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Functional kupffer cells migrate to the liver from the intraperitoneal cavity

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ABSTRACT

We established a method of KC transplantation by intraperitoneal (i.p.) injection using EGFP-expressing cells (EGFP-KCs) and normal KCs. The novel method is easier and less invasive than conventional methods so that it is not only technically advantageous but also ethically preferable for experiments using animals. We demonstrated that KCs migrated to the liver following i.p. Injection. Engraftment in the liver was not observed for peritoneal macrophages (pMPs). This suggests that KCs migrate to the liver via a sorting mechanism. KC injection decreased the KC number at 24 h and then recovered the KCs at 10 days to a normal level. Additionally, recovery to the normal level by KC injection was observed in mice with KC depletion induced by $GdCl_3$. These results suggest that a regulatory mechanism exists for controlling the number of KCs.

1. Introduction

Kupffer cells (KCs) are resident macrophages in the liver. They localize mainly in the hepatic sinusoids and can move around in the liver. They have a high phagocytic capability, allowing them to take out foreign substances, such as viruses and bacteria, apoptotic cells, and cellular debris. Thus, KCs have an important role as gatekeepers in the innate immune response [1]. KCs represent 5%–15% of liver cells and constitute 80% of resident macrophages in the body. However, the origin of KCs has been complicated because KCs consist of heterogeneous lineages coming from at least two tissues, bone marrow and yolk sac [2–5]. KCs play a major role in immunity and tissue injury and repair. KCs produce various inflammatory mediators, including cytokines (TNF-α), prostaglandins, and reactive oxygen species, namely, through NADPH oxidase or inducible NO synthase (iNOS) activities [6,7]. KC dysfunction contributes to the pathogenesis of the nonalcoholic fatty liver disease (NAFLD) [8,9]. However, the role of KCs in the regulation of liver metabolism and the occurrence of the metabolic disease remains enigmatic [10]. Therefore, if KCs were transplanted successfully in the liver with engraftment and survival over the long term, major issues could be addressed regarding their roles in microbial clearance, antigen presentation, and tissue inflammation or repair [3,4,11]. Several studies have shown that KC transplantation is useful. Simone et al. showed that KC transplantation has potential in cell or gene therapy [5]. They reported that KCs could be transplanted by intravenous injection [12]. Portal vein and intravenous injection are usually used for KC transplantation [5,12,13]. Using the portal vein is reliable, but it is highly invasive, and the skill level is difficult. Then, following intravenous injection, KCs must pass through the blood circulation to the liver.

KCs will meet with antigens and experience some stress in the blood vessel before arriving in the liver. It has been reported that inflammatory macrophages can migrate from the peritoneum to lymph nodes [14]. It is supposed to be a mechanism for the fate of the inflammatory macrophages during the resolution of inflammation. Besides, the report clearly shows the existence of the route from the peritoneum to the circulation system. Thus, we speculate if the residential macrophages have the potency to migrate to the proper place, the KCs might migrate to the liver by intraperitoneal (i.p.) injection through the circulation system. Therefore, we considered injection as an alternative method for the KCs transplantation into the liver. A previous study reported that i.p. Injection of KCs in rats reduced liver injury and raised immune tolerance against liver transplantation [15]. However, whether KCs can engraft in the liver is still unclear. In this report, we showed that i.p. Injection was useful for KC transplantation and that KCs maintained their functions in mice.

We introduced KC transplantation experiments. It was important to establish a simple and useful method for the transplantation of KCs.

Abbreviations: KCs, Kupffer cells; pMP, peritoneal macrophages; i.p., intraperitoneal.

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Here, we show an available method using intraperitoneal injection. We investigated the dynamics of exogenous KCs using several materials, including phagocytic cell labeling tracers and KCs prepared from green fluorescent protein (EGFP) transgenic mice.

2. Materials and methods

2.1. Animals and diets

Wild-type (WT) and Nucling-Knockout (KO) male mice (6–8 weeks of age) (ref. for KO mice.) were prepared. The background of the Nucling-KO (control, n = 4; transplantation, n = 3) and WT (control, n = 3–5) mice was C57BL/6J. Enhanced green fluorescent gene transgenic mice (EGFP mice with a C57BL/6 N background) were provided by the Center for Animal Resources and Development (CARD) at Kumamoto University, Japan. Mice were fed a normal chew diet (54.4% CHO, 23.6% protein, 5.3% fat; NCD) and water ad libitum. The animals were kept under constant environmental conditions with a 12-h light-dark cycle. All animals received humane care in compliance with the institutional guidelines. Mice were sacrificed after all the tests were finished. All animal experiments comply with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Cell isolation and transplantation

Kupffer cells (KCs) were isolated from mouse (7 weeks old) livers using a liver dissociation kit and MS columns (MACS, USA) for transplantation. Peritoneal macrophages (pMPs) were isolated using the method of Avijit Ray and Bonnie N. Dittel [16]. Whole cells isolated from one mouse were transplanted into a recipient mouse by i.p. Injection. The average number of transplanted cells was $6.3x10^5$/mouse. After 10 days, the transplanted cells were collected for further experiments. (See the supplementary data for the characterization of isolated cells from control and transplanted mice.)

