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Development of a cytokine-producing immortalized murine Kupffer cell line

Zheng-Yu Wang [a,*], Christopher Burlak [a], James E. Klaunig [b], Lisa M. Kamendulis [b]

[a] Department of Surgery, Indiana University School of Medicine, MS B005, 635 Barnhill Dr., Indianapolis, IN 46202, United States
[b] Department of Environmental Health, Indiana University School of Public Health, 925 E 7th, Bloomington, IN 47405, United States

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ABSTRACT

Kupffer cells (KC) play a critical role in both liver physiology and the pathogenesis of various liver diseases. Isolated primary KC have a limited lifespan in culture, and due to the relatively low number obtained, limit their study *in vitro*. Here, a cytokine-producing immortalized KC (ImKC) line was established from transgenic mice that express the thermolabile mutant tsA58 of the Simian virus 40 large T antigen under the control of the *H-2k*^{b} promoter. Primary KC were obtained using a three step procedure: liver perfusion, centrifugal elutriation, and sorting for F4/80$^+$ cells. ImKC were identified within the small-intermediate population of KC that maintained stable expression of F4/80, and the surface antigens CD11b, CD14 and TLR4. ImKC grow at IFNγ-independent manner at 37 °C and exhibited a doubling time of ~24 h when cultured in RPMI 1640 with 5% FBS. Our observations indicate that both activation of telomerase and expression of P53 are markedly increased, suggesting that enhanced telomerase activity and P53 expression may contribute to the immortalization of this cell population. ImKC cells maintained a high capacity to phagocytose FITC-latex beads, and bind/phagocytose erythrocytes. In addition, similar to primary KC, ImKC responded to stimulation with lipopolysaccharide (LPS: 0.1–1 μg/ml) by upregulating mRNA levels of TNFα (23-fold), IL-6 (28-fold), and IL-1β (1459-fold), as measured by qRT-PCR. Protein levels of TNFα and IL-6 were also increased, 10-fold and 12-fold, respectively. Reactive oxygen species (ROS) and nitric oxide (NO) production were significantly enhanced in ImKC following an LPS challenge. Furthermore, LPS elicited a marked increase in mitogen activated protein kinase (MAPK) phospho-(ERK1/2, JNK) and NF-κB p50 with decreased IκBα in ImKC, as assessed by Western blot. Collectively, these results demonstrate that the ImKC line retains critical characteristics of primary KC, and thus provides a useful tool to assess the role of KC in liver injury and chronic diseases.

1. Introduction

Kupffer cells (KC), the resident liver macrophages, constitute the largest number of tissue macrophages in the body [1]. KC possess critical functions including phagocytizing particles and foreign materials, antigen presentation, and modulating innate immune responses [2]. Upon activation, KC produce a number of biologically active molecules including proinflammatory cytokines, nitric oxygen (NO) and reactive oxygen species (ROS); all of which have been shown to contribute to the pathogenesis of liver injuries elicited by chemical substances and pharmacological agents [2,3], ischemia–reperfusion [4], as well as hepatic carcinogenesis [5].

Isolation and culture of primary KC has been used to study cellular and molecular analysis of Kupffer cell function. However, there are several limitations inherent in using primary KC for *in vitro* studies, including low yield, difficulty in obtaining pure populations, lack of proliferation, and a limited life span in culture. To address these issues, researchers have developed immortalized KC (ImKC) lines, both from rats and from H-2K(b)-tsA58 transgenic mice [6,7]. Although the immortalized mouse KC cell line developed retains several functional features inherent to primary KC, the cell line lacks the capacity to produce inflammatory mediators, including TNFα [6], in response to stimulation with lipopolysaccharide (LPS), an established activator of KC [3,8,9]. Hence, the development of a mouse KC cell line that could be activated and

Abbreviations: LPS, lipopolysaccharides; FBS, fetal bovine serum; TNFα, tumor necrosis factor alpha; IL-6, interleukin-6; NO, nitric oxygen; ROS, reactive oxygen species.

* Corresponding author. Tel./fax: +1 317 274 3251 (O).
E-mail address: zywang@iupui.edu (Z.-Y. Wang).

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release inflammatory mediators upon LPS stimulation, would be useful for studying the mechanistic role of the KC in liver injuries.

KC are comprised of a heterogeneous cell population within the liver, and exhibit differences in their function across regions of the liver lobule [10,11]. The H-2K(b)-tsA58 transgenic mouse harbors a temperature-sensitive mutation of the SV40 large tumor antigen gene under the control of an interferon gamma-inducible H-2K(b) promoter element [12]. In present study, a cytokine-producing subpopulation of ImKC cells were isolated and characterized from the liver of H-2K(b)-tsA58 transgenic mice. We report that this novel ImKC line retains many characteristics and functions of primary KC, including the expression of KC surface markers, phagocytic capability, and the ability to respond to LPS by the production of inflammatory mediators through MAPK signaling.

2. Materials and methods

2.1. Materials

Male H-2K^b-tsA58 transgenic or male C65BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Liberase Blendzyme 3 and DNase 1 were purchased from Roche Applied Science (Indianpolis, IN). Mouse recombinant Interferon-gamma (IFNγ) was from eBioscience (San Jose, CA). LPS (from *Escherichia coli* serotype 026:B6) was purchased from Sigma (St. Louis, MO). MTS cell proliferation kit was from Promega (Madison, WI). Antibodies were obtained from the following: anti-SV40 T Ag antibody (Applied Biological Materials Inc., BC, Canada), rat anti mouse F4/80 and CD31 (AbD SeroTec, Raleigh NC); CD11b (clone M1/70), CD14 (clone Sa2-8) (BD Biosciences, San Jose, CA), mouse monoclonal TLR4 (Abcam Inc. Cambridge, MA); Phospho-p53 (Ser15), total p53, phospho-ERK1/2 (Thr202/Thr204), ERK1/2, I-kappa B beta (I-κB)β, β-Actin, and anti-rabbit IgG-peroxidase (Cell Signaling Technology, Inc., Danvers, MA); phospho-p38 MAPK (pT180/pY182), mouse anti-p38, phospho-JNK/SAPK (pT183/pY185), pan-JNK/SAPK1, and anti-mouse IgG-peroxidase (BD Transduction Laboratories, Sparks, MD).

