

Isolation of murine hepatic lymphocytes using mechanical dissection for phenotypic and functional analysis of NK1.1⁺ cells

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Abstract

AIM: To choose an appropriate methods for the isolation of hepatic lymphocytes between the mechanical dissection and the enzymatic digestion and investigate the effects of two methods on phenotype and function of hepatic lymphocytes.

METHODS: Hepatic lymphocytes were isolated from untreated, poly (I:C)-stimulated or ConA-stimulated mice using the two methods, respectively. The cell yield per liver was evaluated by direct counting under microscope. Effects of digestive enzymes on the surface markers involved in hepatic lymphocytes were represented by relative change rate [(percentage of post-digestion - percentage of pre-digestion)/percentage of pre-digestion]. Phenotypic analyses of the subpopulations of hepatic lymphocytes and intracellular cytokines were detected by flow cytometry. The cytotoxicity of NK cells from wild C57BL/6 or poly (I:C)-stimulated C57BL/6 mice was analyzed with a 4-h ⁵¹Cr release assay.

RESULTS: NK1.1⁺ cell markers, NK1.1 and DX5, were significantly down-expressed after enzymatic digestion and their relative change rates were about 28% and 32%, respectively. Compared with the enzymatic digestion, the cell yield isolated from unstimulated, poly (I:C)-treated or ConA-treated mice by mechanical dissection was not significantly decreased. Hepatic lymphocytes isolated by the mechanical dissection comprised more innate immune cells like NK, NKT and $\gamma\delta$ cells in normal C57BL/6 mice. After poly (I:C) stimulation, hepatic NK cells rose to about 35%, while NKT cells simultaneously decreased. Following ConA injection, the number of hepatic NKT cells was remarkably reduced to 3.67%. Higher ratio of intracellular IFN- γ (68%) or TNF- α (15%) NK1.1⁺ cells from poly (I:C)-treated mice was obtained using mechanical dissection method than control mice. There was no difference in

viability between the mechanical dissection and the enzymatic digestion, and hepatic lymphocytes obtained with the two methods had similar cytotoxicity against YAC-1 cells.

CONCLUSION: There is no difference in the cell yield and viability of the hepatic lymphocyte isolated with the two methods. The mechanical dissection, but not the enzymatic digestion, may be suitable for the phenotypic analysis of hepatic NK1.1⁺ cell.

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INTRODUCTION

In recent decades, isolated lymphocytes from human or murine liver were universally used to explore the immune mechanisms in the defense of pathogens such as hepatitis virus^[1,2] and in the pathogenesis of liver diseases, especially in the autoimmune hepatitis^[3] and liver transplantation^[4]. Several lymphocyte subpopulations reside in the normal adult human liver. These cells mainly include a large number of T cells, B cells, natural killer (NK) cells and natural killer T (NKT) cells, which are distinct from the peripheral blood lymphocytes (PBL)^[5-7]. Up to now, mechanical dissection and enzymatic digestion are two main techniques for isolation of hepatic lymphocytes. The former method started in the early of 1980 and has been used yet. Reportedly, the viability of lymphocyte with this method is poor, and this method also leads to low yield. The latter methods was through incubation with digestive enzymes, 0.5 g/L collagenase IV and 0.01 g/L DNAase I^[8] and was considered to have a relative low contamination of PBL. Because the manipulation of the method is difficult to handle for tenderfoots, the prevalent use is limited. Some investigators also discovered that two digestive enzymes used could influence and decrease the percentage of surface markers of human hepatic lymphocytes such as CD56 molecule^[9]. However, the effects of two digestive enzymes on the surface markers of murine hepatic lymphocytes especially NK1.1⁺ cells, remain obscure. Yet there was not any report exclusively focused on the difference between these two methods. To compare these two methods for suitably selecting an appropriate method to analyze the NK1.1⁺ cells in the liver, we used these methods to isolate the murine hepatic mononuclear cells for the phenotypic and functional analysis.

MATERIALS AND METHODS

Animal

Female C57BL/6 (H-2^b), 6 to 8 week-old, was purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). All mice were maintained under controlled conditions (22 °C, 55% humidity, and 12-h day/night

rhythm) in compliance with the regulations of animal care of University of Science and Technology of China.

