

Characterization of Natural Killer Cells in the Liver of Young Mice

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OBJECTIVE To determine the quantity and quality of liver NK cells from young and adult mice and compare their characteristics.

METHODS C57BL/6 mice were used at 2 weeks (young) and 8 weeks (adult) of age. The percentage and absolute number of NK cells in the liver and spleen were analyzed. The cytotoxicity of NK cells in the liver and spleen against various targets were detected by a 4 h ⁵¹Cr-release method. FACS-can was used to analyze the expression of CD69, Mac-1, Ly49C/I and CD94 on the NK cells. Perforin mRNA levels were analyzed by the reverse transcription polymerase chain reaction (RT-PCR).

RESULTS The percentages of NK cells in the liver of young and adult mice were similar ($11.9\% \pm 1.7\%$ vs. $9.9\% \pm 1.6\%$, $P > 0.05$), but the absolute number per liver weight was higher in the young animals ($11.6 \pm 2.5 \times 10^5/g$ vs. $3.4 \pm 0.8 \times 10^5/g$, $P < 0.05$). The level of NK cytotoxicity was extremely high in the liver of young compared to adult mice ($71.0\% \pm 5.5\%$ vs. $23.8\% \pm 4.4\%$, $P < 0.05$), but this difference was not observed in the spleen. Phenotypes of the liver NK cells from young and adult mice were completely different from each other. The liver NK cells from young mice were CD69^{high} Mac-1^{low} Ly49C/I^{low}, whereas NK cells from older mice displayed inverse antigen levels (CD69^{low} Mac-1^{high} Ly49C/I^{high}). The expression levels of other NK cell-related markers were similar in both groups. The perforin mRNA level in the liver lymphocytes from young mice was consistently greater compared to adult mice.

CONCLUSION From 2 to 8 weeks C57BL/6 mice liver NK cells undergo age-associated changes. At 2 weeks of age the liver NK cells showed a high level of NK cytotoxicity and a unique phenotype which was not apparent at 8 weeks of age.

KEYWORDS: NK cells, liver, aging, cytotoxicity.

INTRODUCTION

Natural killer (NK) cells are a critical component of the innate immune response to viral and obligate intracellular parasitic infections^[1]. NK and T cells appear to differentiate from a common progenitor. The process of NK cell maturation occurs primarily within the bone marrow microenvironment and requires cell-to-cell interactions and soluble factors derived from bone marrow stromal cells^[2]. It has been reported that NK cells are generated in the fetal liver^[3], which may explain why NK cells are abundant in both mouse and human liver.

Age-associated changes in NK cells from the spleen, cord blood and peripheral blood have been widely described^[4,5]. However, little is known about liver NK cells, especially at a very young age.

In the present study, we compared the NK cells present in the liver of young mice with that of adult mice. A unique phenotype

and high cytotoxicity are described for the liver NK cells of young mice.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were used at 2 (young) and 8 (adult) weeks of age. All of the mice were maintained under specific pathogen-free conditions in the animal facility of Harbin Medical University.

Cell preparation

Hepatic lymphocytes were isolated by a previously described method^[6]. Briefly, mice anaesthetized with ether, were sacrificed by exsanguination via a cardiac puncture. To obtain lymphocytes, the liver was removed, pressed through a 200-gauge stainless steel mesh, and then suspended in Eagle's minimum essential medium supplemented with 5 mM HEPES and 2% heat-inactivated newborn calf serum. After being washed once with this medium, the cell pellet was resuspended in the same medium. Lymphocytes were isolated from the parenchymal hepatocytes, the nuclei of hepatocytes, and Kupffer cells by the Percoll-gradient method (35% Percoll containing 100 U/ml heparin).

Splenocytes were obtained by pressing the spleen through a 200-gauge stainless steel mesh; erythrocytes in the spleen were lysed with 0.83% NH₄Cl-Tris buffer (pH 7.6).

Immunofluorescence tests

Lymphocyte phenotypes were identified by two or three-color immunofluorescence^[6]. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin-conjugated mAbs were used and biotin-conjugated reagents were developed with TRI color-conjugated streptavidin (Caltag Lab., San Francisco, CA). The mAbs used included anti-CD3 (145-2C11), anti-NK1.1 (PK136), anti-CD69 (H1.2F3), anti-Ly49C/I (5E6), anti-CD94 (18d3), and anti-Mac-1 (M1/70) mAbs (PharMingen Co., San Diego, CA). Cells were analyzed by flow cytometry (Becton Dickinson Co.). To prevent non-specific binding of mAbs, CD16/32 (2.4G2) (PharMingen) was added before staining with labeled mAb. Forward scatter, side scatter and propidium iodide gating excluded dead cells.