2.3. Phagocytotic fluorescence test

Isolated KCs and pMPs were dyed using the PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. The labeled cells were immediately injected intraperitoneally into recipient mice. Ten days after injection, KCs and pMPs were collected and analyzed using a fluorescence microscope (Nikon, Japan).

2.4. Real-time quantitative PCR

Mice were killed 24 h after KC or saline injection following single $GdCl_3$ or saline administration. The liver was carefully dissected and immersed in liquid nitrogen and stored at −80°C. Total RNA was extracted using TRI reagent (Sigma-Aldrich, USA). cDNA was prepared by reverse transcription of 1 μg total RNA using Prime Script RT Master Mix (Perfect Real Time) (Takara, Japan). Real-time PCR was performed using a 7300 RT-PCR System (Applied Biosystems, USA) with SYBR Premix Ex Taq II (Takara, Japan). GAPDH RNA was chosen as a housekeeping gene. All samples were run in duplicate in a single 96-well reaction plate. Thermal cycling conditions were 30 s at 95°C followed by 40 cycles at 95°C for 5 s and 61°C for 31 s. The expression of each gene was normalized to GAPDH mRNA and calculated relative to the baseline control using the comparative cycle threshold method (ΔΔCt). Primers for GAPDH (control) were 5′-ACCACAGTCCATGCCATCAC-3' (Forward) and 5′-TCCACCACCCTGTTGCTGTA-3' (Reverse). Primers for TNFα were 5′-CTGTAGCCCACGTCGTAGC-3' (Forward) and 5′-TTGAGTTCCATGCCGTTG-3' (Reverse). Primers for IL-1β were 5′-TGTAATGAAAGACGGCACACC-3' (Forward) and 5′-TCTTCTTTGGGTATTGCTTGG-3' (Reverse). Primers for IL-6 were 5′-CCACTTCACAAGTCGGAGGCTTA-3' (Forward) and 5′-CCAGTTTGGTAGCATCCATCATTTC-3' (Reverse).

2.5. Flow cytometry

EGFP KCs were injected into WT and Nucling-KO mice. Ten days after injection, KCs were isolated using a MACS kit and MS columns followed by flow cytometric analysis. The cells were fixed for 30 min with 4% paraformaldehyde and centrifuged at 400 g for 10 min to remove the buffer. Cells were diluted in 100 μl cold PBS with 10% FBS and 1% sodium azide (NaN_3) spun at 400 g for 10 min and, where necessary, incubated with F4/80 antibody with 3% BSA (final con. 10 μg/ml, Sigma, USA) for 1 h at 4°C. Cells were washed with cold PBS with 5% FBS and 20 mM NaN_3 3 times. Fluorescently labeled (Alexa Fluor 594) goat anti-rat IgG was added to the samples for 1 h at 4°C in the dark, and the wash step was repeated 3 times. Propidium iodide (PI) (2 μg/ml) was used as a viability marker for FACS. EGFP- and F4/80-positive cells were measured by Guava® easyCyte™(Luminex, USA).

2.6. Immunohistochemistry

The perfused livers of mice were fixed in 4% paraformaldehyde at 4°C for 2 h, washed in PBS, and suspended in a 10% sucrose gradient for 2 h followed by 20% sucrose for 2 h and then 30% sucrose at 4°C overnight. Tissue was then flash-frozen in Tissue-Tek®OCT embedding compound in 100% ethanol with dry ice and stored at −80°C. Five-micrometer-thick sections were cut using a cryostat (Leica Biosystems, Germany). Following blocking with PBS containing 5% FBS at RT for 45 min, sections were incubated with rat anti-mouse F4/80 antibody (Sigma, USA) at 37°C for 30 min and then incubated with Alexa Fluor 594 or 488 goat anti-rat IgG antibody (Invitrogen), 1:400, diluted in blocking solution for 37°C for 30 min. The images were viewed by using a BZ-X710 fluorescence microscope (Keyence, Japan).

2.7. Gadolinium chloride ($GdCl_3$) treatment

To confirm the effect of the Nucling-KO background on the phenotype of transplanted KCs, we alternatively transplanted KCs into WT mice (n = 4) with or without gadolinium chloride ($GdCl_3$, Wako, Japan; n = 3) (15 mg/ml in PBS) treatment. Saline-injected (PBS, n = 3) mice were prepared as control mice. After 24 h, KCs were collected for further experiments.

2.8. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTPnick-end labeling) assay

The TUNEL assay was performed on frozen sections (5 μm thick) using the in situ Apoptosis Detection Kit (Takara, Japan).

