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Macrophages promote polycystic kidney disease progression

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Abstract

Renal M2-like macrophages have critical roles in tissue repair stimulating tubule cell proliferation and, if they remain, fibrosis. M2-like macrophages have also been implicated in promoting cyst expansion in mouse models of autosomal dominant polycystic kidney disease (ADPKD). While renal macrophages have been documented in human ADPKD, there are no studies in autosomal recessive polycystic kidney disease (ARPKD). Here we evaluated the specific phenotype of renal macrophages and their disease-impacting effects on cystic epithelial cells. We found an abundance of M2-like macrophages in the kidneys of patients with either ADPKD or ARPKD and in the cystic kidneys of *cpk* mice, a model of ARPKD. Renal epithelial cells from either human ADPKD cysts or non-cystic human kidneys promote differentiation of naive macrophages to a distinct M2-like phenotype in culture. Reciprocally, these immune cells stimulate the proliferation of renal tubule cells and microcyst formation *in vitro*. Further, depletion of macrophages from *cpk* mice indicated that macrophages contribute to PKD progression regardless of the genetic etiology. Thus M2-like macrophages are two-pronged progression factors in PKD promoting cyst cell proliferation, cyst growth, and fibrosis. Agents that block the emergence of these cells or their effects in the cystic kidney may be effective therapies for slowing PKD progression.

Keywords

clodronate; M2 macrophage; polycystic kidney disease

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Disclosures

None

Introduction

In response to acute or chronic renal injury, recruited macrophages are important factors both in tissue repair and fibrosis[1, 2]. Of the renal macrophage subpopulations that appear following injury, it is macrophages polarized (differentiated) toward an alternatively activated or "M2-like" phenotype that mediate the early repair and regenerative processes, including the stimulation of tubule epithelial cell proliferation[3, 4]. These macrophages are M2-like in that they share one or more characteristics with *bona fide* M2 macrophages, defined as those that arise from exposure to Th_2-type cytokines IL-4 and/or IL-13[5, 6]. However, because macrophages can alter their phenotype depending on surrounding signals, these renal macrophages are likely to possess distinct phenotypic properties arising from exposure to a complex constellation of stimuli within the kidney microenvironment[6]. With chronic injury, M2-like macrophages may persist to promote fibrosis and scarring. Hence, M2-like macrophages predominate in fibrotic lesions of chronic kidney disease of many different etiologies, where they foster progression to end stage renal disease[7, 8]. In mice, renal M2-like macrophages have been shown to arise from differentiation of inflammatory monocytes that infiltrate the kidney in response to injury[4, 9, 10]. However, the specific renal environmental cues that trigger this differentiation process are unknown.

Polycystic kidney disease (PKD) is a common genetic disorder that is characterized by fluid filled tubular cysts that grow steadily over decades, leading to massive enlargement and distortion of the kidney and progression to renal failure[11, 12]. PKD kidneys reside in a state of chronic injury due to progressive cyst expansion and the resultant compression of the surrounding parenchyma[11, 13]. The autosomal dominant form (ADPKD), which results from mutations in *PKD1* or *PKD2*, encoding the cilia associated polycystin-1 and polycysti- 2, respectively, affects up to 1 in 500 individuals and accounts for about 7.3% of U.S. patients on renal replacement therapy[14, 15]. The autosomal recessive form (ARPKD) affects around 1 in 20,000 and is caused by mutations in *PKHD1*, encoding fibrocystin, which is also cilia-associated. While macrophages have been detected in ADPKD kidneys[16, 17] and shown to comprise approximately 20% of all interstitial cells[16], the specific phenotype of these macrophages have not been examined. In ARPKD kidneys, neither the presence nor characteristics of macrophages have been assessed.

M2-like macrophages have been identified in the kidneys of mouse models of PKD. In orthologous models of ADPKD, which carry deletions in *Pkd1* or *Pkd2*, renal cysts develop postnatally (PN) and at early stages (PN day 24) are accompanied by large numbers of interstitial macrophages, most of which are M2-like[18]. Notably, systemic depletion of macrophages in affected pups by treatment with clodronate liposomes results in kidneys with reduced cystic indices[18]. These findings suggest that macrophages, particularly M2-like macrophages, can promote cyst growth, perhaps by stimulating nearby cyst lining epithelial cells. However, there are no studies in which potential functional interactions between macrophages and PKD cyst epithelial cells have been examined.

Renal M2-like macrophages are also suggested to be present in the cystic kidneys of *cpk/cpk* (congenital polycystic kidney*)* mice[19], a well studied model of ARPKD. The *cpk/cpk* mice, which result from homozygous mutation in *Cys1*, encoding the cilia associated protein

cystin, exhibit rapidly progressing cystic disease that leads to renal failure and death typically by three weeks[20-23]. Notably, gene-expression profile analysis of kidneys from these mice revealed upregulation of genes present in M2 macrophages[19]. However, neither the number of macrophages nor the macrophage phenotype in these cystic kidneys has been examined directly. Also, the potential contribution of renal macrophages to disease progression in this type of PKD has yet to be evaluated.

In this study, we show that large numbers of macrophages expressing the M2 marker CD163 are present in kidneys of patients with both ADPKD and ARPKD. These macrophages are found in interstitial areas, some of which are closely apposed to cysts and, in some areas, infiltrate cyst epithelium. Moreover, we demonstrate that ADPKD cyst epithelial cells promote macrophage differentiation toward a distinct M2-like phenotype and that these macrophages promote proliferation and microcyst formation of ADPKD cyst cells *in vitro*. We also demonstrate that increased macrophages, particularly M2-like macrophages, are present in the cystic kidneys of *cpk/cpk* mice, and that these cells contribute to renal disease progression. These results imply that macrophages are relevant to PKD progression in general, regardless of the genetic abnormality underlying the disease.

Results

M2-like macrophages are present in ADPKD and ARPKD cystic kidneys

To identify macrophages within human PKD kidneys, we performed immunohistochemical analysis using the macrophage antibody HAM56[24] on sections from both ADPKD and ARPKD kidneys. (Figure 1A-B; Supplementary Figure S1A-F). Macrophages were scattered throughout the interstitium, including sites adjacent to cysts; macrophages were found infiltrating occasional cysts (Figure 1A-B, arrows). To determine whether these macrophages were M2-like, we stained consecutive serial sections with an antibody to CD163, a prototypic human M2 macrophage marker[25, 26] that has been associated with chronic injury[26]. The staining pattern was similar to that observed for HAM56 (Figure 1A-B; Supplementary Figure S1A-F). The total number of CD163+ cells in each field was similar to the number of HAM56+ cells in the same field of the adjacent serial section (Supplementary Figure S1H), suggesting that most HAM56+ macrophages were also CD163+. These data confirm the presence of macrophages within both ADPKD and ARPKD kidneys and indicate that many, if not most, exhibit an M2-like phenotype. Sections from non-cystic human kidneys (NHK) were also stained with HAM56 and anti-CD163 (Figure 1C; Supplementary Figure S1G). While far fewer macrophages were present relative to PKD, the ratio of CD163+/HAM56+ cells was similar (Supplementary Figure S1H).

