Lack of Chemokine Receptor CCR5 Promotes Murine Fulminant Liver Failure by Preventing the Apoptosis of Activated CD1d-Restricted NKT Cells[1]

Maureen N. Ajuebor,* Alex I. Aspinall,* Feng Zhou,* Tai Le,* Yang Yang,† Stefan J. Urbanski,*‡ Stéphané Sidobre,§ Mitchell Kronenberg,§ Cory M. Hogaboam,¶ and Mark G. Swain[2]*

Fulminant liver failure (FLF) consists of a cascade of events beginning with a presumed uncontrolled systemic activation of the immune system. The etiology of FLF remains undefined. In this study, we demonstrate that CCR5 deficiency promotes the development of acute FLF in mice following Con A administration by preventing activated hepatic CD1d-restricted NKT cells (but not conventional T cells) from dying from activation-induced apoptosis. The resistance of CCR5-deficient NKT cells from activation-induced apoptosis following Con A administration is not due to a defective Fas-driven death pathway. Moreover, FLF in CCR5-deficient mice also correlated with hepatic CCR5-deficient NKT cells, producing more IL-4, but not IFN-γ, relative to wild-type NKT cells. Furthermore, FLF in these mice was abolished by IL-4 mAb or NK1.1 mAb treatment. We propose that CCR5 deficiency may predispose individuals to the development of FLF by preventing hepatic NKT cell apoptosis and by regulating NKT cell function, establishing a novel role for CCR5 in the development of this catastrophic liver disease that is independent of leukocyte recruitment. *The Journal of Immunology,* **2005, 174: 8027–8037.**

Fulminant liver failure (FLF)[3] is a devastating liver disease that is associated with significant mortality (40–80%) worldwide (1–3). The incidence of FLF has increased in the last decade accounting for >2000 deaths annually in the United States alone (2) and represents the reason for 11 and 30% of all liver transplants in Europe and the United States, respectively (1–3). FLF is a clinical syndrome that is characterized by the sudden onset of severe acute hepatitis with associated symptoms, including jaundice and hepatic encephalopathy in a patient with no previous history of liver disease (2, 3). Viruses, drugs (such as acetaminophen), and toxins have all been identified as trigger factors of FLF (2, 3); however, in many patients (~20%), the etiology of FLF remains unknown (1). Although complex events are likely involved in the pathogenesis of FLF, current theories suggests that regardless of the etiology of FLF, an uncontrolled systemic activation of the immune system as an early initiating event (1–3). Despite today's advanced medical management, the factors leading to the development of systemic immune activation and ultimately FLF are poorly understood.

CCR5 is a CC chemokine receptor that is expressed on various cell types, including NKT and $CD4^+$ T cells (4, 5). A number of studies have suggested a role for CCR5 in the progression of liver diseases (6–8). Although increased hepatic expression of CCR5 ligands (CCL5, CCL4, and CCL3) are observed in biopsies from patients with FLF (9), the contribution of CCR5 ligands or CCR5 to the development of FLF remains unknown. In the present study, we have determined the effects of CCR5 deficiency in murine fulminant hepatitis induced by Con A administration. Con A-induced fulminant hepatitis mimics many aspects of human FLF, including a) severe acute hepatitis (10–12), b) elevated hepatic chemokine (CCL2, CCL5, CCL4, and CCL3) levels (9, 10, 13), c) TNF-α and Fas-driven hepatocyte death (14–16), and d) systemic immune activation and infiltration of the liver by activated T cells (10–12). The role of CCR5 was initially thought to be restricted to leukocyte recruitment. However, in the present study, we report that CCR5 deficiency promotes murine FLF following Con A administration by preventing NKT cell apoptosis, as well as by regulating NKT cell function, and establishes a new role for CCR5 in the development of liver diseases that is independent of leukocyte recruitment.

Materials and Methods

Mice

Male B6129PF2 mice and *CCR5*-deficient mice (B6129PF2 background) ages 8–10 wk were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions and were 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 conformed to the guidelines established by the Canadian Council on Animal Care.

*Gastrointestinal Research Group, †Diabetes and Endocrine Research Group, and ‡Department of Histopathology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada; §Division of Developmental Immunology, La Jolla Institute For Allergy and Immunology, San Diego, CA 92121; and ¶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 Northwest, Calgary, Alberta, T2N 4N1 Canada. E-mail address: swain@ucalgary.ca

[3] Abbreviations used in this paper: FLF, fulminant liver failure; WT, wild type; ALT, alanine aminotransferase; α-GalCer, α-galactosylceramide; FasL, Fas ligand; AICD, activation-induced cell death.

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Con A hepatitis model

Con A-induced hepatitis is widely used as an animal model of fulminant hepatitis (12, 13, 17, 18). *CCR5*-deficient mice and corresponding wild-type (WT) mice were injected i.v. with a single dose of freshly prepared Con A (13.5 mg/kg; Sigma-Aldrich) reconstituted in sterile PBS. At 90 min and 8 h after Con A administration and under halothane anesthesia, blood was collected for measurement of plasma alanine aminotransferase (ALT) levels (commercial kit; Sigma-Aldrich), and livers were perfused with ice-cold sterile PBS to remove blood elements. Liver sections were then processed and stained with H&E, according to standard protocols for histological evaluation of liver injury. Liver damage was evaluated histologically by light microscopy in a blinded fashion by a histopathologist (S.J.U.). Liver damage was semiquantitatively scored for inflammation and damage using the following criteria (none denoted as grade 0; mild denoted as grade 1 in which hepatocyte death is <20%; moderate denoted as grade 2 in which hepatocyte death is between 20 and 50%; severe denoted as grade 3 in which hepatocyte damage is >50%). For Ab-blocking experiments, CCR5-deficient mice received a single i.v. injection of anti-NK1.1 mAb (0.2 mg/mouse; clone PK136; BD Pharmingen), IL-4 mAb (0.3 mg/mouse; clone 11B11; BD Pharmingen), or corresponding isotype controls (BD Pharmingen) 24 h before Con A administration, and all mice were sacrificed 8 h post-Con A treatment.

Flow cytometry

Hepatic lymphocytes were isolated as described previously (13, 19). For staining of NKT cells, isolated lymphocytes were preincubated with anti-mouse CD16/32 (clone 2.4G2) mAb (BD Pharmingen) to block FcγRs and then incubated simultaneously with PE-labeled CD1d-αGalCer tetramers and PerCP-labeled CD3ϵ mAb (clone 145-2C11; BD Pharmingen) or FITC-conjugated anti-$\alpha\beta$TCR (clone H57-597; BD Pharmingen), as described previously (20, 21). Three-color staining was used to assess CCR5$^+$ and cytokine (IL-4 or IFN-γ)-producing CD1d-tetramer$^+$ NKT cells. Briefly, CD1d-tetramer$^+$ NKT cells were permeabilized with Cytofix/Cytoperm plus (13, 19, 21) and stained using either FITC-labeled CCR5 mAb (C34-3448), FITC-labeled IL-4 mAb (clone IIB11), or FITC-labeled IFN-γ mAb (clone XMG1.2), according to the manufacturer's instructions (19, 22). For FACS analysis, the lymphocyte population was gated using forward and side scatter characteristics and analyzed using CellQuest software (BD Biosciences).

Apoptosis assay

CD1d-tetramer$^+$ NKT cells were studied for apoptosis using a) an annexin V-FITC Apoptosis kit and b) FITC-conjugated anti-active caspase-3 Ab, according to the manufacturer's instructions (BD Pharmingen). Briefly, isolated tetramer$^+$ NKT cells were stained extracellularly with annexin V as recommended by the manufacturer (BD Pharmingen). For active caspase-3 staining, tetramer$^+$ NKT cells were fixed and permeabilized with Cytofix/Cytoperm (19, 21) and then stained with FITC-conjugated anti-active caspase-3 mAb. All cells were analyzed by flow cytometry.