2.2. Isolation of primary KC and the ImKC line

A three-step procedure was followed for the isolation of KC. In the first step, the liver was perfused and digested with a buffer containing 0.068 U/ml Liberase Blendzyme 3. Hepatocytes were removed by centrifugation (50*g* for 3 min), then the supernatant (enriched with non-parenchymal cells; NPC), was collected for the purification of KC. In the second step, the NPC fraction was subjected to elutriation centrifugation at 2500 rpm. The KC enriched fraction was eluted and collected at a flow rate between 31 and 60 ml/min. In the third step, KC were further purified by flow cytometry using the KC cell surface marker F4/80. For the development of the ImKC line, the enriched KC from a H-2K^b-tsA58 transgenic mouse (Step 2) were cultured in RPMI 1640 with 10% FBS and 100 U/ml penicillin/streptomycin in the presence with IFNγ (50 U/ml) at 33 °C. After 4 weeks of *in vitro* culture, the cells were sorted using F4/80 marker (Step 3), and grown in RPMI 1640 medium in the absence of IFNγ at 37 °C. The medium was changed every 2–3 days. ImKC at passage 8–30 was used in following experiments. In contrast, primary KC from C65BL/6 mice were sorted immediately following elutriation centrifuge and cultured in RPMI 1640 medium at 37 °C for 2–3 days.

2.3. Growth conditions for the ImKC line by MTS assay

To evaluate the conditions required for the optimal growth, ImKC were cultured at densities of 0.25, 0.5, 1 and 2×10^4/well in 96 well plates in the presence or absence of 10% FBS for 48 h at both 33 °C and 37 °C. To assess cell number, MTS (20 μl/well) was added to the plates for the last 2 h of culture, then the absorbance at 490 nm was read on a Tecan plate reader.

2.4. Assessment of phagocytotic index and RBC binding/phagocytosis

To assess the phagocytic capacity of ImKC, the cells (5×10^5/6-well) were cultured for 15 min with a 1:500 dilution of fluorescein isothiocyanate (FITC)-labeled latex beads (1 μM; Polysciences, Warrison, PA). The cells were then imaged using fluorescence microscopy (using a FITC filter), and the fluorescence intensity analyzed by flow cytometry. In addition, RBC binding assay was also used as a measure of phagocytic activity. Briefly, fresh mouse or human whole blood in a tube containing acid citrate dextrose (ACD) was mixed with an equal amount of PBS, then were centrifuged at 200*g* for 10 min. The RBCs (1×10^7) were washed and labeled with 25 μM Far-Red fluorescent dye (Invitrogen, Carlsbad, CA) for 15 min. ImKC were then cultured with the labeled mouse or human RBC at 1×10^6 cell/ml for 90 min. The fluorescence intensity of labeled RBC and RBC-binding/phagocytosis by ImKC was analyzed by flow cytometry.

2.5. Assessment of surface antigen expression and ROS/RNS production

To evaluate the ability of ImKC to produce ROS and RNS, the cells (5×10^5/6-well) were stimulated with LPS (100 ng/ml) or medium control for 1 h, or with tert-butyl hydroperoxide (10 mM) for 10 min, then ROS and NO were quantified following incubation with 10 μM dihydrorhodamine 123 (DHR123, Invitrogen) for 15 min, and 10 μM 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM, Invitrogen) for 30 min, respectively. The percentage of positive cells was analyzed using flow cytometry.

2.6. Determination of cytokine production in KC

To assess the ability of ImKC and primary KC to produce cytokines upon activation, ImKC or primary KC (1×10^5/96-well) were cultured for 3 days, then were treated with LPS (100 ng/ml) for 6 h. Supernatants were collected and stored at −20 °C until assayed for TNFα and IL-6 protein levels by ELISA (R&D Systems, Minneapolis, MN). In addition, TNFα, IL-6 and IL-1β gene expression was measured using qRT-PCR. For these studies, ImKC or primary KC (1×10^6/dish) were cultured for 3 days, and then treated with LPS (100 ng/ml) for 2 h or 6 h. Cell pellets were collected, and total RNA was extracted using the QiaShredder and RNeasy Mini kits (Qiagen, Valencia, CA). Next, cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers protocol. The primers used were 5′-CCCC AAAGGGATGAGAAGTTC-3′ and 5′-TGAGGGTCTGGGCCATAGAA-3′ for TNFα; 5′-CGCTATGAAGTTCCTCTCTGCAA-3′ and 5′-CACCAGCAT CAGTCCCAAGA-3′ for IL-6; 5′-GACGGACCCCAAAAGATGAAG-3′ and 5′-GTGCTGCTGCGAGATTTGAAG-3′ for IL-1β; and 5′-CTCGTCC CGTAGACAAAATGG-3′ and 5′-TGACCAGGCGCCCAATA-3′ for GAPDH. qRT-PCR was conducted on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using FastStart Universal SYBR Green Master (ROX) (Roche) in a 25 μl final reaction mixture. The cycling conditions were an incubation at 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Experiments were performed in triplicate for each sample. TNFα, IL-6 and IL-1β were normalized to GAPDH and the fold difference was calculated by $2^{-\Delta\Delta Ct}$ as previously described [13].

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