Article

TLR3 Ligand-Induced Accumulation of Activated Splenic Natural Killer Cells into Liver

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It has been revealed that poly I:C is a potent stimulator for NK cells, which can induce NK cell rapid activation and preferential accumulation into liver. However, the process mediating the influx of NK cells remains obscure. In this study, we found that poly I:C administration increased the portion and absolute number of NK cells in liver, but largely decreased those in spleen. There were no obvious changes of these lymphocytes in other immune organs. The results from splenic adoptive transfer and splenectomy showed that the recruited spleen NK cells contributed to the accumulation of NK cells in liver, and this process was regulated by the production of chemokines and the presence of T cells. This investigation will help to understand the enhanced immune cell recruitment in liver upon viral infection. *Cellular & Molecular Immunology*. **2005;2(6):449-453.**

Key Words: liver, spleen, NK, poly I:C, recruitment

Introduction

The liver plays a critical role in first-line host defense against incoming gut-derived foreign antigens that enter *via* the portal veins (1). The hepatic immune system must maintain a well-controlled balance between tolerance and initiation of an immune response. The immune constitution of liver is quite distinct from other sites, containing a large resident macrophages and innate immune lymphocytes such as natural killer (NK) cells. These populations provide immune surveillance against foreign antigens.

NK cells are one of the cellular mediators of innate defense. They are lymphoid cells, without the need for immunization or pre-activation, and can recognize and kill the aberrant cells and rapidly release a large amount of soluble factors which have antimicrobial effects or prime the immune responses of other cells (2). The abundance of NK cells in liver implies a very important role of these cells in liver biology. Several polyribonucleotides have already been adopted in clinical trials for the treatment of cancer and viral diseases. Polyinosinic-polycytidylic acid (poly I:C) is an

artificial mimic of viral RNA, which may trigger the immune response resembling viral infection (3). The previous reports in mice demonstrated that poly I:C potently augmented NK cell activity and accumulated these cells into liver, suggesting that this molecule is an excellent augmentor of liver-associated NK activity (4). However, the origin contributing to the influx of NK cells remains obscure.

In present study, we found that poly I:C administration increased the portion and absolute number of NK cells in liver, but largely decreased those in spleen. There were no obvious changes of these lymphocytes in other immune organs. The results from splenic adoptive transfer and splenectomy showed that the recruited spleen NK cells contributed to the accumulation of NK cells in liver, and this process was regulated by the production of chemokine protein and the presence of T cells. This investigation will help to understand the enhanced immune cell recruitment upon viral infection.

Materials and Methods

Animals
Male C57BL/6, BALB/C and SCID mice with BALB/C gene background (six to eight week-old, weighing 20-24 g) were obtained from Shanghai Experimental Center, Chinese Academy of Sciences and maintained at our animal facility under the specific pathogen-free condition.

Reagents
Poly I:C was purchased from Sigma Chemical Co. and separately dissolved in pyrogen-free phosphate-buffered saline (PBS) at 1 mg/ml. The mAbs used in this study included FITC-conjugated anti-DX5 and anti-NK1.1, PE-

Table 1. Tissue distribution of NK cells

	Liver	Spleen	Bone marrow	Thymus	Lung
0 h	8.55 ± 1.05	5.63 ± 0.37	3.82 ± 0.21	0.39 ± 0.06	10.74 ± 2.01
12 h	$38.96 \pm 2.03^*$	$1.68 \pm 0.44^*$	3.76 ± 0.19	0.41 ± 0.03	11.09 ± 1.60

The lymphocytes from different tissues of C57BL/6 mice were isolated at 0 and 12 h after poly I:C injection (5 μg/g), and their phenotype was detected with anti-NK1.1 and anti-CD3 antibodies by FACS analysis. The portions of NK cells were calculated. Each value represents mean ± SD of results from three mice. * $p < 0.01$ *vs.* corresponding 0 h time point.

conjugated anti-NK1.1 (eBioscience), and PE-CY5-conjugated anti-CD3e (PharMingen), 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Sigma Chemical Co., kept as 0.5 mM stock in DMSO and stored at -20°C in a desiccator box.