Stimulation of splenic NKT cells

Splenocytes were isolated from both naive WT and *CCR5*-deficient mice by conventional methods. Freshly isolated splenocytes were enriched for CD4$^+$ T cells and CD4/CD8 double negative T cells using a CD4$^+$ T cell isolation kit (Miltenyi Biotech). Enriched lymphocytes (5×10^6 cells/well) were stimulated with Con A (20 μg/ml), immobilized anti-CD3 mAb (10 μg/ml), α-galactosylceramide (α-GalCer) (100 ng/ml), rIL-4 (20 ng/ml; R&D Systems), or purified Fas agonistic mAb (1 μg/ml; clone Jo2; BD Pharmingen) for 5 h and then stained for NKT cells as described above. Splenic IL-4-producing CD1d$^+$tetramer$^+$ NKT cells and splenic annexin V$^+$ CD1d-tetramer$^+$ NKT cells were determined as described above. In addition, Fas ligand (FasL) and Fas expression on CD1d-tetramer$^+$ NKT cells were determined using FasL mAb (Kay-10; BD Pharmingen) and Fas mAb (clone Jo2; BD Pharmingen), respectively.

Statistics analysis

All data are shown as mean ± SEM. For comparisons of means between 2 experimental groups a Student unpaired t test was used. Comparison among three or more experimental groups was performed using a one-way ANOVA, followed by either Dunnett's multiple comparison test or Newman-Kuels post hoc test. A value of p [lteq] 0.05 was considered significant.

Results

CCR5 deficiency induces acute FLF

Intravenous injection of a single dose of Con A (13.5 mg/kg) into *CCR5*-deficient mice was associated with the development of FLF as we observed a 50% mortality (three of six mice died) in CCR5-deficient mice within 8 h of Con A administration, whereas all WT mice survived Con A treatment (Fig. 1). The remaining three *CCR5*-deficient mice appeared extremely ill, resulting in termination of the experiment at 8 h. In the *CCR5* gene-deficient mice that survived, we observed markedly exacerbated hepatic injury at 8 h following Con A injection, relative to WT controls, as demonstrated biochemically by a striking augmentation in plasma ALT levels (WT mice, 2,004 ± 715 U/ml vs *CCR5* gene-deficient mice, 22,623 ± 1,365 U/ml; $p < 0.001$; $n = 3$–5/group; Fig. 1*a*). In agreement with biochemical findings, H&E staining of liver sections from Con A-treated *CCR5* gene-deficient mice demonstrated a grade 2 liver damage in which hepatocyte damage was >25% and hepatocellular necrosis of random distribution was observed throughout the liver at 8 h following Con A administration (Fig. 1*b*). In contrast, livers from WT mice exhibited tiny foci of hepatocellular necrosis and grade 1 liver damage in which hepatocyte damage was <10% at 8 h after Con A treatment (Fig. 1*b*). The high mortality in *CCR5* gene-deficient mice at the 8-h time point after Con A treatment precluded us from continuing our experiments at this time point. Therefore, subsequent experiments were conducted at the 90 min post-Con A treatment. Moreover, higher ALT levels in *CCR5* gene-deficient mice at 90 min after Con A administration relative to WT mice (Fig. 1) suggested that events

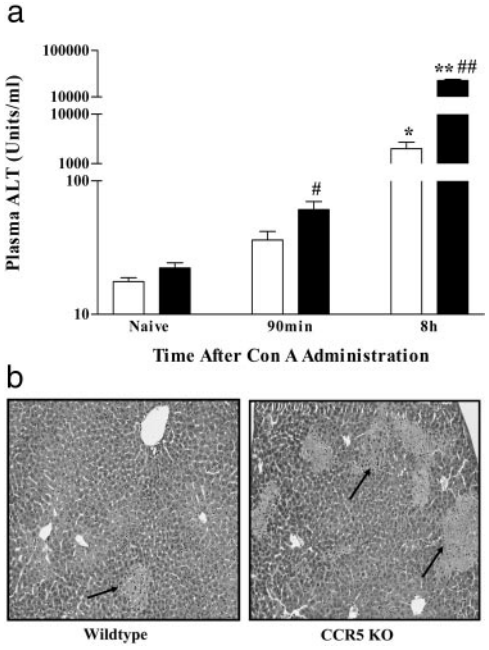

FIGURE 1. *CCR5* deficiency promotes the development of FLF in Con A-treated mice. *a*, ALT levels were determined in CCR5 gene-deficient (■; $n = 3$–5) or WT (□; $n = 4$–6) mice injected with Con A (13.5 mg/kg) or left untreated (naive); *, $p < 0.05$ vs naive WT mice, **, $p < 0.01$ vs naive *CCR5* gene-deficient mice; #, $p < 0.05$ vs Con A-treated WT mice (90 min), ##, $p < 0.01$ vs Con A-treated WT mice (8 h). *b*, Representative H&E staining of liver sections showing confluent hepatocellular necrosis, and hepatocyte damage > 25% could be seen in the liver in Con A-treated *CCR5* gene-deficient mice at 8 h, whereas livers from WT mice exhibited limited foci of hepatocellular necrosis, hepatocyte damage not >10%, and mild inflammatory cell infiltrates at 8 h after Con A treatment.

occurring as early as 90 min may be driving the subsequent development of FLF.

Con A-mediated depletion of CD1d-restricted NKT cells

We next addressed the potential mechanisms underlying the development of FLF in *CCR5*-deficient mice following Con A injection. Con A administration caused a significant and rapid loss of CD1d-tetramer$^+$ NKT cells in the liver within 90 min of injection, which persisted up to 8 h post-Con A administration (Fig. 2, *a* and *b*). Hepatic NKT cell disappearance after Con A treatment is not simply due to a down-regulation of NK1.1 expression on NKT cells (23) because the CD1d-αGalCer tetramer used in our study identifies CD1d-αGalCer-restricted NKT cells regardless of the expression of NK cell surface markers (20). Therefore, our findings are consistent with previous studies (11, 24), suggesting that activation-induced cell death (AICD) may underlie this rapid

