ANIMAL MODELS

Kupffer Cell Transplantation in Mice for Elucidating Monocyte/Macrophage Biology and for Potential in Cell or Gene Therapy

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Kupffer cells (KC) play major roles in immunity and tissue injury or repair. Because recapitulation of KC biology and function within liver will allow superior insights into their functional repertoire, we studied the efficacy of the cell transplantation approach for this purpose. Mouse KC were isolated from donor livers, characterized, and transplanted into syngeneic recipients. To promote cell engraftment through impairments in native KC, recipients were preconditioned with gadolinium chloride. The targeting, fate, and functionality of transplanted cells were evaluated. The findings indicated that transplanted KC engrafted and survived in recipient livers throughout the study period of 3 months. Transplanted KC expressed macrophage functions, including phagocytosis and cytokine expression, with or without genetic modifications using lentiviral vectors. This permitted studies of whether transplanted KC could affect outcomes in the context of acetaminophen hepatotoxicity or hepatic ischemia-reperfusion injury. Transplanted KC exerted beneficial effects in these injury settings. The benefits resulted from cytoprotective factors including vascular endothelial growth factor. In conclusion, transplanted adult KC were successfully targeted and engrafted in the liver with retention of innate immune and tissue repair functions over the long term. This will provide excellent opportunities to address critical aspects in the biogenesis, fate, and function of KC within their native liver microenvironment and to develop the cell and gene therapy potential of KC transplantation. (Am J Pathol 2016, 186: 539–551; http://dx.doi.org/10.1016/j.ajpath.2015.11.002)

The ability to reconstitute Kupffer cells (KC) in the liver by the cell transplantation approach will be significant for studies of the biology and pleiotropic functions of KC. Replacement of hepatocytes and liver sinusoidal endothelial cells has been helpful for biological studies and cell therapy, including for coagulation defects, as in hemophilia A.†‡§ Because KC represent 5% to 15% of liver cells and constitute 80% of resident macrophages in the body,¶ having a better understanding of their roles in tissue homeostasis, injury, or repair is clinically very relevant, but the origin of KC has been complex, since KC may arise within the liver as well as outside of the liver [ie, from bone marrow–derived monocyte/macrophage (BMDM) lineages].¶†‡§ If candidate macrophage populations 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, tissue

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The views expressed in this article are the authors’ own and not an official position of the institutions or funders.

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inflammation or repair, ischemia—reperfusion (IR) pre-conditioning, for example, in which complex cytokine, chemokine, or receptor networks are involved but are difficult to reproduce in conditions other than *in vivo*. Success in transplanting KC also will offer tools to determine whether cell types of interest may be cotransplanted for protecting transplanted cells and thereby improving liver repopulation. For instance, transplantation of adenovirally transduced nonparenchymal liver cells (NPC) expressing superoxide dismutase decreased acute IR injury in rats.

Recently, KC were discovered to express coagulation factor VIII, and transplantation of healthy KC improved blood clotting in hemophilia A mice. On the other hand, putative markers have been useful, especially when examining cell types sharing these markers are eliminated. Therefore, before digesting liver to release KC, we cleared cells from liver sinusoids to avoid contamination with neutrophils, monocytes, or other blood cells. Also, we used mouse BM-derived monocyte/macrophage progenitors, BMDM, peritoneal macrophages (PM), and tail-tip fibroblasts (TTFs) for comparisons. To improve cell engraftment, we depleted KC with gadolinium chloride before cell transplantation, and we also transplanted KC after genetic-modification with lentiviral vectors (LV).

This study determined whether KC could be transplanted successfully in the liver. Although homogeneous cell populations should be helpful for this purpose, phenotypic markers often overlap in given cell populations. In case of macrophages, CD11b, F4/80, CD68, and other markers have been useful, especially when contaminating cell types sharing these markers are eliminated. Therefore, before digesting liver to release KC, we cleared cells from liver sinusoids to avoid contamination with neutrophils, monocytes, or other blood cells. Also, we used mouse BM-derived monocyte/macrophage progenitors, BMDM, peritoneal macrophages (PM), and tail-tip fibroblasts (TTFs) for comparisons. To improve cell engraftment, we depleted KC with gadolinium chloride before cell transplantation, and we also transplanted KC after genetic-modification with lentiviral vectors (LV).

For hepatic IL-6 expression, 5.5 mg/kg lipopolysaccharide (Sigma Chemical Co.) was given i.p. in saline 6 hours before analysis.

