Supplementary Methods

Histology and Immunohistochemistry

All human kidney tissue was supplied by the IRB-approved PKD Biomaterials Research Core laboratory at the KUMC. Sections (4 µm) from formalin-fixed, paraffin-embedded kidney tissues (human or mouse) were stained with hematoxylin and eosin (Richard-Allan Scientific, Kalamazoo, MI). Cortical cystic indices were quantified using ImageJ (v1.43, NIH). Briefly, images were acquired using Spot (v4.6.1, Spot Imaging) from mid-sagittal sections of kidneys of each of two animals using the 2x objective. Renal cortex was defined in the sections, and total cortical area was measured using ImageJ. A common image threshold was identified such that only pixels from non-cystic areas were visible, and non-cystic cortical area was measured. The cortical cystic index was calculated as the ratio of cortical non-cystic area divided by total cortical area.

For immunohistochemistry, de-paraffinized sections were steamed in 0.01 M citrate buffer (pH 6.0) for 20 min (steamer #HS900, Black & Decker, Madison, WI). Sections were then incubated in 3% H₂O₂ followed by serum from the host animal in which the relevant secondary antibody was generated. Samples were incubated with the following primary antibodies: HAM56 (MA1-91009, Thermo Scientific, Rochester, NY), anti-F4/80 (MCA497, AbD Serotec, Raleigh, NC), anti-CD163 (MS-1103, Thermo Scientific, Rochester, NY), or anti-Ki-67 (RM-9106, Thermo Scientific, Rochester, NY) either for 1 h at room temperature (HAM56, CD163, Ki-67) or overnight at 4 °C (F4/80). Appropriate secondary antibodies (ImmPRESS, Vector Laboratories, Burlingame, CA) were then applied for 30 min at room temperature, followed by incubation with DAB substrate (Vector Laboratories) and hematoxylin counterstain, prior to visualization by light microscopy. In situ detection of apoptosis (by TUNEL) was measured using the TACS® kit (Trevigan #4810-30-K, Gaithersburg, MD) on formalin-fixed, paraffin embedded kidney sections according to the manufacturer’s protocol.

For Ki-67 stained sections, the number of positively-stained cells was counted in images acquired from 5 non-overlapping hpf (40x objective) per specimen. To calculate the number of
positive cells/mm² of tissue, the area of cystic space was subtracted from the total area using ImageJ.

For CD163 and HAM56 quantification, 5 identical fields from consecutive serial sections of each case stained by HAM56 and anti-CD163 were imaged, and CD163+ and (non-endothelial) HAM56+ cells were counted. The ratio of HAM56+ cells that are CD163+ (reported as percent) was calculated for each field. The mean ratio ± SEM of CD163+/HAM56+ cells per case is reported.

For F4/80 quantification, images were acquired from 5 consecutive, non-overlapping, hpf in sections from each of two different animals. The mean F4/80 staining area was measured using ImageJ (v1.43, NIH). Briefly, a common display threshold for each image was set such that only pixels from the immunostain were visible. The total area of these pixels contributed by the F4/80 stain relative to the total area of the image was calculated.

