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Higher Activities of Hepatic Versus Splenic CD8$^+$ T Cells in Responses to Adoptive T Cell Therapy and Vaccination of B6 Mice with MHC Class-1 Binding Antigen

Mohamed Labib Salem[1,2], Randa E. El Naggar[1], Sabry A. El Naggar[1,2], Maysa A. Mobasher[3,4], Mohamed H. Mahmoud[5,6], and Gamal Badr[7]

[1] Immunology and Biotechnology Unit, Department of Zoology, Faculty of Science, Tanta University, Tanta, Egypt
[2] Center of Excellence in Cancer Research, Tanta University, Tanta, Egypt
[3] Biochemistry Division, Department of Pathology, College of Medicine, Aljouf University, Sakaka, Saudi Arabia
[4] Department of Clinical Pathology, El Ahrar Educational Hospital, Ministry of Health, Zagazig, Egypt
[5] Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia
[6] Food Science and Nutrition Department, National Research Center, Dokki, Cairo, Egypt
[7] Laboratory of Immunology and Molecular Biology, Zoology Department, Faculty of Science, Assiut University, Assiut, Egypt

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ABSTRACT

The liver has unique microenvironment which is known to induce tolerance of cytolytic CD8$^+$ T cells to hepatic and extra hepatic antigens, resulting in persistence of infection of the liver by the hepatitis B and C viruses. However, under some conditions, functional immune responses can be elicited in the liver in particular to show preferential retention of activated CD8$^+$ T cells. It is not clear whether this retention depends on the type of the exogenous immunostimulatory or the endogenous innate immune cells.

The T cell receptor (TCR) transgenic OT-1 (CD8$^+$) mouse model was used in which OT-1 cells were harvested from the spleen of the donor and transferred into recipient mice followed by immunization with OVA peptide followed by injection of GM-CSF, CCL21 chemokine, or cytokines (IL-2, IL-12, or IL-15), or the toll-like receptor 3 agonist poly(I:C). Co-administration of any of these immunostimulatory agents relatively augmented the retention of CD8$^+$ T cells with different levels of effects. Compared to spleen, the Ag-specific CD8$^+$ T cells in the liver showed higher activities including expansion, proliferation, apoptosis and memory responses as well as cytolytic function. While depletion of natural killer cells significantly decreased the hepatic retention of the antigen-specific T cells, depletion of Kupffer cells showed opposite effect.

Taken together, the antigen reactive T cells in the liver have higher activities than their counterparts in the peripheral tissues such as spleen. These data have important clinical implications for designing immunotherapeutic protocols toward the liver diseases.

Keywords: CD8+ cyclophosphamide; Kupffer cells; Natural killer cells; Ovalbumin-specific OT-I; OVA; Poly(I:C); T cells; Toll-like receptor; Vaccination

Corresponding Author: Mohamed Labib Salem, PhD; Immunology and Biotechnology Unit, Department of Zoology, Faculty of Science, Tanta University, Tanta 31111, Egypt. Tel: (+20 0127) 427 2624, E-mail: mohamed.labib@science.tanta.edu.eg, mohamedlabibsalem@yahoo.com

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INTRODUCTION

During typical systemic immune response, primed T cells are activated and undergo a massive clonal expansion followed by a rapid contraction leaving behind a small population of responding quiescent memory cells.[1-3] T cell expansion of effector T cells occurs mainly in the draining lymph nodes (DLN) and then the cells recirculate via the blood to non-lymphoid compartments (mainly the liver and lung), where they undergo apoptosis and contraction.[4] Giving that trafficking and retention of $CD8^+$ T cells in the liver[5] and how the liver microenvironment dictates the outcome of intrahepatic $CD8^+$ T cell responses[6] has been well described, finding a way to sustain appreciated numbers of functional $CD8^+$ T cells in circulation and their retention in the hepatic microenvironment is of paramount significance to effective immunity against microbial infection.[7,8]

Indeed, the liver has been considered as an active site (graveyard) for trapping activated $CD8^+$ T cell and their subsequent apoptosis and tolerance.[9] For instance, when a mixture of resting and activated T cells were perfused through a mouse liver, the activated $CD8^+$ T were selectively retained in this organ, and most of the retained $CD8^+$ T cells undergo apoptosis after being in physical contact with KCs.[10] Although hepatocytes can induce functional activation of naive $CD8^+$ T lymphocytes they fail to promote their survival and functions[11-15] but sinusoidal endothelial cells poorly stimulate T cells.[16-18] Recent studies showed that the liver-primed antiviral CD8+ T cells are not functional and expressed a regulatory phenotype (high expression of CD28 and PD-1 over time) and function[14,19-21] preceded by a transient rise of T_{reg} cells in the liver which results in dampening the antigen-specific immunity of $CD8^+$T cells[19,22] Certain hepatic resident cells in particular Kupffer cells (KCs) may mediate the trapping of the infused T cells and their subsequent apoptosis and interfere with antigen presentation in the liver, limiting antigen-induced expansion of the entering activated $CD8^+$ T cells.[16,17,23]

On the other hand, prior studies have shown that the liver can be considered as a site of effective immune responses.[24,25] Recent studies also showed that despite the peripheral deletion of liver-specific $CD8^+$ T-cells, a significant number of these cells accumulated in the liver with the capacity to proliferate, produce cytokines and up-regulate activation markers, resulting in hepatocyte injury and production of auto-antibodies.[15,26,27] Such functional $CD8^+$ T cells were even more in the liver than in the spleen.[28] Having that the liver contains a large numbers of immunopotentiating cells such as NK and NKT cells, and dendritic cells (DCs),[24,25,29] the their rapid release of proinflammatory cytokines such as type I IFNs, IFN-γ, TNF-α, IL-1, IL-6, IL-8, and IL-18 by these cells can initiate and augment bystander as well as antigen specific T cell responses.[25,30,31]

Creation of an immunostimulatory environment in the liver during peptide vaccination is critical since injection of peptide alone has been found to result in a transient expansion of the antigen-specific T cells followed by rapid apoptosis and clearance of these cells.[9,11-13] However, co-administration of adjuvants during vaccination induce inflammatory microenvironment that is favored development of effective T cell responses.[32-34] Indeed, our recent studies showed that adjuvants, in particular the Toll-like receptor 3 (TLR3) agonist poly(I:C), can decrease activation-induced cell death (apoptosis) of T cells in secondary lymphoid tissues,[35] and to elicit the accessory functions of innate immune cells in the liver in particular NK cells and DCs.[36-38] It is not clear, however, whether provision of potent adjuvants during vaccination induces activation of $CD8^+$ T cells retained in the liver with enhanced cytolytic function, which can then serve as a reservoir for functional effector T cells.

The present study aimed to determine the impact of the type of adjuvant on the quality and quantity of $CD8^+$ T cells retained in the liver versus spleen as well as to determine the roles of NK cells and KCs in the hepatic retention of T cells. The data showed that the hepatic retention of the antigen-specific $CD8^+$ T cells is relatively augmented upon co-administration of different adjuvant systems. Depletion of NK cells and KCs decreased and increased the hepatic $CD8^+$ T cell retention, respectively. These data help to understand the biology of intrahepatic antigen-specific T cell responses, as the liver is implicated in inducing T cell tolerance as well as the site of viral infection and metastasis of different cancer cells.