Liver Injury Models

For hepatic IR, the porta hepatis of 8- to 10-week-old C57BL/6 male mice was clamped for 15 minutes followed by reperfusion. After 5 minutes, 2.5 to 3 × 10^6 GFP+ KC, 5 × 10^6 GFP+ CD11b+ BM cells, or vehicle phosphate-buffered saline (PBS) were injected intraportally (n = 3 to 4). For APAP injury, 8- to 10-week-old C57BL/6J male mice were fasted overnight and given LD50 dose of 500 mg/kg APAP i.p. After 6 to 8 hours, 2.5 to 3 × 10^6 GFP+ KC, 5 × 10^6 GFP+ CD11b+ BM cells, TTFs, or vehicle were given intraportally (n = 3 to 4). Mice were sacrificed after 1 day and 7 days for histology and cell engraftment evaluations.

Cell Isolation and Culture

Mouse liver was perfused as described previously. Cell viability was determined by trypan blue dye exclusion. Hepatocytes were removed by pelleting under 50 × g for 5 minutes. Red blood cells were lyzed. Remaining NPC were incubated with biotin-conjugated anti-CD11b and biotin-conjugated anti-F4/80 (eBioscience, San Diego, CA) for 20 minutes at 4°C followed by incubation with Streptavidin MicroBeads (Miltenyi Biotec, San Diego, CA). For determining phenotype-specific effects of transplanted KC within the context of cytokine expression in liver microenvironment, we used acetaminophen (APAP)- or IR-induced liver injury models.

Materials and Methods

Animals

Animal Care and Use Committees of Albert Einstein College of Medicine and University of Piemonte Orientale approved studies. Mice were 7 to 10 weeks old. Donors were wild-type C57BL/6J mice, C57BL/6-Tg(CAG-EGFP) 131Osb/LeySopJ mice with ubiquitous green fluorescent protein (GFP) expression or CD45.1 B6.SJL-Ptprc<sup>a</sup> Pepe<sup>b</sup>/BoyJ mice (The Jackson Laboratory, Bar Harbor, ME). Recipients in C57BL/6J background were DPPIV-mice or IL6<sup>tm1Kopf/J</sup> mice (The Jackson Laboratory). Mice were anesthetized with isoflurane. For cell transplantation, 1 to 2 × 10^6 of KC, BMDM, or PM, or 5 × 10^6 of CD11b+ BM cells were injected into portal vein or tail vein in 0.3 mL serum-free Dulbecco’s Modified Eagle Medium.

To deplete KC, 10 mg/kg GdCl<sub>3</sub> (Sigma Chemical Co.), or 100 to 200 μL liposomal clodronate (ClodronateLiposomes.org, Amsterdam, the Netherlands), were given via tail vein in saline. Controls received empty liposomes or saline. Residual KC were analyzed by flow cytometry in NPC isolated by collagenase digestion. KC phagocytosis was analyzed by 1 hour pulse of carbon 24 hours after gadolinium chloride or clodronate as described. Cells were transplanted 24 hours after gadolinium chloride administration.

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cells were flushed out of tibias and femurs from mice with Dulbecco’s modified Eagle’s medium with 5% FBS. Red blood cells were lysed and CD11b⁺ BM cells were isolated as described previously in this paragraph. Moreover, 0.8 to 1 × 10⁶ of total BM cells/cm² plastic were differentiated in Iscove’s Modified Dulbecco’s Medium containing 10% FBS and 5 ng/mL recombinant mouse macrophage colony-stimulating factor for 5 to 7 days, after which cells were released by Versene (Gibco). TTFs were isolated from 3- to 4-week-old GFP⁺ mice. Tails were sterilized in 70% ethanol, skinned, and minced thoroughly. Minced pieces were placed under glass coverslip in 6-well plates (one tail-tip in each well) for culture in Dulbecco’s modified Eagle’s medium with 10% FBS. After 5 to 7 days in culture, coverslips and minced pieces were removed, and TTFs were expanded with fresh medium every other day until confluent. For transplantation, 1 to 2 × 10⁶ freshly isolated KC or 3 to 5 × 10⁶ CD11b⁺ BM cells, BMDM, PM, and TTFs were administered per mouse.

Fluorescence-Activated Cell Sorting

Cells were incubated with antibodies (Table 1), washed, and resuspended in 2% FBS in PBS, pH 7.4. For each sample, 1 × 10⁵ events were acquired by FACScalibur (BD Biosciences). Data were analyzed by Windows Multiple Document Interface for Flow Cytometry (winMDI, v. 2.9; Joseph Trotter, The Scripps Institute, La Jolla, CA).

Biodistribution Studies

Cells (1.5 to 2 × 10⁶) were incubated in 2 mL saline with 370 to 555 MBq ⁹⁹ᵐTc (Ultratag and Ceretec kits; Mallinckrodt, St. Louis, MO) or 15 MBq ¹¹¹In-oxine for 30 minutes at room temperature, as described previously. Labeling efficiencies were measured. Cell viability was evaluated by trypan blue dye. Total body static images were obtained 30 minutes after cell transplantation. Liver, spleen, lungs, kidneys, and muscle were collected after 1 hour for radioactivity.