Reagent

Collagenase IV and DNase I were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in the pyrogen-free RPMI 1640 at the concentration of 0.5 g/L and 0.01 g/L, respectively.

Poly (I:C) or ConA treatment

Polyinosinic-polycytidylic acid sodium [Poly (I:C)] and Concanavalin A (Con A) were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in the pyrogen-free saline at the concentration of 1 mg/mL. For *in vivo* stimulation of NK cells, mice were intraperitoneally injected with Poly (I:C) (7.5 µg/g·b.m.) for 6 h. For *in vivo* stimulation of NKT cells, mice were intravenously injected with ConA (7.5 µg/g·b.m.) for 6 h.

Protocols for isolation of mononuclear cells (MNC) from liver

Under deep ether anesthesia, mice were euthanized by exsanguinations from the subclavian artery and vein. A needle was inserted into the portal vein. The liver was perfused with 20 mL pH 7.0 PBS, and then the liver was removed. Isolation of hepatic lymphocytes with the mechanical dissection was carried out as follows: step 1, the liver was thoroughly dissected and gently passed through a 200-gauge stainless steel mesh and then suspended in RPMI 1640 medium containing 100 mL/L fetal calf serum (FCS). Step 2, the above cell suspension was centrifuged at 1 500 r/min. The pellet was resuspended in 40% Percoll solution containing 100 U/mL heparin, and then loaded on the layer of 70% Percoll solution followed by centrifugation at 2 000 r/min for 20 min at room temperature. Step 3, the cells were aspirated from the Percoll interface and harvested by centrifugation and washed twice with Hanks' balanced salt solution (HBSS) containing 50 mL/L FCS before use. The procedures for the enzymatic digestions were as follows: step 1, the liver was dissected into 1 mm³ pieces and 5 mL digestive solution was added. The mixture was incubated at 37 °C for 1 h. The supernatant was collected and diluted 1/2 in complete RPMI 1640. Step 2 and 3 were similar to the mechanical dissection.

Assessment of yield and viability of hepatic lymphocytes

Isolated hepatic lymphocytes from different groups (4 mice per group) were diluted 1:20 with 20 mL/L acetic acid, and cell numbers were assessed by direct counting under microscope. Cell viability was assessed by staining with trypan blue, and the stained-positive cells were enumerated to dead cell, while negative cells to viable lymphocyte.

Effects of digestive enzymes on the surface markers

Effects of digestive enzymes, collagenase IV and DNase I, on the surface markers were determined by the percentage in the pre-digestion and post-digestion. It was represented as relative change rate (%). Relative change rate=[(percentage of post-digestion - percentage of pre-digestion)/percentage of pre-digestion].

Immunofluorescence

The phenotype of lymphocytes was analyzed using monoclonal antibody (mAb) in conjunction with two-color or three-color immunofluorescence. The mAb used in this study included fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or Cy5-conjugated gamma/delta T cell receptor (γδ TCR), anti-CD3e, anti-CD25, anti-CD69, anti-IFN-γ, anti-DX5, and anti-NK1.1 mAb (PharMingen, San Diego, CA). For Intracellular cytokine staining, liver mononuclear cells were incubated in the presence of brefeldin A (5 µg/mL; BD PharMingen) and phorbol myristate

acetate (PMA) (20 ng/mL; BD PharMingen) for 3 h, and then stained with FITC-conjugated anti-NK1.1 mAb and Cy-5 conjugated anti-CD3e mAb. After fixation with fixation solution and permeabilization with permeabilization solution (Bioscience, Camarillo, USA), intracellular cytokine staining was performed using PE-conjugated anti-IFN-γ or anti-TNF-α mAb. To prevent nonspecific binding, respective isotype antibodies were used as control. Stained cells were acquired by FACSCalibur and analyzed with WinMDI2.8.

Cytotoxicity assay

Target cells used in NK cytotoxicity assay were YAC-1 cells, which were propagated in RPMI 1640 medium supplemented with 100 mL/L heat-inactivated FCS, 2 mmol/L L-glutamine and 25 mmol/L NaHCO₃ in a humidified atmosphere containing 50 mL/L CO₂ at 37 °C. Cytotoxicity assay was carried out as described previously^[10]. Labeled target cells (10⁵/well) were incubated in a total volume of 200 µL with effector cells in RPMI 1640 containing 100 mL/L FCS in 96-well round-bottom microtiter plates at various cell densities in order to achieve effector-to-target (E/T) ratios. The plate was incubated for 4 h, and the supernatant was collected after centrifugation and then counted in a gamma counter. The cytotoxicity was calculated as the percentage of releasable counts after subtraction of spontaneous release. The spontaneous release was less than 15% of the maximum release.