MATERIALS AND METHODS

Mice

B6.SJL (Ly5.1), and C57BL/6 (Ly5.2) mice were purchased from The Jackson Laboratory (Bar Harbor,

ME, USA). OT-1 TCR transgenic (Vα2/Vβ5) mice were bred with B6.SJL mice to generate $Ly5.1^+/Ly5.1^+$ mice heterozygous for the OT-1 TCR transgene. Presence of the transgene was confirmed by flow cytometry with mAb specific for Vα2. $CD8^+$ T (OT-1) cells from these mice can specifically recognize the MHC class-I peptide from ova-albumin (OVAp). All animals were housed under specific pathogen-free conditions at the Medical University of South Carolina (USA) in accordance with institutional and federal guidelines.

Cell Lines

EL-4 is a lymphoma derived from the C57BL/6 mouse ($H\text{-}2^b$). EL4 cells were maintained in vitro in complete RPMI medium (RPMI 1640, 0.1% penicillin/streptomycin (Cellgro, Herndon, VA, USA), 10% FBS (HyClone Laboratories, Logan, UT, USA), 0.2%
L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 0.05% 2-mercaptoethanol, 0.01% sodium pyruvate (Life Technologies, Grand Island, NY, USA), 0.1% (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES, and 0.1% nonessential amino acids.

Antibodies

Anti-CD16/CD32, and fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), and cytochrome-conjugated monoclonal antibodies (mAbs), including anti-Ly5.1, anti-NK1.1, anti-CD11b, anti-CD8, anti-CD44, anti-CD62L, anti-annexin-V, anti-BrdU and mAb specific for Vα2 were purchased from Pharmingen (San Diego, CA, USA). SIINFEKL, an OVA MHC class-I peptide (OVAp), was purchased from American OVAp Company, Inc. (Sunnyvale, CA). OVAp were dissolved in 10% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) and diluted in PBS to the indicated concentrations.

Chemical and Reagents

Cyclophosphamide (CTX) and polyinosinic-polycytidylic acid (poly(I:C)) (Sigma, St. Louis, MO, USA) were stored at -70^0C, and reconstituted in phosphate buffer saline (PBS) before use. Recombinant murine cytokines (IL-2, IL-12, IL-15, GM-CSF, and secondary lymphoid tissue chemokine (CCL21/SLC) were purchased from R&D Systems and reconstituted in 0.1% BSA (Sigma-Aldrich, USA). Bromodeoxyuridine (BrdU), rabbit anti-mouse asialo GM1 (anti-AGM1) polyclonal antibody, and gadolinium chloride ($GdCl_3$) were purchased from Sigma.

Adoptive Transfer of $CD8^+$ T Cells

Spleen and lymph nodes (LN) from OT-1 TCR transgenic B6 $Ly5.1^+$ mice were harvested, homogenized, and washed in HBSS (Cellgro, Herndon, VA, USA). Pooled cells were then passed over a CD8-negative selection column from R&D Systems. Naïve $CD8^+Ly5.1^+$ OT-1 T cells (1.5×10^6) were adoptively transferred through intravenous (i.v.) injection into naïve syngenic C57BL/6 $Ly5.2^+$ recipient B6 mice. The cells were then quantitated in the peripheral blood and other compartment by flow cytometry after staining the cells with anti-Ly5.1 and anti-CD8 mAbs. Typically, these donor $CD8^+$ T cell populations represented approximately 0.3% of cells in the recipients' lymphoid organs. Recipient mice were rested for 24 h and then vaccinated with different vaccination protocols as described in each experiment.

Dendritic Cell Generation from Bone Marrow (BM)

Mouse DCs were derived from BM using murine IL-4 and GM-CSF, as we described previously.[39] Briefly, BM cells were flushed from the femurs and tibias of mice and then depleted of RBC by lysis with ACK buffer (Biofluids, Camarillo, CA, USA). Cells were cultured in vitro at 37, 5% CO_2, supplemented with mGM-CSF (20 ng/mL) and mIL-4 (20 ng/mL) and plated out at 1×10^6 cells/mL. On day 4 of culture, complete medium with 20 ng/mL mGM-CSF and 20 ng/mL mIL-4 was added to increase the total volume by 50%. On day 7, non-adherent and loosely adherent cells were harvested, washed and, except in control conditions, pulsed with 5 μg/mL OVAp for 3 hours. The cells were then washed three times in complete medium and their maturity and phenotype were confirmed by flow cytometry by expression of CD80, CD86, CD40, and CD11c and the lack of CD45R expression. Naïve C57BL/6 mice were vaccinated with 1×10^6 DCs pulsed with or without OVAp, via tail vein injection.

Vaccination and Adjuvant Administration

Different adjuvant treatment protocols that have been successfully established in our previous studies were utilized, including: 1) single subcutaneous (s.c.) injection of 100 μg of SIINFEKL in 100 μL PBS;[37]

2) single i.v. injection of 1 x 10^6 SIINFEKL-pulsed BM-generated DCs;[39] or 3) single s.c. injection of F2 gel matrix-loaded OVAp (F2 gel/SIINFEKL)[40]. F2 gel matrix is composed of polysaccharide polymer that we have developed to provide slow delivery of peptide antigens and cytokines.[36] Different adjuvant systems were used, including: 1) intraperitoneal (i.p.) administration of 200 µg poly (I:C)[37,38] in 300 µL PBS; 2) incorporation of GM-CSF[40] into F2 gel/SIINFEKL; 3) incorporation of IL-12[36] or SLC or both into F2 gel/SIINFEKL; 4) i.p. administration of 1 µg/mouse IL-2 or IL-15[39] twice a day for one week; or 5) i.p. injection of 200 mg/Kg CTX[38] 2 days before T cell adoptive transfer.

Cell Preparation

Single cell preparation of spleens was performed by pressing the spleen tissue between the 2 rough ends of microscopic slides. Then, the tissue was filtered and washed twice with PBS after RBCs had been lysed by ACK buffer. Peritoneal exudates cells (PEC) were harvested by collecting the peritoneal fluid after washing the peritoneal cavity with 12mL PBS. Cells were washed and resuspended in PBS. Intrahepatic lymphocytes were isolated by dissecting the liver out of the abdominal cavity and homogenized the tissues by forcing them through a fine metal strainer. After washing 3-4 times with PBS at 22°C, the liver cell suspension was overlaid with histopaque (Sigma, USA) (1:1 v/v) and centrifuged at 13,000 rpm (no brakes) for 20 min at 22°C (Falcon, Franklin Lake, NJ, USA). The cells at the interface were collected, washed with PBS, and processed for flow cytometry analysis.

Flow Cytometry Assay

Fresh 1 x 10^6 cells were treated with anti-CD16/CD32 for 5 min on ice. Cells were then stained with the indicated conjugated mAb, and incubated for 30 min on ice. The cells were washed twice and resuspended in 0.2 mL of 0.5% BSA, 0.02% sodium azide solution. All analyses were performed on gated $Ly5.1^+CD8^+$ T cells, which represent donor antigen-specific OT-1 T cells. Cells were analyzed by flow cytometry using the Cell Quest software package (Becton Dickinson, San Jose, CA, USA).