Primary epithelial cells from ADPKD cysts and NHK tubules promote macrophage conversion to a distinct M2-like phenotype

Since mouse renal tubular epithelial cells have been shown to promote M2-like differentiation in co-cultured macrophages[4], we assessed whether human ADPKD cyst cells have similar effects. Primary cells isolated from ADPKD cysts or NHK tubules were co-cultured with primary, bone marrow derived, mouse macrophages (BMDM) or mouse RAW 264.7 cells (RAW; a macrophage like-cell line). Mouse macrophages are known to produce

and respond to many species-cross-reacting factors[27], and their use facilitated simultaneous analysis of epithelial and macrophage transcripts using species specific primers. Co-culture of BMDM with both ADPKD and NHK cells resulted in striking upregulation (>50 fold) of *Arg1*, a prototypic mouse M2 macrophage marker (Figure 2A). This stimulatory effect was especially elevated in ADPKD cell co-cultures (3 fold > NHK). These results indicate that both ADPKD and NHK cells induce macrophages to express at least one prototypic M2 marker and thus could be capable of contributing to macrophage polarizing cues *in vivo*.

To expand the analysis of macrophage phenotype, we assessed expression of other markers, including prototypic M1 markers[5, 6]*iNos* and *Il6*, and the prototypic M2 marker *Mrc1* in addition to *Arg1*[5]. Co-cultures of RAW macrophages with ADPKD or NHK cells robustly induced *Arg1* but had no stimulatory effect on *Mrc1*, while treatment with IL-4/IL-13 induced both *Arg1* and *Mrc1*, as expected. Co-culture did not promote *iNos* or *Il6* expression, both of which were induced by IFN-γ (Figure 2B). We also assessed expression of M2-like cytokine *Il10*. As for *Arg1*, *Il10* was upregulated in RAW cells following co-culture with either ADPKD or NHK cells, with an especially robust effect in ADPKD cell co-cultures (11-fold versus 6-fold in NHK co-cultures; Figure 2C). Collectively, these results indicate that both ADPKD and NHK cells can induce a macrophage phenotype that is M2-like but distinct from the canonical M2 state elicited by IL-4/IL-13.

To assess effects of co-culture on non-naïve macrophages, we co-cultured ADPKD cells with RAW cells that had been first polarized to M1 or M2 by treatment with either IFN-γ or IL-4/IL-13, respectively. For M1 macrophages, co culture with ADPKD cells resulted in robust induction of *Arg1* and a diminishment in the M1 marker *Il6* (Figure 2D, lane 4). For M2 macrophages, ADPKD co-culture enhanced *Arg1* and dramatically reduced *Mrc1* induction (Figure 2D, lane 6). These results indicate that ADPKD cells can promote M2-like conversion of polarized, as well as naïve, macrophages and suggest that renal tubule cells may promote phenotypic conversion of previously polarized macrophages *in vivo*.

M2-like macrophage polarizing activity is mediated by soluble factor(s)

If tubular cells promote M2-like macrophage polarization *in vivo*, then it is likely that soluble factors produced by these cells would mediate this effect, since most of the M2-like macrophages in ADPKD and NHK kidneys are not in direct contact with epithelial cells (Figure 1; Supplementary Figure S1). To test this, RAW cells were treated with conditioned media (CM) from ADPKD and NHK cells. As with direct co-culture, ADPKD CM promoted upregulation of *Arg1* but not *Mrc1* and had no stimulatory effect on the M1 marker *iNos* (Figure 3A). Murine IL-10 secretion was also measured by ELISA. RAW cells without treatment (bar 1, Figure 3B) produced very little IL-10, and neither CM contained significant IL-10 (Figure 3B, bars 3 and 5). However, there was a significant increase in RAW cell IL-10 production following treatment with ADPKD CM, NHK CM, or IL-4/IL-13 (Figure 3B, bars 2, 4, and 6). These results indicate that the M2 like polarizing activity of ADPKD and NHK cells can be mediated by soluble factors and suggest that these cells may provide a source of the differentiation cue(s) that induce the appearance of the M2-like macrophages *in vivo*.

Macrophages promote the proliferation of ADPKD cells in vitro

Co-cultures were also used to assess macrophages effects on cyst cell proliferation, an essential element of disease progression. RAW macrophages were co-cultured with ADPKD or NHK cells for 3 days and proliferation determined by direct cell counting. Proliferation of both ADPKD or NHK cells was significantly stimulated by RAW cells, compared to parallel cultures grown in identical culture media alone, and reached respective levels of 58% and 55% maximal proliferation (i.e., that achieved above basal levels in cultures containing 10% sera; Figure 4A-B). In similar assays, using ADPKD cells from five different kidneys, there were significant proliferative effects stimulated by macrophages in four cases achieving between 45-75% maximal proliferation (Supplementary Figure S2A).

Since *in situ* macrophage conversion to the distinct, M2-like phenotype described above is undoubtedly occurring during the incubation period of these proliferation assays, it was of interest to determine whether the converted M2-like cells were uniquely capable of promoting renal tubule cell proliferation. However, this question is difficult to address because, as was demonstrated (Figure 2D), M2- like conversion is induced not only in naïve macrophages but also in those that have been previously polarized to other phenotypes. Nevertheless, we assessed the proliferative effects of M1 and M2 macrophages for comparison. M1 macrophages had small pro-proliferative effects but none reached statistical significance (Supplementary Figure S2A). On the other hand, M2 macrophages stimulated significant proliferation of ADPKD cells in 2/4 cases (Supplementary Figure S2A). These experiments suggest that within a 3 day co-culture period with ADPKD cells, macrophages that start out initially as naïve (or possibly M2) may be more likely to effect significant proliferation than those polarized previously to an M1 phenotype. We also tested the proliferative effects in these co-cultures of the M2 like macrophages that were previously programmed by exposure to ADPKD cells but found no stimulation beyond that effected by naïve macrophages (not shown). These results are consistent with the idea that the enhanced proliferative effects in these co-culture experiments are mediated by macrophages of the distinct, M2-like phenotype, which arise sooner *in situ* from naïve or M2 versus M1 cells.

Soluble macrophage factor(s) promote ADPKD and NHK cell proliferation

To determine whether the proliferative effects of macrophages are mediated by soluble factors, transwell co-cultures were conducted in which RAW macrophages and ADPKD or NHK cells were physically separated by a membrane that allows free exchange of large, soluble macromolecules while disallowing direct cell-cell contact. In these co-cultures, RAW macrophages significantly stimulated proliferation of ADPKD and NHK cells (39% and 21% maximal proliferation, respectively) (Figure 4C-D; Supplementary Figure S2B). Human macrophages differentiated from a human monocyte like cell line (THP-1) also significantly stimulated ADPKD cell proliferation in transwell co-culture (Supplementary Figure S2C). These combined results indicate that macrophages can promote proliferation of ADPKD or NHK cells via soluble factors. Demonstration of these factors in isolated CMs from macrophage cultures has not been successful thus far (not shown). It may be that the factors mediating these effects are unstable or rapidly inactivated under the conditions used.