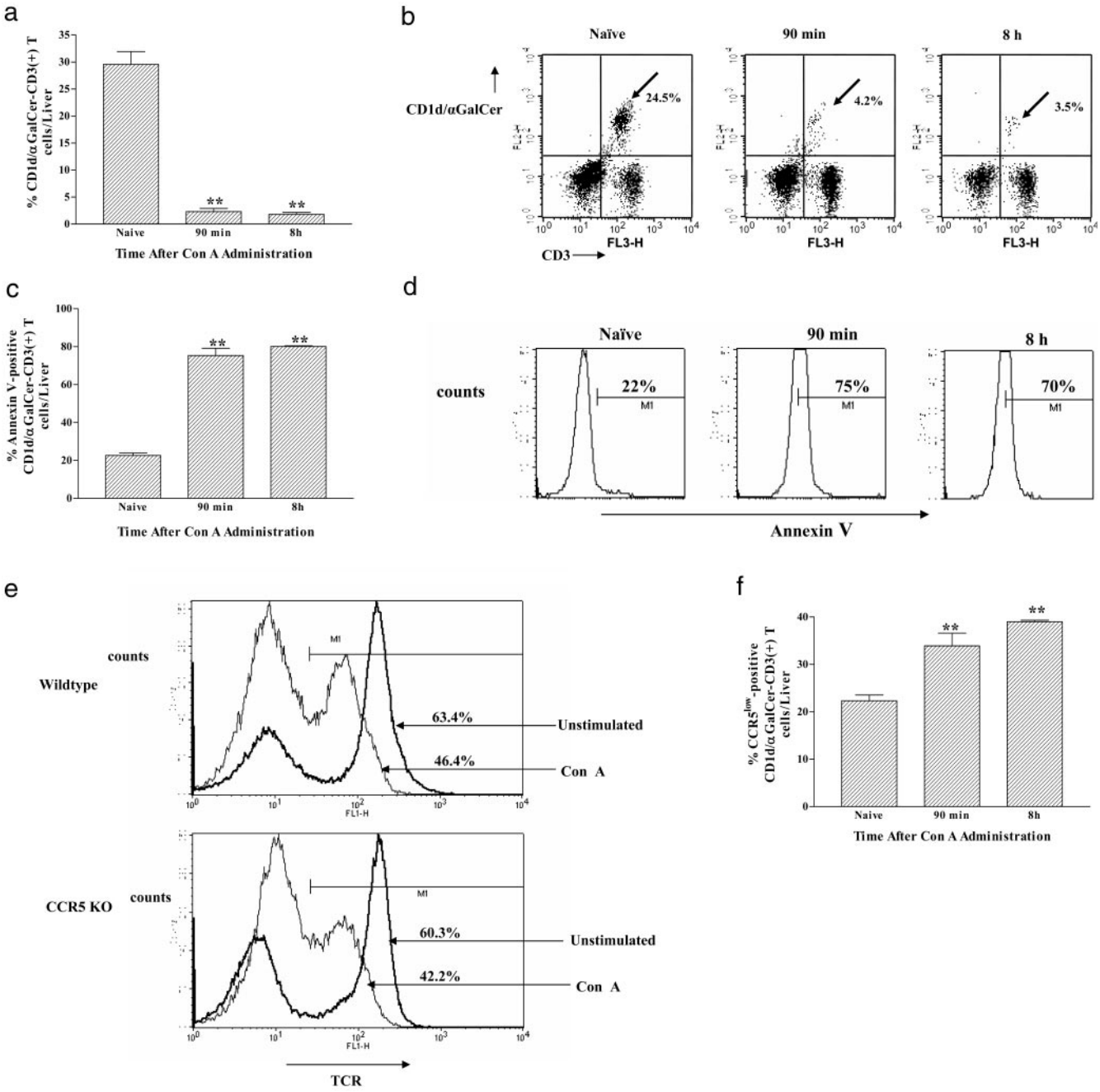

FIGURE 2. Analysis of hepatic CD1d-tetramer$^+$ NKT cells in WT mice during Con A-induced fulminant hepatitis. *a*, Reduced number of CD1d-tetramer$^+$ NKT cells in the liver of Con A-treated WT mice ($n = 4$–7); **, $p < 0.01$ vs naive mice. *b*, A representative FACS dot plot depicting the loss of CD1d-tetramer$^+$ NKT cells (arrows in *upper right quadrant*) in the liver after Con A treatment of WT mice vs naive mice. *c*, Increased annexin V expression on CD1d-tetramer$^+$ NKT cells in the liver of Con A-treated WT mice ($n = 5$); **, $p < 0.01$ vs naive mice. *d*, A representative FACS profile of annexin V expressing CD1d-tetramer$^+$ NKT cells in the liver of naive WT mice 90 min and 8 h after Con A treatment of WT mice. *e*, A representative FACS profile depicting the down-regulation of TCR on splenic CD1d-tetramer$^+$ NKT cells from naive WT mice (*top panel*) and CCR5-deficient mice (*bottom panel*) after stimulation with Con A (20 μg/ml) in vitro for 5 h. *f*, Enrichment of CCR5^{low}-bearing CD1d-tetramer$^+$ NKT cells in the liver of Con A-treated WT mice ($n = 4$–7); **, $p < 0.01$ vs naive mice.

depletion of NKT cells after Con A treatment because hepatic NKT cells isolated from Con A-treated mice showed significantly increased annexin V expression relative to those obtained from naive mice (Fig. 2, *c* and *d*). Annexin V is widely used to detect the early events in cells undergoing apoptosis (22, 25). In addition to apoptosis, down-regulation of the TCR could also account for the apparent reduction in hepatic CD1d-tetramer$^+$ NKT cells after Con A treatment. Specifically, we observed that Con A stimulation of splenic CD1d-tetramer$^+$ NKT cells from naive WT or *CCR5*-deficient mice resulted in a significant TCR down-regulation (Fig. 2*e*). We next determined CCR5 expression on hepatic NKT cells before and after Con A administration. We observed significant increases in CCR5^{low}-expressing CD1d-tetramer$^+$ NKT cells in the liver at 90 min and 8 h after Con A treatment compared with naive mice (Fig. 2*f*). These data suggest that CD1d-tetramer$^+$ NKT cells that survived depletion after Con A treatment (i.e., remnant NKT cells) are mostly CCR5^{low}. In contrast, the numbers of liver-infiltrating CCR5-bearing CD4$^+$ T cells were not increased by Con A treatment when compared with that observed in naive mice (19). Thus, we speculated that activated resident hepatic T cells (specifically NKT cells) may be of central importance in the development of FLF in *CCR5*-deficient mice following Con A administration.

CCR5-deficient hepatic CD1d-restricted NKT cells are resistant to apoptosis in vivo

Previous studies have demonstrated that during Con A-induced fulminant hepatitis hepatic NKT cells are lost through apoptosis (Fig. 2, *c* and *d*; Refs. 11, 24). Therefore, we determined whether *CCR5* gene-deficient mice were more susceptible to FLF following Con A administration because Con A-activated hepatic CD1d-tetramer$^+$ NKT cells are resistant to apoptosis. Annexin V and active caspase-3 were used to determine whether hepatic CD1d-tetramer$^+$ NKT cells were undergoing apoptosis. We observed significant increases in the percentage and absolute number of annexin V expressing hepatic CD1d-tetramer$^+$ NKT cells isolated from *CCR5* gene-deficient and WT mice at 90 min after Con A administration when compared with their respective naive controls (Fig. 3, *a–c*). However, the percentage and absolute number of annexin V expressing hepatic CD1d-tetramer$^+$ NKT cells isolated from *CCR5* gene-deficient mice was significantly lower than that observed in WT mice at 90 min after Con A treatment (Fig. 3, *a–c*). Active caspase-3 staining was used as a second approach to confirm the resistance of *CCR5*-deficient hepatic NKT cells to AICD during Con A-induced hepatitis. As shown in Fig. 3*d*, ~32% of isolated hepatic WT NKT cells was positive for active caspase-3, whereas only 10% of isolated hepatic *CCR5*-deficient NKT cells were active caspase-3 positive. The percentage of tetramer$^+$ hepatic NKT cells expressing active caspase-3 in naive WT mice was 3.21 $\pm$ 0.2 vs 3.98 $\pm$ 0.45% in naive *CCR5*-deficient mice; $n = 3$/group. The resistance of Con A-treated *CCR5*-deficient NKT cells to apoptosis is consistent with our observation that more *CCR5*-deficient NKT cells survived AICD after Con A administration relative to WT NKT cells (Fig. 3*e*). Therefore, we hypothesized that depletion of CD1d-tetramer$^+$ hepatic NKT cells should rescue *CCR5*-deficient mice from FLF following Con A treatment.

Depletion of hepatic CD1d-restricted NKT cells prevents acute FLF in CCR5-deficient mice

Indeed, the depletion of hepatic NKT cells with a NK1.1 mAb (26, 27) ameliorated the development of FLF in Con A-treated CCR5-deficient mice as shown by significantly lower plasma ALT levels (Fig. 3*f*) and markedly improved hepatic histology (Fig. 3*g*). In accordance with the biochemical findings, H&E staining of liver sections from control IgG-treated *CCR5* gene-deficient mice represented a grade 2 liver damage because hepatocyte damage was >25%, and hepatocellular necrosis of random distribution was observed throughout the liver at 8 h following Con A administration (Fig. 3*g*). In contrast, livers from NK1.1 Ab-treated *CCR5*-deficient mice exhibited a grade 0 liver damage in which essentially no hepatocyte damage or hepatocellular necrosis was observed at 8 h after Con A treatment (Fig. 3*g*). These results indicate that hepatic NKT cells play a central role in the development of FLF in *CCR5*-deficient mice following Con A administration. A FACS dot profile confirming depletion of hepatic CD1d-tetramer$^+$ NKT cells after anti-NK1.1 mAb treatment is shown in Fig. 3*h*.

