IL-6 Prevents T Cell-Mediated Hepatitis via Inhibition of NKT Cells in $CD4^+$ T Cell- and STAT3-Dependent Manners

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The hepatoprotective effect of IL-6 on various forms of liver injury including T cell-mediated hepatitis has been well documented, and it is believed that induction of antiapoptotic proteins is an important mechanism. In this study, we provide evidence suggesting an additional mechanism involved in the protective role of IL-6 in T cell-mediated hepatitis. In NKT cell-depleted mice, Con A-induced liver injury is diminished; this can be restored by the adoptive transfer of liver mononuclear cells or NKT cells from wild-type mice, but not from IL-6-treated mice. In vitro IL-6 treatment inhibits the ability of mononuclear cells to restore Con A-induced liver injury in NKT-depleted mice, whereas the same treatment does not inhibit purified NKT cells from restoring the injury. The addition of $CD3^+$ T cells or $CD4^+$ T cells can restore the inhibitory effect of IL-6 on purified NKT cells, whereas the addition of $CD3^+$ T cells from CD4-deficient mice fails to restore this inhibitory effect. The expression of IL-6R was detected in 52.6% of hepatic $CD3^+$ T cells and 32.7% of hepatic $CD4^+$ T cells, but only in 3.9% of hepatic NK and 1.5% of hepatic NKT cells. Finally, treatment with IL-6 induces STAT3 activation in hepatic lymphocytes and hepatic T cells, and blocking such activation abolishes the inhibitory effect of IL-6 on hepatic lymphocytes to restore liver injury. Taken together, these findings suggest that in addition to its antiapoptotic abilities, as previously well documented, IL-6/STAT3 inhibits NKT cells via targeting $CD4^+$ T cells and consequently prevents T cell-mediated hepatitis. *The Journal of Immunology*, **2004, 172: 5648–5655.**

Interleukin-6 is a pleiotropic cytokine that has been shown to promote liver regeneration (1) and protect against liver injury induced by Con A (2, 3), alcohol (4), Fas (5), carbon tetrachloride (CCl_4) (6), ischemia/reperfusion (7), acetaminophen (8), hemorrhagic shock (9), partial liver transplantation (10), and fatty liver transplantation (11). In vitro, IL-6 has been shown to protect against hepatocyte death induced by TGF-β (12) or alcohol plus TNF-α (4) and to prevent hypothermia/rewarming-induced necrapoptosis in hepatic sinusoidal endothelial cells (11). It is believed that the broad protective effect of IL-6 in the liver is mediated through binding to the IL-6R (gp80), followed by dimerization of the gp130 protein and activation of the Janus kinase (JAK)[2]-STAT3 signaling pathway. Studies from liver-specific knockout mice suggest that IL-6/gp130/STAT3 promote cell proliferation (13) and protect against liver injury induced by partial hepatectomy, (14), injection of CCl_4 (15), or LPS (16). Blocking STAT3 activation using dominant negative DNA also abolished the protective action of IL-6 against TGF-β-induced hepatocyte death (12). Furthermore, the overexpression of constitutively activated STAT3 protects against Fas-induced liver injury (17, 18). The protective effect of IL-6/STAT3 is therefore believed to be mediated through induction of various antiapoptotic proteins, including Bcl-x_L, Bcl-2, and FLIP, and redox-associated protein redox factor-1 (17).

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[2] Abbreviations used in this paper: JAK, Janus kinase; ALT, alanine aminotransaminase; ASGM-1, asialo GM-1; AST, asparate aminotransferase; Fas L, Fas ligand; NKT, NK T; MNC, mononuclear cell.

Previously we have shown that an injection of IL-6 prevents Con A-induced hepatitis (2), an established model to study T cell-mediated hepatitis (19). Our data suggest that IL-6 protects against Con A-induced liver injury via induction of the antiapoptotic protein Bcl-x_L and suppression of IFN-γ signaling (2). In this paper we demonstrate an additional mechanism that may be involved in the protective effect of IL-6 in T cell-mediated hepatitis. This includes IL-6 suppression of NKT cell activation, a critical step in the initiation of Con A-induced T cell hepatitis (20–22). We also provide evidence suggesting that IL-6 inhibition of NKT cell activation in vivo is mediated by $CD4^+$ T cell- and STAT3-dependent mechanisms.

Materials and Methods

Materials

Recombinant human IL-6 was produced by recombinant DNA technology, followed by purification through solublization, gel filtration chromatography, refolding, and cation exchange chromatography conducted at the Institute of Immunology (School of Life Science, University of Science and Technology of China). The purified IL-6 had the expected amino acid sequence, as confirmed by compositional analysis, and the correct pI range of 7.0–7.1. The biological activity of the recombinant protein was measured by a proliferation assay using 7TD1 cells, which demonstrated that human IL-6 was active at $>2 \times 10^8$ U/mg. Anti-STAT1, anti-phospho-STAT1 (Tyr^{701}), anti-phospho-STAT3 (Tyr^{705}), and anti-STAT3 Abs were obtained from Cell Signaling Technology (Beverly, MA). The STAT3 inhibitor (a cell-permeable analog of STAT3-Src homology 2 domain-binding phosphopeptide) and JAK2 inhibitor AG 490 were obtained from Calbiochem (San Diego, CA).

Animals and T cell-mediated hepatitis model

Seven- to 8-wk-old male C57BL/6J mice, BALB/cJ mice, and $CD1d^{-/-}$ mice (BALB/c background), were purchased from The Jackson Laboratory (Bar Harbor, ME). To induce hepatitis, mice were i.v. administered various doses of Con A. After 12–24 h, mice were sacrificed, and serum and livers were collected for in vitro experiments.

Determination of liver injury

Liver injury was determined by either H&E staining of liver sections or measuring aminotransaminase activities. For H&E staining, livers were

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fixed with 10% formalin/PBS, then stained with H&E. Alanine aminotransaminase (ALT) and asparate aminotransferase (AST) activities were measured using a kit purchased from Drew Scientific (Cumbria, U.K.).

Isolation of liver mononuclear cells (MNCs)

Mouse livers were removed and pressed through a 200-gauge stainless steel mesh. The liver cell suspension was collected, suspended in RPMI 1640 medium (Life Technologies, Gaithersburg, MD), and centrifuged at $50 \times g$ for 5 min. Supernatants containing MNCs were collected, washed in PBS, and resuspended in 40% Percoll (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium. The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at $750 \times g$. MNCs were collected from the interphase, washed twice in PBS, and resuspended in RPMI 1640 medium for cytotoxicity assay and FACS analysis.