Statistical analysis

Data were expressed as mean±SD. Statistical analysis was performed using *t* test. Difference between the groups was considered statistically significant when *P* value was less than 0.05.

RESULTS

Effects of enzymatic digestion on surface molecules of hepatic lymphocytes

To understand the effect of collagenase IV and DNase I on murine hepatic lymphocytes, we detected the percentage of lymphocyte-related markers, CD3, CD4, CD8e, CD25, CD69, NK1.1, DX5 and γδ TCR in the enzyme-treated and untreated hepatic lymphocytes. Surface markers, CD3, CD4, CD8e, CD25, CD69 and γδ-TCR, remained unchanged and two markers associated with NK and NKT cells, NK1.1 and DX5, were significantly decreased in the enzyme-treated group compared to untreated group and their relative change rates were about 28% and 32% in NK1.1 and DX5 groups respectively (Figure 1). The percentages of NK and NKT cells isolated with the mechanical dissection were higher than those in the enzymatic digestion (Table 1, *P*<0.05). This finding suggested that the enzymatic digestion might decrease the proportion of NK1.1⁺ cells in murine hepatic lymphocytes by decreasing the NK or NKT-related surface molecules, and should be avoided in the study of NK1.1⁺ in the liver.

Table 1 Subpopulations of hepatic lymphocytes isolated by two the methods (%)

Group	T cell	NK cell	NKT cell	γδ T cell
Mechanical dissection	38.56±5.63	13.35±4.61	20.42±4.65	13.6±3.15
Enzymatic digestion	36.96±4.68	8.35±2.69 ^a	13.48±4.25 ^a	12.6±2.74

^a*P*<0.05 vs dissection.

Cell yield of the two methods

In normal C57BL/6 mice, the total cell number obtained with the mechanical dissection was about 2.6×10⁶ per liver and that

obtained with the enzymatic digestion was 2.9×10^6 per liver, showing no significant difference (Figure 2A). To further investigate the cell yield in the stress condition, two stimuli, Poly (I:C) and ConA, were used to trigger hepatic lymphocytes, respectively. About 7×10^6 hepatic lymphocytes per liver were obtained in Poly (I:C)-stimulated or ConA-stimulated mice using either the mechanical dissection or the enzyme digestion, without any significant difference between the two isolation methods (Figures 2B,C). These results suggested that the mechanical dissection was as effective as the enzymatic digestion in the cell yield.

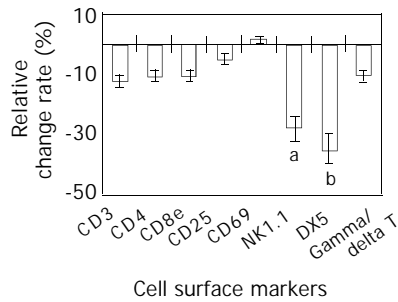


Figure 1 Effects of enzymatic digestion on surface molecules of hepatic lymphocytes. ^a $P < 0.05$, NK1.1 vs other groups except DX5; ^b $P < 0.01$, DX5 vs other groups except NK1.1.

Hepatic lymphocytes isolated with mechanical dissection were suitable for phenotypic analysis

Using mechanical dissection, we isolated the hepatic lymphocytes for analyzing the subpopulations of hepatic lymphocytes. Different from other immune organs, hepatic

lymphocytes of normal C57BL/6 mice comprised more innate immune cells like NK, NKT and $\gamma\delta$ cells, which accounted for 18.60%, 16.62% and 18.60% respectively (Figure 3A). Following Poly (I:C) stimulation, hepatic NK cells increased to about 35% and NKT cells simultaneously decreased, which was consistent with other reports^[11] (Figure 3B). Following ConA injection, hepatic NKT cells were remarkably reduced to 3.67%. In addition, after Poly (I:C) stimulation intracellular IFN- γ and TNF- α were significantly augmented to about 68% and 25% within NK (CD3⁺NK1.1⁺) cells, respectively (Figure 3C). Taken together, the results indicated that hepatic lymphocytes obtained with the mechanical dissection could be used for the antibody-based phenotypic analysis of NK1.1⁺ cells. On the other hand, the liver had the predominance of more innate immune cells.