CCR5-deficient splenic CD1d-restricted NKT cells are resistant to apoptosis in vitro

We reported above (see Fig. 3, *a–e*) that hepatic *CCR5*-deficient NKT cells are resistant to apoptosis relative to WT cells following Con A administration. Additional experiments using splenic NKT cells demonstrated that Con A stimulation of CD1d-tetramer$^+$ NKT cells from *CCR5* gene-deficient or WT mice caused striking increases in annexin V expression relative to their respective naive controls in vitro (Fig. 4*a*). Moreover, annexin V expression by *CCR5*-deficient Con A-stimulated splenic CD1d-tetramer$^+$ NKT cells was significantly lower than that seen in WT NKT cells (Fig. 4*a*). Additional studies were undertaken to determine whether similar findings would be observed in NKT cells activated with anti-CD3 mAb, a specific TCR activator also reported to cause NKT cell apoptosis (25). Anti-CD3 mAb stimulation of splenic NKT cells from WT mice caused significant increases in annexin V expression relative to unstimulated WT or *CCR5*-deficient NKT cells (Fig. 4*b*), an effect that was reduced by *CCR5* deficiency (Fig. 4*b*). Activation of splenic CD1d-tetramer$^+$ NKT cells from WT and *CCR5*-deficient mice with α-GalCer, a NKT cell-specific ligand, did not cause apoptosis (Fig. 4*c*); an observation that is consistent with other recent reports (22, 28).

Fas-driven apoptosis on CCR5-deficient NKT cells is not defective

The contribution of Fas to the pathology of Con A-induced hepatitis is well documented (11, 24, 29). We observed above (see Figs. 3 and 4) that *CCR5*-deficient NKT cells are resistant to AICD after stimulation with Con A or anti-CD3 mAb (direct stimulators of the TCR). Based on this, we speculated that a defective Fas-driven death pathway on *CCR5*-deficient NKT cells may underlie this response. Unexpectedly, we found that NKT cells from naive CCR5-deficient mice are significantly enriched in Fas expression relative to NKT cells from naive WT mice (Fig. 5, *a* and *b*). Furthermore, we observed increased annexin V expression in *CCR5*-deficient NKT cells after stimulation in vitro with agonistic Fas mAb for 5 h (Fig. 5*c*) relative to WT NKT cells. These results would seem to suggest that the resistance of *CCR5*-deficient NKT cells to AICD after Con A or anti-CD3 mAb stimulation is not due to a defective Fas-driven apoptosis pathway.

Preferential IL-4 production by hepatic CCR5-deficient CD1d-restricted NKT cells

Con A-activated NKT cells have been shown to produce large amounts of IL-4 and little IFN-γ (11). Both cytokines play important proinflammatory roles in Con A hepatitis. Development of FLF in *CCR5* gene-deficient mice after Con A treatment could not be explained by enhanced IFN-γ production because IFN-γ production by activated hepatic CD1d-tetramer$^+$ NKT cells was similar in *CCR5* gene-deficient and WT mice at 90 min post-Con A

