only in the CCR5 KO, but not WT mice, 8 hours post-Con A. Moreover, immunohistochemistry demonstrated that hepatocytes seemed to be the main source of CCL5 production in both CCR5 KO and WT mice; however, CCL5 expression was markedly increased in the livers of CCR5 KO versus WT mice and seemed to be confined mainly to damaged areas of the liver and around central veins. Interestingly, this effect of CCR5 deficiency on hepatic CCL5 levels was not generalized to all CCR5 ligands, because hepatic levels of CCL3 in CCR5 KO mice were not significantly different from those observed in WT mice 8 hours after Con A treatment. Therefore, our results suggest that CCR5 signaling negatively regulates CCL5 production during Con A-induced hepatitis in WT mice, a finding that is comparable with observations made in other experimental models of inflammation.^{2,56} A previous study suggested that CCL5 interacts exclusively with the chemokine receptor

Figure 6. Schematic summary of events of how NK cells may promote severe hepatitis in CCR5-deficient mice following Con A administration. IL-4, in conjunction with IFN- γ , produced by activated hepatic NKT cells directly promotes early (ie, 90 minutes post-Con A) NK cell activation after Con A treatment (as reflected by increased NK cell IFN- γ production) and then indirectly mediate later (ie, 8 hours post-Con A) hepatic NK cell recruitment to and activation within the liver mediated by increased hepatic levels of hepatocyte-derived CCL5 acting via CCR1 expressed on NK cells. Activation of antigen-presenting cells during the development of Con A hepatitis may result in the increased production and release of IL-12 and IL-18 within the liver, and both of these cytokines may subsequently promote further NK cell activation and IFN- γ production within the liver. It is the combination of these events that drives the development of severe liver injury in CCR5-deficient mice following Con A administration.

CCR5, but not CCR1, during inflammatory responses in mice.⁵⁷ However, we propose that in the specific setting of CCR5 deficiency, CCL5 interacts with CCR1 to promote NK cell recruitment into the livers of mice after Con A treatment. This suggestion is based on our observation that Met-RANTES (a CCR1 and CCR5 antagonist) treatment of CCR5 KO mice ameliorated hepatitis and prevented the recruitment of CCR1-expressing NK cells into the livers of these mice after Con A administration. Moreover, anti-CCL5 Ab treatment of CCR5 KO mice almost completely prevented Con A-induced liver injury and was accompanied by a significant reduction in hepatic NK cell recruitment and activation. Interestingly, our *in vitro* data indicate that enhanced activation of NK cells in CCR5 KO mice is not modulated by CCL5, suggesting that CCR1 activation by CCL5 is most important for NK cell recruitment to the liver and not the subsequent activation of these cells within the liver. It is likely that in CCR5 KO mice the activating effects of increased numbers of IL-4-producing NKT cells, coupled with IFN- γ released from NKT cells, may explain the observed increase in hepatic CCL5 levels in CCR5 KO mice, but not WT mice, 8 hours after Con A injection. This suggestion is based on our finding that treatment of CCR5 KO mice with a neutralizing IL-4 mAb significantly decreased hepatic levels of CCL5 in these mice at 8 hours after Con A injection. In summary, our findings suggest that IL-4 and IFN- γ released from activated hepatic NKT cells might drive early NK cell activation via CCL5-independent pathways and subsequent later NK cell recruitment to the liver via CCL5-dependent pathways in the setting of CCR5 deficiency after Con A injection (Figure 6).

In summary, the present study demonstrates that NK cells, which normally have a “bystander” role during Con A-induced hepatitis, become major proinflammatory ef-

factor cells in the specific setting of CCR5 deficiency. These findings demonstrate that in the absence of a given chemokine receptor, innate cellular immune responses can be directly altered and, moreover, alternate chemokine receptors can be used during the innate immune response with unforeseen consequences—consequences that should be considered in the future development of chemokine receptor blockers as clinical therapeutics.

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