Cytotoxicity assay

Cytotoxicity assays were performed as previously described^[7]. YAC-1, syngeneic thymocytes and EL-4 target cells were labeled with sodium [⁵¹Cr] chromate for 2 h and washed three times with RPMI 1640 me-

dium. Effector cells were serially diluted and mixed with [⁵¹Cr]-labeled target cells (1×10⁴ or 2×10⁴ cells) in 96-well U-bottomed micro culture plates, and incubated for 4 h at 37°C. At the end of the culture, 100 μl supernatant was counted in a gamma counter.

Detection of perforin mRNA by RT-PCR analysis

To assess the expression of mRNA for perforin, a cytotoxic RNA was reverse-transcribed (RT) using primers noted below and the cDNA then amplified by the PCR method^[8]. Briefly, total RNA was prepared from various MNC by an acid-guanidinium thiocyanate-phenol-chloroform method. cDNA was synthesized using 1 μg of RNA with a first-strand cDNA synthesis kit (Pharmacia Biotech, US).

The following primers were used: perforin sense 5'-GCT CCT TCC CAG TGA ACA CA-3', antisense 5'-GTA GTA AGC ATG CTC TGT GG-3'; G3PDH: sense 5'-ACC ACA GTC CAT GAA ATC AC-3', antisense 5'-TCC ACC ACC CTG TTG CTG TA-3'.

Statistical analysis

All parameters were expressed as the mean±SD. The difference between the values was determined by the Student's *t*-test. *P*<0.05 was considered to be statistically significant.

RESULTS

Age-related changes in the percentage and number of NK cells in the liver and spleen

Lymphocytes were isolated from the liver and spleen of mice at the ages of 2 (young) and 8 (adult) weeks, and the lymphocyte number determined in the tissues. By two-color staining for CD3 and NK1.1, NK cells were identified as CD3-NK1.1+ cells in the liver and spleen. With aging the percentage of NK cells remained unchanged in the liver (11.9%±1.7% vs. 9.9%±1.6%, *P*>0.05) but increased in the spleen (1.4%±0.4% vs. 5.5%±1.3%, *P*<0.05)(Fig. 1A), whereas the number of both liver and spleen NK cells increased significantly with aging (liver: 2.2±0.8×10⁵ vs. 4.6±1.3×10⁵, spleen: 3.5±1.1×10⁵ vs. 28.6±5.5×10⁵ *P*<0.05). Since it is possible that the number of NK cells was related to the weight of the liver and spleen, we calculated the number of NK cells per organ weight. The number of NK cells per organ weight was significantly higher in the young liver as compared to the adult liver (13.6±2.5×10⁵/g vs. 3.4±0.8×10⁵/g *P*<0.05)(Fig. 1B), but in the spleen there were more NK cells per organ weight in the adult mice (8.5