Hepatic lymphocytes isolated with mechanical dissection were suitable for the functional analysis

In order to understand whether hepatic lymphocytes obtained with mechanical dissection were suitable for functional assays, cell viability was assessed through staining with trypan blue. The cell viability of hepatic lymphocytes from normal C57BL/6 mice accounted for 90% using mechanical dissection method, which was similar to enzyme digestion method (Figure 4A). Next, we examined NK cells cytotoxic function against an NK-sensitive cell line, YAC-1 cells, and found that there was no difference between the two methods (Figure 4B). In the absence of Poly (I:C) stimulation, the cytotoxicity of NK cells to YAC-1 cells was about 15% at the E:T ratio of 1:12.5. After Poly (I:C) stimulation, it increased to 35% at the same E:T ratio. These results indicated that the isolated lymphocytes with the two different methods had the similar viability and were suitable for functional analysis of hepatic lymphocytes prior to or after immune stimulation.

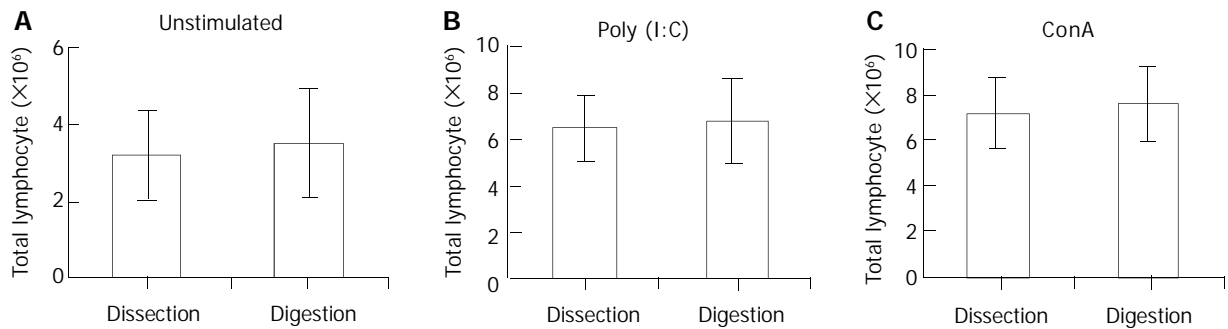


Figure 2 Cell yields between the two isolating methods. Hepatic lymphocytes were isolated using mechanical dissection method and the enzymatic digestion method, respectively, from normal C57BL/6 mice (A), Poly (I:C)-treated C57BL/6 mice (B) or ConA-treated C57BL/6 mice (C).

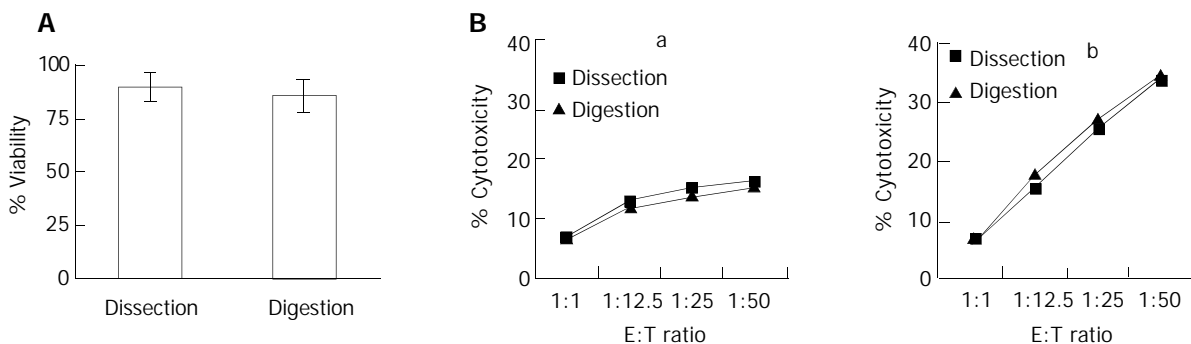


Figure 4 Hepatic lymphocytes isolated with mechanical dissection were suitable for the functional analysis of NK1.1⁺ cells. A: Viability of hepatic lymphocytes isolated with two methods; B: Cytotoxicity analysis of hepatic lymphocytes. Hepatic lymphocytes were isolated by two different methods, respectively, from control B6 mice (a) or Poly (I:C)-treated B6 mice (b). Their cytotoxicity against YAC-1 cells was tested at the (E/T) ratios.