Macrophages promote ADPKD cell microcyst growth

Effects of macrophages on cyst cell growth were also examined in co-culture within a collagen matrix. This "3 D" culture system facilitates ADPKD cell microcyst formation[28] and provides a model thought to be more relevant to cystic disease. The presence of RAW macrophages in low numbers (ADPKD:RAW ratios of 6000:240 and 6000:1200) promoted a dramatic increase in the total surface area of microcysts formed (Figure 5A). The presence of high RAW cell numbers (ADPKD:RAW=6000:6000), however, promoted only a minimal and insignificant increase in microcyst area (Figure 5A). Since the media in these co-cultures with high RAW numbers rapidly became acidified between changes (not shown), it seems likely that an environment less conducive to growth may develop with RAW cell overcrowding. Co-cultures in which total microcyst area was significantly enhanced showed no significant increase in the surface area/cyst (Figure 5B), but rather a marked increase in the total number of microcysts (Figure 5C). The most likely explanation for this is that the macrophage pro-proliferative effects may diminish after the expansion of RAW cells and the resultant overcrowding and accompanying negative effects on the growth environment. Regardless, the data indicate that RAW macrophages can promote ADPKD cell microcyst formation *in vitro*. Collectively, these effects of macrophages on proliferation and microcyst growth manifested *in vitro* suggest that renal macrophages may promote ADPKD cyst expansion *in vivo*.

Cystic kidneys of *cpk/cpk* mice have elevated levels of M2-like macrophages

While the characteristics and effects of renal macrophages in mouse cystic kidneys with *Pkd1* or *Pkd2* loss have been recently documented[18], a direct assessment of renal macrophages from cystic kidneys of mice with PKD arising from other genetic lesions has not been conducted. Cystic kidneys of *cpk/cpk* mice show overexpression of M2 macrophage markers[19], suggesting that this type of macrophage is present. To directly assess renal macrophages in *cpk/cpk* mice, cells were isolated from cystic kidneys and analyzed by flow cytometry for F4/80 (murine monocyte/macrophage marker), Cd11c (dendritic cell marker), and Ly6C. Ly6C can distinguish M1-like ($Ly6C^{high}$) from M2-like ($Ly6C^{low}$) macrophages[10] in $F4/80^{+}Cd11c^{-}$ cell populations. There was a marked elevation in renal macrophages ($F4/80^{+}Cd11c^{-}$) in cystic *cpk/cpk* kidneys compared to wild-type (WT) (20% versus 6% of total single cells; Figure 6). Of these *cpk/cpk* kidney macrophages, most (66%) were M2-like ($F4/80^{+}Cd11c^{-}$ $Ly6C^{low}$)(Figure 6; Supplemental Figure S3). These results indicate that, as for cystic kidneys of ARPKD and ADPKD patients, *cpk/cpk* kidneys contain elevated levels of macrophages, most of which are M2-like.

Depletion of macrophages in *cpk/cpk* mice restrains pathological enlargement of kidneys

To assess the contribution of macrophages to *cpk/cpk* disease progression, macrophages were systemically depleted from *cpk/cpk* mice using liposome-encapsulated clodronate, a bisphosphonate commonly used for this purpose[29, 30]. Since cystic disease progresses rapidly in the *cpk/cpk* mice, clodronate or vehicle was administered to 3 day-old offspring of *+/cpk* mating pairs. On PN day 10, the animals were weighed, sacrificed, and prepared for analysis. The *cpk/cpk* mice were significantly smaller than *+/cpk* or WT littermates as expected[22] (75% of *+/cpk* mice and 83% of WT mice; Supplementary Figure S4A) and

clodronate treatment had no effect on the total body weight for all genotypes (Supplementary Figure S4B). Kidneys from vehicle-treated *cpk/cpk* mice were large, pale, and spongy, as expected[22]; kidneys from clodronate-treated animals were smaller and less spongy, with some preservation of the red-brown color characteristic of normal kidneys (Figure 7A). Clodronate treatment significantly reduced the two-kidney to body weight ratio (2K/TBW) of the *cpk/cpk* mice (24% decrease) compared to control (Figure 7B), while there was no significant effect of clodronate on 2K/TBW in *+/cpk* and WT mice.

Macrophage depletion in *cpk/cpk* mice reduces the kidney cortical cyst load and preserves renal function

To assess the mechanisms by which clodronate attenuated cystic kidney enlargement, tissues from *cpk/cpk* mice treated with clodronate or vehicle were analyzed by light microscopy. Macrophage depletion was confirmed using immunohistochemical staining for F4/80, which showed significant depletion of F4/80+ macrophages in the renal cortical regions (54% of controls, Figure 8A-B) and livers (Supplementary Figure S5) of clodronate–treated animals. Examination of kidneys from clodronate-treated *cpk/cpk* mice revealed a marked reduction in cystic area in the kidney cortices compared to vehicle controls (Figure 8C-D). There was no significant difference in the number of cortical cysts in clodronate versus vehicle-treated mice (not shown) indicating that this reduction in cystic area is due to a reduction in the sizes of cysts rather than fewer cysts. This relative preservation of cortex likely accounts for the red-brown, rather than pale color of these kidneys. However, medullary regions showed marked cystic change regardless of treatment (not shown). Thus, the clodronate effects on kidney preservation were mostly apparent in the cortex.

To determine whether the effects of macrophage depletion in *cpk/cpk* mice on cortical cystic index result from decreased cell proliferation, we stained kidney sections with an antibody to Ki-67 antigen, which is present within the nuclei of proliferating cells[31]. The number of Ki-67+ cells per mm^2 of tissue was significantly reduced in kidney sections from the clodronate treated versus vehicle-treated *cpk/cpk* mice (Figure 8E-F). In WT kidneys, clodronate had no significant effect on the number of Ki-67+ cells (Supplementary Figure S6). We also assessed apoptosis by TUNEL staining, but only occasional positive cells were identified within kidneys from both clodronate- and vehicle-treated mice, with no difference between them (Supplementary Figure S7).

The effects of clodronate treatment on renal function were examined. Blood urea nitrogen (BUN) was measured in clodronate and vehicle-treated *cpk/cpk* mice and littermates. Vehicle-treated *cpk/cpk* mice demonstrated markedly elevated BUN levels compared to WT and heterozygous littermates; clodronate treatment resulted in a significant reduction in serum BUN levels (Figure 9). Thus, in addition to promoting preservation of renal cortex, clodronate treatment also preserved renal function.