Cytotoxicity assay

To assay the cytotoxicity of hepatic MNCs against primary mouse hepatocytes, a 4-h AST release assay, described previously (23), was used. Briefly, primary mouse hepatocytes were isolated by perfusion with EGTA solution (5.4 mM KCl, 0.44 mM KH_2PO_4, 140 mM NaCl, 0.34 mM Na_2HPO_4, 0.5 mM EGTA, and 25 mM tricine, pH 7.2) and DMEM (Life Technologies) and digestion with 0.075% collagenase solution. The isolated mouse hepatocytes were cultured in Hepato-ZYME-SFM medium (Life Technologies) in rat tail collagen-coated plates for 24 h, then cultured in serum-free DMEM. Hepatic MNCs isolated from 2-h Con A-treated mice were added to the cultured hepatocytes at the indicated E:T cell ratios. After 4 h, the supernatant was harvested, and AST activity was measured. The specific cytotoxicity was calculated as: $AST_{\text{experimental}} - AST_{\text{spontaneous}} / AST_{\text{maximum}} - AST_{\text{spontaneous}} \times 100\%$.

Flow cytometric analysis of NKT cells, T cells, Fas ligand (Fas L) expression, and IL-6R expression in liver

NKT cells, T cells, and Fas L expression in the livers of C57BL/6 mice were determined by anti-NK1.1, anti-CD3, and anti-Fas L Abs (BD PharMingen, San Diego, CA) with a FACSCalibur (BD Biosciences, Mountain View, CA). For detection of IL-6R expression, liver MNCs were stained using anti-CD3, anti-NK1.1, anti-CD4 (BD PharMingen), and anti-mouse IL-6R (BioLegend, San Diego, CA) and analyzed with a FACSCalibur (BD Biosciences).

Depletion of NK1.1$^+$ cells

To deplete NKT and NK cells, mice were injected i.p. with 0.5 ml of PBS containing 250 μg of anti-NK1.1 (mAb PK136; American Type Culture Collection (ATCC), Manassas, VA). After 40 h, depletion of NKT ($NK1.1^+CD3^+$) and NK ($NK1.1^+CD3^-$) cells was confirmed by flow cytometry.

Purification of NK1.1$^+$CD3$^+$ T cells (NKT) and various types of T cells

For purification of NKT cells, mice were injected with anti-asialo GM-1 (ASGM-1; WAKO, Richmond, VA) to deplete NK cells ($NK1.1^+CD3^-$). Twenty-four hours later, liver lymphocytes were isolated from these NK-depleted mice, stained with FITC-conjugated anti-NK1.1 mAb (BD PharMingen), and incubated with anti-FITC Microbeads (Miltenyi Biotec, Auburn, CA) for 15 min at 4°C. $NK1.1^+$ cells were enriched by positive MACS according to the manufacturer's protocol. Approximately 92% of the MACS-purified cells were NK1.1 and CD3 positive.

For purification of $CD3^+$ T cells and $CD4^+$ T cells, C57BL/6 mice were injected with anti-NK1.1 mAb (PK136; ATCC) to deplete $NK1.1^+$ cells. Forty hours later, $CD3^+$ T cells and $CD4^+$ T cells then were purified from these NK1.1-depleted mice by positive MACS according to the manufacturer's protocol (Miltenyi Biotec). For purification of $CD3^+CD4^-$ T cells, CD4-deficient mice on a C57BL/6 background (The Jackson Laboratory) were injected with anti-NK1.1 mAb (ATCC) to deplete $NK1.1^+$ cells. Forty hours later, $CD3^+CD4^-NK1.1^-$ T cells were purified from these NK1.1-depleted CD4-deficient mice by positive MACS according to the manufacturer's protocol (Miltenyi Biotech). For purification of $CD4^+CD25^-$ T cells, C57BL/6 mice were injected with anti-NK1.1 mAb to deplete $NK1.1^+$ cells. Forty hours later, $CD25^-$ T cells were purified from these NK1.1-depleted mice by negative MACS, then $CD4^+CD25^-NK1.1^-$ T cells were subsequently purified by positive MACS using anti-CD4 mAb according to the manufacturer's protocol (Miltenyi Biotec).

Adoptive transfer of liver MNCs

Adoptive hepatic MNC transfer was performed as described previously (20). Briefly, 50 μl of hepatic MNCs (5×10^6 cells) or purified NKT (1×10^6 cells) in saline were injected into the lateral left lobe of the liver at a rate of 10 μl/s using a 29-gauge needle attached to a 1-ml syringe, followed by i.v. injection of Con A (15 μg/g for B6 mice and 20 μg/g for BALB/c mice). Liver injury was assessed 9 h later by measuring serum ALT levels and by histological analysis.

For adoptive transfer of liver NKT and T cells (Fig. 5), purified NKT cells (1×10^6) and various subtypes of T cells (1×10^6) were resuspended in serum-free DMEM solution in the absence and the presence of IL-6 (500 ng/ml; the various combinations of these cells are indicated in Fig. 5). After incubation for 4 h at 37°C, cells were washed three times with saline and then resuspended in 50 μl of saline, followed by adoptive transfer into anti-NK1.1 mAb-treated mice. Liver injury was assessed 9 h later by measuring serum ALT levels.

Western blotting

Tissues were homogenized in lysis buffer (30 mM Tris (pH 7.5), 150 mM sodium chloride, 1 mM PMSF, 1 mM sodium orthovanadate, 1% Nonidet P-40, and 10% glycerol) at 4°C, vortexed, and centrifuged at 16,000 rpm at 4°C for 10 min. The supernatants were mixed in Laemmli loading buffer, boiled for 4 min, and then subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary Abs for 16 h. Membranes were washed with 0.05% (v/v) Tween 20 in PBS (pH 7.4) and incubated with a 1/4000 dilution of HRP-conjugated secondary Abs for 45 min. Protein bands were visualized by ECL reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis

For comparing values obtained in three or more groups, one-factor ANOVA was used, followed by Tukey's post-hoc test. Statistical significance was taken at the $p < 0.05$ level.