2.9. PCR assay

Genomic DNA was extracted from KCs using extraction buffer (10 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS) and Proteinase K (20 mg/ml) to digest the cells at 50°C for 2–3 h. TE buffer with phenol was added and mixed slowly before centrifugation at 1200*g* for 2 min. The supernatant was then collected and supplemented with chloroform/isoamyl alcohol (24:1) and mixed slowly for 30 min. The solution was centrifuged at 1200 g for 2 min. Then, the supernatant was collected and supplemented with a 1/10 volume of 3 M CH_3COONa and two volumes of ethanol and mixed. The solution was centrifuged at 1000 g for 10 min, and the supernatant was discarded. The resulting DNA pellet was rinsed with 70% ethanol and dried. DNA was then dissolved with TE buffer. One nanogram of genomic DNA was used as a template for PCR using DNA polymerase KOD FX Neo (TOYOBO, Japan) with 35 cycles at 94°C for 2 min, 98°C for 10 s, 62°C for 30 s, 68°C for 120 s/kb, and final elongation at 68°C for 7 min. PCR products were resolved in 2% agarose gels. Primers for WT mice were 5′-TCCTCTACCTCATCTATGTGTACC-3′ (Forward) and 5′-TATCTCTGTGTTGCCTCCGAA-3′ (Reverse). Primers for Nucling were 5′-CCGGTGGATATAGAATGTGTGCGAGG-3′

(Forward) and 5′-CTCCGCGTATCTCTGTGTTGCCTCCGA-3′ (Reverse) (Eurofins, Japan).

2.10. Reagent

Proteinase K and NaN_3 were obtained from Wako, Japan. Tissue-Tek® OCT compound was from MILES, USA. Ethidium bromide (EtBr) was obtained from Sigma, USA.

2.11. Statistical analysis

Data were expressed as means ± SE. Differences were analyzed by Student's t-test. P values < 0.05 were considered significant.

3. Result

I.p. Injected donor KCs were confirmed in the livers of the recipient mice.

We checked whether KCs could migrate to the liver following i.p. Injection. We prepared KCs from the livers of EGPF mice (EGFP-KCs) and then transplanted them into WT mice by i.p. Injection. After 10 days, we collected KCs from the livers of treated mice. Flow cytometric analysis (FACS) revealed that EGFP-positive cells accounted for ~2.3 % of KCs in EGFP-KC-transplanted mouse livers (Fig. 1A). The number of EGFP-positive cells was significantly different between PBS- and KCs-injection groups (Fig. 1B). In addition, immunohistochemical analysis revealed the migration of the injected KCs into the liver of WT mice (Fig. 1C). Moreover, we prepared KCs from the livers of WT mice and then transplanted them into Nucling-KO WT mice by i.p. Injection because the Nucling gene can be a marker for transplanted KCs in KO mice. After 10 days, we collected KCs from the livers of treated mice. PCR using the genomic DNA of the KCs revealed that the transplanted KCs successfully migrated to the liver (Fig. 1D). Thus, we concluded that i.p. Injection is available for KCs to transplant into the liver.

FACS revealed that EGFP-positive cells accounted for 16.3 % of the transplanted cells and F4/80-positive cells accounted for 12.1 % (Fig. 2A). On the other hand, EGFP-positive cells accounted for 2.3 % of KCs in the transplanted mouse livers (Fig. 1A). The total number of KCs