Cell preparation
Livers were passed through a 200-gauge stainless steel mesh. Washing once with PBS, the cells were resuspended in 40% Percoll (Sigma) and then gently overlaid on 70% Percoll and centrifuged at 2,400 rpm for 30 min at room temperature. Hepatic mononuclear cells (MNCs) were collected from the interphase and washed twice in PBS. To obtain lung mononuclear cells (MNCs), the lung was minced, suspended in medium containing 0.05% collagenase (Sigma) and 0.01% DNase I (Sigma), and then shaken for 20 min in a 37°C water bath. Thereafter, the lung specimens were passed through a 200-gauge stainless steel mesh, and MNCs were isolated with Percoll solution as described above. The splenocytes and thymocytes were obtained by forcing tissues through stainless steel mesh, and the bone marrow cells were isolated form mouse femur and tibia. Before using, the erythrocytes were removed from the cell suspension by lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, and 170 mM Tris, pH7.3).

Tracking the recruitment of splenic lymphocytes
Under sterile conditions, the spleen cells were separated and used after erythrocyte lysing. The splenic lymphocytes were washed with ice cold PBS, resuspended at 1×10^7 cells/ml in ice cold PBS and then incubated with 0.5 μM CFSE for 10 min at 37°C. After labeling, the cells were immediately centrifuged and washed three times with ice cold PBS. Suspension containing 5×10^6 cells in 100 μl PBS was gently injected into the inferior tip of spleen with hemostasis obtained by ligating the injection site (5). At a given time after transfer, the hepatic lymphocytes were prepared, and the CFSE$^+$ cell frequency and phenotype were determined.

Splenectomy
Splenectomy was performed as previously described (6). Briefly, mice were anesthetized by inhalation of ether before surgery. Under sterile conditions, a left-sided laparotomy was performed, then the spleen was gently mobilized, exteriorized and the vascular supply was cut off by two

ligatures (4-0 resorbable suture) placed around the vessels on the upper and lower poles of the spleen. The peritoneal wall and the skin were closed by separate sutures. Mice were allowed to recover from surgery for at least 5 days.

Flow cytometry
After blocking with anti-FcγR (eBioscience), the cells were incubated with saturating amount of the indicated fluorescence-labeled mAbs in darkness at 4°C for 30 min, and then washed twice. The stained cells were analyzed by FACSCalibur, (Becton Dickinson) and the data were analyzed by WinMDI 2.8 software.

Detection of specific mRNA expressions by RT-PCR
PCR primers for detecting mRNAs for VLA, VCAM-1, LFA-1, ICAM-1, FKN, MCP-1, MIP-1, IP-10 and β-actin were designed by us to be 18-24 nucleotides long and to have a 100% homology with the particular regions of the genes according to gene sequences. The RT-PCR method was used as previously described (7).

Statistical analysis
The results were analyzed using the Student's *t* test or analysis of variance where appropriate. $p < 0.05$ was considered significant.

Results

Poly I:C injection preferentially accumulated NK cells in liver
About 8% NK cells were resident in the liver of normal C57BL/6 mouse, while poly I:C treatment significantly elevated this portion to around 40% 12 hours after administration. The portion of NK cells diminished largely in spleen (from 6% to 2%), but remained unchanged in bone marrow, thymus and lung (Table 1).