Results

IL-6 treatment inhibits hepatic MNC and NKT killing against hepatocytes in Con A-induced hepatitis

Hepatic MNC killing against hepatocytes has been suggested to play an important role in the pathogenesis of Con A-induced hepatitis (20), and we previously showed that treatment with IL-6 protected against Con A-induced hepatitis (2). Thus, we wondered whether the protective effect of IL-6 in Con A-induced hepatitis was partly due to inhibition of the cytotoxicity of hepatic MNCs against hepatocytes. As shown in Fig. 1, *A* and *B*, MNCs or NKT isolated from IL-6-treated mice were less cytotoxic against hepatocytes than those isolated from saline-treated mice. Moreover, the percentage of NKT ($NK1.1^+CD3^+$) cells was rapidly decreased after injection of Con A, and this depletion was suppressed in IL-6-treated mice (Fig. 1*C*). The total number of NKT cells was significantly higher at 3 and 6 h post-Con A injection in the IL-6-treated group than in the saline-treated group (Fig. 1*D*). Finally, the effects of IL-6 on Con A-induced Fas L expression on NKT cells were also examined. As shown in Fig. 1*E*, injection of Con A increased the total number of NKT cells expressing Fas L, which was significantly inhibited in IL-6-treated mice compared with saline-treated mice at 3 h post-Con A injection.

Adoptive transfer of MNCs restores Con A-induced hepatitis in NKT-deficient mice, which is significantly suppressed by in vivo and in vitro IL-6 treatment

It has been shown that Con A-induced liver injury is abolished in NKT-deficient mice and can be restored by adoptive transfer of MNCs (20). In this study we also show that injection of Con A failed to induce a significant elevation of ALT serum levels in two strains of mice that lack NKT cells or contain low levels of NKT cells, including NK1.1-depleted mice and CD1d-deficient mice. As shown in Fig. 2*A*, pretreatment with anti-NK1.1 Ab for 40 h completely depleted both NK ($NK1.1^+CD3^-$) and NKT ($NK1.1^+CD3^+$) cells. Injection

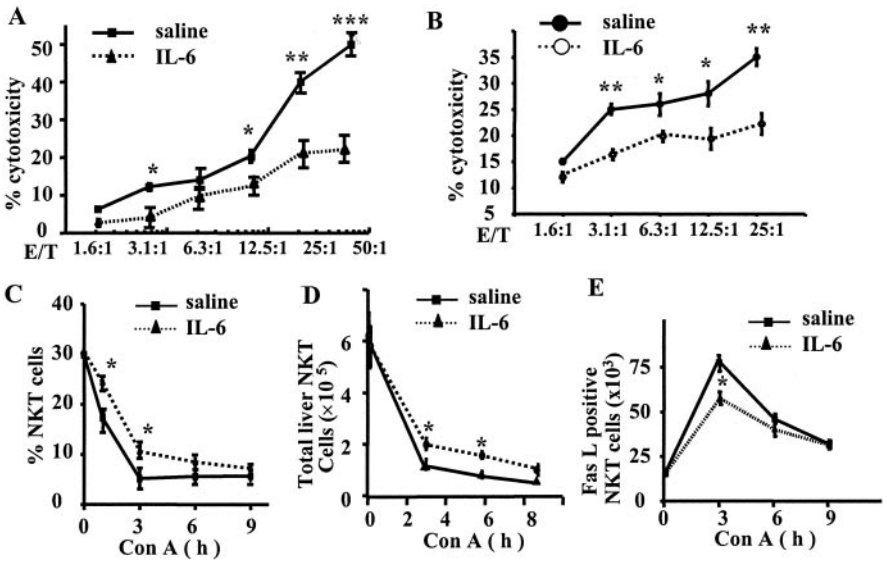

FIGURE 1. IL-6 treatment reduces the cytotoxicity of Con A-treated hepatic MNCs and NKT cells against primary mouse hepatocytes. *A* and *B*, C57BL/6 mice were given i.v. saline or IL-6 (2 μg/g), followed 6 h later by injection (i.v.) of Con A (15 μg/g). After 2 h, hepatic MNCs (*A*) or hepatic NKT cells (*B*) were isolated, and their cytotoxicities were tested against primary hepatocytes from C57BL/6 mice at the indicated E:T cell ratio as described in *Materials and Methods*. *C–E*, Mice were injected i.p. with saline or IL-6 (2 μg/g), followed 6 h later by injection of Con A (15 μg/g). At various time points, hepatic MNCs were isolated and subjected to FACS analyses of NKT cells (*C* and *D*) or Fas L expression on NKT cells (*E*). *C*, Percentage of NKT cells. *D*, Total number of NKT cells. Values are shown as the mean ± SEM from three mice at each time point from one of three independent experiments. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (compared with corresponding saline-treated groups).

of Con A slightly induced elevation of serum ALT levels in these NK- and NKT-depleted mice, whereas the same injection caused significant liver injury (elevation of ALT) in wild-type mice (Fig. 2*B*). Adoptive transfer of hepatic MNCs isolated from wild-type mice restored Con A-induced liver injury in anti-NK1.1 Ab-treated mice (elevation of serum ALT levels; Fig. 2*B*). However, adoptive transfer of MNCs from IL-6-treated mice did not restore Con A-induced liver injury in those mice (Fig. 2*B*). Similarly, adoptive transfer of hepatic MNCs isolated from wild-type mice, but not from IL-6-treated mice, restored Con A-induced liver injury in CD1d$^{-/-}$ mice (Fig. 2*C*), suggesting that in vivo IL-6 treatment inhibits the ability of hepatic MNCs to cause liver injury after injection of Con A.

To examine whether IL-6 directly inhibits hepatic MNCs, hepatic MNCs were isolated and treated with IL-6 in vitro, then transferred to anti-NK1.1 Ab-treated mice or CD1d$^{-/-}$ mice. As shown in Fig. 2, *B* and *C*, in vitro treatment with various doses of IL-6 significantly attenuated the ability of hepatic MNCs to restore Con A-induced liver injury in anti-NK1.1 Ab-treated mice (Fig. 2*B*) or CD1d$^{-/-}$ mice (Fig. 2*C*). These results were further confirmed by liver histology. As shown in Fig. 2*D*, injection of Con A did not induce necrosis in NK1.1-depleted mice, and adoptive transfer of wild-type mouse hepatic MNCs significantly restored Con A-induced necrosis in these mice, whereas adoptive transfer of IL-6-treated mouse hepatic MNCs failed to restore such injury. IL-6-treated hepatic MNCs were washed three times with saline to remove IL-6 before injection, indicating that the absence of liver injury in NKT-deficient mice adoptively transferred with IL-6-treated MNCs was not due to the possible contamination of IL-6 in the liver during the transfer.