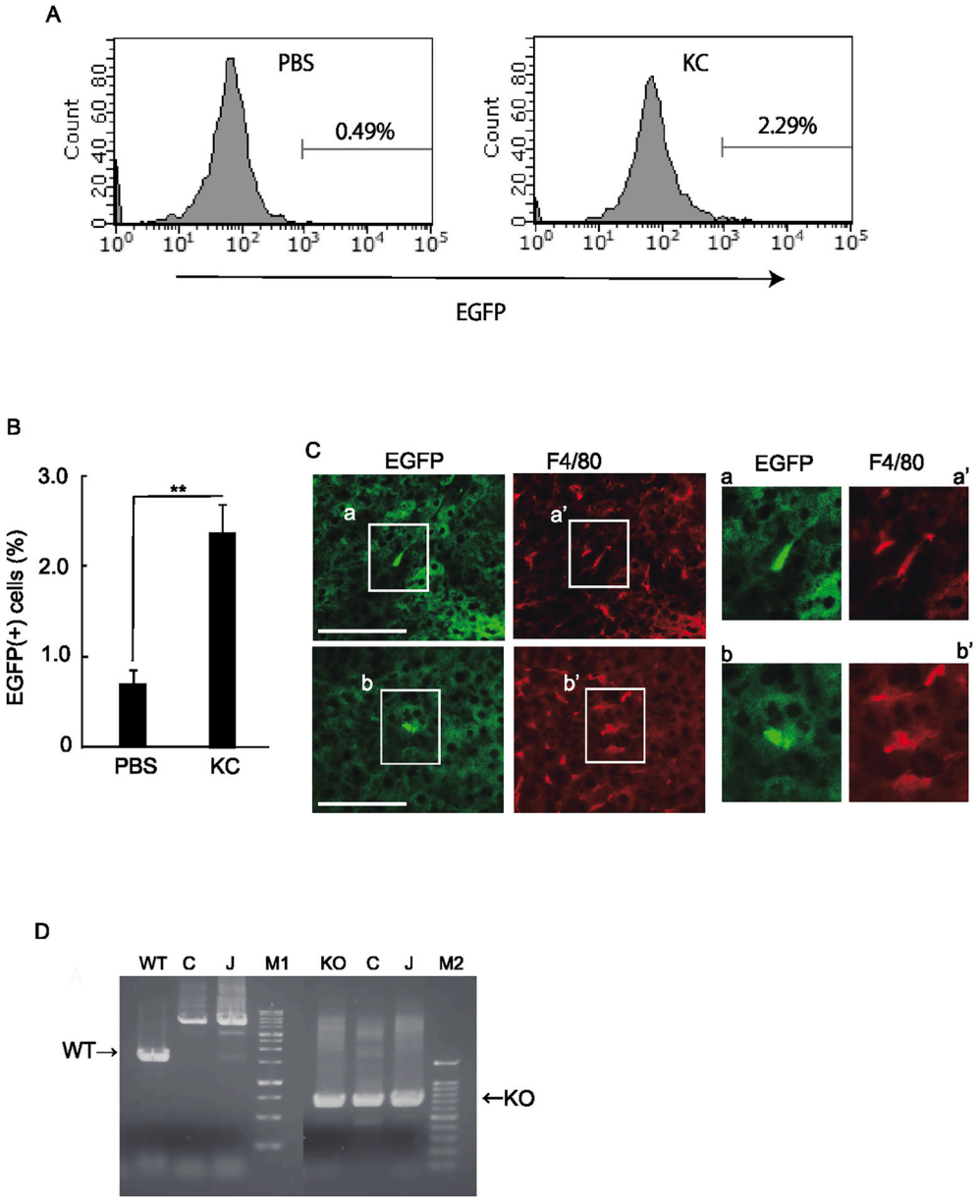

Fig. 1. Exogenous KCs migrate into the liver of transplanted mice.
(A) Representative EGFP expression in KCs prepared from the liver 10 days after EGFP-KCs i.p. Injection into WT (WT-KC, n = 3) mice. Saline-injected WT (WT-PBS, n = 3) mice were prepared as controls. The percentage of EGFP-positive cells was higher in the transplantation groups (WT-KC). (B) The frequency of EGFP (+) cells among WT-PBS and WT-KC (n = 3, 3 experiments) (*p<0.05, **p<0.01). (C) IHC was performed to clarify the distribution of F4/80-positive (red) cells and EGFP-positive (green) cells in WT mice. Some of the positive cells are shown with magnified images an (a')-b (b')) as representatives. Scale bar; 100 μm. (D) KCs prepared from WT mice were transplanted intraperitoneally into Nucling-KO mice. The transplanted KCs were confirmed by detecting the Nucling gene using PCR. (M1, ExcelBand™1KB DNA ladder (SMOBIO, Taiwan); M2, ExcelBand™ 100bp DNA ladder (SMOBIO, Taiwan); WT, wild type (untreated, n = 3); C, control (PBS injected KO mouse, n = 3); J, KC injected KO mouse, n = 3; KO, Nucling knockout (untreated), n = 4). Arrows, specific bands for WT or Nucling-KO genome. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