Consistently, after poly I:C injection the number of total NK cells in liver was dramatically increased in liver and peaked at 12 h with 4-fold increase, while those in spleen was significantly decreased and kept steady after 18 h post injection (Figure 1A). As shown in Figure 1B, nearly all the accumulated NK cells in liver expressed activation molecule CD69 after poly I:C administration and remained at a high level till 24 hours, indicating a lasting activation of NK cells induced by poly I:C stimulation.
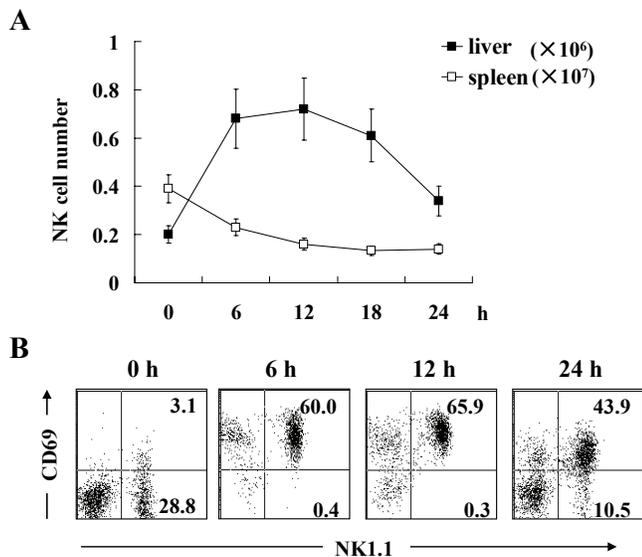
A

B

Figure 1. poly I:C preferentially assembled NK cells in liver. (A) The lymphocytes from liver and spleen of C57BL/6 mice were isolated at the indicated time points after poly I:C injection and stained with anti-NK1.1 and anti-CD3 antibodies. The absolute number of NK cells was calculated. Each value represents mean ± SD of results from three mice. (B) The hepatic lymphocytes were prepared at the indicated time points post poly I:C administration, and then stained with anti-NK1.1, anti-CD69 and anti-CD3 antibodies.

A

B

Figure 2. Splenic NK cells were recruited into liver by poly I:C stimulation. (A) C57BL/6 mice received adoptive transfer of CFSE-labeled spleen cells, and then were injected with poly I:C. The portion and phenotype of liver CFSE positive cells were determined by FACS analysis with anti-NK1.1 and anti-CD3 antibodies. (B) Mice were splenectomized and then treated with poly I:C. Twelve hours later, the liver lymphocytes were harvested and analyzed by FACS using anti-NK1.1 and anti-CD3 antibodies. The absolute number of NK cells was calculated. Each value represents mean ± SD of results from three mice. * $p < 0.05$ *vs.* PBS-treated or sham-operated group.

Recruited spleen NK cells contributed to the accumulation of NK cells in liver

The evidence that the portion and absolute number of NK cells were markedly increased in liver but significantly decreased in spleen after poly I:C injection raised a possibility that NK cells infiltrated from spleen to liver following poly I:C challenge. To further confirm this conclusion, we performed splenic adoptive transfer of labeled cells. Gated on CFSE positive transferred cells in liver, though T cells were increased to nearly 2 fold of that in PBS-treated group, NK cells were the major enhanced population with about 6-fold increase (Figure 2A), suggesting the recruitment of NK cells from spleen into liver. As shown in Figure 2B, when mice were splenectomized, the number of liver NK cells in poly I:C-treated mice was significantly reduced compared to sham-operated group, indicating that spleen supplied additional NK cells to liver and largely contributed to their recruitment into liver upon poly I:C stimulation.