Discussion

We provide evidence that the vast majority of ADPKD renal macrophages have an M2-like phenotype, which is reported to be the predominant macrophage phenotype in other types of chronic kidney disease[7]. M2-like macrophages also predominate in the cystic kidneys of

orthologous ADPKD mouse models[18]. Extending these results, we show that macrophages within cystic kidneys of both ARPKD patients and ARPKD model mice (*cpk/cpk*) are primarily M2-like. These results suggest that M2-like macrophages may be a prevalent feature of PKD arising from any genetic abnormality.

Most of the resident macrophages in NHKs appear to be M2-like as well (Figure 1C; Supplemental Figure S1G), though there are markedly fewer compared to ARPKD and ADPKD kidneys[16]. M2-like resident renal macrophages have also been identified in WT littermates of mice from ADPKD models[18] and other normal mouse kidneys[10]. Resident M2-like renal macrophages, however, have been shown to be functionally distinct from those M2-like macrophages that arise from infiltration into the kidney and subsequent phenotypic conversion following acute injury[10]. Thus, while the macrophages present in both cystic and non-cystic kidneys express M2 markers, they are likely to be functionally distinct.

Infiltrated M2-like macrophages appear in the kidney in response to injury around day 3-5 post-injury[4, 9, 10]. These macrophages arise by conversion of monocyte precursors recruited from the bone marrow. The cues that promote this differentiation reside within the renal environment, and *in vitro* studies indicate that tubular epithelial cells likely contribute to these cues[4, 9]. The current study demonstrates that epithelial cells both from ADPKD cysts and NHKs convert naïve macrophages to an M2-like phenotype. Furthermore, this M2-like macrophage- converting activity of ADPKD and NHK cells is mediated, at least in part, by soluble factors, which supports the idea that cyst cells can direct M2-like polarization of macrophages *in vivo*, even without direct contact.

Since these M2-like macrophage-converting factors have yet to be identified, it is unknown whether they are expressed by cyst/tubule cells *in vivo*. Notably, though, PKD kidneys are known to highly express injury-associated proteins *in vivo* that, while not found at high levels in uninjured NHKs[32], are expressed by isolated NHK epithelial cells (and PKD cyst cells) *in vitro*[33]. It has been postulated that the isolation procedures for these cells mimic injury conditions[33] and induce expression of injury-associated markers in NHK cells that are already present in PKD cyst cells. Thus, if the M2-like macrophage-converting activity is specifically expressed under injury conditions, its expression in NHK cells *in vitro* may not reflect behavior *in vivo.* While this hypothesis is unproven, it is consistent with the likelihood discussed above that resident M2-like macrophages in NHKs are functionally different from the infiltrating M2-like macrophages present following injury[10] or in chronic kidney diseases like PKD.

Another important finding of this study is that M2-like macrophages promote *in vitro* proliferation of both ADPKD and NHK cells and microcyst formation of the ADPKD cyst cells. Furthermore, the proliferative effect of these M2-like macrophages does not require cell-cell contact but can be mediated via soluble factor(s). Since ADPKD cyst expansion depends on proliferation of the cyst lining cells, proliferative effects of similar M2-like macrophages present in ADPKD kidneys can potentially promote cyst growth. In addition, growth of non-cystic tubule cells in these kidneys could be similarly stimulated, given the similar proliferative effect of these M2-like macrophages on NHK cells *in vitro*. Indeed, an

increase in Ki-67 labeling has been demonstrated in both the cyst-lining cells and non-cystic tubules of ADPKD kidneys[34]. In contrast, very few tubule cells in NHKs are positive for proliferation markers[16, 34], which supports the notion that resident M2-like macrophages are functionally distinct from the M2-like macrophages in ADPKD or injured kidneys. Alternatively, they may be present at levels too low to elicit proliferative changes.

The normal function of renal M2-like macrophages in injury is to promote tissue remodeling and regeneration. Depletion of macrophages delays repair and diminishes tubular cell regeneration and proliferation[4, 35]. Also, by reintroducing macrophages into mice during the repair phase, M2 macrophages have been shown to have the capacity to stimulate tubular cell proliferation[4]. As wound healing agents, then, renal M2-like macrophages stimulate tubule epithelial cell regeneration to promote repair. Our data suggest that in the setting of ADPKD M2-like macrophages become pathologic promoters of disease progression. In this regard, it is notable that acute kidney injury has been shown to accelerate the onset of cystogenesis in mouse models of PKD[36-38], but the contribution of macrophages to this effect has not been determined.

The proposed role of M2-like macrophages in PKD cyst expansion and disease progression suggested by these studies is supported by studies in an orthologous ADPKD mouse model[18]. As with this study in *cpk/cpk* mice, the kidneys in that model contain abundant M2-like macrophages, and systemic depletion of them restrains cyst growth and partially preserves renal function. The current study extends these observations to demonstrate both the presence of M2-like macrophages in human PKD and the functional interactions between cyst cells and macrophages. Collectively, the data point to the presence of macrophages, in particularly M2-like macrophages, as promoters of cyst expansion and disease progression in PKD, regardless of the specific genetic abnormality underlying the disease. Moreover, the data imply that the processes that regulate the appearance and pro-proliferative effects of these M2-like macrophages in PKD may provide effective therapeutic targets to slow disease progression.