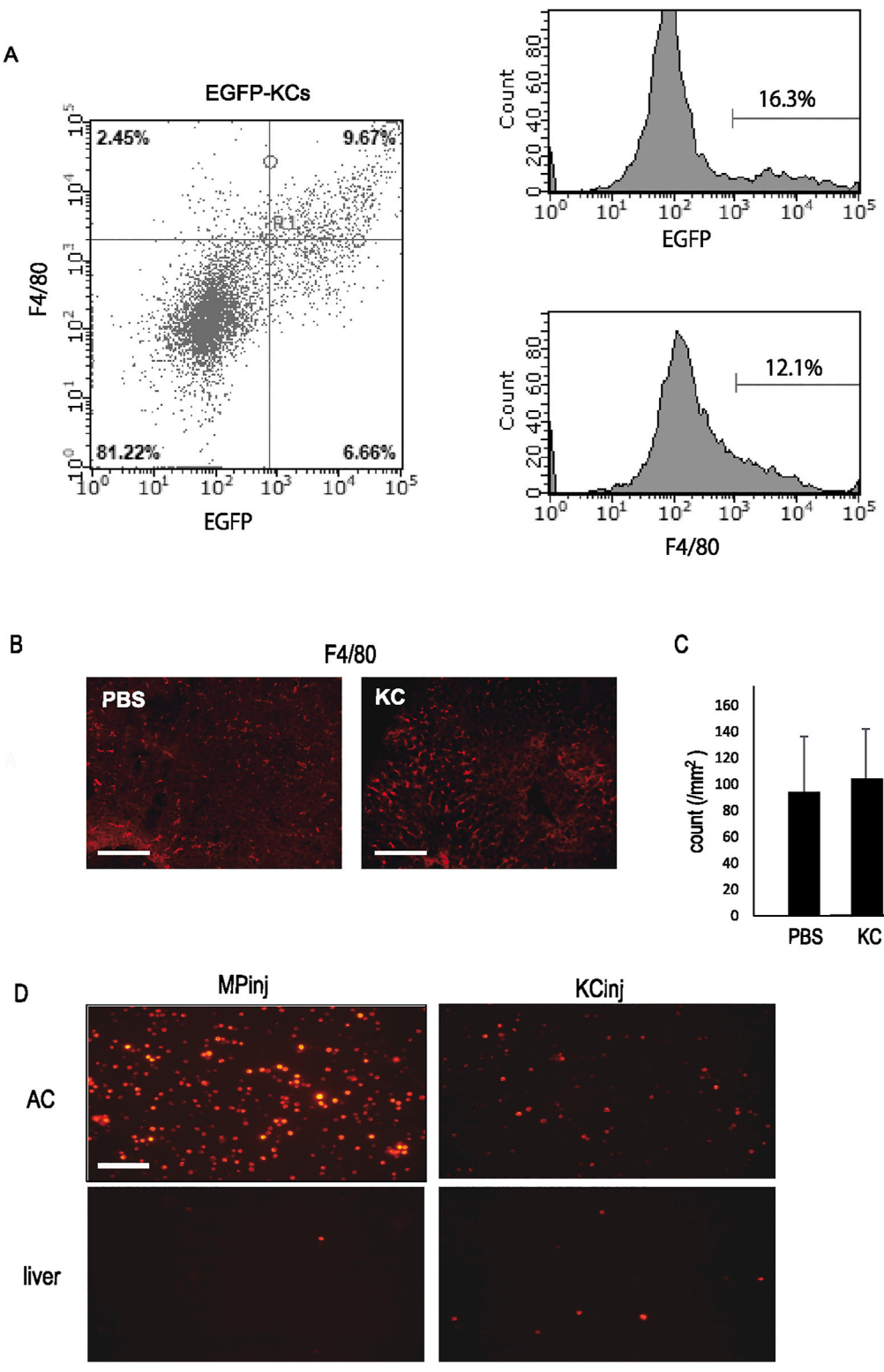

Fig. 2. F4/80-positive cells were increased after KC injection.
(A) To characterize the prepared KCs, we checked the ratio of EGPF-positive cells and F4/80-positive cells following isolation. Representative dot plots are shown here to depict the EGFP^{high} cells and F4/80-positive cells, accompanied by each ratio (%) of cells in four quadrants. EGFP-positive cells accounted for 16.3 % of the transplanted cells and F4/80-positive cells accounted for 12.1 %. EGFP-KCs, KCs prepared from EGFP mouse. (B) Immunohistochemistry (IHC) was performed to clarify the distribution of F4/80-positive (red) cells in the liver 10 days after i.p. Injection into WT (WT-KC, n = 3) mice. Saline-injected WT (WT-PBS, n = 3) mice were prepared as control mice. (C) The number of F4/80-positive cells was counted in IHC images. F4/80-positive cells were not different between PBS and WT-KC. Scale bar; 100 μm. (D) We used the PKH26 Red Fluorescent Cell Linker Kit to dye isolated KCs and macrophages (MPs), and cells were collected and observed by fluorescence microscopy. On KC injection, approximately 60% (ratio of red fluorescent cells) remained in the abdominal cavity. The results showed that most KCs remained in the abdominal cavity in the MP group (n = 3), and approximately 11.7% migrated to the liver in the KC group (n = 3) ($p<0.01$). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was not changed by the transplantation (Fig. 2B–C). Thus we concluded that the engraftment ratio of KCs in the liver was approximately 14% (=2.3/16.3). Most of the migrated KCs are conceivable as maintaining physiological functions. We investigated the engraftment rate of transplanted KCs with retention of macrophage functions in the liver by the i.p. method. We prepared KCs and pMP from WT mice. The cells were labeled with the fluorescent dye PKH26 red using phagocytotic activity and transplanted by i.p. Injection. After 10 days, we collected KCs and pMPs from the recipient mice, observed the cells by fluorescence microscopy, and counted the number of labeled cells. The results showed that only 1 cell was detected by fluorescence microscopy in a 100 μl cell solution when we calculated the KC number from the livers of the MP injection group. Most of the injected pMPs stayed in the abdominal cavity (0.004%; 243 cells vs. 1 cell/100 μl; $p<0.01$). However, a few cells (mean 7 cells) were detected in the 100 μl cell preparation of KCs from the livers of the KC injection group. 60 cells were detected in the

MP solution. The KC engraftment ratio was approximately 11.7% in the liver (60 cells vs. 7 cells/100 μl; $p<0.01$) (Fig. 2D). The estimation ratio was almost the same as that using the data of EGFP-KCs transplantation. Thus we concluded that most of the migrated KCs were retaining phagocytotic functions.

Transplanted KCs induced KC depletion, and the activation of KCs was correlated with endogenous KCs.

