Ly6c\textsuperscript{hi} Infiltrating Macrophages Promote Cyst Progression in Injured Conditional \textit{Ift88} Mice

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Key Points

- Ly6c\textsuperscript{hi} infiltrating macrophage numbers are increased in injured, conditional \textit{Ift88} mice, compared with controls.
- Loss of Ly6c\textsuperscript{hi} infiltrating macrophages slows injury-accelerated cystic disease.
- Ly6c\textsuperscript{hi} infiltrating macrophages drive cystic disease in non-Pkd1–deficient cystic models.

Introduction

Cystic kidney diseases are commonly inherited genetic disorders caused by mutations in proteins that localize to the primary cilia (e.g., polycystin 1 and polycystin 2) (1). On the basis of data showing that depletion of all phagocytic cells (using liposomal clodronate) reduced cystic severity, it has been proposed that mononuclear phagocytes can accelerate cyst growth (2,3). Importantly, renal mononuclear phagocytes comprise a diverse array of cell types, including macrophages and dendritic cells (4). Macrophages can be separated, on the basis of their functional properties and ontologic origins, into infiltrating and tissue-resident populations (4–6). Infiltrating macrophages can be further subtyped on the basis of cell-surface expression of Ly6c (Ly6c\textsuperscript{hi} versus Ly6c\textsuperscript{int/lo}). In the kidney, Ly6c\textsuperscript{hi} infiltrating macrophages accumulate in a Ccl2/Ccr2-dependent manner during initial periods after injury, and they produce proinflammatory cytokines that promote tissue damage (7). In contrast, Ly6c\textsuperscript{int/lo} infiltrating macrophages are derived from Ly6c\textsuperscript{hi} infiltrating macrophages, and they promote wound healing and resolution of injury (8).

Although data indicate that resident macrophages promote cystic disease in adult-induced, injury accelerated, \textit{Ift88} and \textit{Pkd1} models of cystogenesis (9,10), the involvement of infiltrating macrophages in cystic disease is controversial. Inhibition of infiltrating macrophage recruitment to the kidney, using a pharmacologic inhibitor of CCL2, the major CCR2 ligand, failed to reduce cystic disease in \textit{pck} (Pkd1\textsuperscript{-/-} mutation) rats (11). Likewise, deficiency of CCR2 failed to affect cystic severity in \textit{pck} (Cys1 mutation) mice, although these mice were protected from cardiac lethality (12). In contrast to these studies, deletion of \textit{Ccl2} significantly reduced cyst severity in the adult-induced, noninjured, \textit{Pkat1fl/fl};\textit{Pax8-rTA};\textit{TetO-Cre} mouse model of renal cystogenesis (13,14). These data suggest that the type of cilia/cystogenic gene mutation may be important in determining macrophage involvement in cyst progression.

Although the type of cilia-related genetic mutation may determine the involvement of infiltrating macrophages and the rate of cyst progression, external environmental factors, such as renal injury, can also influence the rate of cyst progression (15). However, the involvement of infiltrating macrophages in injury-accelerated models of cystic disease is unknown. In this study, we test the hypothesis that Ccr2-deficient, Ly6c\textsuperscript{hi} infiltrating macrophages promote cyst progression in the injury-accelerated, adult-induced, conditional \textit{Ift88} model of cystic disease.

Materials and Methods

Mice

Male and female, 8-week-old, CAGG-Cre/\textit{Esr1}/\textit{5Amc/J Ift88fl/fl} (referred to as conditional \textit{Ift88} mice) C57BL/6j mice were bred in house. CCR2\textsuperscript{rfp/rfp} mice (B6.129[Cg]-Ccr2tm2.1Ifc/J; stock number 017586) were purchased from The Jackson Laboratories and crossed to the conditional \textit{Ift88} mice to generate the CCR2-deficient, cilia mutant mouse. Animals were maintained in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with the Institutional

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Animal Care and Use Committee regulations at the University of Alabama at Birmingham (UAB; approval numbers, 10130 and 21072).

**Induction of Cilia Loss**

Conditional Ift88 mice, 8–10 weeks old, were given an intraperitoneal injection of tamoxifen at 6 mg per 40 g body wt, once daily for 3 consecutive days. This dosing scheme results in deletion of Ift88 within a week, and loss of primary cilia in the kidney within 3 weeks, as previously published (9). Loss of the Ift88 gene was confirmed by PCR.

**Renal Ischemia-Reperfusion Injury**

At 3 weeks after tamoxifen induction, cilia wild-type (cre-Ift88fl/fl mice) or conditional Ift88 (cre+Ift88fl/fl) mice were subjected to unilateral ischemia-reperfusion (IR) injury by clamping the left renal pedicle for 30 minutes, under 1% isoflurane, followed by reperfusion, as previously published (9). Mice were injected with buprenorphine (0.05 mg/kg) for pain relief and allowed to recover on a 37°C heating pad. Mice were euthanized at different points after IR injury to analyze the number of Ly6chi infiltrating macrophages in renal cystic disease.