Adoptive transfer of NKT cells restores Con A-induced hepatitis in NK1.1-depleted mice, which is suppressed by in vivo IL-6 treatment, but not by in vitro IL-6 treatment

The above data show that IL-6 is able to inhibit hepatic MNCs to prevent Con A-induced T cell hepatitis. Next we examined whether IL-6 directly inhibited NKT cells. As shown in Fig. 3*A*, normal B6 mouse liver lymphocytes contain 31.05% NKT cells. Injection of anti-AGSM1 depleted NK cells without affecting NKT cells. After purification, ~91.23% of the MACS-purified cells were NKT cells (NK1.1 and CD3 positive). Adoptive transfer of purified NKT cells significantly restored Con A-induced liver injury in NK1.1-depleted mice (Fig. 3*B*), whereas adoptive transfer of NKT cells from IL-6-treated mice failed to restore such injury. These findings suggest that in vivo IL-6 treatment inhibits the ability of NKT cells to restore Con A-induced liver injury in NK1.1-depleted mice. In contrast, in vitro treatment with IL-6 only slightly, but not significantly, inhibited the ability of purified NKT cells to restore Con A-induced liver injury (~10% inhibition; Fig. 3*B*), suggesting that IL-6 does not directly inhibit NKT cells.

In Fig. 1*E*, we showed that in vivo IL-6 treatment down-regulated Fas L expression on NKT cells. We examined the effects of IL-6 treatment on Fas L expression on adoptively transferred NKT cells. As shown Fig. 3*C*, neither in vitro nor in vivo treatment with IL-6 alone altered Fas L expression on NKT cells. Injection of Con A up-regulated Fas L expression on adoptively transferred NKT cells (Fig. 3, *D* and *E*, black line shifted to the right compared with IgG control thin line), which was significantly inhibited in the in vivo IL-6-treated group (Fig. 3*D*, gray line shifted to the left compared with saline control black line), but was not suppressed in the in vitro IL-6-treated group (Fig. 3*E*, gray line overlapped with saline control black line). These findings suggest that in vivo, but not in vitro, IL-6 treatment down-regulates Con A-induced Fas L expression on adoptively transferred NKT cells, which is consistent with the above findings showing that in vivo, but not in vitro, treatment with IL-6 inhibited the ability of adoptively transferred NKT cells to restore Con A-induced liver injury.

Expression of IL-6R is detected on a large percentage of CD3$^+$ and CD4$^+$ T cells, but on only a small percentage of NKT and NK cells

The above data suggest that IL-6 does not directly inhibit NKT cells, but may indirectly inhibit NKT cells via targeting other cells. To answer this question, we analyzed the expression of IL-6R on hepatic MNCs. As shown in Fig. 4*A*, IL-6R expression was detected in 52.6% of hepatic CD3$^+$ T cells and 32.7% of hepatic CD4$^+$ T cells, but in only 3.9% and 1.5% of hepatic NK cells and NKT cells, respectively. Next, the effects of IL-6 treatment and Con A injection on IL-6R expression were examined. As shown in Fig. 4*B*, a large percentage of CD3$^+$ T cells expressed IL-6R. IL-6 treatment markedly down-regulated the fluorescence intensity (the IL-6R$^+$ peak gray line in the IL-6-treated group shifted to the left

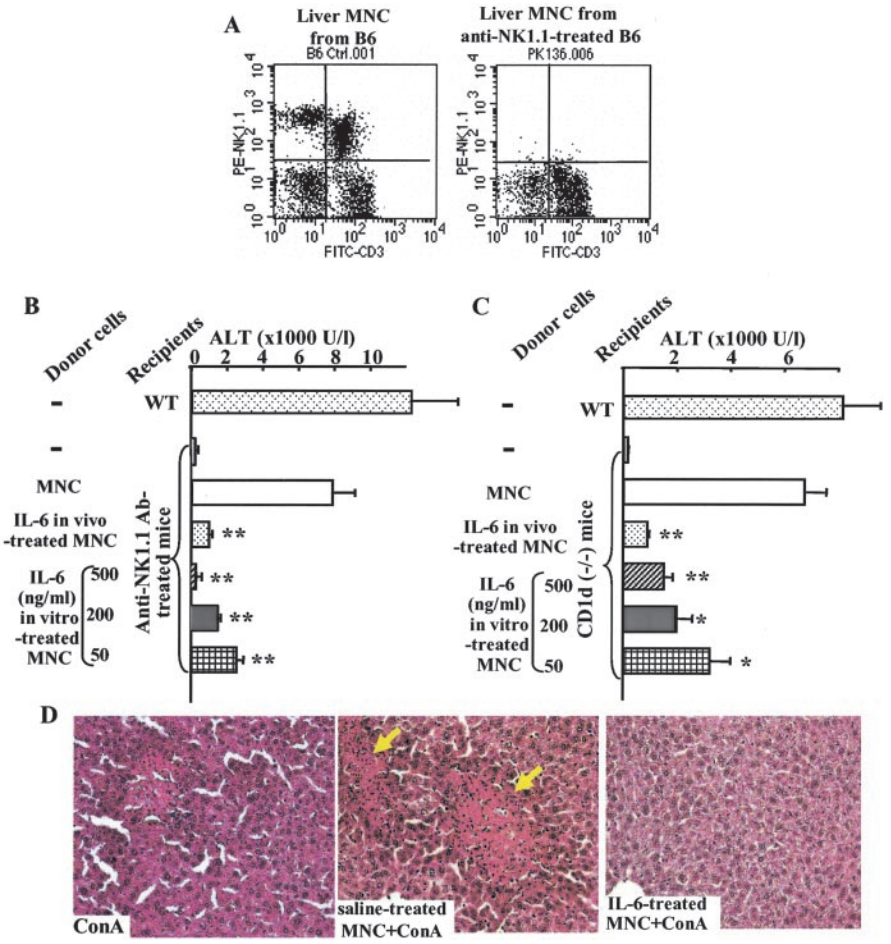

FIGURE 2. Adoptive transfer of hepatic MNCs, but not IL-6-treated MNCs, restores Con A-induced liver injury. *A*, Anti-NK1.1 Ab treatment depletes NK and NKT cells. C57BL/6 mice were injected i.p. with 0.5 ml of PBS containing 250 μg of anti-NK1.1 Ab, and 40 h later, depletion of NKT ($NK1.1^+CD3^+$) and NK ($NK1.1^+CD3^-$) cells was confirmed by flow cytometry. *B* and *C*, Various forms of donor cells (5×10^6 cells) were adoptively transferred into NK1.1 Ab-treated mice and $CD1d^{-/-}$ mice, followed by i.v. injection with 15 and 18 μg/g of Con A, respectively. After 24 h, mice were killed, and serum levels of ALT were measured. These donor cells included hepatic MNCs isolated from wild-type mice, hepatic MNCs isolated from wild-type mice treated i.p. with IL-6 (2 μg/g) in vivo for 6 h, and hepatic MNCs treated with various concentrations of IL-6 in vitro for 4 h. Values are shown as the mean $\pm$ SEM from four mice. *, $p < 0.01$; **, $p < 0.001$ (compared with corresponding mice transferred with wild-type MNCs (□)). *D*, NKT-depleted mice were adoptively transferred with total MNCs (5×10^6 cells) that were treated with saline or IL-6 (500 ng/ml) in vitro for 4 h, followed by injection of Con A (15 μg/g) for 24 h. Liver histology was determined by H&E staining. Yellow arrows indicate the massive necrosis observed in the liver.

compared with the black line in the saline-treated group). The expression of IL-6R was detected in a very small percentage of NKT cells and was not affected after in vitro treatment with IL-6. The expression of IL-6R on $CD4^+$ T cells was also slightly down-regulated after IL-6 treatment (data not shown). Furthermore, administration of Con A did not affect IL-6R expression on $CD3^+$ T and NKT cells (Fig. 4*C*) and $CD4^+$ T cells (data not shown).