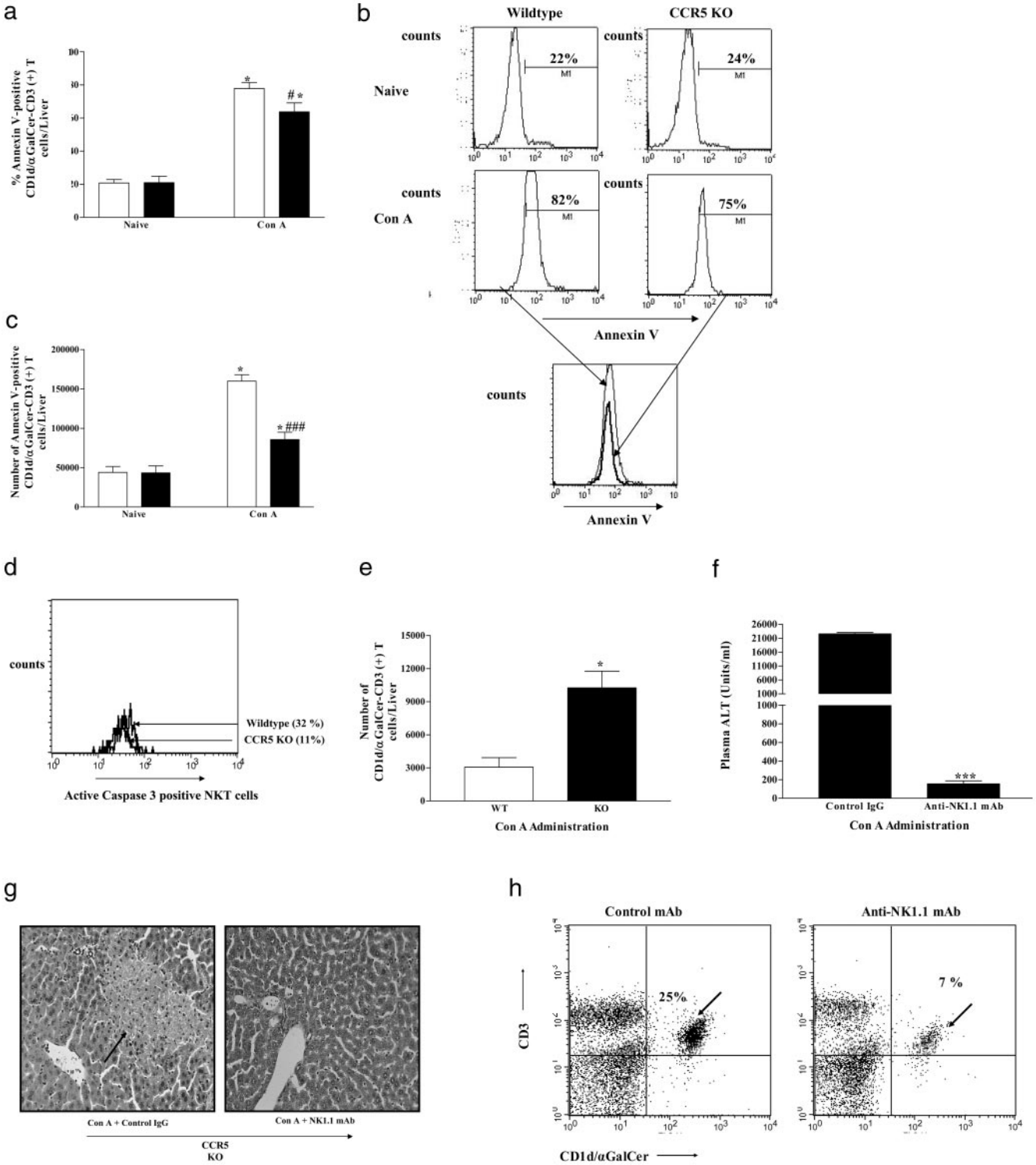

FIGURE 3. *CCR5*-deficient CD1d-tetramer$^+$ NKT cells are resistant to apoptosis during Con A-induced FLF. *a*, Percentage of annexin V expression on isolated hepatic CD1d-tetramer$^+$ NKT cells before and 90 min after Con A administration ($n = 3$–4); ∗, $p < 0.05$ vs naive WT (□); ∗, $p < 0.05$ vs naive *CCR5* gene-deficient mice (■); #, $p < 0.05$ vs Con A-treated WT mice. *b*, A representative FACS profile depicting lower annexin V expressing CD1d-tetramer$^+$ NKT cells in the liver of Con A-treated *CCR5*-deficient mice relative to Con A-treated WT mice. *c*, The absolute number of hepatic annexin V expressing CD1d-tetramer$^+$ NKT cells before and at 90 min after Con A administration ($n = 4$); ∗, $p < 0.05$ vs naive WT mice; ∗, $p < 0.05$ vs naive *CCR5* gene-deficient mice; ###, $p < 0.001$ vs Con A-treated WT mice. *d*, A representative FACS profile depicting lower percentage counts of active caspase-3 expressing CD1d-tetramer$^+$ NKT cells in the liver of *CCR5*-deficient mice relative to WT mice after 90 min of Con A treatment. *e*, Survival (depicted as absolute number) of isolated hepatic CD1d-tetramer$^+$ NKT cells in WT and CCR5 gene-deficient mice 90 min after Con A administration ($n = 4$). ∗, $p < 0.05$ vs WT mice. *f*, Lower ALT levels in *CCR5*-deficient mice pretreated with NK1.1 mAb and ALT levels determined 8 h after Con A administration ($n = 4$); ∗∗∗, $p < 0.001$ vs control IgG-treated *CCR5*-deficient mice. *g*, Representative H&E-stained liver sections demonstrating markedly improved hepatic histology (virtually normal) in NK1.1 mAb treated *CCR5*-deficient mice relative to control IgG-treated CCR5 deficient mice in which hepatocyte damage $> 25\%$ is observed at 8 h post-Con A administration. *h*, An illustrative FACS profile demonstrating depletion of isolated hepatic CD1d-tetramer$^+$ NKT cells in WT mice after anti-NK1.1 mAb treatment for 24 h relative to control Ab-treated WT mice.

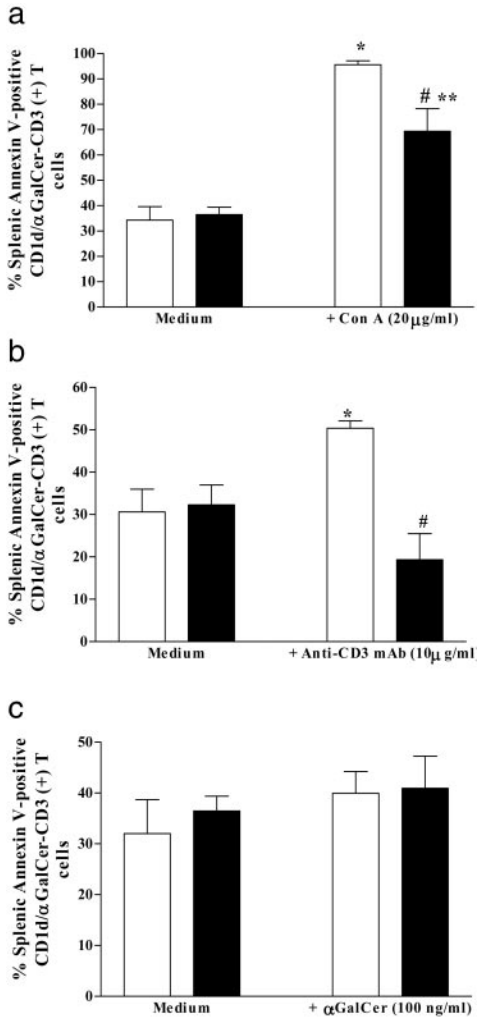

FIGURE 4. Effects of CCR5 deficiency on ligand-activated splenic CD1d-tetramer$^+$ NKT cells in vitro. *a*, Percentage of splenic Con A-activated NKT cells expressing annexin V after 5 h of incubation in vitro (n = 3); ∗, $p < 0.05$ vs medium-treated WT cells (□), #, $p < 0.05$ vs medium-treated CCR5-deficient cells (■), ∗∗, $p < 0.01$ vs Con A-treated WT cells. *b*, Percentage of annexin V expression on anti-CD3 mAb-activated splenic NKT cells after 5 h of incubation; ∗, $p < 0.05$ vs medium-treated WT or CCR5-deficient cells, #, $p < 0.05$ vs anti-CD3 mAb-treated WT NKT cells (n = 3–4). *c*, Percentage of annexin V expression on splenic α-GalCer activated splenic NKT cells after 5 h of incubation.

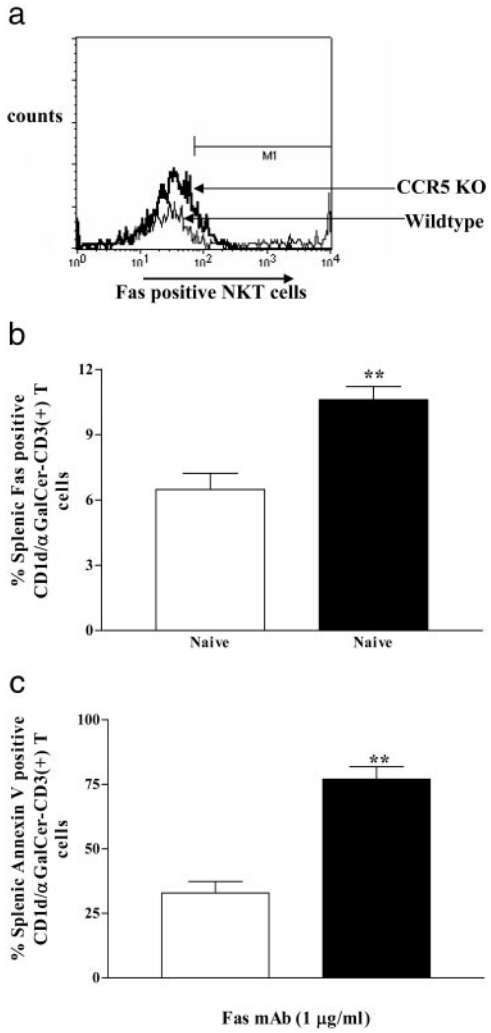

FIGURE 5. Effects of CCR5 deficiency on agonistic Fas mAb-activated splenic CD1d-tetramer$^+$ NKT cells in vitro. *a* and *b*, FACS profile and graph depicting increased Fas expression on splenic NKT cells from naive CCR5-deficient mice (■) relative to naive WT mice (□). ∗∗, $p < 0.01$ vs naive WT cells (n = 3–4). *c*, A graph depicting increased annexin V expression on CCR5-deficient CD1d-tetramer$^+$ NKT cells (■) after agonistic Fas mAb stimulation in vitro for 5 h relative to WT cells (□). ∗∗, $p <$ 0.01 vs naive WT cells (n = 3–4).

treatment (Fig. 6, *a* and *b*). However, the percentage of IL-4-producing NKT cells was significantly higher in CCR5-deficient mice relative to NKT cells isolated from WT mice after Con A administration (Fig. 6, *c* and *d*). Based on this, we next determined whether neutralization of endogenous hepatic IL-4 could ameliorate FLF in *CCR5*-deficient mice post-Con A administration. The development of FLF in Con A-treated *CCR5*-deficient mice was abrogated by IL-4 mAb treatment as demonstrated by a significantly reduced plasma ALT levels (Fig. 6*e*) and a significant improvement in hepatic histology (Fig. 6*f*). H&E staining of liver sections from control IgG-treated *CCR5* gene-deficient mice represented grade 2 liver damage as hepatocyte damage was >25%, and hepatocellular necrosis of random distribution was observed throughout the liver at 8 h following Con A administration (Fig. 6*f*). In contrast, liver sections from IL-4 Ab-treated *CCR5*-deficient mice exhibited a grade 0 liver damage in which essentially no hepatocyte damage or hepatocellular necrosis was observed at 8 h after Con A treatment (Fig. 6*f*).