Measuring Cell Apoptosis

Cells were prepared and stained as above, and washed twice with Annexin-V binding buffer, and 5 µL Annexin-V. Cells were incubated at 22°C in the dark for 15 min, washed twice with Annexin-V binding buffer, and re-suspended in the same buffer for flow cytometry.

T Cell Proliferation Assay

Basically, the analysis of responses of the antigen-specific $CD8^+$ T cells in the spleen and the liver was compared, in certain experiments, however, T cell analysis was performed also in peripheral blood (PBL), draining lymph node (DLN), and peritoneal exudate cavity (PEC). To determine cell proliferation, mice were injected i.p. with 1 mg/mouse BrdU the day before harvesting cells. Cell proliferation was then measured by flow cytomerty using anti-BrdU mAb using intracellular staining assay. In brief, cells were stained for surface markers as above and then fixed, permeablized, and stained with anti-BrdU mAb to detect intracellular levels of these markers. Cells were analyzed by flow cytometry using the Cell Quest software package (Becton Dickinson, San Jose, CA, USA). For NK cell depletion, mice were i.p. injected with 300 µL PBS containing 50 µL anti-AGM1 Ab 1 day before adoptive transfer of OT-1 cells. To deplete KCs, mice were i.v. injected with 10 mg/day/mouse $GdCl_3$ for 3-5 consecutive days before OT-1 T cell transfer.

Cytotoxic T Lymphocyte (CTL) Assay

The lytic activities of spleen and hepatic mononuclear cells obtained on day 7 post vaccination were determined using a standard 4-h ^{51}Cr release assay. In brief, serial dilutions of effector cells (from the liver or spleen) were plated in 96-well U-bottom plates with 5 x 10^3 ^{51}Cr-labeled EL-4 cells pulsed with or without OVAp. After 4-h incubation at 37°C, 25 µL of culture supernatant was removed from each well and radioactivity was determined by gamma counter. The percentage of specific ^{51}Cr release was calculated according to the following equation: percentage of specific lysis=[(experimental release–spontaneous release)/(maximum release–spontaneous release)] x100. Maximum target release was determined by treatment of cells with 9% Triton X-100 solution (Sigma-Aldrich, USA).

Statistics

Numerical data obtained from each experiment were expressed as mean ± SD and statistical differences

between experimental and control groups were assessed using the Student's t-test. *p* values less than 0.05 were considered statistically significant.

RESULTS

Higher Homoeostatic Proliferation of CD8$^+$ T Cells in the Liver

We first asked if naïve CD8$^+$ T cells also preferentially home to the liver and proliferate there in absence of vaccination. To this end, OT-1 cells were infused i.v. into naïve mice and then their relative numbers in the liver was compared to that in PBL, spleen, LN, and PEC after 24 h. As shown in Figure (1A), equal numbers of naïve CD8$^+$ T cells homed to PBL, spleen, LN, and PEC. However, more cells homed to the liver. Further, the homeostatic proliferation of these cells was tested in a host rendered lymphopenic by CTX treatment two days before cell infusion. As shown in Figure 1(B), when naïve OT-1 cells were transferred into CTX-treated mice they showed dramatic proliferation in the liver as compared to naïve mice. This cell proliferation was not transient since it was observed at days 2, 4, and 6 post infusions (Figure 1B).

Adjuvants Plus Peptide-based Vaccination Augments Hepatic Retention of Antigen-Specific CD8$^+$ T Cells

The numbers, phenotype, functions, apoptosis, and proliferation of the antigen-specific CD8$^+$ T cells were measured in the liver upon peptide vaccination and co-administration of different adjuvants. First, we tested the expansion of OT-1 cells in response to OVAp vaccination plus different cytokine adjuvants, including F2 gel/GM-CSF, F2 gel/GM-CSF+ systemic IL-2 treatment, F2 gel/IL-12, F2 gel/SLC, and F2 gel/SLC/IL-12. As shown in Figure 2A, F2 gel/OVAp/GM-CSF vaccination induced higher number antigen-specific CD8$^+$ T cells in the liver than in spleen when analyzed on days 3, 5, and 7 post vaccinations. Although addition of IL-2 to this vaccination regimen further increased their numbers the liver still showed higher number of these cells (Figure 2A). Similar results were obtained with IL-12 when it was incorporated with F2gel/OVAp (Figure 2B).

SLC is chmeotactic for mature DCs.[41] Thus, we tested if addition of this chemokine alone or in combination with IL-12 can alter the trafficking of antigen-specific CD8$^+$ T cells into the liver. Interestingly, addition of SLC to vaccination induced substantial higher numbers of antigen-specific CD8$^+$ T cells in the liver than in PBL, spleen, and DLN (Figure 2C). Of note, combination of IL-12 and SLC did not enhance the effect of SLC on the number of antigen-specific CD8$^+$ T cells in the liver (Figure 2C). Similar results of increased numbers of CD8$^+$ T cells in the liver were obtained after vaccination protocol based on immunization with OVAp-pulsed DCs with or without systemic administration of IL-2 or IL-15 cytokines (Figure 2D).

The TLR3 agonist poly (I:C) is another adjuvant which target mostly innate immune cells such as macrophages, dendritic cells and NK cells. We found that co-administration of the TLR3 ligand poly(I:C) with peptide vaccination associated with significant increases in the numbers of the antigen-specific CD8$^+$ T cells in the liver compared to spleen and DLN (Figure 3, middle panel). Interestingly, poly(I:C) also induced a marked increase, although lesser than in the liver, in the numbers of these cells in PEC, another non-lymphoid compartment (Figure 3, middle panel). Similarly, preconditioning the recipient mice with CTX three days before OT-1 adoptive transfer and OVAp vaccination augmented the increase in the numbers of the antigen-specific CD8$^+$ T cells in both the liver and PEC as compared to spleens (Figure 3, right panel) associated with higher activity as evidenced by the increased expression of the activation marker CD11b (Figure 3).

Antigen-Specific Effector T Cells Retained in the Liver Responded to Recalling Antigen

Interestingly, when the number of antigen-specific CD8$^+$ T cells was analyzed 60 days (resting memory phase) post vaccination there were more numbers of cells in the liver than in PBL, spleen, DLN, and PEC (Figure 4A). This increased numbers was further augmented by co-administration of poly(I:C) at the time of antigen priming. To further understand if these quiescent memory cells in the liver can respond to the recalling antigen, mice were re-vaccinated with OVAp alone after 60 days post the first vaccination. When mice primed with F2 gel/OVAp were boosted with OVAp, more antigen-specific CD8$^+$ T cells observed in the liver than in PBL, spleen or DLN (Figure 4B). These effects were further increased after addition of IL-12 (F2 gel/ OVAp /IL-12) (Figure 4B).