To confirm the effect of the phenotype of transplanted KCs, we alternatively transplanted WT-KCs into mice with or without gadolinium chloride ($GdCl_3$) treatment. $GdCl_3$ is known to deplete KCs and inhibit KC activation. Interestingly, simple transplantation of WT-KCs decreased the number of KCs in the control (PBS-injected) mice ((1) vs (2), in Fig. 3A) at 24 h. In contrast, transplantation of KCs into $GdCl_3$-treated mice recovered the number of KCs in the liver ((4) in Fig. 3A). In addition, inflammatory cytokines (TNFα, IL-1β, and IL-6) were upregulated in the KCs of control mice after KC transplantation ((2) in Fig. 3B), which indicates the activation of KCs. Such upregulation of cytokines was not observed in the $GdCl_3$-treated mice ((4) in Fig. 3B). These data suggest that exogenous KCs induced KC depletion and that the activation of KCs was correlated with endogenous KCs. We concluded that KC transplantation led to inflammatory reactions by interacting exogenous KCs with endogenous cells.

To confirm the effect of transplanted KCs, we transplanted KCs into WT mice (WT-KC, n = 4) with or without gadolinium chloride ($GdCl_3$, 15 mg/kg, n = 3) treatment. Vehicle (PBS)-injected (n = 3) mice were prepared as a control. (A) The number of KCs was counted to clarify the distribution of KCs in the liver 24 h after WT KCs and $GdCl_3$ i.p. Injection into WT. (B) Quantitative RT-PCR was performed to check the expression of cytokines (TNFα, IL-1β, and IL-6). TNFα and IL-6 expression was significantly upregulated in WT mice by KC injection. (*$p<0.05$; **$p<0.01$)

Next, to confirm the effect of apoptosis of transplantation KCs. TUNEL assay was performed to assess apoptosis following KC (WT-KC) injection. TUNEL-positive cell numbers were not different among them (Fig. 4). This may suggest that the regulatory mechanism of the KC population consists of earlier stages, including stages before migration into the parenchymal area of the liver.

IHC was performed to clarify the distribution of F4/80-positive (green) and apoptotic TUNEL-positive (red, white arrowhead) cells in the liver 10 days after i.p. Injection into WT (WT-KC, n = 3) mice. Vehicle-injected WT (WT-PBS, n = 3) mice were prepared as a control. Scale bar; 100 μm. (B) F4/80-positive cells and TUNEL-positive cells were counted in IHC images. F4/80-positive cells were increased after transplantation compared to the control (*, $p<0.05$). On the other hand, there was no significant difference in the number of TUNEL-positive cells among any groups.