Tissue Studies

Liver samples were fixed in formalin for hematoxylin and eosin staining or in 4% paraformaldehyde in PBS, equilibrated in 15% and 30% sucrose, embedded in optimum cooling temperature compound (Tissue-Tek, Sakura Finetek, Torrance, CA), and frozen at −80°C. Cryosections (5 μm) were post-fixed with paraformaldehyde for 5 minutes at room temperature, blocked with 5% goat serum, 1% bovine serum albumin, and 0.1% Triton X-100 (Sigma-Aldrich) in PBS.

Table 1  Antibodies Used for Fluorescence-Activated Cell Sorting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Format</th>
<th>Incubation time (on ice, minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16/32</td>
<td>BD Pharmingen</td>
<td>2.4G2</td>
<td>Purified</td>
<td>1</td>
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<tr>
<td>F4/80</td>
<td>Invitrogen</td>
<td>BM8</td>
<td>PE</td>
<td>30</td>
</tr>
<tr>
<td>CD80</td>
<td>eBioscience</td>
<td>16-10A1</td>
<td>APC</td>
<td>30</td>
</tr>
<tr>
<td>CD14</td>
<td>Biologend</td>
<td>Sa14-2</td>
<td>APC</td>
<td>30</td>
</tr>
<tr>
<td>NK-cells</td>
<td>Immunotools</td>
<td>PK136</td>
<td>PE</td>
<td>30</td>
</tr>
<tr>
<td>CD19</td>
<td>Miltenyi Biotec</td>
<td>LT19</td>
<td>PE</td>
<td>30</td>
</tr>
<tr>
<td>CD11b</td>
<td>BD Pharmingen</td>
<td>M1/70</td>
<td>PE</td>
<td>30</td>
</tr>
<tr>
<td>CD11c</td>
<td>eBioscience</td>
<td>N418</td>
<td>PE</td>
<td>30</td>
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<tr>
<td>I-A/I-E</td>
<td>e-Bioscience</td>
<td>M5/114.15.2</td>
<td>PE</td>
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<tr>
<td>CD3e</td>
<td>Immunotools</td>
<td>145-2C11</td>
<td>FITC</td>
<td>30</td>
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<tr>
<td>B220</td>
<td>eBioscience</td>
<td>RA3-6B2</td>
<td>PE</td>
<td>30</td>
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<td>Gr-1</td>
<td>Immunotools</td>
<td>RB6-8C5</td>
<td>APC</td>
<td>30</td>
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</tbody>
</table>

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Table 2  Antibodies Used for Immunofluorescence

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Dilution</th>
<th>Incubation time at room temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>F4/80</td>
<td>AbD Serotec (Bio-Rad, Raleigh, NC)</td>
<td>CI-A3-1</td>
<td>1:500</td>
<td>1 hour</td>
</tr>
<tr>
<td>CD68</td>
<td>AbD serotec</td>
<td>FA-11</td>
<td>1:500</td>
<td>1 hour</td>
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<tr>
<td>GFP</td>
<td>Molecular Probes</td>
<td>Rabbit polyclonal</td>
<td>1:300</td>
<td>1 hour</td>
</tr>
<tr>
<td>CD45.1</td>
<td>eBioscience</td>
<td>A20</td>
<td>1:50</td>
<td>1 hour</td>
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<tr>
<td>VEGF</td>
<td>Santa Cruz Biotechnology, Inc. (Dallas, TX)</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>1 hour</td>
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<tr>
<td>Ki-67</td>
<td>Vector Laboratories (Burlingame, CA)</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
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<td>Secondary antibodies</td>
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<td></td>
<td></td>
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<tr>
<td>Goat anti-rat IgG</td>
<td>Molecular Probes</td>
<td>Alexa Fluor 546 or 488-conjugated</td>
<td>1:500</td>
<td>45—60 minutes</td>
</tr>
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<td>Molecular Probes</td>
<td>Alexa Fluor 488-conjugated</td>
<td>1:500</td>
<td>45—60 minutes</td>
</tr>
</tbody>
</table>
and incubated with rabbit anti-GFP or anti-vascular endothelial growth factor (VEGF) or rat anti-mouse F4/80. For CD45.1 staining, sections were postfixed for 10 minutes in ice-cold acetone, blocked, and probed with anti-mouse CD45.1 labeled with Zenon Alexa Fluor 488 Mouse IgG2a kit (Molecular Probes, Thermo Fisher Scientific, Waltham, MA). Sections were incubated with Alexa Fluor 488 conjugated goat anti-rabbit IgG and/or Alexa Fluor 546 conjugated goat anti-rat IgG (Molecular Probes), with DAPI-Antifade (Molecular Probes) for nuclear counterstaining. For cell proliferation, sections were permeabilized for 10 minutes in ice-cold 1% Triton X-100 in PBS, blocked as above, and stained with rabbit anti-Ki67 and rat anti-mouse F4/80 antibodies. Localization used Alexa Fluor 488-conjugated goat anti-rat IgG and Alexa Fluor 546-conjugated goat anti-rabbit antibodies. Primary and secondary antibodies are listed in Table 2. Sections were counterstained with DAPI or TO-PRO-3 and examined by Observer.Z1 fluorescence microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) or Leica confocal microscopy TSP2 (Leica Microsystems, Buffalo Grove, IL).