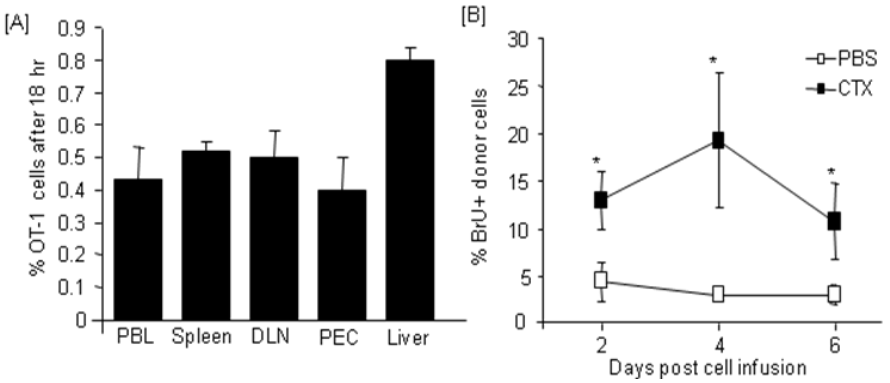

Figure 1. Increased homing and homoeostatic proliferation of adoptively transferred naïve OT-1 cells in the liver. Un-fractionated pooled spleen and lymph node cells (1 x 10^6) from naïve B6.SJL (Ly5.1) OT-1 mice were adoptively transferred by lateral tail vein injection into either untreated naïve C57BL/6 (Ly5.2) mice (A) or transferred into mice treated 1 day before with 4mg/mouse cyclophosphamide (CTX; 4mg/mouse) through intraperitoneal injection to induce leukopenia (B). All mice were sacrificed 1 day (A) or 2, 4, and 6 days (B) after cell infusion and the numbers of the infused cells (Ly5.1$^+$) was visualized in the recipient mice (Ly5.2$^+$) by flow cytomtery.

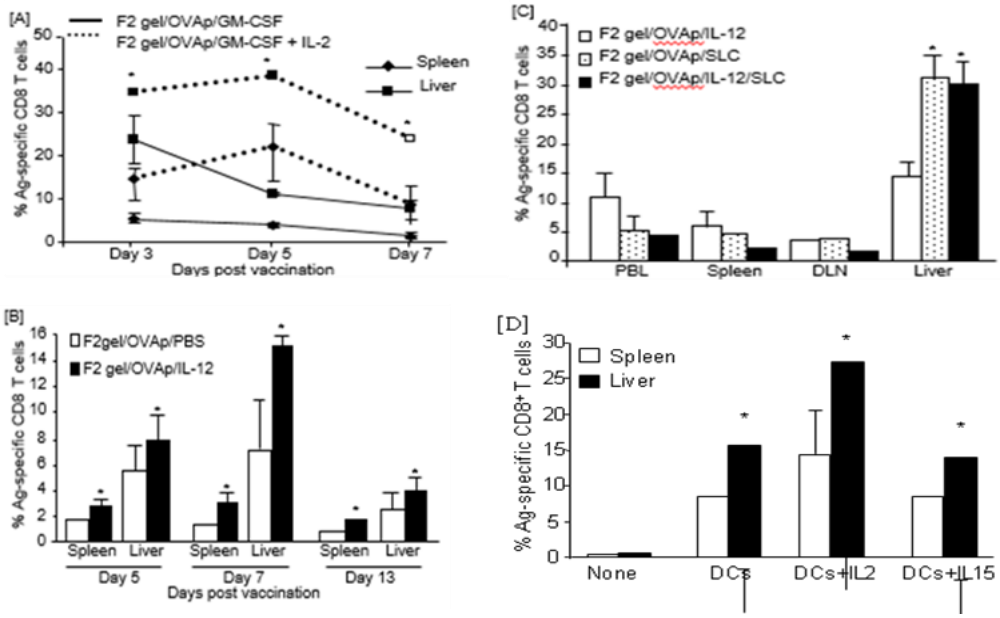

Figure 2. Co-administration of adjuvants along with OVAp vaccination increases the numbers of antigen-specific CD8+ T cells in the liver. Following adoptive transfer of OT-1 cells as described in the legend of Figure 1, B6 mice (4/group) were treated with different vaccine formulations, including (A) s.c. injection of F2 gel/OVAp/GM-CSF or s.c. injection of F2 gel/OVAp/GM-CSF plus systemic i.p. injection of 1µg/day IL-2 for 6 days starting from the day of vaccination, or (B) s.c. injection of F2 gel/OVAp or s.c. injection of F2 gel/OVAp/IL-12, (C) s.c. injection of F2 gel/OVAp/IL-12, s.c. injection of F2 gel/OVAp/SLC or s.c. injection of F2 gel/OVAp/SLC/IL-12. The livers, spleens, PBL, and DLN were harvested on days 3, 5, and 7 (A); days 5 and 7 (B); or day 7 (C) following vaccination and the percentage of donor OT-1 T cells in these compartments was analyzed by flow cytometry. (D) Mice were vaccinated with OV-pulsed DCs, OVAp-pulsed DCs plus systemic i.p. injection of 1 µg/day IL-2 or IL-15 for 6 days starting from the day of vaccination. The livers and spleens were harvested on day 7 following vaccination and the percentage of donor OT-1 T cells in these compartments was analyzed.

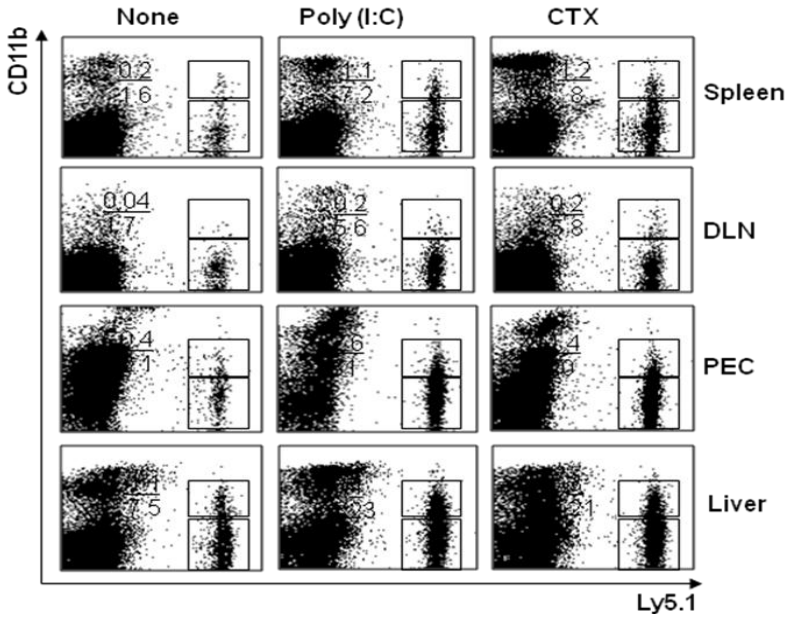

Figure 3. Co-administration of poly(I:C) along with OVAp vaccination following CTX chemotherapy increases the numbers of the antigen-specific CD8$^+$ T cells in the liver. B6 recipient mice (n= 4/group) were i.p. treated with PBS (left panel) or 4 mg/mouse cyclophosphamide (CTX) (right panel), transferred 3 days later with 1.5 x 10^6 OT-1 T cells, rested for 24 hour and vaccinated with 100 µg/mouse OVAp. Half of the phosphate buffer saline PBS)-treated vaccinated mice were immediately treated i.p. with 200 µg/mouse poly(I:C) (middle panel). The recipient mice were sacrificed on day 7 following vaccination and the numbers of OT-1 T (Ly5.1) cells were determined in spleen, liver, draining lymph node (DLN), and peritoneal exudate cavity (PEC) by flow cytometry.