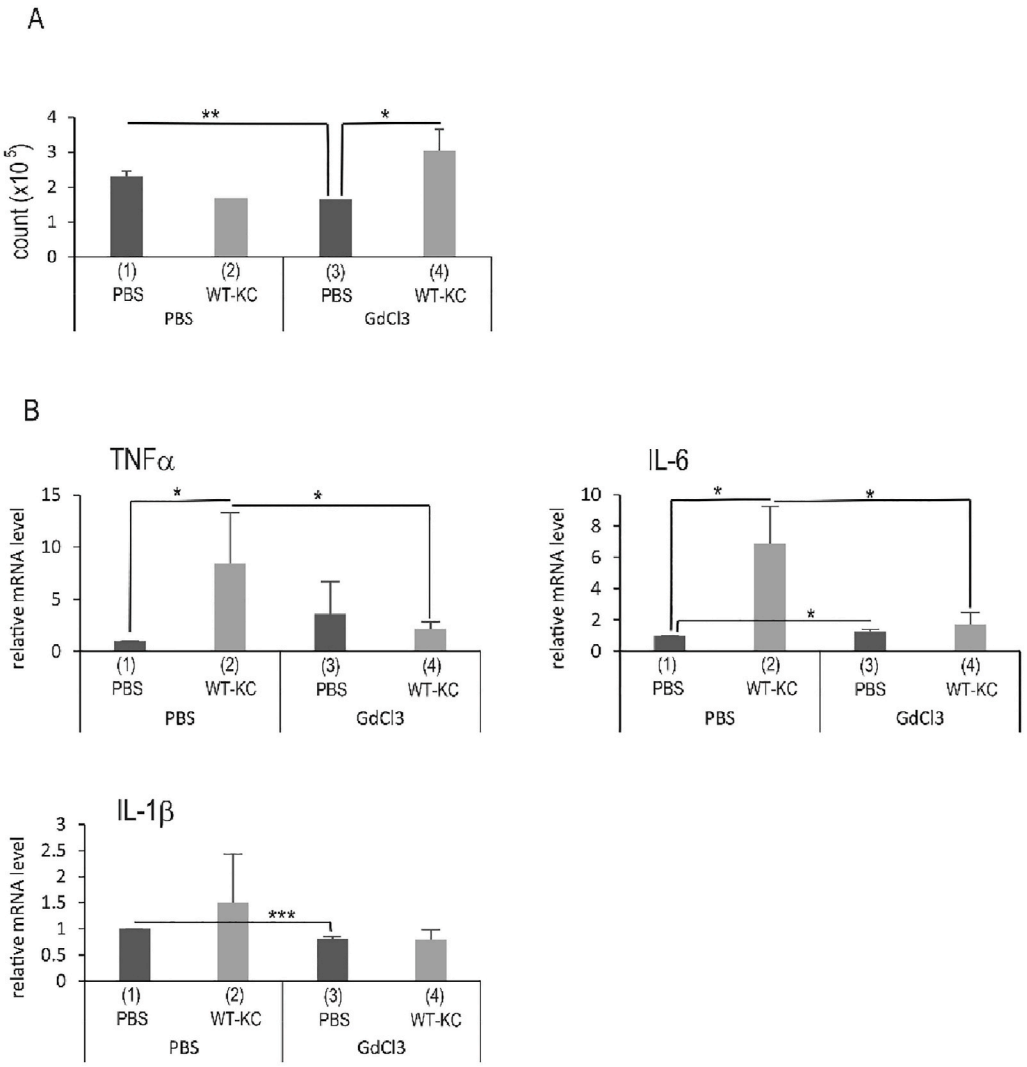

Fig. 3. Number and functionality of KCs isolated from WT KC-injected mice pretreated with or without $GdCl_3$.

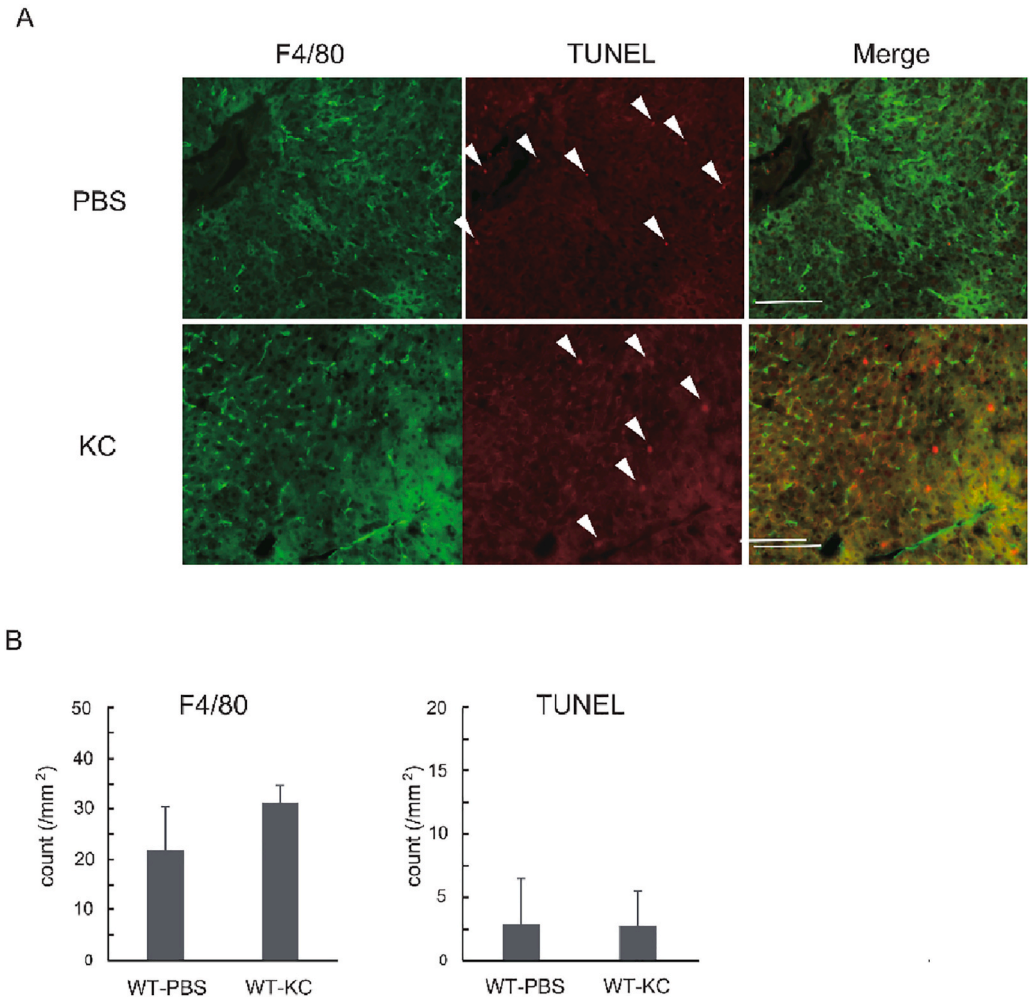

Fig. 4. Apoptosis in the liver of KC-transplanted mice.

4. Discussion

In general, KC transplantation is performed through the portal vein or tail vein because conventional methods are reliable and achieve a high migration rate to the liver [5]. However, these methods require highly sophisticated skills. Here, we performed i.p. Injection to transplant KCs in the liver. This novel method is easier and less invasive than conventional methods. Hoppo et al. reported that liver cells could be transplanted by i.p. Injection. They developed hepatized lymph nodes with liver function in the abdominal cavity to improve the lethal hepatic failure, but the expression of KC has not been found in these lymph nodes [17].

