GASTROENTEROLOGY 2006;130:435–452

# BASIC–LIVER, PANCREAS, AND BILIARY TRACT

## Natural Killer Cells Ameliorate Liver Fibrosis by Killing Activated Stellate Cells in NKG2D-Dependent and Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Dependent Manners

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*Background & Aims:* Viral hepatitis infection, which is a major cause of liver fibrosis, is associated with activation of innate immunity. However, the role of innate immunity in liver fibrosis remains obscure. *Methods:* Liver fibrosis was induced either by feeding mice with the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet or by injecting them with carbon tetrachloride. The Toll-like receptor 3 ligand, polyinosinic-polycytidylic acid, was used to activate innate immunity cells and mediators, including natural killer cells and interferon γ. *Results:* In the mouse model of DDC-induced liver fibrosis, natural killer cell activation by polyinosinic-polycytidylic acid induced cell death to activated hepatic stellate cells and attenuated the severity of liver fibrosis. Polyinosinic-polycytidylic acid treatment also ameliorated liver fibrosis induced by carbon tetrachloride. The observed protective effect of polyinosinic-polycytidylic acid on liver fibrosis was diminished through either depletion of natural killer cells or by disruption of the interferon γ gene. Expression of retinoic acid early inducible 1, the NKG2D ligand, was undetectable on quiescent hepatic stellate cells, whereas high levels were found on activated hepatic stellate cells, which correlated with the resistance and susceptibility of quiescent hepatic stellate cells and activated hepatic stellate cells to natural killer cell lysis, respectively. Moreover, treatment with polyinosinic-polycytidylic acid or interferon γ enhanced the cytotoxicity of natural killer cells against activated hepatic stellate cells and increased the expression of NKG2D and tumor necrosis factor–related apoptosis-inducing ligand on liver natural killer cells. Blocking NKG2D or tumor necrosis factor–related apoptosis-inducing ligand with neutralizing antibodies markedly diminished the cytotoxicity of polyinosinic-polycytidylic acid–activated natural killer cells against activated hepatic stellate cells. *Conclusions:* Our findings suggest that natural killer cells kill activated hepatic stellate cells via retinoic acid early inducible 1/NKG2D-dependent and tumor necrosis factor–related apoptosis-inducing ligand–dependent mechanisms, thereby ameliorating liver fibrosis.

Viral hepatitis, which affects half a billion people worldwide, is a major cause of liver injury, fibrosis, and cirrhosis; however, the cellular and molecular mechanisms underlying the progression of liver disease during infection are not fully understood.[1,2] Emerging evidence suggests that natural killer (NK) cells, which are particularly enriched in the liver and activated by hepatitis viruses, play crucial roles in inducing antiviral immunity in the liver and inducing liver injury from elimination of virally infected hepatocytes.[3–8] NK cells are also involved in drug-induced hepatotoxicity[9,10] and in suppressing liver regeneration via an interferon (IFN)-γ–dependent mechanism[11]; however, the role of NK cells in the development and progression of liver fibrosis remains unknown.

Liver fibrosis is a common scarring response to chronic liver injury, regardless of etiology, including viral hep-

*Abbreviations used in this paper:* Ab, antibody; ASGM-1, anti–asialo GM-1; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FACS, fluorescence-activated cell sorting; FasL, Fas ligand; HSC, hepatic stellate cell; IFN, interferon; MNC, mononuclear cells; NK, natural killer; NKT, natural killer T cell; PDGF, platelet-derived growth factor; poly I:C, polyinosinic-polycytidylic acid; RAE1, retinoic acid early inducible 1; RT-PCR, reverse-transcription polymerase chain reaction; α-SMA, α-smooth muscle actin; TGF, transforming growth factor; TLR3, Toll-like receptor 3; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.


0016-5085/06/$32.00
doi:10.1053/j.gastro.2005.10.055

atitis infection, alcohol abuse, nonalcoholic steatohepatitis, and autoimmune hepatitis.[12,13] Activation of hepatic stellate cells (HSCs) (formerly known as lipocytes, Ito cells, fat-storing cells, and perisinusoidal cells) is a key step in the development of liver fibrosis. Once activated, resident HSCs become fibrogenic myofibroblasts (activated HSCs), which express α-smooth muscle actin (α-SMA; a hallmark for activated HSCs) and produce large amounts of extracellular matrix proteins, such as collagen, resulting in liver fibrosis.[12,13] HSCs are activated by a variety of cytokines and growth factors, including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), tumor necrosis factor α, interleukin 1, angiotensin II, and leptin,[12,13] but are inhibited by antifibrogenic cytokines, such as interleukin 10, adiponectin, IFN-α/β, and IFN-γ.[12,13] For example, IFN-γ–deficient mice are more susceptible to liver fibrosis induced by carbon tetrachloride,[14] and the antifibrogenic effect of IFN-γ is believed to be mediated via inhibiting HSC activation and TGF-β signaling.[15–19] In this article, we show that NK cells act as antifibrogenic cells by killing activated HSCs. We also show that the cytotoxicity of NK cells is regulated by IFN-γ and the Toll-like receptor 3 (TLR3) ligand, polyinosinic-polycytidylic acid (poly I:C). Poly I:C is a potent stimulator for NK cells[20] and recognizes TLR3.[21]