Preferential IL-4 production by splenic CCR5-deficient CD1d-restricted NKT cells

In agreement with our in vivo data (Fig. 6, *c* and *d*), additional experiments using splenic NKT cells demonstrated a significant augmentation in IL-4-producing splenic *CCR5*-deficient NKT cells after Con A activation in vitro relative to that seen in WT cells after 5 h of stimulation (Fig. 7*a*). Previous studies have documented increases in IL-4 production by NKT cells following activation with anti-CD3 mAb (25) and α-GalCer (22, 28, 30); therefore, we also examined whether this response is enhanced by CCR5 deficiency. Splenic *CCR5*-deficient NKT cells were enriched significantly in IL-4 after in vitro activation with anti-CD3 mAb for 5 h (Fig. 7*b*) but not with α-GalCer (Fig. 7*c*) at a similar time point, relative to stimulated WT NKT cells. However, treatment of NKT cells with α-GalCer over a longer period of time (i.e., 21 h) did increase NKT cell IL-4 production (M. N. Ajuebor and M. G. Swain, unpublished observation).

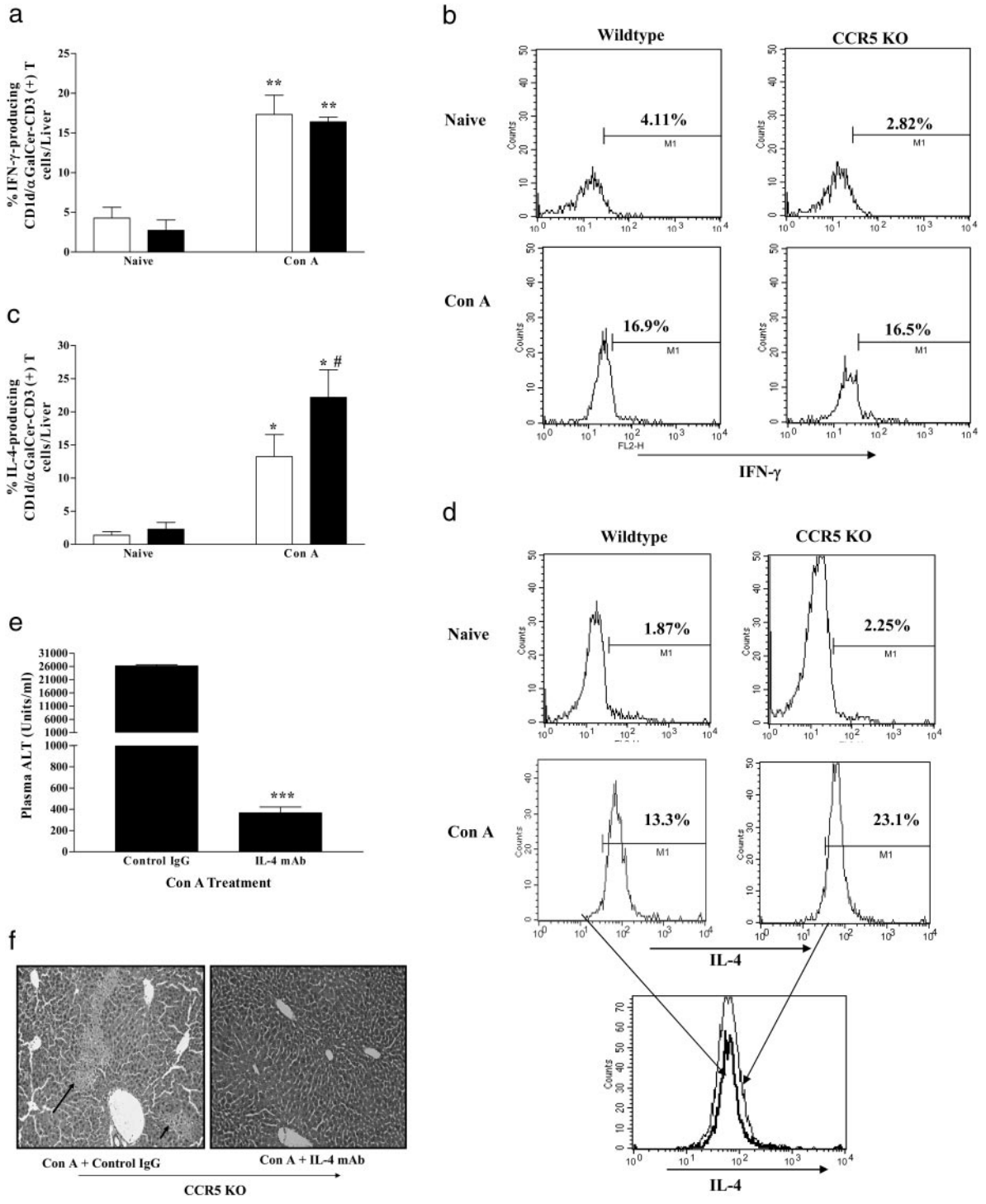

FIGURE 6. Effects of CCR5 deficiency on hepatic cytokine production by Con A-activated NKT cells. *a*, Percentage of IFN-γ-producing hepatic NKT cells during Con A-induced fulminant hepatitis at the 90-min time point ($n = 4$–5); **, $p < 0.01$ vs respective naive WT (□) or CCR5-deficient controls (■). *b*, A representative FACS histogram depicting IFN-γ-producing hepatic NKT cells during Con A-induced fulminant hepatitis at the 90-min time point. *C*, Percentage of IL-4-producing hepatic NKT cells during Con A-induced fulminant hepatitis at the 90-min time point ($n = 3$–5); ∗, $p < 0.05$ vs respective naive WT or CCR5-deficient mice, #, $p < 0.05$ vs Con A-treated WT mice. *d*, Representative FACS profile illustrating enriched IL-4-producing hepatic NKT cells in CCR5-deficient mice during Con A-induced fulminant hepatitis at the 90-min time point. *e*, Reduced ALT levels in CCR5-deficient mice pretreated with IL-4 mAb and ALT levels determined 8 h after Con A administration ($n = 4$), ***, $p < 0.001$ vs control Ig G-treated CCR5-deficient mice. *f*, A representative H&E-stained liver sections demonstrating markedly improved hepatic histology in IL-4 mAb-treated CCR5-deficient mice relative to control IgG-treated CCR5-deficient mice at 8 h post-Con A administration.

IL-4 treatment of CCR5-deficient splenic CD1d-restricted NKT cells enhances FasL expression in vitro

IL-4 is known to contribute to Con A-mediated liver damage by promoting increased FasL expression on NKT cells. Specifically, it has been demonstrated that IL-4 produced by Con A-activated resident hepatic NKT cells acts on NKT cells in an autocrine fashion to induce the up-regulation of FasL expression on these cells, ultimately resulting in an enhancement of NKT cell-mediated