Since a considerable amount of NK cells were recruited into liver, it is possible that poly I:C locally enhanced the liver expression or release of some effector molecules to trap NK cells. As shown in Figure 3, poly I:C injection did rapidly induce much greater production of FKN and a slight increase of VCAM-1 in liver, but the levels of other detected chemokine molecules and adhesion proteins were not significantly elevated.
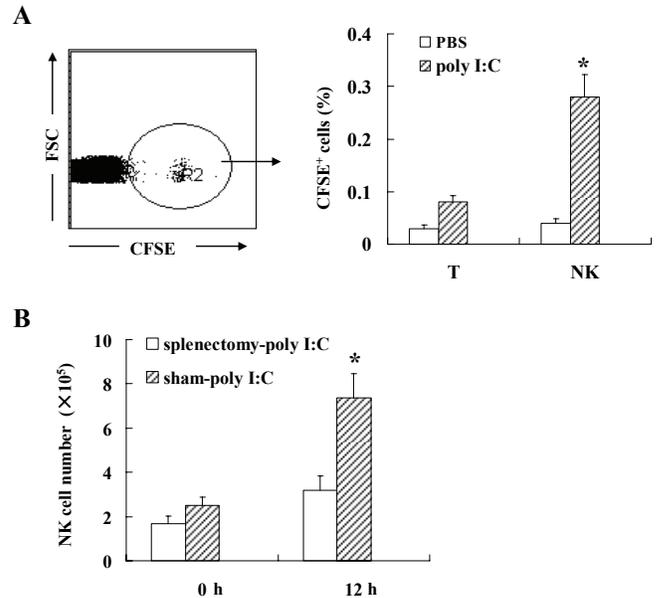
The recruitment and activation of NK cells required the presence of T cells

Next, we investigated whether NK cells were solely sufficient for their own infiltration and activation after poly I:C challenge. In T cell-deficient SCID mice, the early recruitment of NK cells was significantly reduced (Figure 4A), and this absence of T cells also blunted the expression of CD69 on NK cells as indicated by the decreased percentage of CD69$^+$ NK cells in liver compared to control BALB/C mice (Figure 4B), suggesting that to some extent T cell existence was important to the effect of poly I:C on NK cells.

Discussion

Liver contains an unusual population of resident lymphocytes, among which CD8$^+$ T cells usually out-number CD4$^+$ T cells, and both natural killer (NK) and natural killer T (NKT) cells are enriched relative to their proportions in other lymphoid tissues (8). These resident and migratory populations of lymphocytes and microphages set a powerful defense line against invading antigens, and can be rapidly expanded in response to infection or injury by recruiting leucocytes from the circulation and other lymphoid organs (9).
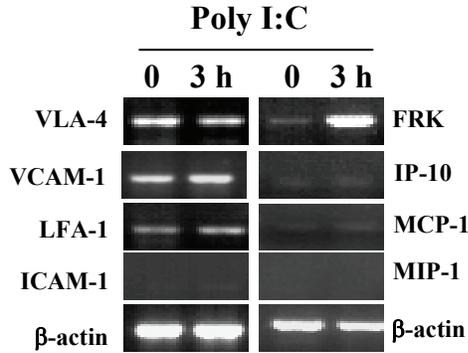
Poly I:C

Figure 3. The expression of effector molecules may direct the infiltration of NK cells. The mRNA were extracted from liver tissue at the indicated time points after poly I:C injection, and the expressions of the indicated genes were determined using RT-PCR.

Figure 4. The presence of T cells assisted NK cell recruitment and activation. The liver lymphocytes from BALB/C and SCID mice were isolated at the indicated time points after poly I:C injection, and then stained with anti-DX5, anti-CD69 and anti-CD3 antibodies. The number of total NK cells (A) and CD69^{+} NK cells (B) were calculated. Each value represents mean ± SD of results from three mice. * $p < 0.05$ *vs.* the corresponding 0 h time point.

Double-stranded RNA (dsRNA) and the viral RNA mimic, poly I:C, are recognized by Toll-like receptor 3 (TLR3) that mediates the innate immune response to viral infections (10). It has been well documented that poly I:C, as a potent stimulator for NK cells, can highly activate and accumulate these innate immune cells into liver, and also enhance their natural cytotoxic activity and cytokine production (4). Consistent with the previous observations, our results showed that poly I:C did preferentially accumulate NK cells in liver, with a rapid increases of absolute number counting and cell portion in total hepatic lymphocytes. Interestingly, NK cell distribution in spleen was significantly reduced after poly I:C injection, but no measurable alterations were detected in other lymphoid tissues. Considering the close location of spleen and liver in physiology, the results raised a possibility of the recruitment of NK cells from spleen to liver. Cell-trafficking experiment, with fluorescence-labeled cells, directly showed that compared to T cells, poly I:C induced a more greater influx of spleen NK cells into liver. The further results from splenectomized mice supported our conclusion: liver recruited splenic NK cells upon poly I:C stimulation. But the splenectomy did not completely inhibit poly I:C-induced accumulation of NK cells in liver, suggesting that there probably exist other sources supplying NK cells to liver, such as the peripheral circulation. Our preliminary experiment showed a slight decrease of NK cells in peripheral blood from poly I:C-treated mice (data not shown).