IL-6 inhibits NKT cells in a $CD4^+$ T cell-dependent manner

As only 1.5% of NKT cells express IL-6R, whereas 52.6% T cells express IL-6R, it is likely that IL-6 inhibition of NKT cells may be mediated via targeting T cells. To test this hypothesis, we performed adoptive transfer experiments with NKT cells plus T cells. As shown in Fig. 5, in vitro IL-6 treatment did not inhibit the ability of purified NKT cells to restore Con A-induced liver injury. Adoptive transfer of NKT cells plus $CD3^+$ T cells caused more severe liver injury in NK1.1-depleted mice, but was significantly inhibited by in vitro IL-6 treatment. These findings suggest that IL-6 inhibits NKT cells via a $CD3^+$ T cell-dependent mechanism. $CD4^+CD25^+$ regulatory T cells have been shown to suppress NKT cell function (24). Thus, we wondered whether IL-6 inhibition of NKT cells depended on $CD4^+CD25^+$ regulatory T cells. To test this hypothesis, we first examined the role of $CD4^+$ T cells in IL-6 inhibition of NKT cells. As shown in Fig. 5, adoptive transfer of NKT cells plus $CD3^+CD4^-$ T cells ($CD3^+$ T cells were isolated from CD4-deficient mice) caused severe liver injury in NKT1.1-depleted mice, which was not attenuated by in vitro IL-6 treatment. In contrast, in vitro IL-6 treatment markedly inhibited the ability of NKT cells plus $CD4^+$ T cells to restore Con A-induced liver injury in NK1.1-depleted mice. These findings suggest that IL-6 inhibits NKT cells via a $CD4^+$ T-dependent mechanism. As the $CD25^+$ cell population is very low in the liver, it is very difficult to obtain enough $CD4^+CD25^+$ regulatory T cells for adoptive transfer. Instead, we examined the effect of CD25 depletion on IL-6 inhibition of NKT cells. As shown in Fig. 5*A*, adoptive transfer of NKT cells plus $CD4^+CD25^-$ regulatory T cells restored Con A-induced liver injury in NK1.1-depleted mice, which was markedly suppressed by in vitro IL-6 treatment. In addition, as shown in Fig. 5, *B* and *C*, IL-6 treatment did not alter CD25 expression on hepatic $CD4^+$ T cells. Collectively, these findings suggest that $CD25^+$ T cells may not be important for IL-6 suppression of NKT cells.

IL-6 inhibits hepatic MNCs to restore Con A-induced hepatitis in NK1.1-depleted mice via a STAT3-dependent mechanism

To understand the molecular mechanism underlying IL-6-mediated inhibition of MNCs in Con A-induced hepatitis, we examined IL-6 activation of signaling pathways. As shown in Fig, 6*A*, IL-6 treatment caused significant activation of STAT3, but weak activation of STAT1. Fig. 6*A* also showed that IL-6 induced significant activation of STAT3 in liver $CD3^+$ T cells. Next, we examined the effects of STAT3 blockade on the ability of adoptive transfer of MNCs to restore Con A-induced liver injury in NK1.1-depleted mice. As shown in Fig. 6*B*, adoptive transfer of MNCs treated in vitro with IL-6 failed to restore Con A-induced liver injury in NK1.1-depleted mice, whereas inhibition of STAT3 activation with the STAT3 inhibitor or the JAK2 inhibitor abolished the inhibitory effect of IL-6 on NKT cells.