**Fixation and Tissue Processing**

After euthanasia, mouse kidneys were immersion fixed in 4% (wt/vol) paraformaldehyde (PFA) overnight at 4°C, switched to 70% ethanol overnight, embedded in paraffin, sectioned at 5 μm, and stained using hematoxylin and eosin. The cystic index was quantified using ImageJ software, and was calculated by determining the total cystic area divided by the total kidney area.

**Immunofluorescence Microscopy**

After overnight fixation, kidneys were switched to 30% sucrose for cryopreservation and optimal cutting temperature compound–embedded tissues were sectioned at 8 μm. Tissue sections were fixed with 4% PFA for 10 minutes, permeabilized with 1% Triton X-100 in PBS for 8 minutes, and incubated in 500 μl blocking solution (PBS with 1% BSA, 0.3% Triton X-100, 2% vol/vol donkey serum, and 0.02% sodium azide) for 30 minutes at room temperature. Sections were incubated in 200 μl primary antibody overnight at 4°C, washed with PBS, and incubated with the appropriate secondary antibodies in blocking solution for 1 hour at room temperature. The primary antibody was a rat anti-mouse F4/80 antibody (diluted 1:200 in blocking solution, clone BMS, catalog number 14-4801; eBioscience). The secondary antibody was an Alexa Fluor 647–conjugated anti-rat (1:250 dilution in blocking solution, catalog number 712-606-153; Jackson ImmunoResearch). After the addition of secondary antibody, nuclei were stained using Hoechst nuclear stain (1:1000 dilution in PBS; Sigma-Aldrich) and samples were mounted using Immu-Mount (Thermo Fisher). All fluorescence images were captured on a Nikon T2i Eclipse (Nikon Instruments) spinning disk confocal microscope equipped with a Yokogawa ×1 disk (Yokogawa) on an Orca Flash4.0 sCMOS (Hamamatsu), using a 40× Plan Fluor 1.3 NA (Nikon Instruments) objective. Confocal images were processed and analyzed in NIS Elements version 5.0 software.

**Flow Cytometry**

After perfusion of the mouse with PBS, kidneys were minced and digested in 1 ml of RPMI 1640 containing 1 mg/ml collagenase type I (catalog number C1030; Sigma-Aldrich) and 100 U/ml DNase I (catalog number D5025; Sigma-Aldrich) for 30 minutes, at 37°C, with agitation. Kidney fragments were passed through a 70-μm mesh (Falcon; BD Biosciences), yielding single-cell suspensions. Red blood cells were lysed, and cells were resuspended in 1 ml PBS containing 1% BSA with Fc blocking solution (1:200 dilution, catalog number BE0307; BioXcell) for 30 minutes on ice. Live cells (2 × 10^6) were stained for 30 minutes, at room temperature, with the following primary antibodies: allophycocyanin anti-mouse CD11b (1:200, M1/70, catalog number 17-0112-82; Thermo Fisher), allophycocyanin–Cy7 anti-mouse GR1 (1:100, RB6-8C5, catalog number 557661; BD Pharmingen), FITC anti-mouse Ly6c (1:100, AI-21, catalog number 553104; BD Pharmingen), Pacific Blue anti-mouse F4/80 (1:200, BMS, catalog number 48-4018; Thermo Fisher), PerCP-Cy5.5 anti-mouse CD45 (1:200, 30-F11, catalog number 45-0451-82; Thermo Fisher), and LIVE/DEAD dye (1:500, L34965; Life Technologies Corporation). Cells were washed with PBS, spun (220 × g), fixed in 2% PFA for 30 minutes on ice, spun (220 × g), and resuspended in FACS staining buffer. After immunostaining, cells were analyzed on a BD LSR II flow cytometer. Data analysis was performed using FlowJo version 10 software.

**Statistical Analysis**

Data was presented as mean ± SEM. ANOVA, analysis of covariance, and t tests were used for statistical analysis, and differences were considered significant for P values <0.05.