The recruiting process is dependent on the ability of lymphocytes to recognize, bind to and migrate across the endothelial cells that line the vasculature. Liver sinusoidal endothelial cells constitutively express intercellular adhesion molecule-1 (ICAM-1) and leukocyte function associated antigen (LFA-1) (1, 9, 11), and can be induced to express vascular cell adhesion molecule (VACM) to retain the target cells with VLA expression (9, 12, 13). Thus, hepatic endothelium could trap the circulating lymphocytes through the intera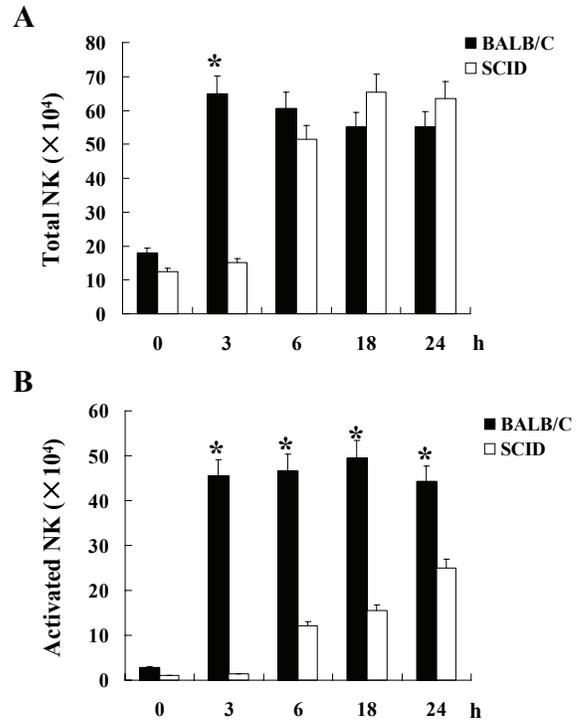ction of these adhesion proteins. We only detected a slight increase of poly I:C-induced VCAM-1 expression in liver, which is partially consistent with the observation of Fogler WE, et al. (14). But the expressions of other adhesion molecules showed no distinct changes compared to those of control group. In addition, chemokines have great potential to promote NK cell chemoattraction and migration. Some studies have shown that expression of many CC chemokines including macrophage inflammatory protein 1 (MIP-1) and monocyte chemotactic protein 1 (MCP-1), as well as CXC chemokines such as interferon γ-inducible protein 10 (IP-10) in the liver (15, 16). A newly identified CX3C-chemokine, fractalkine, expressed on activated endothelial cells also plays an important role in leukocyte adhesion and migration (17, 18). Fractalkine plays an important role not only in the binding of NK cells to endothelial cells (19) or chemoattraction of NK cells into tumor sites (20), but also activation of NK cells (21, 22), all of which play important roles in elimination of pathogens and cancer cells. Our results showed that a dramatic elevation of fractalkine was induced by poly I:C injection, while other chemokines remained unchanged. This pattern of chemokine expression provided evidence for specific pathways of poly I:C-caused NK cell recruitment into liver.

Furthermore, the absence of T cells markedly retarded the

early liver recruitment of NK cells, and also downregulated NK cell activation in liver, suggesting that the infiltration and activation of NK cells induced by poly I:C was T cell-dependent. Our preliminary experiment also found that pre-activated T cells could boost NK cell activation and finally lead to an aggravated liver injury (our unpublished data).