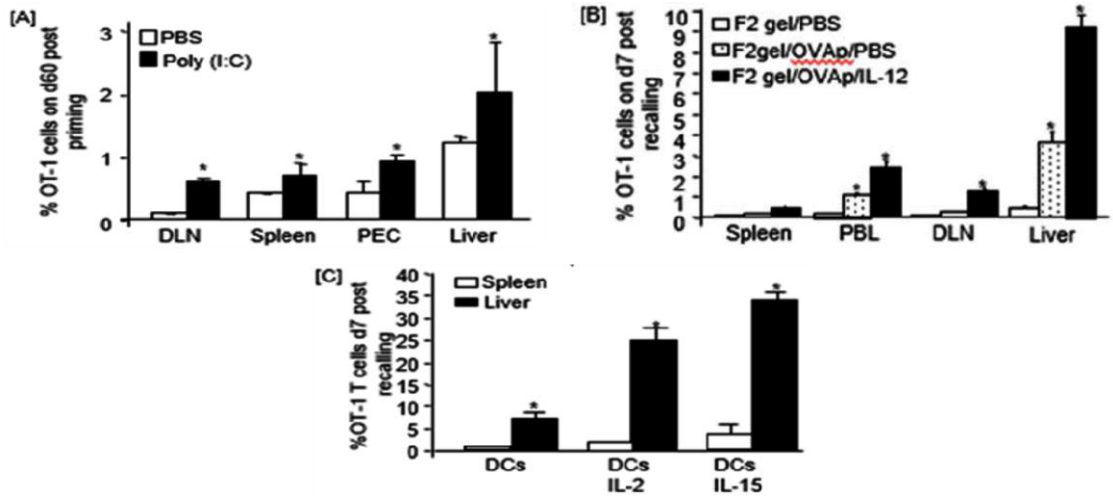

Figure 4. Antigen-specific effector CD8$^+$ T cells retained in the liver respond to the antigen recalling. (A) Following adoptive transfer, B6 mice (4/group) were treated with 100 µg/mouse OVAp, then half of them were i.p. treated with 200 µg/mouse poly(I:C). Mice were bled 60 days later and the numbers of OT-1 T (Ly5.1) cells were determined in spleen and liver. (B) Mice were vaccinated with F2 gel/PBS, F2 gel/OVAp/PBS, or F2 gel/OVAp/IL-12; (C) or with OVAp-pulsed DCs ± systemic i.p. injection of 1 µg/day IL-2 or IL-15 for 6 days starting from the day of vaccination. Mice in (B) and (C) were revaccinated s.c. with free OVAp 60 days post vaccination and the mice were sacrificed 7 days later to determine the number of effector memory antigen-specific CD8$^+$ T cells in livers, spleens, PBL, and DLN (B) or in the livers and spleens (C).

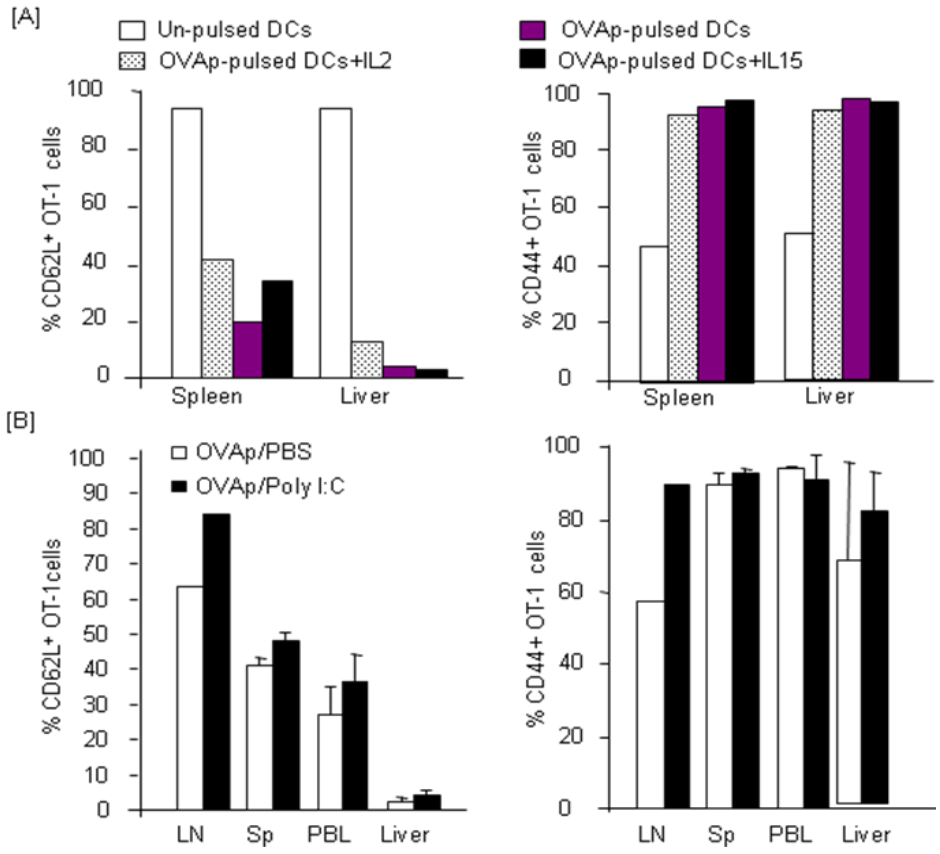

Figure 5. Hepatic antigen-specific OT-1 T cells showed lower expression levels of the homing molecule CD62L. Following adoptive transfer of OT- cells (1 x 10^6) via lateral tail injection, recipients B6 mice were vaccinated with OVAp-pulsed DCs along with either IL-2 or IL-15 (A) or OVAp along with poly(I:C) (B). The mice were sacrificed 7 days post vaccination for flow cytometry analysis of CD62L (left panel) and CD44 (right panel) in the livers and spleens (A) and in the livers, spleens, peripheral blood (PBL), and draining lymph node (LN) (B).

Similar profile of hepatic retention of memory $CD8^+$ T cells was also observed after DC-based vaccination plus IL-2 or IL-15 (Figure 4C), and after vaccination plus poly (I:C) or CTX (data not shown).

Hepatic Antigen-specific T Cells Showed Downregulation of CD62L Expression

CD62L is a typical homing receptor that directs T cell trafficking to secondary lymphoid organs in particular DLN to induce anti-tumor immunity.[42] Thus, lower expression of CD62L on T cells decreases their homing to DLN cells and directs them to the liver. When phenotypic analysis was performed on the gated OT-1 cells 7 days post vaccination with DCs+IL-2 or IL-15, hepatic antigen-specific $CD8^+$ T cells showed dramatic downregulation in the CD62L expression compared to those cells in the spleen (Figure 5A). These $CD8^+$ T cells in both the liver and spleens showed the typical memory phenotype evidenced by high expression of CD44 (Figure 5B, right panel). Similar profiles of CD62L (Figure 5B, left panel) and CD44 (Figure 5B, right panel) expression were also observed after vaccination plus IL-12 and CTX (data not shown).

