Research Article

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Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice

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Background & Aims: Liver Kupffer cells have been suggested to be heterogeneous macrophage lineage cells. We explored this possibility by classifying the mouse Kupffer cells into subpopulations and characterizing them by their phenotype and function.
Methods: Liver mononuclear cells (MNCs) from C57BL/6 mice were isolated and their phenotypes and functions were analyzed. The effects of clodronate liposomes and gadolinium chloride ($GdCl_3$) on Kupffer cells were also investigated.
Results: Approximately 25% of liver MNCs were F4/80$^+$ Kupffer cells. Of these, 46% were CD11b$^-$CD68$^+$, 22% were CD11b$^+$CD68$^-$, and 6% were CD11b$^+$CD68$^+$. CD68$^+$ cells showed potent phagocytic activity and reactive oxygen species (ROS) production capacity after lipopolysaccharide (LPS) stimulation, whereas CD11b$^+$ cells did not. CD11b$^+$ cells showed a strong capacity for the production of cytokines (TNF and IL-12), which was much less prominent in CD68$^+$ cells. At 24h after LPS or *Escherichia coli* injection into mice, the proportions of CD11b$^+$CD68$^-$ and CD11b$^+$CD68$^+$ cells increased but that of CD11b$^-$CD68$^+$ cells decreased. The increase in CD11b$^+$CD68$^+$ cells appeared to be derived from the CD11b$^+$CD68$^-$ subset. Although the CD11b$^+$ cells augmented phagocytic activity after LPS injection, they did not increase ROS production, suggesting their weak lytic activity. Injection of clodronate or $GdCl_3$ into mice depleted the CD68$^+$ cells but increased CD11b$^+$ cells proportionally because CD68$^+$ cells may phagocytose these toxic reagents and undergo apoptosis. $GdCl_3$-treated mice also consistently increased serum TNF after LPS challenge.
Conclusions: Two F4/80$^+$ Kupffer cell subsets may exist, a CD68$^+$ subset with phagocytic activity and a CD11b$^+$ subset with cytokine-producing capacity.

Keywords: Phagocytosis; Reactive oxygen species; Cytokines; CD68; CD11b; Immunohistochemistry.
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Abbreviations: ROS, reactive oxygen species; $GdCl_3$, gadolinium chloride; LPS, lipopolysaccharide; CRP, C-reactive protein; *E. coli*, *Escherichia coli*; PBS, phosphate-buffered saline; MNCs, mononuclear cells; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay.

Introduction

Functional characterization and classification of mouse Kupffer cells have yet to be fully elucidated. Our recent studies have revealed that gadolinium chloride ($GdCl_3$), which reportedly depletes Kupffer cells or inhibits their functions, suppresses lipopolysaccharide (LPS)-induced superoxide production but augments TNF production [1]. In addition, C-reactive protein (CRP) augments phagocytosis by Kupffer cells but suppresses their TNF-producing capability [2]. We also recently reported that $GdCl_3$ pretreatment completely inhibited concanavalin-A-induced hepatitis by decreasing superoxide-producing CD68$^+$ Kupffer cells without suppression of serum TNF levels [3]. These findings raise the possibility that mouse Kupffer cells can be classified into subpopulations. Several previous studies using rats have demonstrated heterogeneity of Kupffer cells depending upon their size and/or location in the liver [4,5], and $GdCl_3$ pretreatment selectively depleted large Kupffer cells [6]. However, the relationship between cytokine production capacity and either cell size or surface markers of Kupffer cells in both rats and mice still remains unclear.

F4/80 is a representative surface marker of mouse mononuclear phagocytes. It is a stable antigen and is not usually present in other types of leukocytes [7,8]. CD11b, a C3b receptor, is present on the surface of monocytes/macrophages, granulocytes, and NK cells [9]. CD68 (macrosialin) is also used as a marker of macrophages, including Kupffer cells. This antigen is predominantly localized in the cytosol of macrophages [10] but is expressed on the cell surface upon activation. Based on these findings, we have studied the subclassification of Kupffer cells according to their surface markers and function.

Materials and methods

This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Japan.