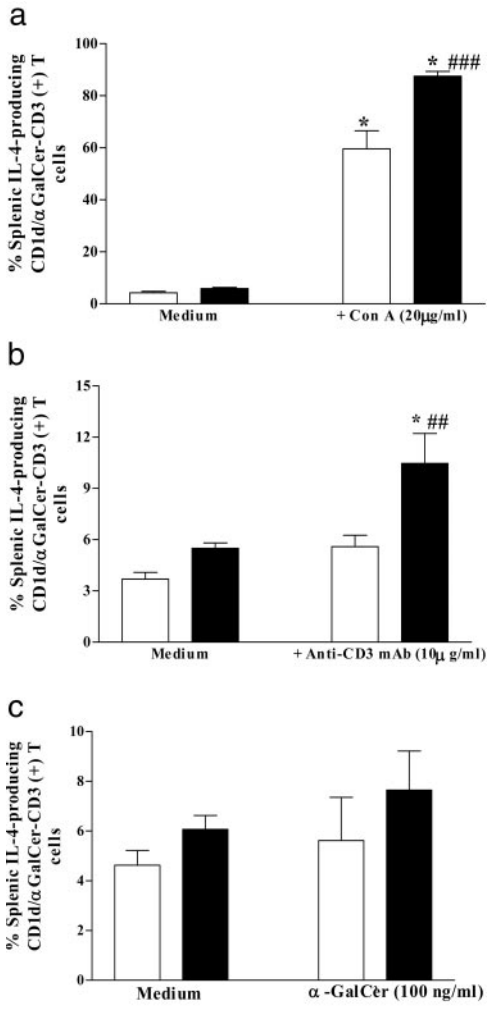

FIGURE 7. Effects of CCR5 deficiency on IL-4 production by activated splenic NKT cells. *a*, Graph demonstrating increased percentage of splenic IL-4-producing CCR5-deficient NKT cells after Con A activation in vitro for 5 h ($n = 3$–4), *, $p < 0.05$ vs respective medium-treated WT (□) or CCR5-deficient NKT cells (■), ###, $p < 0.001$ vs Con A-treated WT cells. *B*, Percentage of IL-4-producing splenic NKT cells after anti-CD3 mAb activation in vitro for 5 h ($n = 3$), *, $p < 0.05$ vs medium-treated CCR5-deficient or WT NKT cells, ##, $p < 0.01$ vs anti-CD3 mAb-treated WT cells. *c*, Percentage of IL-4-producing splenic NKT cells after α-GalCer activation in vitro for 5 h ($n = 3$–4).

cytotoxicity (11). Moreover, the contribution of FasL to the pathology of Con A-induced hepatitis has been documented previously (11, 29). Therefore, we investigated the effect of CCR5 deficiency on splenic NKT cell FasL expression after rIL-4 treatment in vitro. We observed a significant increase in the number of FasL-expressing CCR5-deficient splenic NKT cells after IL-4 treatment in vitro relative to IL-4 treated WT NKT cells (Fig. 8).

Normal development of CD1d-restricted NKT cells in CCR5-deficient mice

To exclude the possibility that an inherent defect in the development of CD1d-restricted NKT cells in *CCR5*-deficient mice may underlie our observations during Con A-induced fulminant hepatitis, we compared CD1d-restricted NKT cells expression in peripheral blood, spleen, and liver in naive *CCR5*-deficient mice vs naive WT mice. The percentages of CD1d-tetramer$^+$ NKT cells in the liver, spleen, and peripheral blood of *CCR5*-deficient mice were similar to those observed in WT mice (Table I).

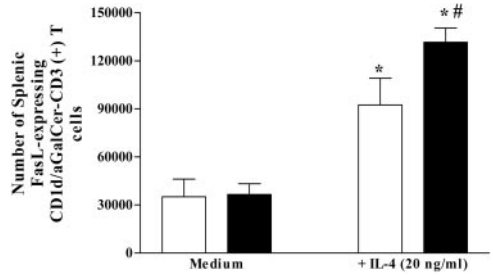

FIGURE 8. Effects of CCR5 deficiency on splenic NKT cell FasL expression after IL-4 treatment in vitro. Absolute number of splenic FasL expressing NKT cells after IL-4 treatment for 5 h in vitro ($n = 4$), *, $p < 0.05$ vs medium-treated WT (□) or CCR5-deficient NKT cells (■), ##, $p < 0.01$ vs IL-4-treated WT cells.

Discussion

NKT cells are a unique T cell lineage that are defined as cells that coexpress the NK cell marker (usually NK1.1) and a highly restricted TCR specific for glycolipid Ag (31–33). NKT cells have the unusual property of recognizing glycolipid Ags in conjunction with the MHC class I-like molecule, CD1d (32–35), and are abundant in the liver, thymus, and to a lesser extent in the spleen and bone marrow (31, 36–39). Previous studies have demonstrated that *CD1d* gene-deficient and *Vα14* gene-deficient mice, which express very few NKT cells, are resistant to Con A-induced hepatitis (11, 24, 40). Furthermore, NKT cell elimination using the NK1.1 Ab has been shown to ameliorate Con A-induced hepatitis (27). Although, viruses, drugs (such as acetaminophen), and toxins have all been identified as trigger factors of FLF (2, 3), uncontrolled systemic activation of the immune system appears to be a central cause of FLF regardless of the etiology. Of note, Con A-induced fulminant hepatitis mimics many aspects of human FLF, including systemic immune activation and infiltration of the liver by activated T cells (10–12), severe acute hepatitis (10–12), and Fas/FasL driven hepatocyte death (14–16), making this model ideal for examining the role of CCR5 in the development of FLF. In this study, we have shown for the first time in Con A-induced fulminant hepatitis, a prototype murine model for human FLF (9–13), that the lack of CCR5 promotes the development of FLF. A novel and interesting finding was our observation that CD1d-restricted NKT cells (and not conventional T cells) from Con A-treated *CCR5*-deficient mice are resistant to apoptosis, as shown by reduced annexin V expression. Annexin V is widely used to detect the early events in cells (including NKT cells (25) and CD4$^+$ T cells (18)) undergoing apoptosis. Specifically, annexin V binds to phosphatidylserine, which is normally confined to the inner plasma membrane of a cell. Phosphatidylserine externalization, and other plasma membrane changes, is an early event in cells undergoing apoptosis and allows phagocytes to recognize and engulf damaged cells before they rupture (41). The importance of caspase-3 in the signal transduction pathway, leading to apoptosis of T cells, has been documented previously (42). Specifically, caspase-3 proenzyme becomes cleaved into its active form during apoptosis.

Table I. *Percentage of CD1d-αGalCer tetramer$^+$ NKT cells*[a]

Treatment	WT	Knockout
Peripheral blood	1.68 ± 0.31	1.67 ± 0.31
Liver	23.76 ± 3.46	24.29 ± 4.09
Spleen	2.35 ± 0.31	2.29 ± 0.35

[a]Percentage of CD1d-tetramer$^+$ NKT cells in the liver, spleen, and peripheral blood of naive WT and naive *CCR5*-deficient mice ($n = 4$).

Therefore, active caspase-3 staining was used as a second approach to corroborate our annexin V data that *CCR5*-deficient hepatic NKT cells resist AICD during Con A-induced hepatitis. Indeed, the percentage of isolated tetramer$^+$ hepatic NKT cells expressing active caspase-3 in *CCR5*-deficient mice was less than that observed in WT mice following Con A administration. Therefore, our data suggests that despite the rapid loss of CD1d-tetramer$^+$ NKT cells in the liver after Con A administration, more CD1d-restricted NKT cells survived AICD in *CCR5*-deficient mice than in WT mice following Con A treatment. These remnant NKT cells, which are resistant to apoptosis, promote FLF. Indeed, we have shown a direct role for these NKT cells in the development of FLF in Con A-treated *CCR5*-deficient mice because FLF in these mice was prevented by NKT cell elimination after NK1.1 mAb treatment. Our findings in murine FLF are consistent with observations in murine autoimmune diabetes (43) and experimental allergic asthma (30) where more severe diabetes and airway inflammation in *CD1d*-deficient NOD mice has been attributed to remnant NKT cells. It is unlikely that an inherent defect in Fas expression or a defective Fas-driven death pathway on *CCR5*-deficient NKT cells may underlie the resistance of these cells to AICD during Con A-induced fulminant hepatitis. Specifically, we found that *CCR5*-deficient NKT cells are enriched in Fas expression. Additionally, direct stimulation of *CCR5*-deficient NKT cells with agonistic Fas mAb caused enhanced AICD relative to stimulated WT NKT cells, thus excluding a defective Fas-driven death pathway as a putative mechanism. However, the mechanism(s) by which *CCR5*-deficient NKT cells resist AICD during Con A-induced fulminant failure warrants additional investigation.