Antigen-specific $CD8^+$ Effector T Cells Retained in the Liver Were Highly Proliferating

The proliferation of hepatic antigen-specific $CD8^+$ T cells was then quantitated on day 5 or 7 post vaccination. OVAp vaccination with F2 gel/OVAp/GM-CSF induced higher number of proliferating antigen-specific $CD8^+$ T cells in the liver

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than spleen both on days 5 and 7 post vaccination with more proliferating cells on day 5 than on day 7 in both organs (Figure 6A). Co-administration of IL-2 further delayed the contraction in the proliferating T cells in both the liver and spleen, where the liver still harboring higher number of these cells (Figure 6A). Typical proliferation profile of the antigen-specific $CD8^+$ T cells in the liver was also observed after co-administration of other adjuvants, including poly (I:C), and CTX (Figure 6B). The numbers of the antigen-specific $CD8^+$ T cells undergoing apoptosis was determined precisely by annexin-V staining and flow cytometry. Upon vaccination with F2 gel/OVAp/GM-CSF about 20% and 12% of the antigen-specific $CD8^+$ T cells undergone apoptosis were found in spleen and the liver, respectively, on day 5 post vaccination (Figure 6C). The number of apoptotic cells on day 7 was slightly increased in spleen while dramatically increased in the liver; 28% in spleen versus 50% in the liver (Figure 6A). Systemic treatment with IL-2 decreased the number of apoptotic cells only in spleens, but not in the liver, analyzed on days 5 and 7 post vaccination (Figure 6A). Similar data were observed with poly (I:C) and CTX treatments (Figure 6B). In order to define the functional status of the antigen-specific $CD8^+$ T cells retained in the liver, the liver and spleen were harvested 7 days post vaccination with F2 gel/OVAp, F2 gel/OVAp/GM-CSF, and F2 gel/OVAp/IL-12 to test their capability to kill target tumor cells. As shown in Figure 7, spleen cells showed significant killing of EL-4 lymphoma cell line pulsed with OVAp but not the non-target cells (un-pulsed EL4 cells). However, interestingly the liver cells showed much higher killer capability than those cells in spleen (Figure 7).

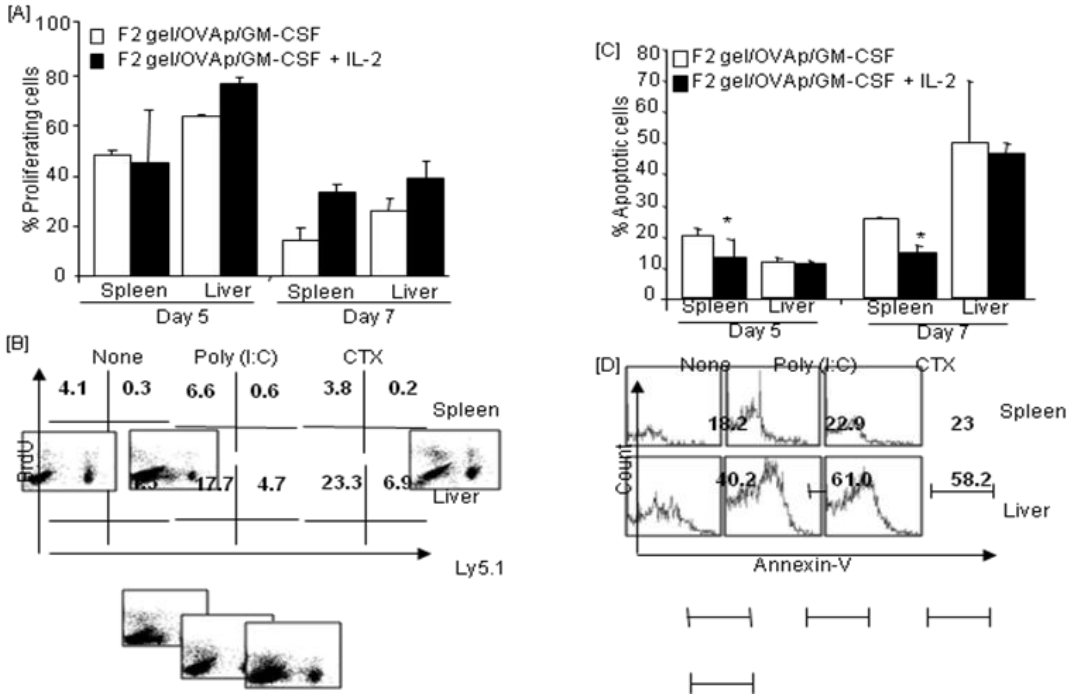

Figure 6. Antigen-specific $CD8^+$ effector T cells retained in the liver are highly proliferating. (A) Following adoptive transfer, mice were vaccinated with F2 gel/OVAp/GM-CSF or F2 gel/OVAp/GM-CSF + IL-2. (B) Recipient mice were pre-treated with cyclophosphamide (CTX) (right panel), transferred 3 days later with 1.5 x 10^6 OT-1 T cells, and vaccinated with OVAp with or without poly(I:C) treatment (middle panel). All mice were injected with 1mg BrdU on days 4 or 6 following vaccination. Mice were sacrificed on days 5 and 7 (A) or on day 7 (B) following vaccination and the numbers of OT-1 T (Ly5.1) cells undergoing proliferation were determined in spleen and liver by flow cytometry. (C and D) Adoptive transfer, vaccination, CTX preconditioning, and poly(I:C) treatment were performed as described in the legend of Figure 6. Mice were sacrificed on days 5 and 7 (C) or on day 7 (D) following vaccination and the numbers of OT-1 T (Ly5.1) cells undergoing apoptosis were determined in the liver and spleen by flow cytometry.

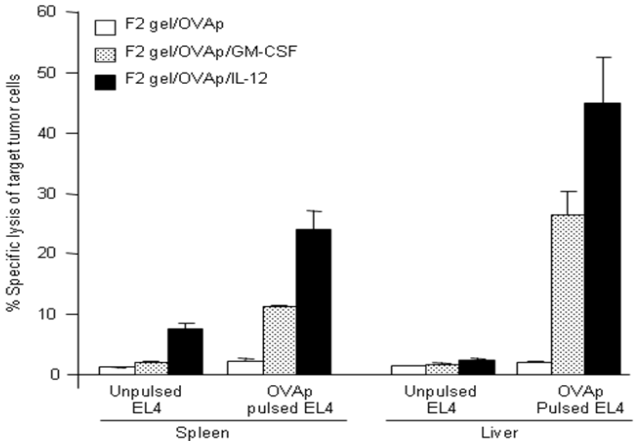

Figure 7. Antigen-specific effector CD8$^+$ T cells retained in the liver are functional. 1.5 x 10^6 OT-1 T cells were adoptively transferred into C57BL/6 mice (n=4/group). Recipient mice were then vaccinated with F2 gel/OVAp, F2 gel/ OVAp/GM-CSF or F2 gel/OVAp/IL-12. The livers and spleens were harvested on day 7 and lytic capacity was determined by the standard ^{51}Cr-release assay using OVAp-pulsed EL-4 cells as target tumor cells. Un-manipulated EL-4 tumor cells were used as a control target.