In conclusion, the studies presented in this work demonstrated that poly I:C induced the migration of NK cells from spleen into liver with elevated fractalkine production and the presence of T cells in liver. These trafficking events may serve to promote host immune responses against viral infection.

References

1. Peters MG. Animal models of autoimmune liver disease. Immunol Cell Biol. 2002;80:113-116.
2. Colucci F, Caligiuri MA, Di Santo JP. What does it take to make a natural killer? Nat Rev Immunol. 2003;3:413-425.
3. Bigger JE, Thomas III CA, Atherton SS. NK cell modulation of murine cytomegalovirus retinitis. J Immunol. 1998;160:5826-5831.
4. Twilley TA, Mason L, Talmadge JE, Wiltrout RH. Increase in liver-associated natural killer activity by polyribonucleotides. Nat Immun Cell Growth Regul. 1987;6:279-290.
5. Ponder KP, Gupta S, Leland F, et al. Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. Proc Natl Acad Sci U S A. 1991;88:1217-1221.
6. John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober. Current protocols in immunology. Boston, MA: John Wiley & Sons, Inc.;1994.
7. Tian Z, Shen X, Feng H, Gao B. IL-1β attenuates IFN-α β-induced antiviral activity and STAT1 activation in the liver: involvement of proteasome-dependent pathway. J Immunol. 2000;165:3959-3965.
8. Norris S, Collins C, Doherty DG, et al. Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. J Hepatol. 1998;28:84-90.
9. Lalor T, Adams D. The liver: a model of organ-specific lymphocyte recruitment. Expert Rev Mol Med. 2002;2002:1-15.
10. Schmidt KN, Leung B, Kwong M, et al. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. J Immunol. 2004;172:138-143.
11. Crispe IN. Hepatic T cells and liver tolerance. Nat Rev Immunol. 2003;3:51-62.
12. Adams DH, Burra P, Hubscher SG, Elias E, Newman W. Endothelial activation and circulating vascular adhesion molecules in alcoholic liver disease. Hepatology. 1994;19:588-594.
13. Yoong KF, McNab G, Hubscher SG, Adams DH. Vascular adhesion protein-1 and ICAM-1 support the adhesion of tumor-infiltrating lymphocytes to tumor endothelium in human hepatocellular carcinoma. J Immunol. 1998;160:3978-3988.
14. Fogler WE, Volker K, McCormick KL, Watanabe M, Ortaldo JR, Wiltrout RH. NK cell infiltration into lung, liver, and subcutaneous B16 melanoma is mediated by VCAM-1/VLA-4 interaction. J Immunol. 1996;156:4707-4714.
15. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol. 1999;17: 189-220.
16. Morris MA, Ley K. Trafficking of natural killer cells. Curr Mol Med. 2004;4:431-438.
17. Bazan JF, Bacon KB, Hardiman G, et al. A new class of membrane-bound chemokine with a CX3C motif. Nature. 1997;385:640-644.
18. Pan Y, Lloyd C, Zhou H, et al. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. Nature. 1997;387:611-617.
19. Yoneda O, Imai T, Goda S, et al. Fractalkine-mediated endothelial cell injury by NK cells. J Immunol. 2000;164: 4055-4062.
20. Lavergne E, Combadiere B, Bonduelle O, et al. Fractalkine mediates natural killer-dependent antitumor responses *in vivo*. Cancer Res. 2003;63:7468-7474.
21. Robinson LA, Nataraj C, Thomas DW, et al. The chemokine CX3CL1 regulates NK cell activity *in vivo*. Cell Immunol. 2003;225:122-130.
22. Guo J, Chen T, Wang B, et al. Chemoattraction, adhesion and activation of natural killer cells are involved in the antitumor immune response induced by fractalkine/CX3CL1. Immunol Lett. 2003;89:1-7.