Mice and reagents

Male C57BL/6 mice were studied (8 weeks old, 20 g, Japan SLC, Hamamatsu, Japan). *Escherichia coli* (*E. coli*) strain B (ATCC 11303, Sigma Co., St. Louis, MO),

lipopolysaccharide (LPS; *E. coli* 0111:B4, Sigma), Fluoresbrite Carboxylate YG (= FITC) microspheres (hereafter called FITC-microspheres; Polysciences, Eppelheim, Germany), aminophenyl fluorescein (APF, Daiichi Chemical, Tokyo, Japan), gadolinium chloride ($GdCl_3$, Sigma), and clodronate (LKT Laboratories, Inc., St. Paul, MN) were also used.

Escherichia coli and LPS challenge

Escherichia coli (1×10^9 CFU), LPS (2.5 mg/kg), or phosphate-buffered saline (PBS; 0.2 ml) was intravenously injected into mice 24 h before the experiments.

Isolation and culture of mononuclear cells (MNCs) or Kupffer cells

Liver MNCs, particularly non-parenchymal cells which include Kupffer cells, were isolated using the previously reported method of *in vitro* 0.05% collagenase incubation of liver specimens [1,3,11–13]. Under deep ether anesthesia, the liver was perfused with Hanks balanced salt solution (HBSS; Invitrogen, Gaithersburg, MD) via the portal vein. Immediately after perfusion, the liver was removed and minced with scissors. After adding 10 ml HBSS containing 0.05% collagenase (Type IV; Sigma), the specimens were shaken for 20 min at 37 °C. The liver specimen was filtered through a stainless steel mesh, suspended in 33% Percoll solution containing 10 U/ml heparin, and centrifuged for 15 min at 500*g* at room temperature. After red blood cell lysing, the cells were washed twice [1,3]. We also isolated liver MNCs using the method of *in situ* collagenase digestion. Briefly, the liver was perfused with HBSS for 5 min and subsequently with HBSS containing 0.05% collagenase at 5 ml/min for 5 min. The liver was then removed and filtered through the mesh. The sample was suspended in HBSS and centrifuged at 50*g* for 1 min at 4 °C. After collecting non-parenchymal cell-enriched supernatant, the non-parenchymal cells were purified using centrifugation with 33% Percoll solution containing 10 U/ml heparin. Splenocytes were also filtered through the mesh, treated with a lysing solution, and washed twice. For *in vitro* LPS stimulation, liver MNCs (5×10^5 cells/200 μl) were cultured with 10 μg/ml LPS in 96-well flat-bottomed plates at 37 °C in 5% CO_2.

Flow cytometric analysis

After incubation with Fc-blocker (2.4 G2; BD PharMingen, San Diego, CA), MNCs were stained with FITC-conjugated anti-F4/80 Ab (eBioscience, San Diego, CA), PE-conjugated anti-CD11b Ab (M1/70, eBioscience), and biotin-conjugated anti-CD68 Ab (FA-11, AbD Serotec, Oxford, UK) with Cy5-streptavidin. Flow cytometry was performed using an EPICS XL (Beckman Coulter, Miami, FL).

Evaluation of microsphere phagocytosis

As described previously [2], liver MNCs including Kupffer cells were incubated with 1 μL FITC-microspheres (1×10^8/μl) in 200 μL for 20 min. Thereafter, the cells were stained with PE-conjugated anti-CD11b Ab or biotin-conjugated anti-CD68 Ab with PE-streptavidin and Cy5-conjugated anti-F4/80 Ab (eBioscience). Phagocytosis of FITC-microspheres was analyzed using the EPICS XL.

Determination of ROS production

Liver MNCs (5×10^5 cells/200 μl) were incubated with 1 μl APF, a fluorescent reagent for detecting ROS [14], and subsequently stimulated by 10 μg/ml LPS, 5 mM ATP, or PBS for 30 min at 37 °C in 5% CO_2. After incubation with Fc-blocker, the cells were similarly stained with anti-CD11b, CD68, and F4/80 Abs. ROS production, as assessed by FITC fluorescent intensity, was analyzed using the EPICS XL.