Little is known about the life span of KCs. Several reports mention the life span of KCs as several days, several weeks, or up to one year [18]. It is becoming a consensus that macrophages consist of heterogenic populations from monocytes, residential progenitors, or self-renewal cells [19]. However, the renewal mechanisms of KCs are unrevealed. Thus it is difficult to identify the renewal span of KCs, meaning that it is difficult to calculate the migration rate of exogenous KCs. So we use the word "engraftment ratio" instead of the word "migration rate." In the experiment, we prepared KCs from EGFP-mice, which contain 16.3% $EGFP^{high}$ cells. All the KCs prepared from one mouse were i.p. Injected into one recipient mouse. Then the KCs were collected from the recipient, and the $EGFP^{high}$ population was investigated. Average 2.3% of the KCs were $EGFP^{high}$. So we concluded that the engraftment ratio of the exogenous KCs was 14% (=2.3/16.3) in ten days. We also checked the migration of KCs in 24h after i.p. Injection of EGFP-KCs into mice. We observed no migration of the exogenous KCs in the liver. On the other hand, we observed 30%–66% of the injected KCs remaining in the peritoneal cavity in 24h (data not shown). The ratio was almost the same level as the ratio at ten days (Fig. 2D), suggesting that approximately half of the transplanted KCs start migrating from the peritoneal cavity in 24h. we speculate that the KCs might migrate to the liver by i.p. Injection through the circulation system. However, we have not confirmed the migration of KCs to lymph nodes by i.p. Injection yet. Further studies are necessary to reveal the migration mechanism of KCs.

The total number of KCs was reduced by transplantation along with the upregulation of inflammatory cytokines (TNFα, IL1β, and IL-6). Such a reduction was suppressed by pretreatment with $GdCl_3$ i.p. Injection. These results suggest that exogenous KCs induced an apoptotic decrease in endogenous KCs. Some studies have indicated that $GdCl_3$ can increase the KC engraftment rate by depleting KCs before transplantation [5,20]. In fact, in WT mice treated with $GdCl_3$, the number of KCs increased than normal levels after transplantation. This means that most of the exogenous cells migrated to the liver. Thus, the method using i.p. Injection is very useful for the transplantation of KCs.

As mentioned, KC transplantation led to a decrease in endogenous KCs in WT mice (Fig. 3A) at 24 h. On the other hand, the KC population was higher than control in WT mice at 10 days (Fig. 1B). The increase in KCs at 10 days can be explained by at least three reasons. 1, KC migration gradually progressed in 10 days. 2, Following the decrease in KCs in 24 h, native KCs were spontaneously supplied. 3, The migrated exogenous KCs proliferated in 10 days. In the experiment using KCs prepared from EGFP mice (EGFP-KCs), we confirmed the migration of

EGFP-KCs into the liver (Fig. 1). However, the population of EGFP^{high} cells was very low (~2.3%). On the other hand, transplanted KCs prepared from EGFP mice contain approximately 16% of the EGFP^{high} population (Fig. 2). From that point of view, it seems to be difficult to explain the proliferation of the transplanted cells. The preparation of KCs from EGFP mice contained approximately 12% F4/80^{high} cells and approximately 10% F4/80^{high}/EGFP^{high} cells (Fig. 2), indicating that 80% of the F4/80^{high} cells were EGFP^{high}. Thus, we concluded that the increase in KCs in 10 days comes from the spontaneous supply of native KCs. We also observed that exogenous peritoneal macrophages had difficulty migrating to the liver. This means that KCs are moved to the liver by some unique mechanism using specific attractants. We checked the apoptotic response during KC transplantation. A TUNEL assay was performed to assess apoptosis following KC (WT-KC) injection. However, TUNEL-positive cell numbers were not different among them (Fig. 4). This may suggest that the regulatory mechanism of the KC population consists of earlier stages, including stages before migration into the parenchymal area of the liver. The liver sinusoidal area or its endothelial cells (LSECs) may play an important role.

These results suggest the existence of a mechanism for regulating the number of KCs. To confirm this hypothesis, we compared the distribution of KCs in the liver between KC-transplanted mice and control (PBS-injected) mice. IHC revealed that KC distribution and cell numbers were not different between KC-transplanted mice and control mice (Fig. 2).

To confirm the KC migration into the liver by genetic approach, we used Nucling-KO mice. On the other hand, we are interested in the KO mice to reveal the importance of KCs to develop inflammatory diseases, including metabolic syndrome. We previously reported that Nucling deficiency led to a decrease of KCs in the liver. Nucling is expressed in both parenchymal cells and nonparenchymal cells including KCs in the liver of mice [21]. In addition, Nucling-KO mice have shown a tendency to suffer from nonalcoholic fatty liver diseases (NAFLD) (unpublished data), which may lead to metabolic syndrome. However, the physiological function of Nucling in the liver is still unclear. We are planning to experiment using Nucling-KO mice to reveal the importance of KCs in developing inflammatory liver diseases.

Here we showed that i.p. Injection is very useful for the transplantation of KCs and that native KCs can be spontaneously supplied through external stimulation. However, the mechanism still needs to be elucidated.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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