At present, it is generally accepted that the cytotoxicity of NK cells against tumor cells and microbially infected autologous cells is determined by the balance between the effects of opposing NK cell receptors.[22–27] NK cells express several well-defined inhibitory receptors that recognize major histocompatibility complex class I molecules and inactivate NK cell functions.[22–26] These inhibitory receptors include inhibitory killer cell immunoglobulin-like receptor KIR, Ly-49A, and CD94/NKG2 receptors. Among the several stimulatory NK cell receptors, the NKG2D receptor is the best defined and is expressed on both human and mouse NK cells, where it is recognized by several ligands, including MHC class I chain–related gene A and UL16-binding protein for human NK cells and retinoic acid early inducible 1 (RAE1), histocompatibility 60, and mouse UL16-binding protein-like transcript 1 for mouse NK cells.[22–26] It has been shown that tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)$^+$ liver NK cells express low levels of the NK inhibitory receptor Ly-49A, which could be an important mechanism contributing to the cytotoxicity of liver NK cells against self hepatocytes[28] and tumors.[29] Here we show that NK cells kill activated HSCs, but not quiescent HSCs, which is likely mediated via an NKG2D/RAE1-dependent mechanism because expression of RAE1, the NKG2D ligand, was detected at high levels on activated HSCs but not on quiescent HSCs. Moreover, the cytotoxicity of NK cells against activated HSCs seems to be dependent on TRAIL also.

## Materials and Methods

### Materials

Poly I:C, Gey's balanced salt solution, and OptiPrep were purchased from Sigma (St Louis, MO). Pronase E and collagenase D were obtained from Roche (Indianapolis, IN).

### Mice

Eight- to 10-week old male C57BL/6J, IFN-$\gamma^{-/-}$ mice (C57BL/6J background), perforin$^{-/-}$ mice (C57BL/6J background), and Fas ligand (FasL)$^{-/-}$ mice (C57BL/6Smn background) were purchased from the Jackson Laboratory (Bar Harbor, ME). CD1d$^{-/-}$ mice (natural killer T cell [NKT] deficient) on a BALB/c background were originally purchased from the Jackson Laboratory and backcrossed with C57BL/6J mice for at least 8 generations. All mice used in this study were housed in a specific pathogen–free facility and were cared for in accordance with National Institutes of Health guidelines.

### Liver Injury and Fibrosis Induced by a 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine Diet and Carbon Tetrachloride

For 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet–induced liver injury, mice were fed a diet containing DDC (0.1% wt/wt in Purina 5015 mouse chow; Bio-Serv, Frenchtown, NJ) for 1–4 weeks. In the carbon tetrachloride–induced liver injury model, mice were injected intraperitoneally (IP) with carbon tetrachloride (10% in olive oil, 2 mL/kg, 3 times a week) for 2–3 weeks. After mice were killed, liver tissues were frozen in liquid nitrogen or fixed in 10% buffered formalin and embedded in paraffin.

### Depletion of Natural Killer Cells by Anti–Asialo GM-1 Antibodies

To deplete NK cells (NK1.1$^+$CD3$^-$), anti-asialo GM-1 (ASGM-1) antibody (Ab; 100 μL per mouse; catalog no. 986-10001; Wako, Richmond, VA) was injected IP into mice. After 24 hours, depletion of NK (NK1.1$^+$CD3$^-$) cells was confirmed by flow cytometry.[11] To chronically deplete NK cells, mice were treated with anti–ASGM-1 every 48 hours for 2 weeks.

### Treatment of Mice With Polyinosinic-Polycytidylic Acid

We have previously shown that injection of mice with poly I:C markedly induces accumulation and activation of NK cells in the liver, with the peak effect occurring at 24 hours and returning to basal levels at 48 hours after injection.[30] Therefore, for chronic treatment with poly I:C, mice were injected IP with poly I:C every 48 hours for 1–2 weeks. For mice in the