**Cell culture**

Primary cells obtained from the cavities of cysts on the surfaces of ADPKD kidneys (ADPKD cells) and those obtained from cortical tubule fragments from non-cystic kidneys (NHK cells) were supplied by the PKD Biomaterials Research Core laboratory at KUMC and cultured up to 3 passages in DMEM/F-12 (Cellgro 15-090-CV, Mediatech, Manassas, VA) supplemented with 5% FBS, 15 mM HEPES, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenite (ITS, BD Biosciences, San Jose, CA) plus penicillin (100 U/ml), streptomycin (130 µg/ml) (Pen/Strep). RAW 264.7 (RAW) cells were obtained from ATCC and cultured in DMEM (D6429; Sigma) containing 10% FBS, 2 mM additional glutamine and Pen/Strep. This media was also used to prepare conditioned media (CM) and for co-culture experiments unless otherwise specified. CM was obtained from ADPKD or NHK cells incubated for 3 d. THP-1 monocytes were maintained in RPMI-1640 media (R8758, Sigma-Aldrich, St. Louis, MO) containing 10% FBS, 200 µM L-glutamine and Pen/Strep and differentiated into macrophages by treatment for 3 d with 200 nM PMA (phorbol myristate acetate, P1585, Sigma-Aldrich) and further incubation for 4 d in maintenance media. Primary bone marrow-derived macrophages were isolated from
adult C57/Bl6 mice as described\textsuperscript{2}. For co-culture experiments, ADPKD or NHK cells (0.5 – 4 x \(10^5\), depending on incubation period of experiment) were seeded into 6-well tissue culture plates and incubated overnight, prior to the addition of RAW cells (0.25 – 20 x \(10^5\)). RAW cells were polarized either toward an M1 phenotype by overnight treatment with 20 ng/ml mouse IFN-\(\gamma\) (575302, BioLegend, San Diego, CA), unless otherwise specified, or toward an M2 phenotype by overnight treatment with 20 ng/ml each mouse IL-4 (214-14, PeproTech, Rocky Hill, NJ) and IL-13 (413-ML, R&D Systems, Inc., Minneapolis, MN).

**Proliferation assays**

For direct, co-culture assays, primary ADPKD cyst epithelial or NHK cells (0.5 x \(10^5\)/well) were seeded into 6-well tissue culture plates and incubated overnight. In initial experiments, kidney cells were then labeled for 30 min with the fluorescent lipophilic cell tracer, Dil (V22885, Life Technologies, Grand Island, NY) and washed according to manufacturer directions. Labeled cells were incubated overnight in ATP-depleting, starvation media (glucose-free DMEM [11966, Life Technologies] containing 2% FBS, 10 mM deoxyglucose and Pen/Strep\textsuperscript{3}) prior to incubation for 3 d with DMEM (D6429, Sigma-Aldrich) supplemented with 200 µM L-glutamine, Pen/Strep and containing either 0.1% FBS (low-serum media) or 10% FBS (high-serum media), or with macrophages washed three times and re-suspended in low-serum media. After co-culture incubation, cells were trypsinized, fixed in 1% paraformaldehyde and counted manually using a hemocytometer and a combination of fluorescence/bright field microscopy. While fluorescence was used in initial experiments to distinguish between renal tubule cells and macrophages, the marked differences in the two cell types allowed accurate epithelial cell counts to be obtained easily in the absence of these tracers. The physical characteristics of fixed RAW cells versus fixed ADPKD/NHK cells allowed them to be easily distinguished: fixed RAW cells are markedly smaller, have a regular, round shape but with a rough-appearing cell surface, whereas, fixed ADPKD and NHK cells are much larger, irregularly shaped and have a smooth-appearing cell surface. Therefore, Dil-loading of kidney cells was not necessary to determine accurate kidney
cell numbers in co-cultures and so was not utilized for subsequent direct, co-culture proliferation assays.

For transwell assays, primary kidney cells were seeded as described for direct co-cultures and incubated overnight, prior to gentle rinsing in Hank's buffer (H6648, Sigma-Aldrich) and followed by 24 h incubation in starvation media. Cells were then treated in the presence of transwell inserts designed to allow passage of large macromolecules (0.4 µm pore-size, BD Biosciences) with low-serum media, high-serum media or with macrophages prepared in low-serum media and seeded inside of transwells. For experiments requiring THP-1 macrophages, differentiated cells were trypsinized, washed (3 times) and resuspended in low-serum media prior to seeding in transwell inserts (1 x 10^5 cells for each 6-well plate insert). Following 3 d incubation at 37°C, cells were either trypsinized and prepared for manual counting or washed in PBS and frozen at -80°C prior to lysis in buffer containing CyQUANT® GR dye (Life Technologies), according to manufacturer’s directions. Quantitative measurement of fluorescence was carried out using a Synergy™ 2 microplate reader (BioTEK Instruments, Inc., Winooski, VT).