Intracellular staining with TNF

Liver MNCs were incubated with LPS (10 μg/ml) for 6 h. BD GoldiStop (0.7 μg/ml, BD PharMingen) was added 4.5 h before staining. After incubation with Fc-blocker, the cells were stained with FITC-conjugated anti-F4/80 Ab and Cy5-conjugated anti-CD11b Ab or biotin-conjugated anti-CD68 Ab with Cy5-streptavidin. Subsequently, the cells were incubated with BD Cytofix/Cytoperm solution (BD Pharmingen) at 4 °C for 20 min and then washed with BD Perm/Wash solution (BD Pharmingen). Thereafter, the cells were stained with PE-conjugated anti-TNF mAb (eBioscience) or isotype rat IgG1 Ab (eBioscience) at 4 °C for 20 min and then analyzed using the EPICS XL.

Cell sorting

As described previously [3], the mice were pretreated with anti-NK1.1 Ab 3 days before sacrifice to deplete NK 1.1^+ cells, because NK cells express CD11b in the present study. We confirmed that isolated liver MNCs contained few $CD31^+$ cells (less than 3%), thereby ruling out a significant contamination by liver sinusoidal endothelial cells. For positive selection, liver MNCs were incubated with Fc-blocker and then stained (1) with PE-conjugated anti-CD11b Ab and magnetically labeled with anti-PE MicroBeads (Miltenyi Biotec, Auburn, CA) or (2) with biotin-conjugated anti-CD68 Ab and magnetically labeled with anti-biotin MicroBeads (Miltenyi Biotec). $CD11b^+$ or $CD68^+$ cells were sorted using the MACS system (Miltenyi Biotec); the purity of magnetic separation was more than 90% and 85%, respectively.

For negative selection, liver MNCs (2×10^6/ml) were seeded in tissue culture dishes and incubated for 2 h at 37 °C in 5% CO_2. Thereafter, plastic-adherent Kupffer cells were obtained from the dish. After staining (1) with PE-conjugated anti-CD11b Ab and magnetically labeled with BD IMag anti-R-PE Particles (BD Pharmingen) or (2) with biotin-conjugated anti-CD68 Ab and BD IMag Streptavidin Particles Plus, $CD11b^+$ cells or $CD68^+$ cells were depleted using the IMag system (BD Pharmingen) to obtain $CD11b^-$ Kupffer cells (>95% depleted) or $CD68^-$ Kupffer cells (>90% depleted), respectively.

Measurement of TNF, IL-12, and IFN-γ

TNF, IL-12, and IFN-γ levels of the samples were measured using a cytokine-specific enzyme-linked immunosorbent assay kit (Endogen, Woburn, MA).

Splenectomy

Splenectomy was performed under deep ether anesthesia through a left-side, lateral, 1.0-cm subcostal incision. Sham operation was performed by the same incision process. The mice were studied 4 weeks after surgery [15].

Pretreatment with $GdCl_3$ or clodronate liposome

$GdCl_3$ (10 mg/kg) or saline (0.2 ml) was intravenously injected into mice 24 h before the experiments. Some $GdCl_3$-treated mice were intravenously challenged with 10 mg/kg LPS. Clodronate was encapsulated into liposomes and 200 μl of a 25 mg/ml suspension was intravenously injected into the mice 24 h before the experiments.

Immunohistochemistry

Fresh frozen sections of the liver were cut 6 μm thick on a cryostat, collected on slides, and immediately dried. The sections were fixed with acetone and washed. After incubation with 1% Block Ace (DS Pharma Biomedical, Tokyo) for 10 min, the slides were incubated overnight with anti-CD68 Ab (FA-11) or anti-CD11b Ab (M1/70) diluted 1:100, followed by incubation with Histofine Simple Stain Mouse MAX-PO (Rat) (Nichirei, Tokyo) for 1 h and Histogreen for 5 min (Abcysonline, Paris, France) and then counterstained. Negative controls were incubated with 1% Block Ace instead of primary Abs.

Statistical analysis

The results were expressed as mean value ± SD. Statistical analyses were performed using the Stat View 4.02J software package (Abacus Concepts, Berkeley, CA). Where appropriate, Student's *t*-test was employed to compare the data of two different groups. $p < 0.05$ were considered to be significant.

Results

Collagenase pretreatment effect on liver MNC isolation

The number of liver MNCs isolated was increased by *in vitro* collagenase pretreatment relative to MNCs isolated without collagenase (from approximately 3×10^6 to 4.5×10^6 per liver). It also increased the proportion of $F4/80^+$ cells in the isolated MNCs