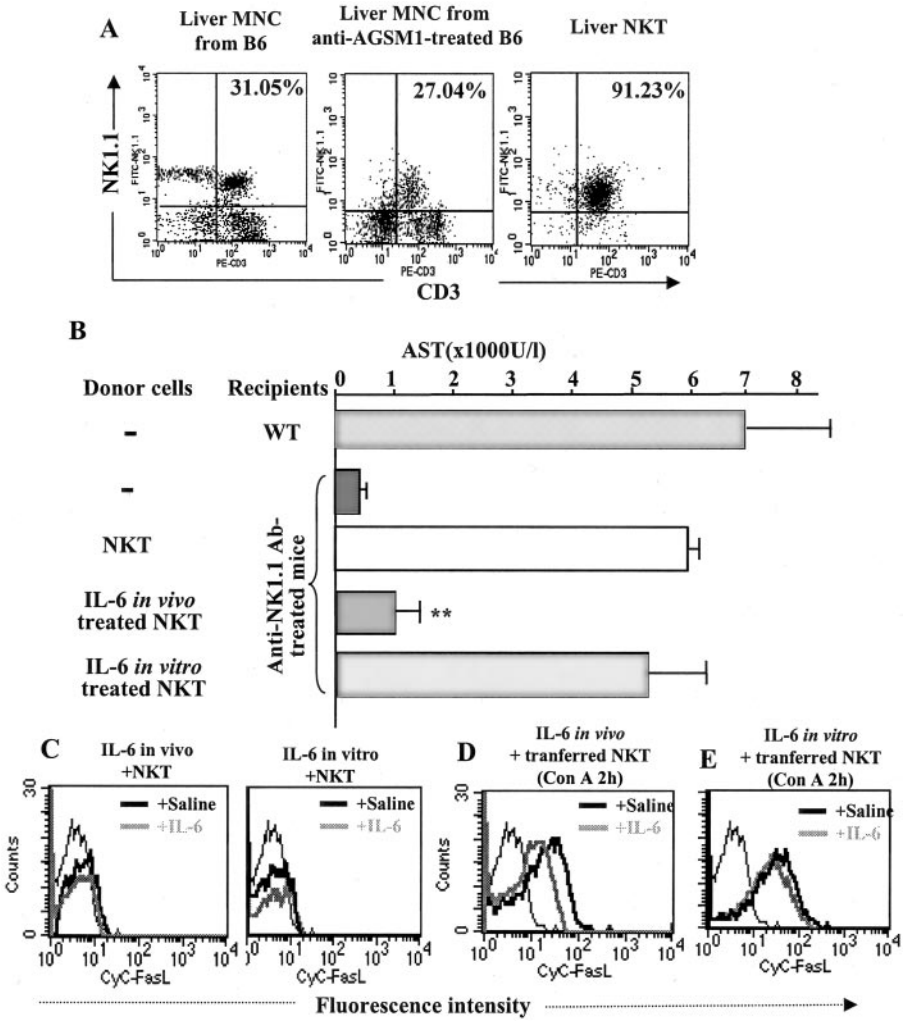

FIGURE 3. IL-6 indirectly inhibits the ability of NKT cells to restore Con A-induced liver injury in NK1.1-depleted mice. *A*, FACS analyses of liver MNCs from C57BL6 mice, liver MNCs from anti-ASGM-1-treated C57BL6 mice, and purified hepatic NKT cells with anti-CD3 and anti-NK1.1 Abs. *B*, NK1.1 Ab-treated mice were adoptively transferred with donor cells (1×10^6 purified hepatic NKT cells from different conditions), followed by injection of Con A (15 μg/g). After 24 h, mice were killed, and serum levels of ALT were measured. These donor cells included hepatic NKT cells isolated from wild-type mice, hepatic NKT cells from wild-type mice that were treated i.p. with IL-6 (2 μg/g) in vivo for 6 h, and hepatic NKT cells that were treated with IL-6 (500 ng/ml) in vitro for 4 h. Values are shown as the mean ± SEM from four mice. **, $p < 0.001$ (compared with corresponding mice transferred with purified NKT cells from wild-type MNCs (□)). *C*, Effects of IL-6 treatment on Fas L expression on NKT cells before Con A stimulation. Fas L expression was examined on hepatic NKT cells that were treated with IL-6 (2 μg/g) in vivo for 6 h or with IL-6 (500 ng/ml) in vitro for 4 h. *D* and *E*, Effects of IL-6 treatment on Fas L expression on adoptively transferred NKT cells after Con A stimulation. NK1.1 Ab-treated mice were adoptively transferred with 1×10^6 purified hepatic NKT cells that were treated with IL-6 (2 μg/g) in vivo for 6 h (*D*) or with IL-6 (500 ng/ml) in vitro for 4 h (*E*), followed by injection of Con A (15 μg/g). After 2 h, mice were killed, and Fas L expression was examined on hepatic NKT cells. Representative histograms in *C–E* for Fas L expression on NKT cells (black line for the saline-treated group and gray line for the IL-6-treated group) are shown overlaid on the IgG control (thin line).

Discussion

In this paper we demonstrate for the first time that IL-6 prevents T cell hepatitis via suppression of NKT cells in addition to its well-documented antiapoptotic action. We provide both in vitro and in vivo evidence suggesting that IL-6 inhibits NKT cells in T cell hepatitis. First, we examined the effect of IL-6 on in vitro hepatic NKT cell killing against hepatocytes, which has been suggested to play an important role in the pathogenesis of Con A-induced hepatitis (20). As shown in Fig. 1, administration of IL-6 markedly attenuated the ability of Con A-activated hepatic MNCs and NKT cells to kill hepatocytes in vitro (Fig. 1, *A* and *B*), which is probably due to inhibition of Fas L expression on NKT cells (Fig. 1*E*). $NK1.1^+CD3^+$ T cells rapidly declined after injection of Con A, which was markedly suppressed by IL-6 treatment (Fig. 1*C*). It was proposed that depletion of $NK1.1^+CD3^+$ T cells was due to apoptosis (20), but increasing evidence suggests that $NK1.1^+CD3^+$ T cells lose NK1.1 expression when they are activated (25–27). This suggests that loss of $NK1.1^+CD3^+$ T cells after injection of Con A may be due to loss of NK1.1 expression and could be an index of NKT cell activation in the liver, and IL-6 prevention of loss of $NK1.1^+CD3^+$ T cells may result from IL-6 inhibition of NKT activation. Although the number of NKT cells was higher in the IL-6-treated group, the expression of Fas L in these remaining NKT cells in this group was much lower than that in the saline-treated group (Fig. 1*E*), which could contribute to the reduced cytotoxicity of NKT cells from the IL-6-treated group compared with the saline-treated control group (Fig. 1*B*). Production of TNF-α, IL-4, and IFN-γ by NKT cells has been shown to play an important role in T cell hepatitis (21–23); however, in vivo IL-6 treatment did not reduce such production (R. Sun and B. Gao,

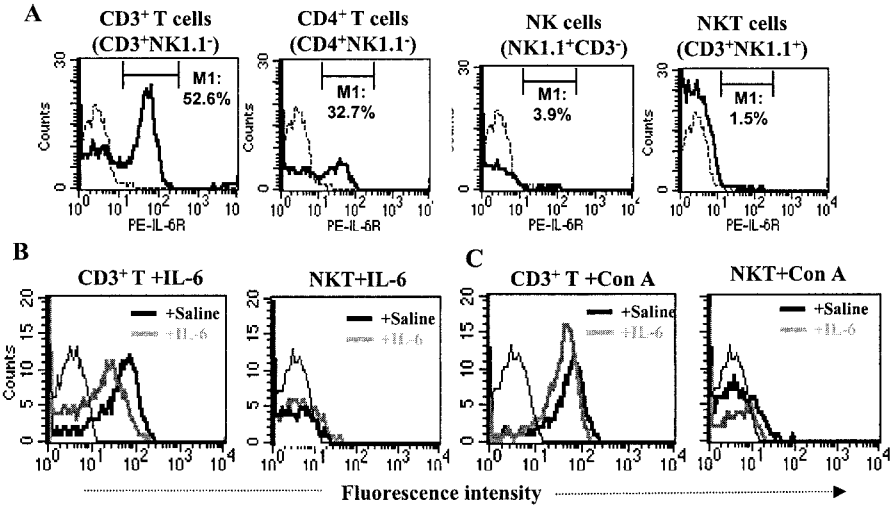

FIGURE 4. FACS analysis of expression of IL-6R on hepatic MNCs. *A*, Hepatic MNCs from C57BL6 mice were isolated and subjected to FACS analyses with anti-CD3, anti-NK1.1, anti-CD4, and anti-IL-6R Abs or control Ig G. Histograms for IL-6R on $CD3^+$ T cells, $CD4^+$ T cells, NK cells, and NKT cells (black solid line) are shown overlaid on the IgG control (thin dotted line). *B*, Hepatic MNCs were treated with IL-6 (500 ng/ml) for 4 h, followed by FACS analysis of IL-6R expression on $CD3^+$ T cells and NKT cells using anti-CD3, anti-NK1.1, and anti-IL-6R Abs. *C*, Hepatic MNCs were isolated from the livers of mice treated with Con A (15 μg/g) for various time periods, followed by FACS analysis of IL-6R expression as described in *B*. Histograms for IL-6R on $CD3^+$ T cells and NKT cells (black line for the saline-treated group and gray line for the IL-6-treated group) are shown overlaid on the IgG control (thin line).