Methods

An expanded version of Methods is included in *Supplementary Methods*. Detailed methods for histology, immunohistochemistry, cell culture, qRT-PCR, and flow cytometry are included. All animal experiments were approved by IACUC, and the use of human tissue was approved by IRB. Data are presented as the mean ± SEM and were compared using the two-tailed *t* test calculated with Prism (v4.0, GraphPad, La Jolla, CA). *P* values of < 0.05 were considered significant, as indicated in the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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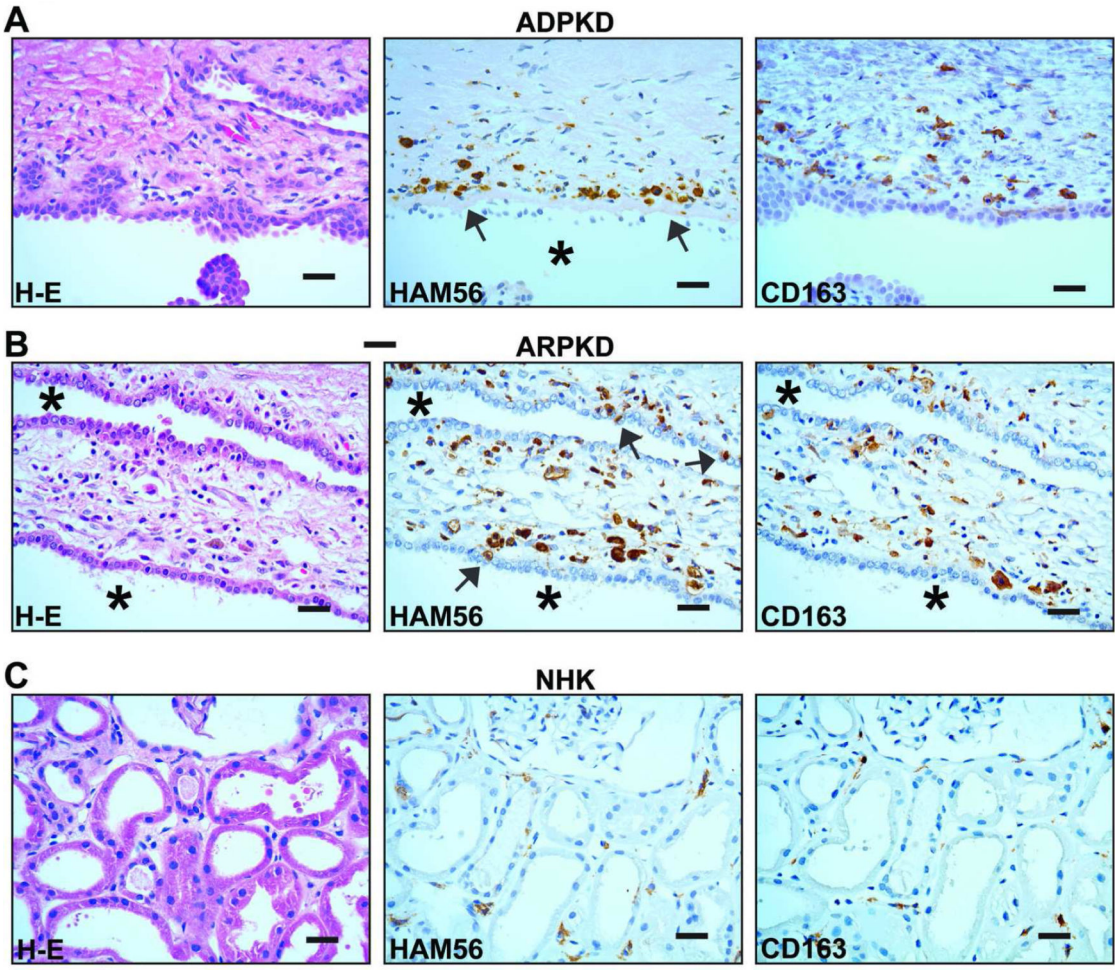

Figure 1. Macrophages are present in ADPKD and ARPKD kidney tissue

Formalin-fixed, paraffin-embedded tissues from ADPKD (kidney K239) (**A**) and ARPKD (kidney K275) (**B**) kidney, as well as non-cystic kidney (**C**) 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 CD163 (**right**). Scale bars represent 25 μm. Cystic space is indicated by *. The arrows indicate macrophage infiltration of cystic epithelium.

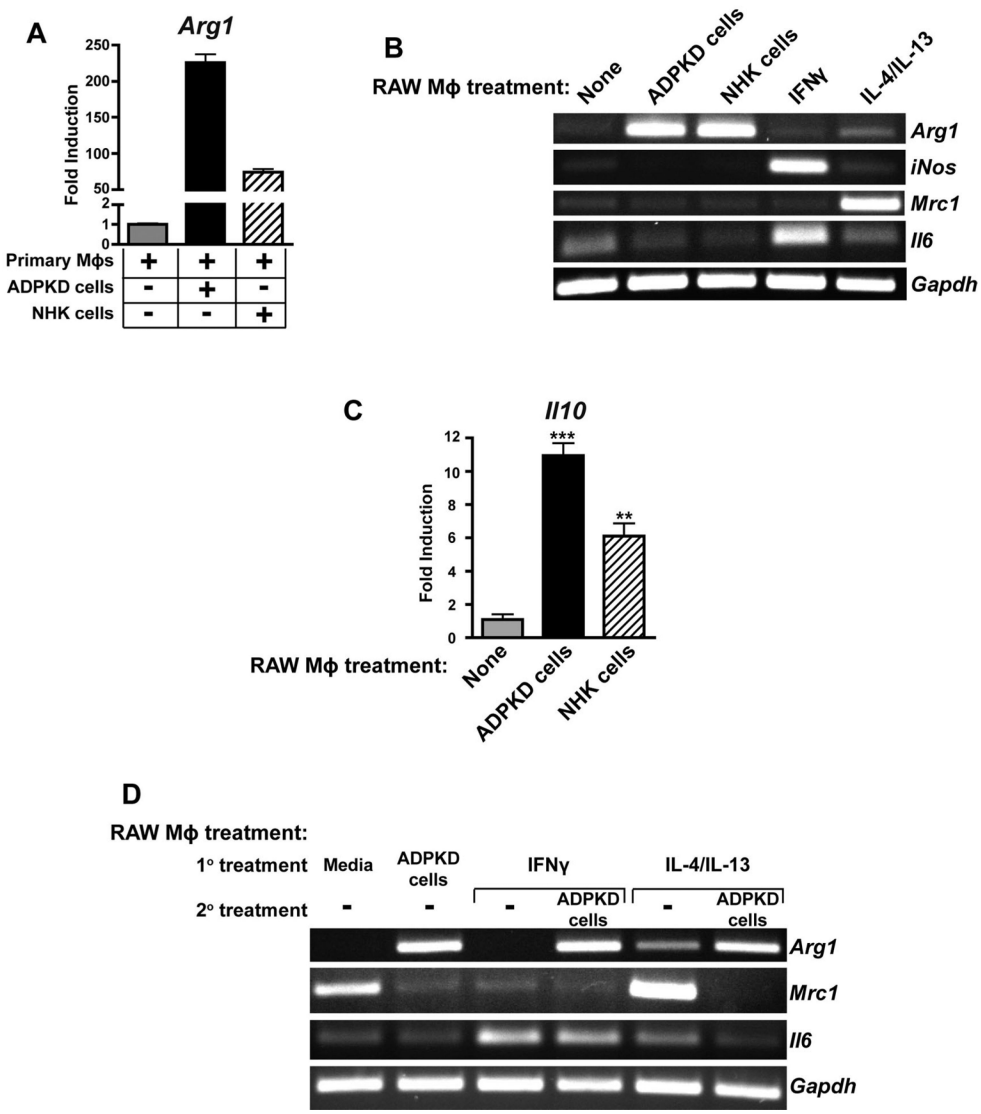

Figure 2. Primary ADPKD or NHK cells promote macrophage conversion to a distinct M2-like phenotype

(A) Primary BMDMs were co-cultured with ADPKD or NHK cells for 4 d prior to RNA isolation and analysis of mouse *Arg1* by qRT-PCR. The data represent the mean ± SEM of 3 determinations. **(B)** Semi quantitative RT-PCR of the indicated mouse gene transcripts following 18 h co-culture of RAW macrophages with ADPKD cells, NHK cells, IFNγ (20 ng/ml) or IL-4 and IL-13 (20 ng/ml each). Similar experiments carried out using ADPKD cells from 6 different kidneys and NHK cells from 3 different kidneys showed similar levels of *Arg1* induction (data not shown). **(C)** RAW cells were co-cultured with ADPKD or NHK cells for 12 h prior to RNA isolation and analysis of mouse *Il10* by qRT-PCR. Data are presented as mean ± SEM and are representative of experiments with ADPKD and NHK cells from two different kidneys each. (**D**) Semi quantitative RT-PCR of the indicated mouse gene transcripts from lysed cell samples following 18 h culture of RAW macrophages with media (lane 1), with ADPKD cells (lane 2), or following treatment with either IFNγ (lanes 3 4) or IL-4 and IL-13 (lanes 5-6) for 18 h (as in **B**) prior to either no further treatment (lanes

3 and 5) or a secondary treatment with ADPKD cell co-culture for 3 d (lanes 4 and 6). ** and *** denote $P<0.01$ and $P<0.001$, respectively.