**Microcyst growth**

Microcyst growth assay was performed as described^4^ with some modifications. Briefly, primary ADPKD cyst cells (6 X 10^3 cells/well) were suspended in cold Type I collagen (Advanced Biomatrix; San Diego, CA), either alone or in the presence of increasing numbers of RAW macrophages prior to dispersal into wells of a 96-well culture plate. Plates were then warmed to 37 °C to allow collagen gel polymerization, and the gel-suspended cells were overlayed with defined media (DMEM/F-12, 15 mM HEPES, ITS, 5 x 10^{-8} M hydrocortisone, 5 x 10^{-5} M triiodothyronine, 2 mM glutamine, Pen/Strep) containing either no further additions or forskolin (5 µM) and EGF (5 ng/ml) to stimulate microcyst growth. After 9-10 days incubation at 37 °C, with media changes every 2-3 days, culture gels were fixed in equal volume 2% formaldehyde, and the outer diameters of spherical cysts with distinct lumens were measured using a digital camera and video analysis software. The surface area (SA) of each cyst, as well
as the total SA of the cysts within each well, was calculated from the individual cyst diameters (cysts >50µm). These experiments were repeated for a total of four times using primary cells from four different ADPKD kidneys.

**qRT-PCR and ELISA.**

Total RNA was isolated from cells (macrophages or macrophages plus renal tubule cells) after 18 h culture (unless otherwise specified) using RNeasy Miniprep kit (Qiagen, Valencia, CA). 1.5 µg RNA was used as template for cDNA synthesis using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) in a 20 µl reaction. For semi-quantitative RT-PCR, 1-2 µl of the cDNA reaction was used in a 20 µl PCR mixture. Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) was used and PCR carried out per manufacturer protocol. Housekeeping gene Gapdh was used as the endogenous control. PCR products after amplification were analyzed by 2% agarose gel electrophoresis. Real-time qRT-PCR was performed on Stratagene Mx3005P using Sybr Green Master Mix (#330529; Qiagen). The thermal cycler conditions were 95 °C for 10 m X 1, then 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and completed with 1 cycle of 95 °C 1 min, 55 °C for 30 s, and 95 °C for 30 s. Expression of mouse gene products was normalized to mouse Gapdh. The following primers were used: Arg1, 5'-CTCCAGGCAAGAACAGTCAA-3' and 5'-GGAGCTGTCATTAGGACATC-3'; iNos (Nos2), 5'-GGTTCAGGCCAAATAAACAG-3' and 5'–GTGGACGGTATGTCAC-3'; Mrc1, 5'-CTCTGTAGGATCTTGGC-3' and 5'–CGGAAGTCTGAGATGAGCC-3'; Il6, 5'-CTGCAAGAGACTTCCATCAAC-3' and 5'–AGTGGTATGACAGGTCTGTTGG-3'; Il10, 5'-GCAGCTCTAGGAGCATGTGG-3' and 5'ACAGCGCGGAGACATAACT-3' and Gapdh, 5'-CCACTCACGCAATCCACACTCA-3' and 5'-GTAGACTCCACGCTTCA-3'.

IL-10 was measured in culture supernatants using an ELISA kit with a murine-specific IL-10 antibody (eBioscience #88-7104-22, San Diego, CA) according to the manufacturer’s protocol.

**Renal macrophage enrichment and quantitative FACS analysis**
Whole kidneys from PN day 16 mice were harvested and minced, prior to digestion for 30 min with collagenase type I (2mg/ml in DMEM) at 37°C with rotation (150 rpm). Equal volume DMEM containing 10% FBS was added to inactivate the collagenase and samples were strained through a 40 µM strainer and centrifuged (300 X g, 5 m), and pellets were washed once and resuspended in PBS. Lymphocytic cells were enriched by density separation using Lympholyte M (Cedarlane, Burlington, NC) following by washing/suspension in PBS containing 2% FBS and 1 mM EDTA. Samples were incubated with mouse Fc receptor-blocking antibodies (Stem Cell Technologies, Vancouver, BC) for 20 min on ice, followed by incubation with the following antibodies (Biolegend): anti-F4/80 (APC-conjugated), anti-CD11c (BV421-conjugated) and anti-Ly6C (PE-conjugated) for 45 min on ice. Samples were washed, resuspended in PBS, fixed in 2% paraformaldehyde in PBS and analyzed using LSRII analyzer (BD Biosciences).