unpublished observations). Taken together, these findings suggest that IL-6 inhibition of T cell hepatitis is partly due to suppression of Fas L expression on NKT cells. Second, we examined the effect of IL-6 on the ability of adoptive transfer of NKT cells in vivo to restore Con A-induced liver injury in two strains of mice that lack NKT cells or contain low levels of NKT cells. Although injection of anti-NK1.1 Ab depleted both NK ($NK1.1^+CD3^-$) and NKT ($NK1.1^+CD3^+$) cells (Fig. 2*A*), adoptive transfer NKT alone was able to restore Con A-induced liver injury in these NK- and NKT-depleted mice (Fig. 3*B*). These findings suggest that NK cells play a minor role in Con A-induced T cell hepatitis, which is consistent with a previous report showing that depletion of NK cells alone by

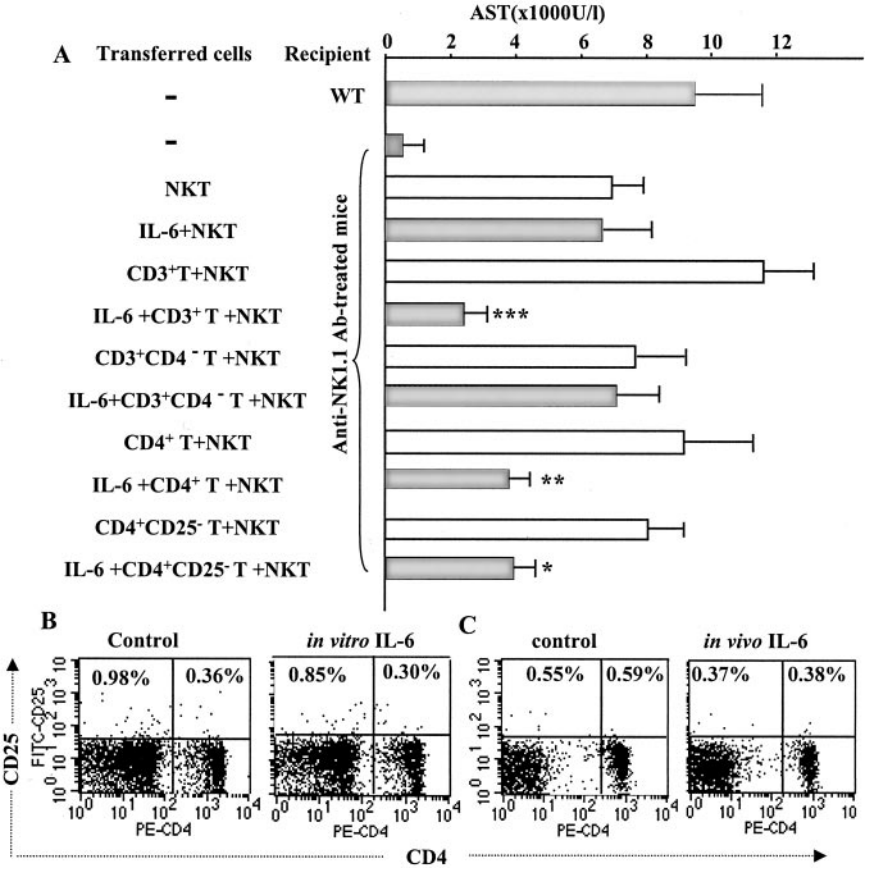

FIGURE 5. IL-6 inhibits the ability of NKT cells to restore Con A-induced liver injury in NK1.1-depleted mice via a $CD4^+CD25^-$ T cell-dependent mechanism. *A*, NK1.1 Ab-treated mice were adoptively transferred with donor cells, followed by injection of Con A (15 μg/g). After 24 h, mice were killed, and serum levels of ALT were measured. These donor cells included NKT (1×10^6) alone and a mixture of NKT cells (1×10^6) with various subtypes of T cells (1×10^6) that were treated with saline or IL-6 (500 ng/ml) in vitro for 4 h. Values shown are the mean ± SEM from three independent experiments. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (compared with corresponding mice transferred with purified NKT cells plus T cells without IL-6 treatment (□)). *B*, Hepatic MNCs were incubated with IL-6 (500 ng/ml) for 4 h, followed by FACS analysis with anti-CD4 and anti-CD25 Abs. *C*, Hepatic MNCs were isolated from mice that were injected i.p. with 1 μg/g of IL-6 or saline for 6 h, followed by FACS analysis with anti-CD4 and anti-CD25 Abs.

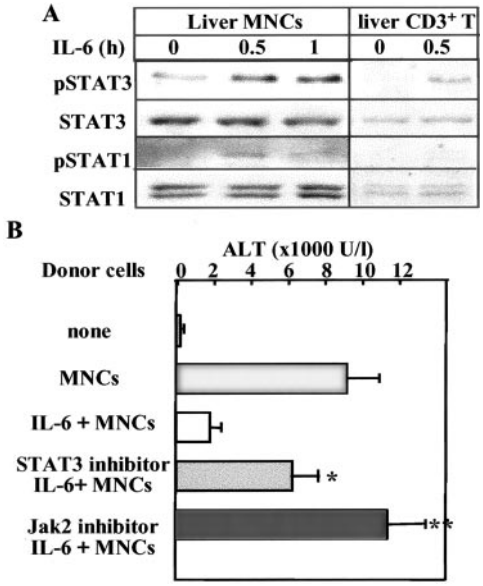

FIGURE 6. IL-6 inhibits the ability of MNCs cells to restore Con A-induced liver injury in NK1.1-depleted mice via an STAT3-dependent mechanism. *A*, Hepatic MNCs or hepatic $CD3^+$ T cells were treated with IL-6 (500 ng/ml) for various time periods. Cell extracts were then subjected to Western blot analyses with various Abs as indicated. *B*, NK1.1 Ab-treated mice were adoptively transferred with donor cells (5×10^6 hepatic MNCs cells from different conditions), followed by injection of Con A (15 μg/g). After 24 h, mice were killed, and serum levels of ALT were measured. These donor cells included hepatic MNCs, hepatic MNCs that were treated with IL-6 (500 ng/ml) in vitro for 4.5 h, and hepatic MNCs that were treated with STAT3 inhibitor (160 μM) or JAK2 inhibitor (AG490; 100 μM) for 30 min, followed by IL-6 (500 ng/ml) in vitro for 4 h. Values shown as the mean ± SEM from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (compared with IL-6-treated MNC group (□)).