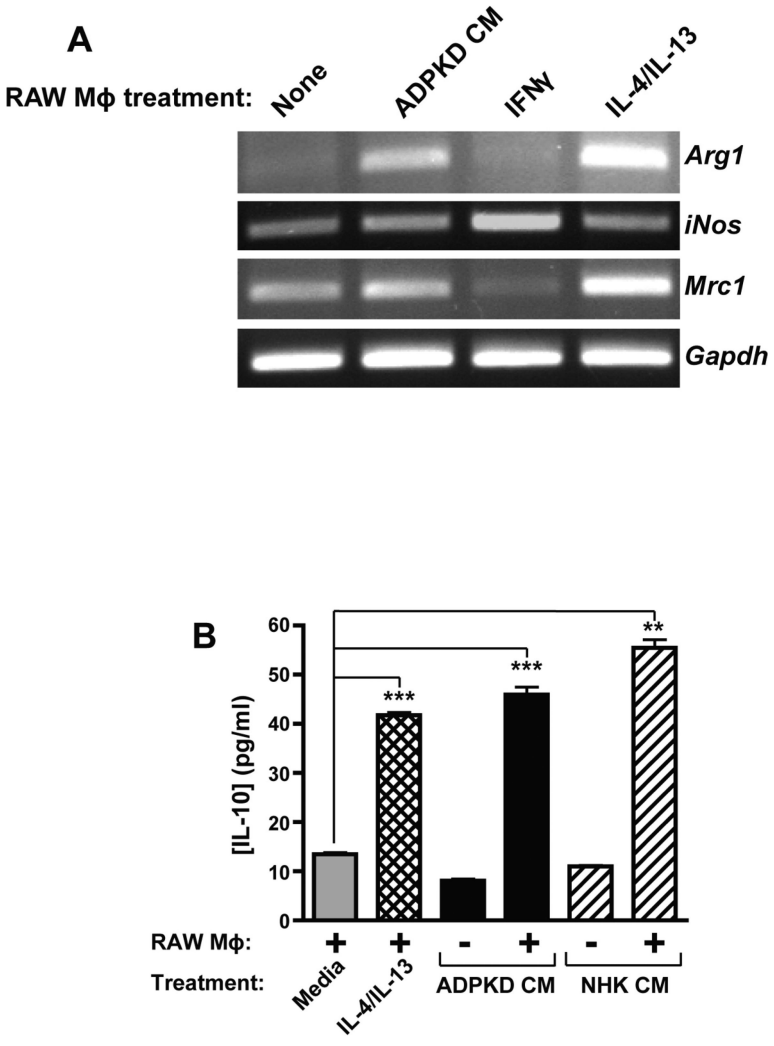

Figure 3.

(A) Semi-quantitative RT-PCR of indicated mouse gene transcripts following 18 h culture of RAW cells with ADPKD CM, IFNγ (20 ng/ml) or IL-4/IL-13 (20 ng/ml each). Experiment was carried out using CMs derived from ADPKD cells from 6 different kidneys and NHK cells from 3 different kidneys, and similar induced levels of *Arg1* were detected. **(B)** Mouse IL-10 concentration was measured in culture supernatants from RAW cells following 18 h incubation in either media with no additions, media containing IL-4/IL-13, ADPKD CM or NHK CM. Data are presented as mean ± SEM. The experiment was repeated using CMs from ADPKD cells derived from two additional different kidneys with similar results. ** and *** denote $P<0.01$ and $P<0.001$, respectively.

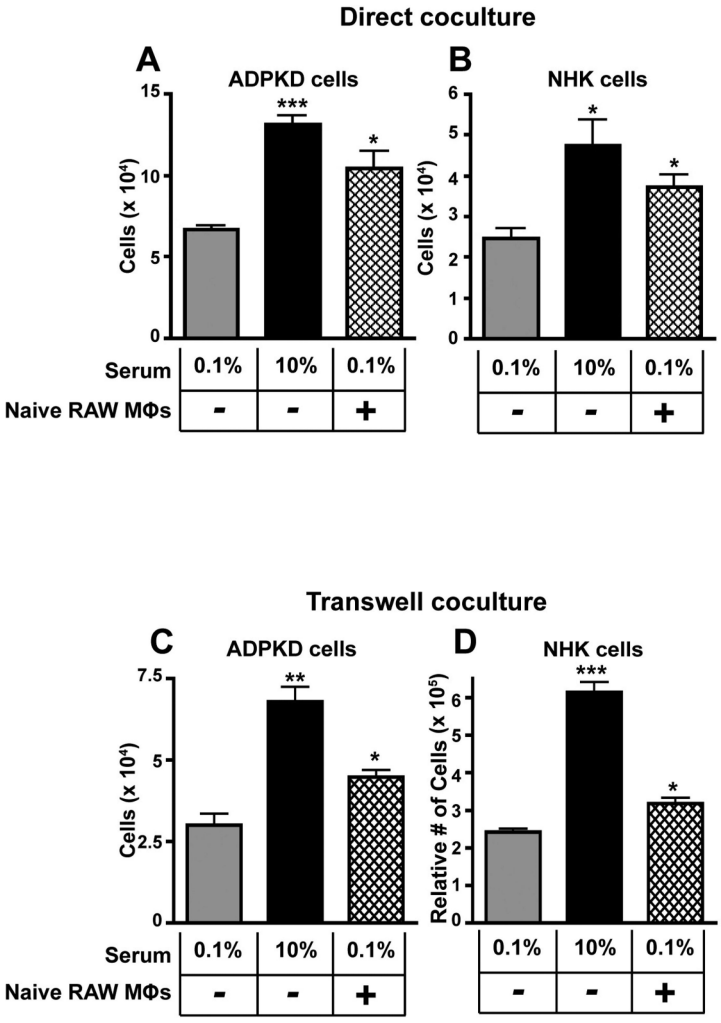

Figure 4. Macrophages elaborate soluble factor(s) that promote ADPKD cyst cell and NHK proliferation