**LV Transduction**

LVs were prepared as described previously. Cells were transduced with 5 to 20 multiplicities of infection of following vectors: pCCLsin.PPT.hPGK.eGFP.pre LV (LV-PGK-GFP) (control); vector substituting hPGK

### Table 3 RT-PCR Primers and Expected Products

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Tm (°C)</th>
<th>PCR cycles</th>
<th>Amplicon (bp)</th>
<th>Polymerase used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human VEGFires-GFP</td>
<td>5'-GGCACGCGTGGTAAA-3'</td>
<td>5'-CACACCGGCTTTATCC-3'</td>
<td>55</td>
<td>35</td>
<td>152</td>
<td>GoTaq Flexi DNA Polymerase (Promega, Madison, WI)</td>
</tr>
<tr>
<td>Mouse IL-1β</td>
<td>5'-GCTGTGCTAGAGAGCATTC-3'</td>
<td>5'-CATGAGTCAGGAGATTG-3'</td>
<td>60</td>
<td>30</td>
<td>199</td>
<td>GoTaq Flexi DNA Polymerase</td>
</tr>
<tr>
<td>Mouse IL-6</td>
<td>5'-GCTGTCAGACCCAGCGCTC-3'</td>
<td>5'-TGTCAGACCCAGCGCTC-3'</td>
<td>60</td>
<td>30</td>
<td>202</td>
<td>GoTaq Flexi DNA Polymerase</td>
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<tr>
<td>Mouse IL-10</td>
<td>5'-CCCTGCTGACGCTGCTCT-3'</td>
<td>5'-TTAGCTTTTCTATTTGTATC-3'</td>
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<td>30</td>
<td>534</td>
<td>GoTaq Flexi DNA Polymerase</td>
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<tr>
<td>Mouse IL-12α</td>
<td>5'-GAATTCGCCGCAGCATACGCA-3'</td>
<td>5'-GAACGCGCTGAAGCATGATGCAG-3'</td>
<td>60</td>
<td>30</td>
<td>198</td>
<td>GoTaq Flexi DNA Polymerase</td>
</tr>
<tr>
<td>Mouse IL-12β</td>
<td>5'-GTGTCATGCCTGCTCT-3'</td>
<td>5'-CAGGGCGCTGCAGGCAGATCGACGCA-3'</td>
<td>60</td>
<td>30</td>
<td>200</td>
<td>GoTaq Flexi DNA Polymerase</td>
</tr>
<tr>
<td>Mouse VEGF</td>
<td>5'-TCTGTAAGAGTGCTCTGCTGCTG-3'</td>
<td>5'-AGGAACATTTACACGTCTGC-3'</td>
<td>60</td>
<td>35</td>
<td>279–351</td>
<td>GoTaq Flexi DNA Polymerase</td>
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<tr>
<td>Mouse β-Actin</td>
<td>5'-GCTGTTAAGGCGGACCAACACGCA-3'</td>
<td>5'-CTTCTTTTTTTTATTTTTTTTCTGC-3'</td>
<td>55</td>
<td>25</td>
<td>540</td>
<td>Platinum PCR Supermix (Invitrogen, Thermo Fisher Scientific)</td>
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</table>

GFP, green fluorescent protein; VEGF, vascular endothelial growth factor.