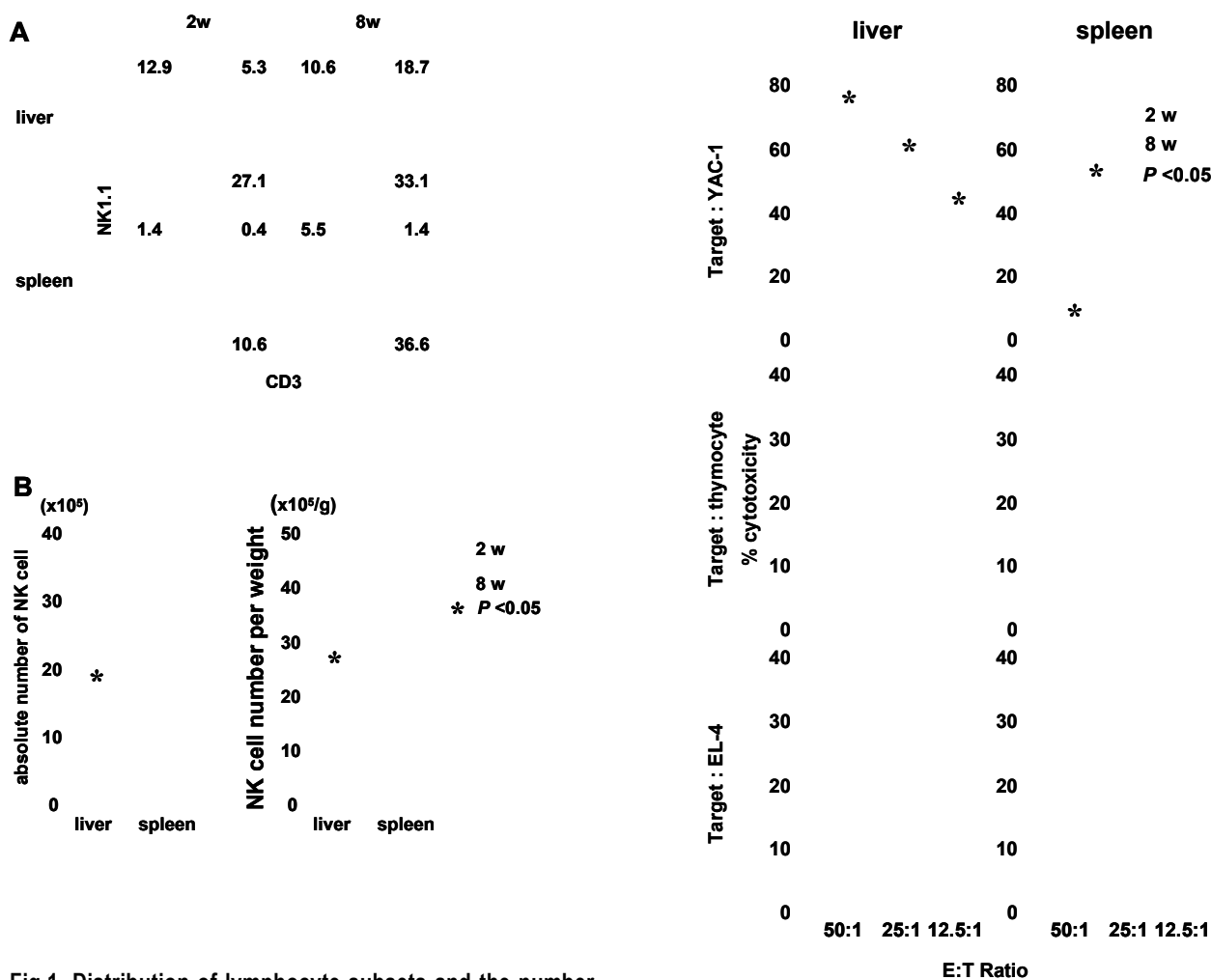


Fig.1. Distribution of lymphocyte subsets and the number of NK cells. [A] Two-color staining for CD3 and NK1.1 was conducted on cells from the liver and spleen of mice at 2 or 8 weeks of age. Numbers in the figure show the percentages of fluorescence-positive cells in the corresponding areas. [B] Age-associated change in the absolute number (or absolute number per weight) of NK cells in the liver and spleen. The means±SD were produced by 4 independent experiments.

Fig.2. Cytotoxicity assays of lymphocytes in young and adult mice. Target cells: YAC-1, syngeneic thymocyte and EL-4. E: T ratio: 50:1, 25:1 and 12.5:1. The means±SD were produced by 4 independent experiments.

±2.0×10⁵/g vs. 38.2±5.1×10⁵/g, P<0.05).

Increased level of NK cytotoxicity in the liver of young mice

NK cell cytotoxicity was examined against YAC-1. NKT activity was also examined against syngeneic thymocytes and CTL activity was examined against EL-4 cells (Fig.2). A prominent increase in the level of NK activity was found in the liver of young mice as compared to the liver of adult mice (71.0%±5.5% vs. 23.8%±4.4%, P<0.05), whereas this did not occur in the spleen. Age-dependent variation was not seen

in NKT and CTL cytotoxicity. Increased NK cytotoxicity was confirmed in repeated experiments (n=4).