anti-ASGM-1 Ab did not affect Con A-induced T cell hepatitis (22). Adoptive transfer of hepatic MNCs restored Con A-induced liver injury in anti-NK1.1-treated mice; however, adoptive transfer of hepatic MNCs treated with IL-6 in vivo and in vitro failed to restore such injury (Fig. 2), clearly indicating that IL-6 inhibits hepatic MNC initiation of T cell hepatitis. The inhibitory effect of IL-6 on the ability of adoptive transfer of MNCs in vivo to restore Con A-induced liver injury was also confirmed using $CD1d^{-/-}$ mice that lack NKT cells (Fig. 2*D*). Furthermore, adoptive transfer of NKT cells purified from wild-type mouse livers, but not from IL-6-treated mouse livers, restored Con A-induced liver injury in NK1.1-depleted mice, suggesting that IL-6 inhibits NKT cells in vivo. Subsequently, we provide several lines of evidence indicating that IL-6 inhibition of NKT cells is mediated via targeting $CD4^+$ T cells. First, in vitro treatment with IL-6 caused 90% inhibition of the ability of liver MNCs to restore Con A-induced liver injury in NK1.1-depleted mice (Fig. 2), whereas the same treatment did not significantly inhibit the ability of enriched NKT cells to restore such injury (Fig. 3). Second, the addition of $CD4^+$ T cells markedly restored the inhibitory effect of IL-6 on the NKT cells, whereas the addition of $CD3^+$ $CD4^-$ T cells did not restore this inhibitory effect (Fig. 5). Third, only a very small percentage of NKT cells express the IL-6R, whereas a large percentage of $CD4^+$ T cells express the IL-6R (Fig. 4). Additionally, neither IL-6 treatment nor Con A injection induced IL-6R expression on NKT cells (Fig. 4*B*). Therefore, it is very unlikely that IL-6 directly targets NKT cells.

The mechanism by which IL-6-activated $CD4^+$ T cells inhibit NKT cells is not clear. Recently, Azuma et al. (24) reported that human $CD4^+CD25^+$ regulatory T cells suppressed the cell proliferation, cytokine production, and cytotoxic activity of $V\alpha24^+$ NKT cells via cell-to-cell contact. Although the immunosuppressive effects of $CD4^+CD25^+$ T cells have been documented, the underlying mechanisms remain largely unknown (28, 29). Liver T cells contain ~1% $CD4^+CD25^+$ regulatory T cells, and this level was not elevated by in vivo or in vitro IL-6 treatment (Fig. 5, *B* and *C*). Adoptive transfer of $CD4^+CD25^-$ T cells plus NKT cells restored Con A-induced liver injury in NK1.1-depleted mice, which was still significantly inhibited by IL-6 in vitro treatment (Fig. 5*A*), suggesting that $CD4^+CD25^+$ regulatory T cells may only play a minor role in the inhibitory effect of IL-6 on NKT cells, and $CD4^+CD25^-$ T cells may have an important role. Currently, it is not clear how IL-6 targets $CD4^+CD25^-$ T cells, which subsequently suppress NKT cells in T cell-mediated hepatitis. Further studies are required to clarify the potential regulatory functions of $CD4^+CD25^-$ T cells in the inhibitory effect of IL-6 on NKT cells. In this paper we also demonstrate that the inhibitory effect of IL-6 on NKT cells requires STAT3 activation. IL-6 activation of STAT3 plays a crucial role in T cell survival (30). As there was no difference in T cell apoptosis between IL-6-treated and untreated T cells during adoptive transfer (R. Sun and B. Gao, unpublished observations), IL-6/STAT3 potentiation of the inhibitory effect of $CD4^+$ T cells on NKT cells is not due to protection against T cell apoptosis.

NKT cells represent a unique T cell population that coexpresses receptors of the NK lineage (e.g., NK1.1) and TCRs (31, 32). Two main subsets of NKT cells have been identified to date, including classical NKT cells, which are restricted by CD1d, and nonclassical NKT cells, which are CD1d-independent. The majority of CD1d-restricted NKT cells express an invariant TCR α-chain ($V\alpha14$-$J\alpha281$) with $V\beta8.2$, $V\beta2$, or $V\beta7$. Con A-induced hepatitis is markedly suppressed in both strains of CD1d- and $J\alpha281$-deficient mice (20, 21), suggesting that CD1d-restricted $V\alpha14$NKT cells play a critical role in Con A-induced hepatitis. The role of CD1d-independent nonclassical NKT cells in the development of Con A-induced hepatitis remains unknown. In this paper we demonstrate that IL-6 is able to inhibit $NK1.1^+CD3^+$ T cells via a $CD4^+$ T cell-dependent mechanism and consequently prevent Con A-mediated hepatitis. As $V\alpha14$NKT cells represent the majority of $NK1.1^+CD3^+$ T cells in murine livers (26, 31, 32), it is likely that IL-6 inhibits $V\alpha14^+$ NKT to develop Con A-induced hepatitis in a $CD4^+$ T cell-dependent manner. However, we do not rule out the potential role of $V\alpha14^-$ $NK1.1^+CD3^+$ T cells in IL-6 inhibition of T cell hepatitis, because a small percentage of $NK1.1^+CD3^+$ T cells in murine livers do not express the typical invariant $V\alpha14$-$J\alpha281$ TCR (26, 31–33). Further studies are required to clarify the role of $V\alpha14^-$ $NK1.1^+CD3^+$ T cells in T cell hepatitis and the possible involvement of these cells in the inhibitory effect of IL-6 in this model.

In addition to the Con A-induced T cell hepatitis model, NKT cells play an important role in the pathogenesis of liver injury in other models, including liver injury by *Salmonella* infection (34), partial hepatectomy (35), α-galactosylceramide (36), and carrageenan (37). It will be interesting to examine whether IL-6 also protects against liver injury in these models via suppression of NKT cells. NKT cells have also been implicated in the pathogenesis of viral hepatitis (38–40) and hepatocellular carcinoma (41, 42), which might be affected by IL-6, as elevation of IL-6 is always associated with these conditions.

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