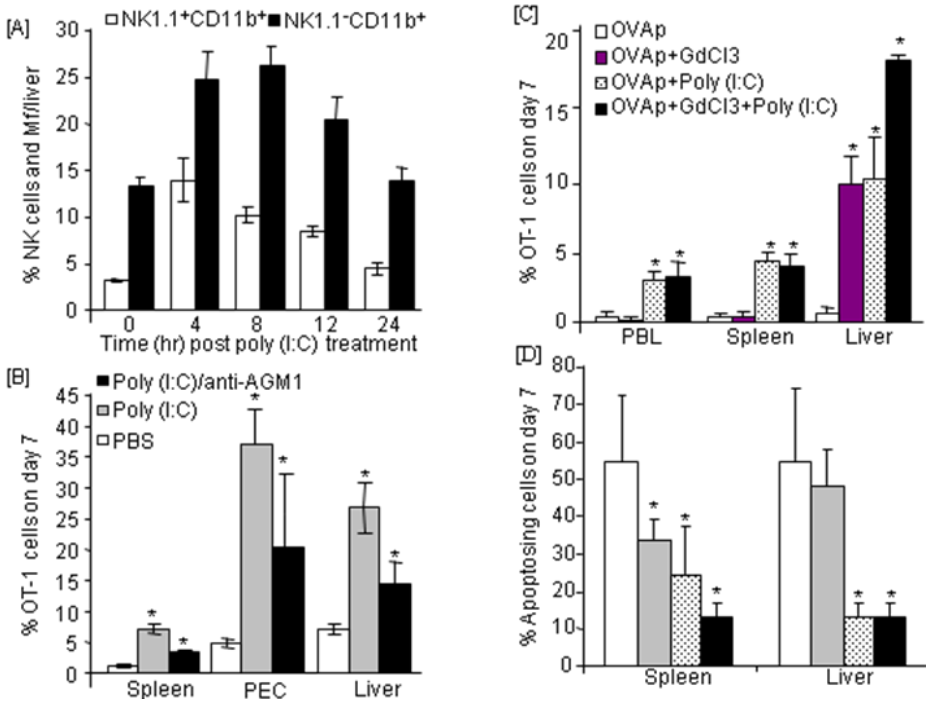

Figure 8. Opposing roles of natural killer (NK) cells and Kupffer cells (KCs) in mediation the hepatic retention of the antigen-specific T cells. Naïve mice were treated with 200 µg/mouse poly (I:C) and then the numbers of NK cells were determined in the liver at multiple time points (A). OT-1 adoptive transfer, vaccination, and poly (I:C) treatments were performed as described in the legend of Figure 5 and 6. The recipient mice were either naïve or depleted of NK cells by an i.p. injection of anti-asialo GM1 (anti-AGM1) (B) or depleted of KCs by an i.p. injection of GdCl3 (C). The numbers of antigen-specific CD8$^+$ T cells was determined by flow cytometry in the liver, spleens, and peripheral blood (PBL) 7 days post OVAp vaccination (B & C). The numbers of the antigen-specific CD8$^+$ T cells undergoing apoptosis were determined in spleen and the liver (D) by flow cytometry.

Opposing Effects of NK Cells and KCs in Mediation the Retention of the Antigen-specific $CD8^+$ T Cells in the Liver

The liver contains different types of innate immune cells such as NK cells, NKT cells, DCs, and KCs[24] that might positively or negatively impact on its retention of the antigen-specific $CD8^+$ T cells. By analyzing the relative numbers of NK cells and macrophages (KCs) in the liver, poly(I:C) adjuvant was found to induce time-dependent increases in the number of these cells as evidenced by the increases in the $NK1.1^+$ (NK cells) and $CD11b^+$ (macrophages) expressing cells (Figure 8A). Then, the roles of NK cells and KCs in mediating the enhanced effects of poly(I:C) on the retention of $CD8^+$ T cells in the liver was tested. Depletion of NK cells by treatment with anti-AGM1 Ab markedly decreased the numbers of the antigen-specific $CD8^+$ T cells in the liver (Figure 8B). Depletion of KCs by i.p. injection of $GdCl_3$ before adoptive transfer and subsequent vaccination had no effect in either PBL or spleen, but strikingly increased the numbers of these cells in the liver (Figure 8C). Interestingly, removal of KCs decreased the number of apoptotic $CD8^+$ T cells in spleen, but not in the liver when analyzed on day 7 of mice vaccinated with OVAp plus poly(I:C) (Figure 8D).

DISCUSSION

The normal liver has been found to retain in vitro and in vivo activated T cells with a strong preference for $CD8^+$ T cells, and has been suggested as a key regulatory organ shaping the nature of T cell responses.[28] Thus, the liver can mediate induction of tolerance as well as effector immunity, depending on the nature of intrinsic factors encoded in the T cells entering the liver and factors residing in the liver microenvironment.[6] Yet, the impact of factors in mediation the hepatic retention of $CD8^+$ T cells has not been defined. We have reported recently on the beneficial effects of different cytokines (IL-2, IL-12, IL-15, G-CSF and GM-CSF),[36,39,40,43-52] the TLR3 ligand poly(I:C)[37,38,44-71] and the preconditioning with the chemotherapeutic drug cyclophosphamide[54,56,57,61,66] to augment $CD8^+$ T cell responses to OVA and melanoma peptides vaccination. Herein, these adjuvants, regardless of their nature, accentuated the retention of the antigen-activated $CD8^+$ T cells in the liver than in spleen or other compartments. These cells were highly proliferating and functional as evidenced by their capability to specifically kill EL4 lymphoma. Further, even though the number of the activated $CD8^+$ T cells retained in the liver contracted with time, higher number of these cells persisted in the liver as quiescent long-lasted memory cells. The latter cells showed superior responses to secondary immunization, indicating that the cells retained in the liver persisted as functional effector memory $CD8^+$ T cells. NK cells and KCs played opposite roles in mediation the hepatic retention of $CD8^+$ T cells; NK cells showed crucial while KCs showed detrimental effects. Therefore, augmentation of hepatic T cell retention by stimulating the host microenvironment having different mode of actions seems to be a global phenomenon, where both intrinsic mechanism, encoded in the retained $CD8^+$ T cells, and extrinsic mechanisms encoded in the liver microenvironment contribute to this process.

Although it is not clear how the adjuvants used in the present study accentuated the hepatic retention of the antigen-activated $CD8^+$ T cells, modulation of intrinsic and extrinsic mechanisms would be suggested as key players. Intrinsic mechanisms, although not mutually exclusive, include preferential proliferation of T cells in hepatic microenvironment; altering apoptosis of the trapped cells; and/or alteration of trafficking of T cells.[5,6] Since it is not possible to test the effect of liver T cell retention on a systemic immune response by removing or bypassing the liver, we tested the proliferation, apoptosis, and homing receptor (CD62L) expression of the trapped cells in the liver. We clearly found that antigen-specific $CD8^+$ T cells retained in the liver showed substantial higher rate of proliferation and apoptosis. The tendency of $CD8^+$ T cells for higher proliferation capability in the liver was further evidenced in the absence of vaccination, where substantial local proliferation (analyzed by BrdU accumulation) of naïve $CD8^+$ T cells was observed in the liver upon infusion of these cells in hosts rendered lymphopenic by pretreatment with CTX. Thus, these data indicate that both naïve and antigen-activated $CD8^+$ T cells entering the liver possess intrinsic capability for high rate of proliferation.