**Results**

**Injured, Adult-Induced, Conditional Ift88 Mice Have Delayed, but Increased, Ly6c^hi Infiltrating Macrophage Accumulation Compared with Injured Cilia Wild-Type Controls**

Our previous data indicate that injured, adult-induced, conditional Ift88 mice have increased numbers of infiltrating macrophages compared with injured cilia wild-type (WT) controls, and that this accumulation occurs before the onset of renal cysts (9). To determine the contribution of the Ly6c^hi subset to the total infiltrating macrophage pool, we performed flow cytometry analysis on Ly6c expression in infiltrating macrophages at different time points after renal injury in cilia WT and conditional Ift88 mice. For these studies, we gated infiltrating macrophages on the basis of differential expression of CD11b and F4/80 (9); Ly6c^hi infiltrating macrophages were gated off of the infiltrating macrophage gate based on differential Ly6c expression (Figure 1A). Our data indicate that conditional Ift88 mice have a significant reduction in the number of Ly6c^hi infiltrating macrophages day 1 postinjury (Figure 1B). However, by day 3 postinjury, the number of Ly6c^hi infiltrating macrophages was increased in the conditional Ift88 mice, compared with WT mice, and became significantly increased in the conditional Ift88 mice by 7 days postinjury (Figure 1B). Thus, on the basis of the number of Ly6c^hi macrophages, it appears that the injury response is delayed in conditional Ift88 mice relative to
controls. After day 7, the number of Ly6c^hi infiltrating macrophages was not different between injured control and conditional injured Ift88 mice.

Loss of Ccr2 Reduces Cyst Severity in Injured, Adult-Induced, Conditional Ift88 Mice

To determine the importance of Ccr2 in controlling Ly6c^hi infiltrating macrophage recruitment to the kidney in injured, adult-induced, conditional Ift88 mice, we quantified the number of Ly6c^hi infiltrating macrophages of injured conditional Ift88 mice on the CCR2 control (CCR2^rfp/wt) or CCR2-deficient (CCR2^rfp/rfp) background. CCR2 is required for emigration of Ly6c^hi monocytes out of the bone marrow and recruitment to the kidney, thereby allowing us to specifically test if Ly6c^hi infiltrating macrophages are required for injury-induced cyst progression (16). We analyzed the kidneys at 56 days postinjury, because this represents the initial stages of cystogenesis in this model. Analysis of flow cytometry data indicates that loss of CCR2 in injured conditional Ift88 mice...
results in a significant reduction in the number of Ly6c\(^{hi}\) infiltrating macrophages in the kidney, as compared with injured, adult-induced, conditional \(\text{Ift88}^+\) mice (Figure 2, A and B).

To determine the spatial relationship between CCR2-RFP\(^+\) macrophages and renal cysts, we costained kidney sections from both experimental groups with the pan macrophage marker F4/80. This analysis revealed a strong accumulation of CCR2-RFP\(^+\), F4/80\(^+\) macrophages adjacent to renal cysts in the conditional \(\text{Ift88}^+\) knockout background (Figure 2C). In contrast, conditional \(\text{Ift88}^+\) mice had reduced CCR2-RFP\(^+\), F4/80\(^+\) macrophage accumulation (Figure 2C), suggesting genetic deletion of Ccr2 significantly reduces infiltrating macrophage recruitment to cystic regions.

To determine the importance of Ly6c\(^{hi}\) infiltrating macrophages in regulating cyst progression, we measured cystic indices (cystic area/total kidney area) and cyst number 56 days postinjury (Figure 2D and E).
days post-renal injury in conditional Ift88 CCR2<sup>fl/fl</sup> and conditional Ift88 CCR2<sup>fl/fl;Pax8-rtTA;TetO-Cre</sup> mice. The data indicate that reduction in Ly6<sup>ch</sup> infiltrating macrophage number in the injured, conditional Ift88, cilia-deficient model significantly reduced cyst severity, but did not affect cyst number (Figure 2, D and E). Collectively, these data suggest Ly6<sup>ch</sup> infiltrating macrophages promote cyst progression in the injured, conditional Ift88 model of cystogenesis.

Discussion
Our data indicate that loss of cilia (conditional Ift88 mice), followed by renal injury, results in a significant, although delayed, accumulation of Ly6<sup>ch</sup> infiltrating macrophages in the kidney. Genetic deletion of Ccr2, which is required for recruitment of Ly6<sup>ch</sup> infiltrating macrophages to the kidney, significantly reduced cystic disease in injured, adult-induced, conditional Ift88 mice, compared with control mice. These data indicate Ly6<sup>ch</sup> infiltrating macrophages are important for cyst progression in an injury-induced, conditional Ift88 model of cystic disease.