(A) Primary ADPKD cyst epithelial cells (kidney K354) or **(B)** NHK cells (kidney K342) were incubated with low (1% FBS) or high (10% FBS) serum media or co-cultured with naïve RAW macrophages suspended in low serum media. After 72 h, cells were collected and fixed and the number of ADPKD cells determined by counting. The data are representative of four similar experiments for ADPKD cells from different kidneys (Supplementary Figure S2). **(C and D)** Cell-impermeable transwell inserts were placed in tissue culture wells previously seeded with primary ADPKD (**C**) or NHK (**D**) cells (Kidneys K338 and K343, respectively). Naïve RAW macrophages in low serum, low serum media alone, or high serum media were placed in these inserts, and the kidney cells were collected after 72 h and counted (C) or relative number of cells determined by lysis and incubation with CyQUANT® GR dye (D). Data are presented as mean ± SEM. *, **, and *** denote $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

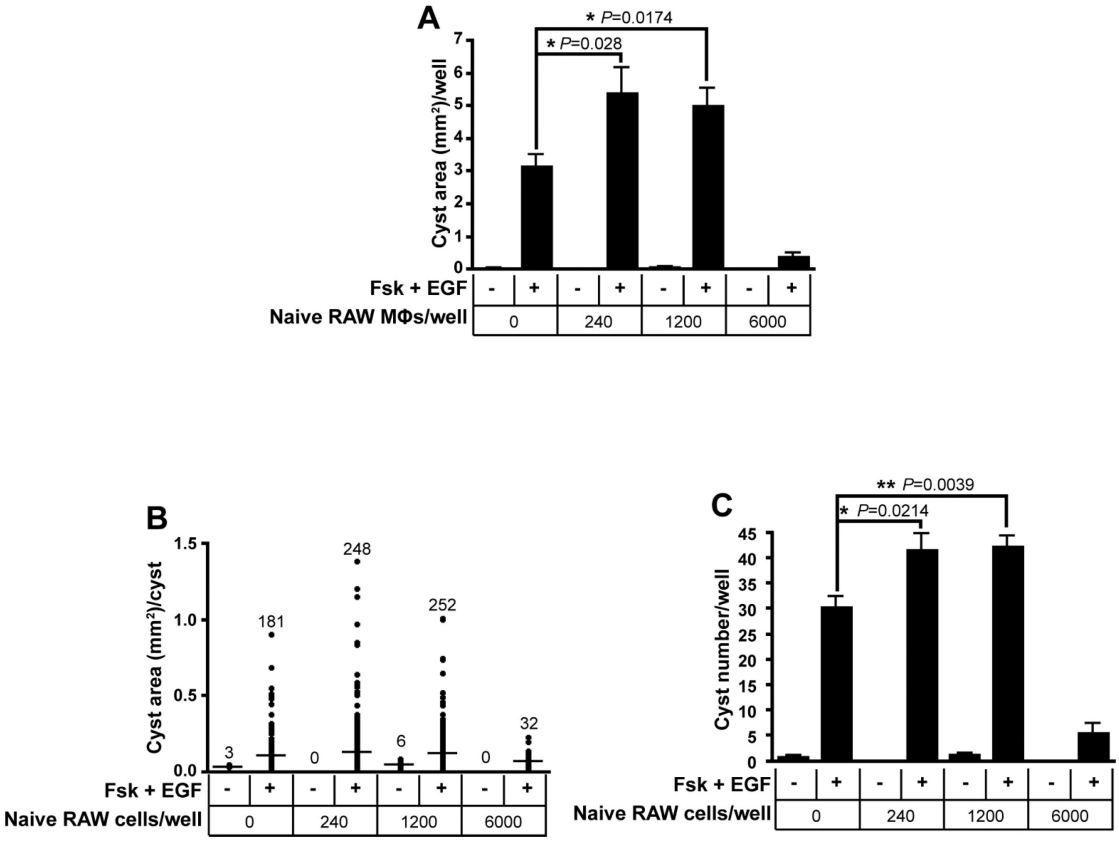

Figure 5. Macrophages promote ADPKD microcyst growth

ADPKD cyst epithelial cells (6000 per sample) and increasing numbers of RAW cells were seeded within a collagen gel and incubated in the presence or absence of forskolin (5 μM) plus EGF (5 ng/ml) for 10 days to allow microcyst formation and expansion. Shown is **(A)** the total surface area of microcysts/well (>50μM in diameter)**; (B)** surface area per microcyst (mean indicated by lines; total number of microcysts shown on top for each sample); and **(C)** number of microcysts/well. The data are representative of 4 independent experiments using cells from different ADPKD kidneys.

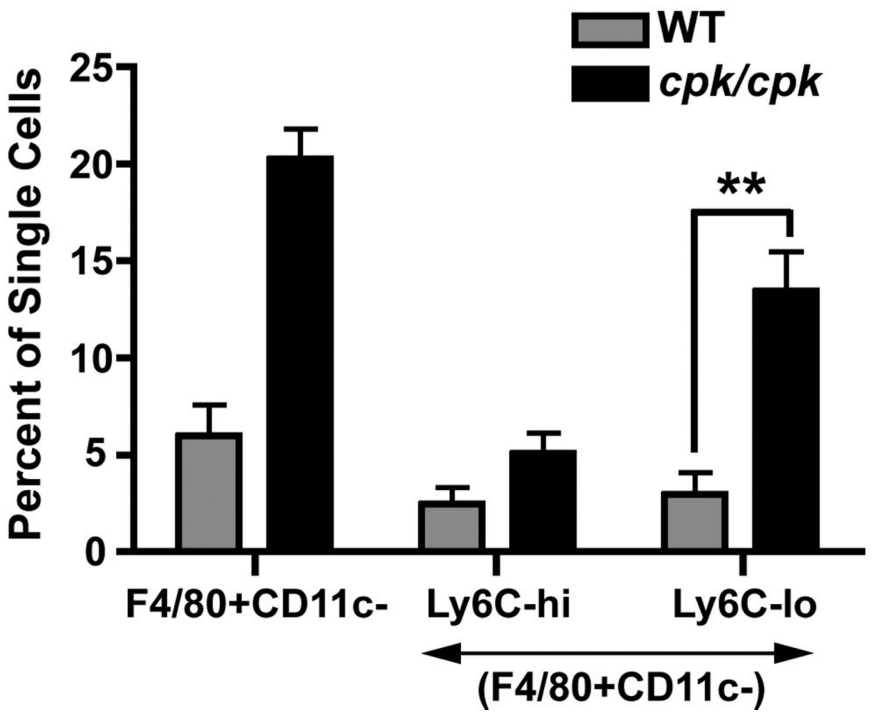

Figure 6. Cystic *cpk/cpk* kidneys contain elevated numbers of macrophages that are mostly M2-like

Quantitative flow cytometry analysis of single, live cells isolated from WT (gray bars) and *cpk/cpk* (black bars) kidneys. Shown are the percentages of single cells that are macrophages ($F4/80^{+}CD11c^{-}$) (**left**), M1-like macrophages ($F4/80^{+}CD11c^{-}$ Ly6C-hi) (**middle**), and M2-like macrophages ($F4/80^{+}CD11c$Ly6C-lo) (**right**). Data are presented as mean ± SEM. Kidneys from a total of 9 *cpk/cpk* and 8 WT mice were analyzed. Representative flow cytometry plots of these analyses are in Supplemental Figure S3. ** denotes $P<0.01$.