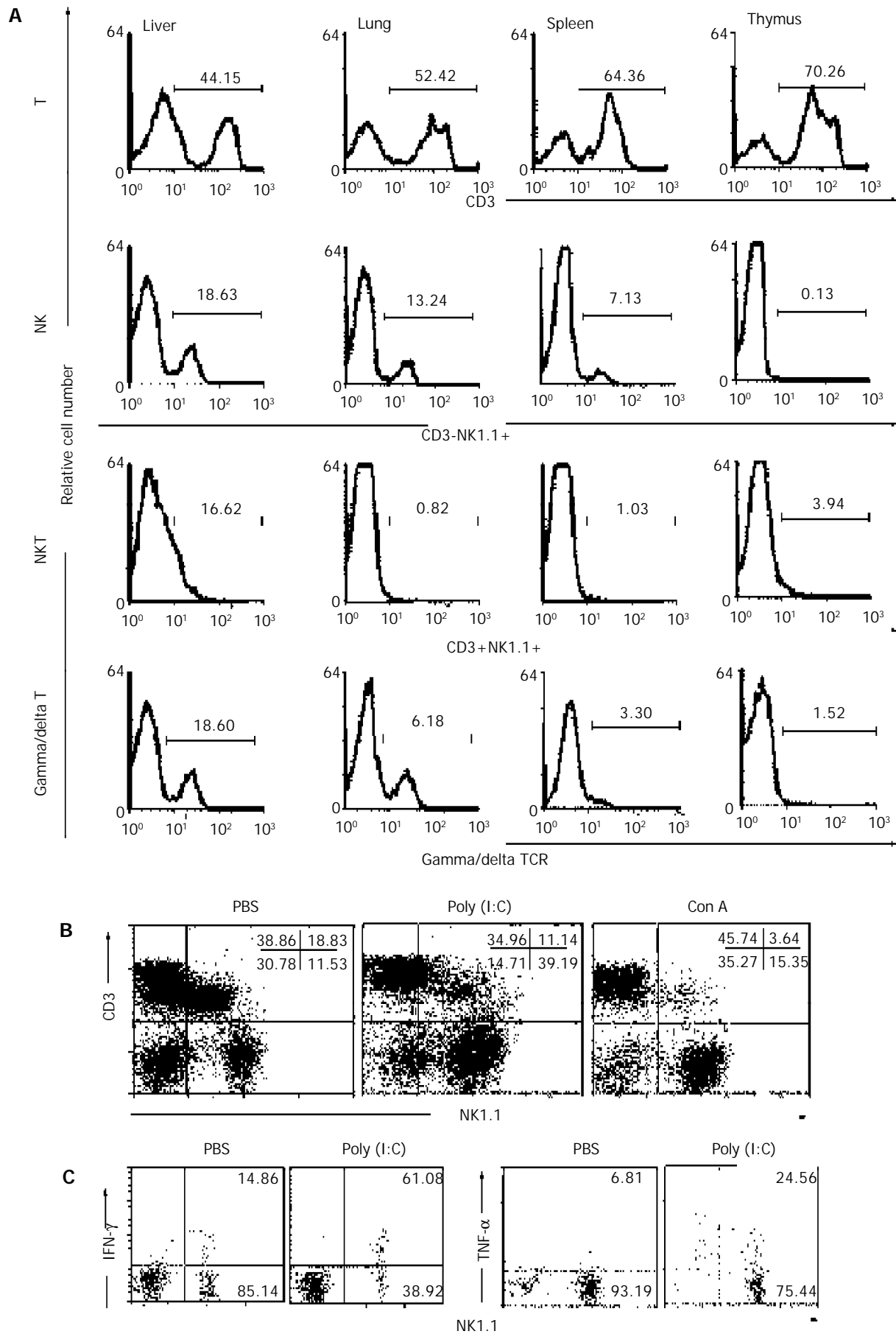


Figure 3 Hepatic lymphocytes isolated with mechanical dissection were suitable for phenotypic analysis of NK1.1⁺ cells. A: Lymphocytes from liver, lung, spleen and thymus of normal C57BL/6 mice were labeled with two-color immunofluorescence; B, C: C57BL/6 mice were injected with PBS, Poly (I:C) or ConA, respectively. Hepatic lymphocytes were isolated and labeled for phenotype (CD3 and NK1.1) and intracellular cytokine (IFN- γ and TNF- α) detection.

DISCUSSION

Liver has a unique dual blood supply with venous blood from the gut via the portal veins and arterial blood delivered via the hepatic arteries. In view of this distinct anatomy character, liver is thus constantly exposed to gut-derived antigens and infectious organisms in the portal blood. So many unidentified mechanisms have been involved to allow rapid and selective immune responses within this tissue^[12,13]. Structurally and functionally, qualification of liver as a lymphoid organ has been reflected by many studies^[14,15], and therefore a new term "hepatoimmunology" was emerged in 2002^[16]. Liver harbors many innate immune cells, such as NK, NKT and $\gamma\delta$ T cells. The predominance of these cells in the liver endows liver with the character of innate immune organ^[17], which was supported by the response of NK cells to the innate immune stimulus, Poly (I:C) in our study. However, the exact roles of these cells in the liver remain unclear. NK1.1 molecule is a member of NKR-P1 family, also named as CD161 in human^[18], and NK1.1⁺ cells often have DX5 molecule on their membrane in mice^[19]. Their remarkable distinction was that NK1.1 was only expressed by certain strain of mice, such as C57BL/6 and NZB, but not BALB/c^[20]. Conventionally, NK1.1⁺ cells mainly included NK cells and NKT cells. However, upon certain stimulation, activated cytotoxic T cell could also upregulate NK1.1 molecule. NK and NKT cells are two important subpopulations in the liver. They exerted their versatile roles in the defense of pathogens like murine cytomegalovirus (MCMV)^[21,22]. Several studies also reported that hepatic NK and NKT cell took part in the antimetastasis of