Phenotypic characterization of NK cells in young and adult mice

Lymphocytes were isolated from the liver and spleen of young and adult mice. Three-color staining was used for CD3, NK1.1 and CD69 (or Mac-1, Fas-L, Ly49C/I, CD94, NKG2). The expression of these markers was compared by gated analysis (Fig.3). Compared to adult liver, NK cells of young mice were found to express a higher level of CD69 (78.0%

A	2w	8w	B	2w	8w
	78.0	26.0		8.1	18.5
	CD69			CD69	
	22.0	71.0		39.0	83.0
	Mac-1			Mac-1	
	19.4	52.6		28.5	49.1
	Ly49C/I			Ly49C/I	
	86.2	56.8		76.7	50.7
	CD94			CD94	

Fig.3. The expression of CD69, Mac-1, Ly49C/I and CD94. Three-color staining for CD3, NK1.1 and CD69 (Mac-1, Ly49C/I, and CD94) was conducted on cells from the liver [A] and spleen [B] of 2- or 8- week old mice.

±5.3% vs. 26.0%±3.8%), but cells from the spleen did not show this difference (8.1%±2.0% vs. 18.5%±4.4%). Expression of Mac-1 on NK cells was lower in young mice (liver: 22.0%±2.4%, spleen: 39.0%±3.5%) compared to adult mice (liver: 71.0%±5.6%, spleen: 83.0%±6.4%). The expression of Ly49C/I was lower in both the liver and spleen of young mice, but CD94 levels were comparable in the liver and spleen, irrespective of age.

The expression of perforin mRNA in the liver

The expression of perforin mRNA in the lymphocytes from the liver of young and adult mice was analyzed by the RT-PCR method. The signal for perforin was greater in the liver lymphocytes of young mice compared to adult mice (Fig.4).

DISCUSSION

In this study, we demonstrated that the quantity and quality of NK cells in the livers of mice changes with age. Although the absolute number of NK cells in the

liver and spleen increased with aging, the number of NK cells per organ weight in young mouse liver was higher than that in adults, perhaps because fetal liver is a hematopoietic organ^[3]. We suggest that there is more potential NK cell activity in the liver of young C57BL/6 mice.

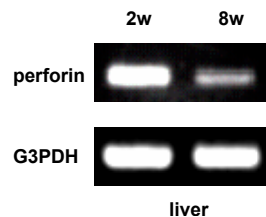


Fig.4. Expression of perforin mRNA. Total RNA was obtained from the liver lymphocytes of 2- and 8-week old mice. The mRNA signal was assessed by the RT-PCR method.

NK cytotoxicity against YAC-1 cells was high in the liver of young mice, but this was not in the spleen. With aging the cytotoxicity against syngeneic thymocytes and EL-4 cells was not changed in the liver or spleen. The results of RT-PCR also showed a high expression level of perforin in the liver of young mice as compared to the adults. It is well known that NK

cells mostly utilize perforin to lyse target cells^[9]. This might partially explain why NK cells in the young liver exhibit a high cytotoxicity.

In our study we compared phenotypes of NK cells. The NK cells in the liver of young mice were CD69^{high} Mac-1^{low} Ly49C/I^{low}, whereas those in the liver of adult mice showed an inverse relationship CD69^{low} Mac-1^{high} Ly49C/I^{high}.

In considering these cell types, we are primarily interested in the expression of CD69 and Mac-1. CD69 is a C-type lectin receptor, with its gene located in the NK gene complex. It is rapidly induced on NK cells in response to cytokines or other activating stimulators^[10] and has been shown to induce cytotoxic activity in activated NK cells^[11]. Our data show that NK cells in the liver of young mice expressed high levels of CD69, suggesting that they have been activated by some unknown stimulator. We suggest that it might be related to the unique environment present in young liver.

Mac-1 is an adhesion molecule thought to be a mark of mature NK cells. An earlier report demonstrated that immature bone marrow NK cells expressed a low level of Mac-1 in comparison with peripheral mature NK cells. These immature NK cells showed only a low level of cytotoxicity^[12]. Contrary to our expectation, results of the present study showed that these Mac-1-low NK cells isolated from the liver of young mice had the highest level of NK cytotoxicity. These results conflict with our previous understanding.

Taken together, our data suggest that NK cells in the liver of young C57BL/6 mice have a high potential for cytotoxicity in terms of their phenotype and cytotoxic molecular expression. Although it is unknown why only hepatic NK cells exhibit this phenomenon, it should be kept in mind for future studies.

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