**Figure 1** Cell characterization. Fluorescence-activated cell sorting for various cell surface markers indicated in freshly isolated Kupffer cells, bone marrow cells, CD11b⁺ BM cells and peritoneal cells. Numbers represent means ± SD of at least five experiments. BM, bone marrow; Gr1, lymphocyte antigen 6 complex, locus 6; KC, Kupffer cells; MHC II, major histocompatibility complex class II; NK, natural killer.
Figure 2  Targeting of radiolabeled cells and engraftment of transplanted green fluorescent protein (GFP)+ cells in liver. A and B: Representative images 30 minutes after i.p. (n = 5) and i.v. (n = 4) injection of 111In-labeled Kupffer cells (KC) (A) or bone marrow–derived monocyte/macrophage (BMDM) (B). Charts indicate distribution of organ activity after 1 hour. I.P. versus I.V. injection was more effective for delivering cells to liver: KC, 88% ± 3% versus 48% ± 17%; BMDM, 94% ± 3% versus 49% ± 7%. More cells were entrapped in lungs after IV versus IP injection: KC, 42% ± 19% versus 6% ± 2%; BMDM, 46% ± 8% versus 1% ± 0.4%. Similarly, more cells entered spleen after IV injection: KC, 7% ± 2% versus 1 ± 0.3; BMDM, 2% ± 0.3% versus 0.4% ± 0.1%. Cells were similarly distributed in kidneys or muscle. C: DPPIV+ recipients 1 week after transplantation of KC or CD11b+ bone marrow cells with and without gadolinium chloride. Panels on extreme right provide magnified views. Identity of GFP+ transplanted cells (green) was verified by F4/80 costaining (red). More cells engrafted after gadolinium chloride. However, CD11b+ BM cells engrafted less well versus KC. D: Morphometric analysis of KC and CD11b+ BM cell engraftment after 1 week. *P < 0.05. Scale bars: 20 µm (C). GdCl₃, gadolinium chloride; Kd, kidneys; Ms, muscle; Lg, lungs; Lv, liver; Sp, spleen.
promoter with myeloid-preferred CD11b promoter (LV-CD11b-GFP), or vector with VEGF cDNA in bicistronic cassette (LV-PGK-VEGFiresGFP). To genetically modify KC in vivo, C57BL/6J mice were given 5 \times 10^8 transduction units of LV to express GFP under monocyte/macrophage specific promoter, CD11b (LV-CD11b-GFP), or 8 \times 10^8 TU of bicistronic LV expressing VEGF, and GFP with internal ribosomal entry site (IRES) under ubiquitous phosphoglycerate kinase (PGK) promoter (LV-PGK-VEGFiresGFP). VEGF cDNA was a gift from late Dr. Bruce Terman (Albert Einstein College of Medicine, New York, NY).

RT-PCR and Quantitative RT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen, Thermo Fisher Scientific) and treated with DNase I (RNase-free DNase; Qiagen, Germantown, MD). cDNAs were generated from 1 \mu g total RNA with Omniscript RT Kit (Qiagen). PCR products were resolved in 2% agarose gels. For quantitative RT-PCR, cDNAs were generated from 2 \mu g total RNA by RT First Strand Kit, followed by RT SYBR Green qPCR Master Mix (SABiosciences, Qiagen), RT qPCR Primer Assay for mouse IL-6 and GAPDH (SABiosciences) with ABIprism7000 software version 1.2.3 (Applied Biosystems, Inc., Thermo Scientific). Primers with expected product sizes are listed in Table 3.

Blood Tests

Blood was collected from animals in various groups. Serum was separated and stored at \(-80^\circ\text{C}\). This was followed by measurement of ALT levels with a commercial kit (ALT-GPT LR; Gesan, Campobello di Mazara, Italy). Differences in animal groups were then compared.

Statistics

Data are shown as means ± SD. Significances were analyzed by Student's t-test, \(\chi^2\), or analysis of variance with Tukey or Bonferroni post-hoc tests by SigmaStat version 3.1 (Systat Software Chicago, IL). \(P < 0.05\) was considered significant.

Results

Characterization of KC, PM, and BMDM

Typically, we isolated 1.5 to 2.5 \times 10^6 KC per mouse with ≥90% viability. KC expressed monocyte/macrophage...
markers, CD11b, and F4/80, at medium-high levels, and CD11c, at a medium level. Low-level expression of CD14 and CD80 in isolated KC indicated that these were distinct from dendritic cells. Isolated KC expressed major histocompatibility complex II marker, but not other leukocyte markers [ie, Gr-1 (granulocytes), B220 or CD19 (B cells), or CD3 (T cells)] (Figure 1). By contrast, freshly-isolated BM cells and CD11b⁺ BM cells showed high levels of CD11b and low levels of F4/80 expression, indicating that these were primarily monocyte/macrophage precursor cell populations (Figure 1). BMDM expressed CD11b at medium-high level and F4/80 and CD11c at low levels. PM expressed CD11b and F4/80 at high levels and CD11c at medium level (Supplemental Figure S1A). After culture, F4/80 expression was readily detected by immunostaining in
KC, BMDM, and PM (Supplemental Figure S1B), which verified macrophage phenotypes.