**Mice and Macrophage depletion**

Offspring from Balb/c-<i,cpk</i>/+ heterozygous matings were given intraperitoneal injections of either vehicle (saline) or 0.1 ml(0.7g)/10g body weight of clodronate liposomes prepared as described<sup>5</sup>,<sup>6</sup> at PN days 3 and 6. At PN day 10, animals were weighed and sacrificed, followed by harvest of tissues, which were weighed and fixed in 10% buffered formalin prior to paraffin embedding. All protocols were approved by IACUC.

**Blood Urea Nitrogen (BUN) Measurement**

Blood was collected at the time of sacrifice on PN day 10. Serum was isolated from each sample (centrifugation 2500 X g for 10 m at room temp), and blood urea nitrogen (BUN) was measured using QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA).
References
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Supplementary Figure Legends

Supplementary Figure S1. Macrophage staining in additional ADPKD (A-E), ARPKD (F), and NHK (G) kidneys. Formalin-fixed, paraffin-embedded kidney tissues were serially sectioned and consecutive sections stained by hematoxylin and eosin (left) or by immunohistochemistry using the macrophage antibody HAM56 (middle) or an antibody to C163 (right). Scale bars represent 25 µm. Cystic space is indicated by *. (H) Five identical fields from consecutive serial sections of each case indicated stained by HAM56 and anti-CD163 were imaged, and CD163+ and (non-endothelial) HAM56+ cells were counted. The ratio of HAM56+ cells that are CD163+ (reported as percent) was calculated for each field. The mean ratio ± SEM of CD163+/HAM56+ cells per case is reported.

Supplementary Figure S2. Naïve and M2-type RAW macrophages and human differentiated THP-1 macrophages promote ADPKD cyst cell proliferation. (A) Direct co-culture proliferation experiments were carried out with RAW macrophages and primary ADPKD cyst epithelial cells from five different kidneys. For each experiment, ADPKD cells were co-cultured for 72 h with low serum (0.1%) media to determine the baseline level of cell proliferation and high serum (10%) media to determine maximal level of proliferation. These controls were cultured in parallel with co-cultures of RAW macrophages prepared in low serum media. Cells were then collected, fixed and counted to determine ADPKD cell numbers. The percent maximal proliferation stimulated by the RAW macrophages co-cultures was then calculated (~100 x {number of ADPKD cells co-cultured with RAW macrophages /[number of ADPKD cells in high serum media – number of ADPKD cells in low serum media]}) and the mean is shown for each experiment. RAW macrophages types used: naïve (untreated); M1.1 (2 h treatment with 2.5 µg/ml LPS + 22h incubation in media with no LPS); M1.2 (48 h treatment with
100 ng/ml LPS + 20 ng/ml IFNγ; M1.3 (18 h treatment with 20 ng/ml IFNγ); M2.1 (18 h treatment with 20 ng/ml IL-4); M2.2 (48 h treatment with 20 ng/ml IL-4); M2.3 (19 h treatment with 20 ng/ml IL-4 + 20 ng/ml IL-13). (B) Transwell proliferation assays were carried out using primary ADPKD cyst epithelial cells from three different kidneys. Control cultures for each experiment included wells containing transwell inserts with low serum (0.1%) media and wells plus transwells with high serum (10%) media. In parallel, naïve RAW macrophages prepared in low serum media were added to transwell inserts. Following 72 h incubation, cells were collected and either counted or assayed for relative proliferation following lysis and incubation with CyQUANT® GR dye. The percent maximal proliferation stimulated by the RAW macrophage co-cultures was then calculated as in (A) and the mean is shown for each experiment. (C) Transwell proliferation assay of ADPKD K353 cyst epithelial cells as described for (B) to test the affects of human differentiated THP-1 macrophages. In this experiment THP-1 macrophages stimulated 80% maximal proliferation of the ADPKD cells. This experiment was repeated using ADPKD cyst cells from two other kidneys with similar results. Data are presented as mean ± SEM. * and ** in (A), (B) and (C) denote P<0.05 and P<0.01, respectively.