CD62L, a typical trafficking molecule from selectin family (L-selectine), which directs the preferential homing of T cells to secondary lymphoid compartments, in particular LN.[42] Thus, decreasing

CD62L expression by T cells significantly decrease their homing to LN associating with their retention in non-lymphoid compartments such as the liver, lung, and PEC. The antigen-specific $CD8^+$ T cells retained in the liver showed dramatic decrease in the CD62L expression compared to their counterparts in the spleen. Therefore, the increased local proliferation and downregulation of CD62L expression by the antigen-specific T cells in the liver would explain, at least in part, some of the intrinsic mechanism mediating their retention in this unique immunological niche. Although these two properties were observed in the absence of adjuvants, they became more pronounced after provision of the adjuvants, indicating that the later are crucial for shaping the intrinsic mechanisms encoding by the T cells.

The induction of T cell activation or tolerance depends on the nature of the immune response and the nature of the engaged hepatic cells.[6,72] Thus, on one hand antigen expressed by the non-bone marrow-derived cells (endothelial cells and hepatocytes) in the liver actively cause $CD8^+$ T cell accumulation and subsequent apoptosis.[16,17] On the other hand, when the professional antigen presenting DCs in the liver are engaged, the liver could be an excellent priming site for naive $CD8^+$ T cells.[20,73] Other mechanisms in the liver might also regulate hepatic retention of the antigen-activated $CD8^+$ T cells include the functional status of NK cells, DCs, T_{reg} cells and KCs as well as and endothelial and parenchymal cells in the liver.[74] The requirement of NK and NK T cells, DCs, and Mϕ for effective immune response has been established in several experimental settings.[75,76] Few studies, however, has reported on the essential function of these cells in hepatic retention of T cells. By analyzing the role of NK cells as one suggested component of the mechanisms, the data of the present study confirmed the importance of these cells in mediating the hepatic retention of T cells. This is evidenced by the finding that poly(I:C) adjuvant treatment selectively induced substantial increases in the numbers of NK ($NK.1.1^+CD11b^+$) cells in the liver, and depletion of NK cells significantly decreased the hepatic retention of the antigen-specific T cells. These data confirm and extend our previous recent finding showing that NK cells significantly contributed to the adjuvant effects of the TLR3 ligand poly(I:C)[38] and IL-12[36] inducing expansion of the antigen-specific $CD8^+$ T cells in PBL, spleen, and DLN. Similarly, it has been reported that the hepatic retention of infused thymocyte-derived $CD8^+$ NKT cells was severely impaired by in vivo depletion of NK cells, but not KCs.[77] Further, this is in consistent with the fact that the liver harbor substantial numbers of NK/NK T cells that home to the liver under the steady state condition as well as in response to several insults.[25,74] Taken together, NK cells are important contributor of the hepatic retention of the activated $CD8^+$ T cells in the liver, and enhancing the number and/or functions of these cells by biological response modifiers would greatly enhance the immune responses in the liver.

In previous studies, KCs have been found to induce apoptosis of activated and anergy of T cells[23] through expression of B7-H1/B7-DC.[78,79] However, these cells can also mediate anti-tumor[25] and anti-viral immunity[79] in particular they are capable of producing cytokines in response to TLR agonists.[24,80] As such, we analyzed the role of KCs as a potential mechanism mediating the hepatic retention of T cells with the hypothesis that removal of these cells from the liver might induce negative impact on the retention of activated hepatic $CD8^+$ T cells. Interestingly, however, we found that absence of KCs in the liver microenvironment markedly increased the retention of the activated $CD8^+$ T cells in the liver, but not in PBL, spleen or DLN. In line with these results, blocking of KCs reversed the suppressive effects of these cells on the delayed type hypersensitivity and autoimmune diseases.[81,82] In the same vein, it has been suggested that interfering with antigen presentation by KCs may be a strategy to limit antigen-induced deletion of the activated $CD8^+$ T cells entering the liver.[23] The enhancement of T cell retention in the liver after KC depletion would explain why the immune response to foreign antigens in the liver is often suboptimal that is clinically relevant in chronic persistence of hepatotropic viruses.

Although the regulatory effects of KCs in hepatic T cell retention were attributed in most cases to the capability of these cells to promote apoptosis of the antigen-specific T cells trapped in the liver, we could not find any difference in the apoptotic rate of the antigen-activated $CD8^+$ T cells in the liver after removal of KCs. KCs can also recognize phosphatidyl serine, a lipid component of the cell membrane that is generally restricted to the inner leaflet of the plasma membrane in the healthy cells, after its exposure on the plasma membrane of the early apoptotic cells resulting in phagocyosis of the early apoptotic cells and their

subsequent clearance.[83] In addition, KCs and endothelial cells both express mannose and galactose receptors,[84,85] which play a dominant role in trapping apoptotic T cells by KCs.[86] Alternatively, several mechanisms could be suggested including: 1) KCs might contribute to the retention of T cells in the liver at early stage of retention, whilst their persistence might limit the T cell retention at later stages as a mechanism required to avoid autoimmunity, 2) removal of KCs might be compensated by other accessory cells such as NK/NK T cells and/or other macrophages subpopulations abundant in the liver tissues that can support the T cell retention, and 3) removal of KCs leaves DCs ($CD11c^+$) as the major antigen presenting cells sustaining survival of antigen-primed T cells.[87] In consistent with this notion, we have found that injection of poly(I:C) into CD11b deficient mice induced higher numbers of NK/NKT cells as well as DCs compared to poly(I:C)-injected wild type mice.[38]

Regardless the mechanism underlying the increase in the number of the antigen-specific $CD8^+$ T cells in the liver after KCs depletion this finding is of significant importance in improving the application directed to optimize the local antigen-specific T cell responses in the liver microenvironment toward liver-borne diseases such as hepatitis, liver cancers, and liver metastasis. We have reported recently that co-administration of poly(I:C) adjuvant with adoptive immunotherapy established preventive anti-tumor effects against EL4 lymphoma grown as interaperitoneal ascites.[37] Accordingly, experimental studies planning on establishing effective vaccination regimens, in particular toward liver metastasis, might consider blocking or inducing KC cell function as a beneficial preconditioning regimen when augmented and suppressed immunity in the liver, respectively, is desirable. The opposite scenario might work for NK cells. Ultimately, defining adjuvant with immunomodulatory effects, such as the TLR3 ligand poly(I:C), on NK cells and KCs would have potential applications in immune-based treatment directed toward infectious, autoimmune, and cancer diseases in the liver.

Taken together, these data of the present study show that antigen reactive T cells in the liver have higher activities that their counterparts in the peripheral tissues such as spleen. These data have important clinical implications for designing immunotherapeutic protocols toward the liver borne diseases such as hepatitis and liver metastasis.

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