Hepatic Targeting and Engraftment of Transplanted Cells

For optimal cell transplantation route, we radiolabeled KC and BMDM and found these incorporated $^{111}$In equally ($65 \pm 4\%$ efficiency) but neither cell type incorporated $^{99m}$Tc. More cells entered the liver after i.p. ($n = 5$) versus i.v. ($n = 4$) injection in 30 to 60 minutes (Figure 2, A and B). Therefore, we injected cells in all studies with i.p. route. After transplantation in DPPIV- mice, GFP$^+$ KC engrafted in liver at 2 hours, 24 hours, 3 days, and 7 days (Figure 2C). To improve cell engraftment, we determined the effects of gadolinium chloride and clodronate on native KC 24 hours after administering these substances. Gadolinium chloride decreased KC to 27% $\pm$ 2% of controls ($P < 0.05$), and carbon uptake to 60% of controls ($P < 0.05$). In low (100 $\mu$L) or high dose (200 $\mu$L), clodronate decreased KC numbers to 20% $\pm$ 5% and 21% $\pm$ 1%, respectively, and carbon uptake to 60% to 70% of controls ($P < 0.05$) (Supplemental Figure S2). Therefore, both gadolinium chloride and clodronate decreased KC phagocytosis; clodronate depleted KC subpopulations without affecting CD11b$^+$ KC previously. In gadolinium chloride—treated mice, GFP$^+$F4/80$^+$ KC engrafted better (Figure 2C). However, GFP$^+$ BMDM engrafted poorly, as these were found in liver after 2 hours but rarely after 24 hours, and not after 3 days or 7 days. Gadolinium chloride did not improve BMDM engraftment (Figure 2C). Similarly, PCR for DPPIV DNA-verified transplanted BMDM were present after 2 hours and not after 24 hours (Supplemental Figure S3A). Next, to determine whether freshly-isolated CD11b$^+$ BM cells might engraft better than BMDM, we isolated CD11b$^+$ BM cells from GFP$^+$ donors followed by transplantation in mice. Transplanted GFP$^+$ BM cells were in liver up to 7 days after transplantation (Figure 2C), although in fewer numbers than transplanted KC at 7 days, 15 $\pm$ 5 versus 25 $\pm$ 4 cells per 100 fields ($P < 0.05$) (Figure 2D). More GFP$^+$CD11b$^+$ BM cells engrafted in the liver of gadolinium chloride—treated mice after 7 days, but this was less than KC (21 $\pm$ 4 versus 53 $\pm$ 5 cells per 100 fields [1.4- versus 2.2-fold increase from controls]) ($P < 0.05$) (Figure 2D). However, BM cells were distributed additionally to the spleen as described in Hepatoprotective Potential of Transplanted KC and CD11b$^+$ BM Cells. Donor BM cells were rarely F4/80$^+$, indicating that they did not produce mature macrophages. When GFP$^+$ PM were transplanted, these engrafted inefficiently in liver, 0 to 5 PM per 100 fields after 3 days, and none were found after 7 days, indicating that KC had predilection for liver (Supplemental Figure S3).

Long-Term Survival of Transplanted KC with Retention of Macrophage Functions

For these studies, we used CD45.1 C57Bl/6 donors to avoid immunogenicity of GFP. In DPPIV— recipients, transplanted CD45.1/F4/80/CD11b$^+$ KC were present in liver after 24...
Therefore, transplanted KC survived essentially 1 hour, 1 week, 1 month, and 3 months (LV-GFP-transduced Kupffer cells (KC) decrease phagocytosis of DH5α Escherichia coli expressing DsRed was identified in transplanted CD45.1+ KC by immunostaining after 2 weeks (Figure 3B). In gadolinium chloride–treated IL-6 knockout mice, transplantation of healthy GFP+ KC led to the appearance of lipopolysaccharide-inducible IL-6 mRNA expression when examined after 5 days, as another property of macrophages (Figure 3C).

**Hepatoprotective Potential of Transplanted KC and CD11b+ BM Cells**

To examine additional biological effects of transplanted KC, we studied mice with APAP hepatotoxicity. We noted differences in distributions of transplanted KC and CD11b+ BM cells in APAP-treated mice 1 week after transplantation. More transplanted KC were in the liver (12 per 20 fields) than spleen (3 per 20 fields), but more transplanted CD11b+ BM cells were in the spleen (35 per 20 fields) than liver (6 per 20 fields) (P < 0.05) (Figure 4, A and B, and Supplemental Figure S4). Given that twice as many BM cells were transplanted than KC, the overall number of transplanted cells in these two organs was not significantly different. After 1 week, transplanted GFP+CD11b+ BM cells were rarely F4/80+, indicating that liver injury had not induced these to differentiate into mature macrophages. Liver injury decreased when either KC or CD11b+ BM cells were transplanted (Figure 4C). The alanine aminotransferase levels were lower, particularly after transplantation of KC (Figure 4D). Morphometric analysis confirmed decreases in liver necrosis in these animals 24 hours after cell transplantation, including recipients of transplanted KC (Figure 4E). By comparison, transplantation of TTFs had no significant effect on either serum alanine aminotransferase levels (Figure 4D) or liver histology (not shown) in APAP-treated mice. To determine whether these beneficial effects of transplanted KC may have involved angiogenic modulators (eg, VEGF36) or anti-inflammatory cytokines (eg, IL-1012,37), we examined this further by transplanting genetically modified KC.