NK1.1. mAb is known to deplete hepatic NK cells in addition to hepatic NKT cells. To determine whether there was a role for hepatic NK cells in the development of FLF in Con A-treated *CCR5*-deficient mice, hepatic NK cells were depleted selectively with anti-asialo GM1 Ab. Unexpectedly, we observed that selective depletion of hepatic NK cells with anti-asialo GM1 Ab prevented FLF in Con A-treated *CCR5*-deficient mice (M. N. Ajuebor and M. G. Swain, unpublished observations). Our observations demonstrating that depletion of *CCR5*-deficient hepatic NK cells prevents FLF following Con A administration is in contrast to previous reports in WT mice (11, 27, 44) where hepatic NK cell depletion did not alter Con A-induced hepatitis. In contrast to activated hepatic NKT cells, which can produce both IL-4 and IFN-γ, activated hepatic NK cells produce only IFN-γ (21, 45). Although, the precise mechanism(s) by which *CCR5*-deficient hepatic NK cells contribute to FLF in *CCR5*-deficient mice following Con A administration remains unknown and warrants an additional detailed investigation. We speculate that the ability of *CCR5*-deficient hepatic NK cells (but not WT NK cells) to exhibit enhanced IFN-γ production may underlie the contribution of these cells to the development of FLF in Con A-treated *CCR5*-deficient mice (M. N. Ajuebor and M. G. Swain, unpublished observations).

There are several mechanisms by which remnant NKT cells in *CCR5*-deficient mice could promote FLF after Con A administration. In recent years, NKT cells have gained significant attention as targets for immunomodulation primarily due to their ability to secrete high levels of cytokines, including IFN-γ and IL-4, within minutes of activation (21, 22, 30). Despite this, the role of NKT cells in the pathology of liver diseases (particularly FLF) remains poorly defined. IL-4 is a Th2 cytokine that exhibits proinflammatory effects during Con A-induced hepatitis as demonstrated by anti-IL-4 Ab or IL-4 gene-deficient mice studies (11, 27). Moreover, a recent study demonstrates a regulatory role for resident hepatic NKT cells in augmenting hepatic damage via the production of IL-4 during Con A-induced fulminant hepatitis. Specifically, Kaneko et al. (11) demonstrated that IL-4 produced by Con A-activated resident hepatic NKT cells acts on NKT cells in an autocrine fashion to induce the up-regulation of FasL expression on these cells, ultimately resulting in an enhancement of NKT cell-mediated cytotoxicity. Indeed, we found that the lack of CCR5 was associated with augmented hepatic IL-4 but not IFN-γ production by Con A-activated CD1d-restricted NKT cells. More importantly, we demonstrate a direct role for IL-4-producing NKT cells in the development of FLF in Con A-treated CCR5-deficient mice because the depletion of NKT cells in these mice using NK1.1 mAb prevented the development of FLF. Moreover, neutralization of IL-4 also abrogated FLF in CCR5-deficient mice following Con A administration. Additionally, increased IL-4-producing NKT cells could directly account for the elevated FasL expression during FLF because treatment of splenic CCR5-deficient NKT cells with rIL-4 augmented the number of FasL-expressing NKT cells relative to WT NKT cells. Thus, Con A-treated *CCR5*-deficient mice are enriched in proinflammatory IL-4-producing, FasL-expressing, CD1d-restricted NKT cells.

There are a number of reports suggesting that CCR5 contributes to the pathogenesis of autoimmune and infectious diseases by promoting a Th1 response via IFN-γ induction (7, 46). In our study, CCR5 deficiency did not augment hepatic IFN-γ production by activated CD1d-restricted NKT cells, suggesting that the development of FLF is not directly dependent on IFN-γ production. Nonetheless, IFN-γ could contribute indirectly to the development of FLF in Con A-treated *CCR5*-deficient mice by prolonging/sustaining the hepatic inflammatory response that ultimately causes FLF in these mice. Moreover, IFN-γ produced by activated T cells (19) is reported to contribute to the pathology of Con A-induced hepatitis by activating the Fas/FasL pathway, thereby resulting in hepatocyte cell death (29). Moreover, NKT cell development in *CCR5*-deficient mice is similar to that observed in WT mice, thus excluding the possibility that inherent differences in WT and *CCR5*-deficient mice used in this study underlies our findings.

The contribution of liver-infiltrating CD4$^+$ T cells (but not CD8$^+$ T cells) to the pathogenesis of Con A-induced hepatitis is well established (11, 12, 24, 27). However, we did not observe augmented increase in CD4$^+$ T cell numbers in the liver of *CCR5*-deficient mice following Con A administration compared with WT mice (M. N. Ajuebor and M. G. Swain, unpublished observations), therefore eliminating a significant role for CD4$^+$ T cells in the development of FLF in *CCR5*-deficient mice following Con A administration.

In agreement with our in vivo findings, in vitro Con A or anti-CD3 mAb activation of splenic WT CD1d-restricted NKT cells caused increased NKT cell apoptosis, an effect that was reduced by *CCR5* deficiency. Moreover, IL-4 production by NKT cells was enhanced by CCR5 deficiency after Con A or anti-CD3 mAb activation in vitro. However, it was apparent that the effects of Con A on NKT cell apoptosis and IL-4 production in vitro were more pronounced than those observed with anti-CD3 mAb treatment. We speculate that the ability of Con A to activate the putative lectin receptor(s) on NKT cells in addition to the TCR may underlie these differences (47, 48). In contrast, the activation of splenic CD1d-restricted NKT cells from WT mice with α-GalCer (a NKT cell-specific synthetic ligand) did not cause NKT cell apoptosis or increase IL-4 production in comparison to untreated WT cells at the 5-h time point. The inability of α-GalCer (a nonphysiological ligand for NKT cells) to promote NKT cell apoptosis in our study might appear to call into question the physiological relevance of our findings. A recent study has used the mCD1d-αGalCer tetramer to demonstrate a role for α-GalCer in NKT cell apoptosis (49). However, in agreement with our findings, a number

of more recent studies have convincingly used a similar mCD1d-αGalCer tetramer to show that α-GalCer is unable to promote NKT cell apoptosis (22, 28, 50). These apparently conflicting data could be reconciled by proposing that differences in the quality and/or dose of α-GalCer probably influenced the extent and kinetics of NKT cell death. Moreover, the reason for the apparent discrepancy of Con A and anti-CD3 mAb vs α-GalCer on NKT cell apoptosis is unknown. It is possible that the ability of Con A and anti-CD3 mAb to both directly activate the TCR (48) to mediate their effects, whereas α-GalCer is known to bind to CD1d before activation of the TCR (32, 36, 39) may underlie this discrepancy. Regardless, our data emphasize the fact that NKT cells are more prone to AICD when directly activated through the TCR.

Collectively, we propose that the following events may underlie FLF in Con A-treated *CCR5*-deficient mice: Con A activates apoptosis-resistant NKT cells and biases these cells to produce more IL-4 while still increasing IFN-γ production. IL-4 in turn acts on NKT cells in an autocrine fashion to induce FasL expression, which subsequently promotes hepatocyte cell death, possibly by interacting with Fas-expressing hepatocytes. In addition, IL-4 directly induces hepatocyte apoptosis (51). This interaction may account for the sudden onset of severe hepatitis and ensuing mortality in patients with FLF. The role of CCR5 was initially thought to be restricted to leukocyte recruitment. However, our results demonstrating that CCR5 deficiency promotes murine FLF by regulating NKT cell function establishes a new role for CCR5 that is independent of leukocyte recruitment. It is noteworthy that ~1% of the Caucasian population is homozygous for the *CCR5Δ32* polymorphism, which renders a nonfunctional *CCR5* receptor (52). Therefore, our findings suggest that *CCR5* deficiency in humans may predispose to the development of FLF in individuals exposed to a hepatotoxic insult that would normally result in a relatively mild and transient hepatitis that resolves spontaneously. Our data provides a better understanding of the etiology of FLF and may lead to the design of novel therapies for the treatment of this devastating liver disease.

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Disclosures

The authors have no financial conflict of interest.

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