tumor^[23-25]. NK1.1⁺ cells were reported to involve in the pathogenesis of many diseases, like human immunodeficiency virus (HIV) infection and liver disease^[26,27]. The demonstration that NK cell or NKT cell could give rise to liver injury had been confirmed by different groups, including our laboratory^[28-30]. Accurate assessment of these NK1.1⁺ cells in the liver required an appropriate technique to purify the hepatic lymphocytes. Various enzymatic or mechanical methods were proposed in the past to isolate lymphocytes from human or murine liver^[9]. The Enzymatic digestion was considered to be a satisfied method in the preparation of human organ-specific lymphocytes^[31]. In addition, this method has been used to isolate cells like hepatocytes^[32]. However, results from different studies appeared to be inconsistent. For example, Trobonjaca *et al.* reported that NKT cells accounted for 35.9% of the total hepatic lymphocytes in normal mice^[8], while Osman *et al.* reported that NKT cell accounted for only 16%^[33]. This difference may contribute to the different isolating methods taken by them. The roles of collagenase on surface makers of peripheral blood lymphocytes were investigated in 1996. They found collagenase could disrupt the surface markers, such as CD3, CD4, CD8, $\alpha\beta$ and $\gamma\delta$ TCR by about 20-40%^[34]. It was also reported that isolation of intestinal lymphocytes using collagenase released cytotoxic factors, which were found to suppress NK cell activity of isolated cell^[35]. Although the mechanical dissection for preparation of hepatic lymphocytes has been used for many years and still used yet, this technique has a defect in cell yield. To make sure the extent, in this study we found that there was no difference in cell yields and viability between the mechanical dissection method and the enzymatic digestion method. We did not observe any decrease in the surface molecules of hepatic lymphocytes in all cases except NK1.1 and DX5, suggesting that enzymatic digestion using collagenase IV and DNase is unsuitable for isolating hepatic lymphocytes for NK1.1⁺ cell analysis.

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