After transduction with LVs, the GFP transgene was expressed efficiently under the PGK promoter (LV-PGK-GFP) in cultured mouse KC and BMDM. Similarly, cultured KC and BMDM expressed GFP under the monocyte/macrophage promoter, CD11b (LV-CD11b-GFP) (Supplemental Figure S5). As freshly isolated KC proved difficult to transduce by LV in vitro (5% to 10%), we gave 5 × 108 transducing units of LV-CD11b-GFP to donor C57BL/6J mice by tail vein injection followed by isolation of KC after 1 week. The cell yield was similar to healthy donor mice, and we isolated approximately 2 × 10⁶ KC from these LV-transduced donor mice. We found 55% ± 9% of isolated KC were positive for both F4/80 and GFP (Supplemental Figure S6). These LV-transduced GFP+ KC were localized in liver 1 week after transplantation in gadolinium chloride–preconditioned C57BL/6J mice.

In animals subjected to IR, analysis of KC isolated after 2 hours showed expression of inflammatory cytokines [IL-1β
before enzymatic digestion to release cells from isolated cells by clearing blood from donor livers eliminated circulating or loosely adherent monocytes or other F4/80 and CD11b, besides cell types such as neutrophils, we multiple macrophage and monocyte populations may express this approach for biological or therapeutic studies. Although functionality of transplanted cells because of the relevance of
determining cell adhesion or other engraftment mechanisms should be appropriate. For instance, adhesion of transplanted hepatocytes to liver sinusoidal endothelial cells via integrin-dependent interactions is an early step in their engraftment. Similarly, macrophage adhesion to endothelium requires CD11b, which forms a subunit of heteromeric zMβ2integrin. ELucidating whether this or similar molecules may contribute to KC engraftment could help in liver repopulation with these cells. Cytokines, such as hepatocyte growth factor, stromal cell-derived factor 1, or insulin-like growth factor 1, promote accumulation of hepatic progenitor cells in liver, but whether these contributed in engraftment of KC is unknown. Microcirculatory changes (eg, release of vasoactive molecules and inflammatory cytokines/chemokines/receptors), interfere with hepatocyte engraftment and may have contributed in clearing of transplanted KC, BMDM, PM, or TTFs. Control of vascular and inflammatory perturbations has been useful for improving hepatocyte engraftment and may be helpful for KC engraftment, too.

After engraftment, transplanted KC survived for at least 3 months in the liver. Such longevity of KC had been unknown previously. This should be relevant for studying the biological roles of KC in immunological memory, antigen presentation, or homeostasis during liver injury. In their ontogeny, KC originate from

Discussion

These studies established that transplanted KC engrafted and survived over the long term in the liver. Retention of appropriate macrophage functions in transplanted KC, particularly in a context-specific manner, indicated that replacement of these cells will provide important means to address the biological and therapeutic potential of monocyte/macrophage cell types.

Our studies did not address the trafficking and exchange of cells from BM, peripheral blood, and tissues, which have been studied in extensive detail by other investigators, including in recent publications. Our focus concerned the fate and functionality of transplanted cells because of the relevance of this approach for biological or therapeutic studies. Although multiple macrophage and monocyte populations may express F4/80 and CD11b, besides cell types such as neutrophils, we eliminated circulating or loosely adherent monocytes or other cells from isolated cells by clearing blood from donor livers before enzymatic digestion to release firmly adherent KC. Moreover, positive selection of KC with immunobeads requiring significant amounts of CD11b and F4/80 expression would have avoided contamination by cells with low-level expression of these markers. In healthy donor livers, we found no evidence of macrophage tissue invasion to consider such contaminants. Therefore, KC isolates lacking markers found in other white blood cell populations, robust macrophage functions such as phagocytosis, bacterial clearance, inflammatory cytokine expression in response to lipopolysaccharide, expression of cytokines observed in macrophages, plus the fact that transplantation of these KC led to their engraftment in liver, whereas other macrophage populations or fibroblasts did not engraft, further corroborated that the cell isolation procedures yielded suitable KC.

We isolated BM cells and generated BMDM with established protocols. The vast majority of BMDM and BM-derived cells were CD11b positive, and when these cells were Gr-1 positive, that was simultaneously with CD11b positivity, which is not typical of mature neutrophils. Generation of BMDM indicated that isolated BM cells contained substantial numbers of mononuclear cells or monocyte precursor cells. Similarly, PM and TTFs were isolated with established methods. Typical morphology and phenotype characterizations confirmed that these cells, too, were suitable for studies.