Supplementary Figure S3. Characterization of macrophage subpopulations in WT and cpk/cpk kidneys. Representative flow cytometry plots of the CD11c− population of single, live cells isolated from WT and cpk/cpk kidneys identifying macrophage populations by the surface expression markers F4/80 and Ly6C. Note that the levels of F4/80+CD11c- cells (all cells above the horizontal line) found in cpk/cpk kidneys versus WT are elevated, and that of these a Ly6Clow population (M2) predominates.
Supplementary Figure S4. Clodronate does not affect body weight. (A) The mean total body weight of WT (n=14), +/-cpk (n=16), and cpk/cpk (n=17) mice measured at PN10. (B) Comparison of the total body weight from WT, +/cpk, and cpk/cpk mice (PN10) treated either with vehicle or clodronate (n=6-10 for each sample set).

Supplementary Figure S5. F4/80-positive cells from mouse liver are depleted by clodronate liposomes. (A) Formalin-fixed, paraffin-embedded liver tissue from PN10 cpk/cpk mice injected with either vehicle (left) or clodronate liposomes (right) was sectioned and stained by immunohistochemistry using a monoclonal antibody against F4/80. A representative image from one of four different mice for each condition is shown. Scale bars represent 25 µm. (B) The mean area (µm²) of F4/80 staining per high-powered field (HPF) in sections was measured as described in Methods from 2 livers each of mice treated with either vehicle or clodronate.

Supplementary Figure S6. Clodronate-treatment does not affect the levels of Ki-67-positive cells in WT mouse kidneys. Formalin-fixed, paraffin-embedded kidney tissues from PN10 WT mice treated with either vehicle (A) or clodronate liposomes (B) were sectioned and stained by immunohistochemistry using a monoclonal antibody against Ki-67. A representative image from one of two different mice for each condition is shown. Scale bars represent 25 µm. (C) Ki-67+ cells/mm² tissue in PN10 WT mice treated with either vehicle or clodronate.

Supplementary Figure S7. Clodronate-treatment does not affect the levels of apoptotic cells in cpk/cpk mouse kidneys. TUNEL staining was performed on formalin-fixed, paraffin-embedded kidney tissue from PN10 cpk/cpk mice treated with either vehicle (A) or clodronate liposomes (B). A representative image from one of two
different mice for each condition is shown. Scale bars represent 25 µm. (C) As a positive control for the technique, TUNEL staining was performed on WT PN10 kidney sections treated with nuclease in parallel to A and B. A representative image from one of two different mice for each condition is shown. Scale bars represent 25 µm.
Supplementary Figure S1A

K236 (ADPKD)

H-E  HAM56  CD163
Supplementary Figure S1D

K315 (ADPKD)

H-E  HAM56  CD163
Supplementary Figure S1E

K259 (ADPKD)

H-E

HAM56

CD163
Supplementary Figure S1F

K275 (ARPKD)

H-E  |  HAM56  |  CD163

* | * | *
Supplementary Figure S1G

K265 (NHK)

H-E  HAM56  CD163
Supplementary Figure S2

A. Direct coculture effects of RAW Mφs on proliferation of ADPKD cells

B. Transwell coculture effects of naive RAW Mφs on proliferation of ADPKD cells

C. THP-1 Mφ transwell coculture with ADPKD K353 cells

Supplementary Figure S2
143x203mm (300 x 300 DPI)
Supplementary Figure S5

A

Vehicle   Clodronate

B

F4/80 staining in liver (um²/HPF)

Vehicle  Clodronate

*** P<0.0001

Supplementary Figure S5
151x143mm (300 x 300 DPI)
Supplementary Figure S6

A  Vehicle

B  Clodronate

C

![Graph showing Ki-67+ cells/mm² (X10^7) for Vehicle and Clodronate.](image)

Supplementary Figure S6
138x142mm (300 x 300 DPI)
Supplementary Figure S7

A  Vehicle

B  Clodronate

C  Positive control

Supplementary Figure S7
138x139mm (300 x 300 DPI)