Previous data indicate that genetic deletion of Ccl2, which is required for recruitment of infiltrating macrophages to the kidney, reduced the severity of renal cysts in Pkd1<sup>fl/fl;Pax8-rtTA;TetO-Cre</sup> mice (13,14). In contrast, data from other investigators showed that inhibition of infiltrating macrophage recruitment in Pck rats (11) or cpk mice (12) did not alter cystic pathology. Because these studies were done in polycystin-deficient and nonpolycystin-deficient models of cystic disease, it suggests that infiltrating macrophage involvement may be dependent on the type of cilia-related genetic mutation. In agreement with this hypothesis, data from Viau et al. (13) showed that deletion of Pkd1 in the renal tubules resulted in increased expression of Ccl2 and increased accumulation of CCR2+ macrophages compared with control mice; deletion of both Pkd1 and Ki67a, which results in a Ki67a-like cystic phenotype, significantly reduced Ccl2 expression and CCR2+ macrophage accumulation. These data suggest that cystogenesis in Pkd1-deficient mice is driven by Ccl2-dependent infiltrating macrophage recruitment, whereas infiltrating macrophages are minimally involved in non-Pkd1/Pkd2 models of cystic disease (Ki67a, Pkd1, and Cys1). Despite the data supporting this hypothesis, the results from our study challenge this hypothesis by showing that loss of Ccr2 reduces cyst severity in the injured, conditional Ift88 model of cystogenesis. These data suggest renal injury is an additional factor that must be accounted for when considering the involvement of inflammatory cells in cystic mouse models.

It is also possible that the involvement of infiltrating macrophages in cystic kidney disease in the mouse is dependent on the severity of the model. For example, as mentioned above, deletion of Ccl2 reduced cyst severity in the moderately severe (approximately 40–50% cyst severity) Pkd1<sup>fl/fl;Pax8-rtTA;TetO-Cre</sup> model (13,14), but had no effect on cyst burden in the extremely severe (approximately 80%) and rapidly progressing Cys1<sup>pk</sup> model (12). Because our model involves mild, slowly progressing cystic disease (approximately 5%), and loss of the Ccl2 receptor (Ccr2) reduces cyst formation, it raises the possibility that Ccl2/Ccr2-dependent macrophages are only involved during the early stages of cyst progression. However, there were also a number of other variables between these studies, including differences in genetic mutations (Pkd1, Cys1, and Ift88) and the presence or absence of injury. Future studies exploring these intricacies are warranted.

In these studies, we also made the unexpected discovery that conditional Ift88 mice, which lack primary cilia, had significantly reduced numbers of Ly6<sup>ch</sup> infiltrating macrophages on day 1 after IR injury, compared with injured WT mice. Because tubulule epithelia are the main cell type affected by Ift88 loss in this model (9), it would suggest that epithelial cells lacking functional cilia have a blunted or delayed response to injury. Why loss of cilia would blunt the initial injury response is unknown, but it may be due to impaired cilia-dependent pathway activation (i.e., you need intact cilia during the acute injury phase to induce chemotactic gene expression, which brings in infiltrating macrophages; when the cilia are gone, the ability to induce chemotactic gene expression is blunted). This idea is in line with recent literature showing that intact cilia are required for induction of cyst-activating pathways in the absence of polycystin proteins (17). It is also likely, although unsubstantiated, that cilia are required for induction of chemotactic gene expression in WT mice. It is unknown which pathways are blunted in the absence of primary cilia, and this needs to be explored.

The mechanism through which macrophages influence cyst growth is incompletely understood. On the basis of published findings, it is proposed that infiltrating macrophages promote cyst progression in Pkd1-deficient mice by inducing oxidative damage in the tubulule epithelium, resulting in epithelial flattening and proliferation-independent cyst expansion (14). Our laboratory and others showed that macrophages may promote cyst growth through production of cytokines, such as IL-6, that induce Stat3 signaling in the epithelium, presumably resulting in Stat3-dependent cyst progression (10). It is also possible that infiltrating macrophages promote cyst growth in cilia mutant mice during the reparative phase of AKI, after switching their polarization status to anti-inflammatory, M2-like macrophages. These M2-like macrophages then produce IL-10, which is required, but not sufficient, for cyst epithelial cell proliferation (18). Beyond these reported functions, macrophages may influence cyst growth through phagocytosis (of apoptotic cystic epithelium) or by influencing T-cell polarization and function. Collectively, our data indicate that Ly6<sup>ch</sup> infiltrating macrophages promote injured-accelerated cyst progression in conditional Ift88 mice.

Disclosures
M. Mrug reports having consultancy agreements with Chinook, Goldilocks Therapeutics, Natera, Otsuka Corporation, and Sanofi; receiving research funding from Chinook, Goldilocks Therapeutics, Otsuka Corporation, and Sanofi; receiving honoraria from Chinook, Natera, Otsuka Corporations, and Sanofi; and serving as a scientific advisor for—or member of—the PKD Foundation, on the Sanofi STAGED-PKD Steering Committee, and on the advisory board for Santa Barbara Nutrients. All remaining authors have nothing to disclose.
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Author Contributions
E. J. Aloria, M. J. Croyle, Z. Li, C. J. Song, and K. A. Zimmerman were responsible for visualization; and all authors conceptualized the study; K. A. Zimmerman wrote the original draft; M. J. Mrug laboratories for suggestions and technical support on the Genomic Core.

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