Remarkably, KC and BMDM incorporated In but not Tc, a property shared with liver sinusoidal endothelial cells, but not with hepatocytes, which incorporated both Tc. Besides the obvious differences in the morphology of hepatocytes and KC or BMDM, this finding confirmed that hepatocytes had not contaminated KC and BMDM preparations. As targeting of radiolabeled KC and BMDM to organs after intraportal or tail vein injection was similar, we could directly compare their fates. Because KC and BMDM largely were entrapped in lunge after tail vein injection, this finding also should be helpful for designing or interpreting cell transplantation studies.

Approximately 20% of transplanted KC engrafted in the liver in mice preconditioned with gadolinium chloride. This efficiency of transplanted cell engraftment recapitulated that, in cases of hepatocytes or liver sinusoidal endothelial cells, prior liver preconditioning involving endothelial or hepatic injury created room for transplanted cells and promoted their integration in niches. As CD11b+ BM cells, BMDM, PM, and TTFs either engrafted poorly or did not engraft in liver, consideration of intrinsic properties determining cell adhesion or other engraftment mechanisms should be appropriate. For instance, adhesion of transplanted hepatocytes to liver sinusoidal endothelial cells via integrin-dependent interactions is an early step in their engraftment. Similarly, macrophage adhesion to endothelium requires CD11b, which forms a subunit of heteromeric zMβ2integrin. ELucidating whether this or similar molecules may contribute to KC engraftment could help in liver repopulation with these cells. Cytokines, such as hepatocyte growth factor, stromal cell-derived factor 1, or insulin-like growth factor 1, promote accumulation of hematopoietic cells in liver, but whether these contributed in engraftment of KC is unknown. Microcirculatory changes (eg, release of vasoactive molecules and inflammatory cytokines/chemokines/receptors), interfere with hepatocyte engraftment and may have contributed in clearing of transplanted KC, BMDM, PM, or TTFs. Control of vascular and inflammatory perturbations has been useful for improving hepatocyte engraftment and may be helpful for KC engraftment, too.

After engraftment, transplanted KC survived for at least 3 months in the liver. Such longevity of KC had been unknown previously. This should be relevant for studying the biological roles of KC in immunological memory, antigen presentation, or homeostasis during liver injury. In their ontogeny, KC originate from
mesodermal precursor cells in yolk sac and later appear in fetal liver. In adult liver, KC likely replenish from mononuclear cells arising in BM. Whether liver-resident KC could self-renew over the long term may be studied now by the cell transplantation approach. The difficulties in analyzing the fate and functions of KC within their native microenvironment led to generation of immortalized macrophage lines. Such immortalized macrophages, including those derived from pluripotent stem cells, gained broad interest. But studies of macrophage differentiation, fate, and function should greatly benefit from cell transplantation under specific liver microenvironment contexts in vivo.

We noted the ability of transplanted KC to express pro- and anti-inflammatory cytokines in a context-specific manner (ie, after lipopolysaccharide or liver injury), implying that transplanted KC flexibly adopted either pro- or anti-inflammatory phenotypes as was needed. Previously, when NPC, including KC, were cotransplanted with hepatocytes in the peritoneal cavity, transplanted hepatocytes showed superior survival. Therefore, cotransplantation of these cell types might be worth considering in applications of hepatocyte transplantation. Also, our results of improved outcomes in liver injury after transplantation of KC, along with promotion of tissue repair through release of relevant cytokines (eg, VEGF) were in agreement with this hepatoprotective ability of KC. We do not imply that VEGF alone accounted for beneficial effects in this setting, because multiple cytokines are involved in hepatic injury. The hepatoprotective role of KC was in agreement with previous studies in which APAP toxicity in mice was worsened by prior depletion through clodronate of native KC with significant decreased expression of multiple regulatory cytokines and molecules, including several interleukins. By contrast, freshly isolated CD11b+ BM cells and BMDM survived only briefly after transplantation without producing mature KC despite depletion of native KC by gadolinium chloride, and so this approach alone should not be effective for replacing KC. Previously, infusion of BMDM in a mouse model of fibrosis was beneficial even though transplanted cells were cleared within 1 week. In our studies, transplanted KC survived longer in the liver and directly supported liver repair or regeneration.

Another aspect of KC transplantation concerns genetic modification of cells before transplantation. Previously, non-integrating adenoviral vectors were used to express hepatoprotective superoxide dismutase or heme oxygenase— genes in NPC, including KC, or BMDM. The ability to permanently transduce KC with LVs and then to successfully return these to the liver will have therapeutic relevance. For instance, healthy native monocytes and KC expressed coagulation factor VIII at low but sufficient levels for ameliorating coagulation defect in hemophilia A. This indicates that cell and gene therapy approaches should merit additional studies in hemophilia and other relevant conditions amenable to KC transplantation.

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Supplemental Data

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