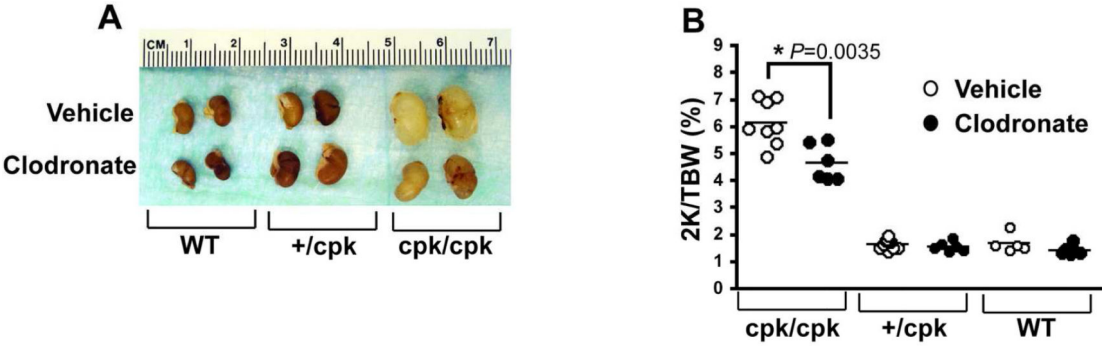

Figure 7. Clodronate liposome treatment restrains cystic kidney enlargement in *cpk/cpk* mice

(**A**) Kidneys were harvested from PN10 mice (WT, +/*cpk*, or *cpk*/*cpk*, as indicated) treated with either vehicle (top row) or clodronate (bottom row). The kidneys are representative of at least 6 animals per condition. (**B**) Shown are the two-kidney/body weight ratios of PN10 kidneys harvested from mice of the indicated genotypes treated either with vehicle or clodronate.

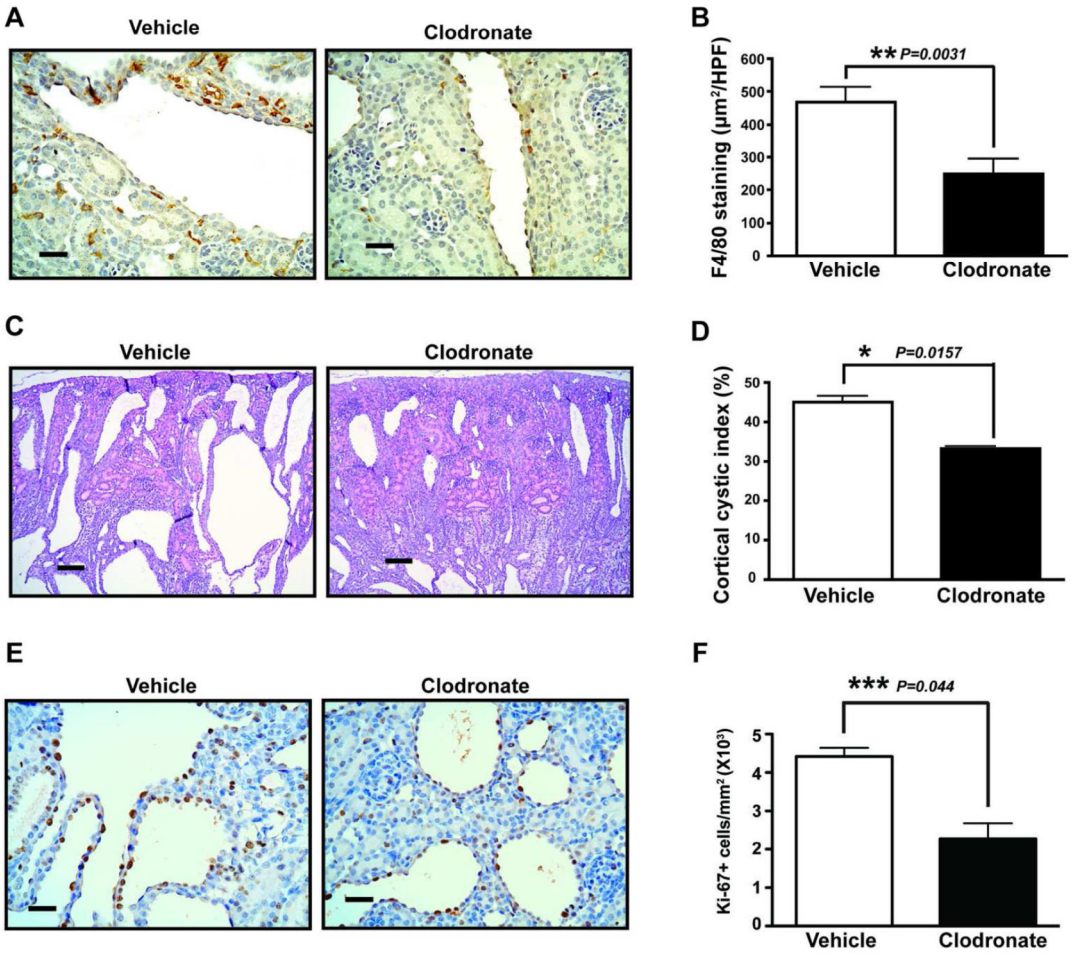

Figure 8. Clodronate liposome treatment of *cpk/cpk* mice reduces macrophage load,renal cortical cyst area, and renal cortical cell proliferation

(**A**) Formalin-fixed, paraffin-embedded kidney tissues from PN10 *cpk*/*cpk* mice treated with either vehicle (**left**) or clodronate liposomes (**right**) were sectioned and stained with hematoxylin and eosin. Each image is a representative field from one mouse of four examined per condition. Scale bars represent 100 µm. (**B**) Average cortical cystic index (cortical cyst area/total cortical area) was calculated from measurements of kidneys from animals treated with vehicle or clodronate liposomes, two per condition. (**C**) Formalin-fixed, paraffin-embedded kidney tissues from PN10 *cpk*/*cpk* mice treated with vehicle (**left**) or clodronate (**right**) were sectioned and stained by immunohistochemistry using a monoclonal antibody against F4/80. A representative image from one of two different mice for each condition is shown. Scale bars represent 25 µm. (**D**) The mean area (μm^2) of F4/80 staining per high-powered field (HPF) in sections of kidney cortex described in **C** was measured from 2 kidneys each of mice treated with either vehicle or clodronate liposomes. (**E**) Formalin-fixed, paraffin-embedded kidney tissues from PN10 *cpk*/*cpk* mice treated with vehicle (**left**) or clodronate (**right**) or were stained with an antibody to Ki-67. A representative image from one of two mice per condition is shown. Scale bars represent 25 µm. (**F**) Ki-67+ cells/mm^2 tissue in PN10 *cpk*/*cpk* mice treated with either vehicle or clodronate.

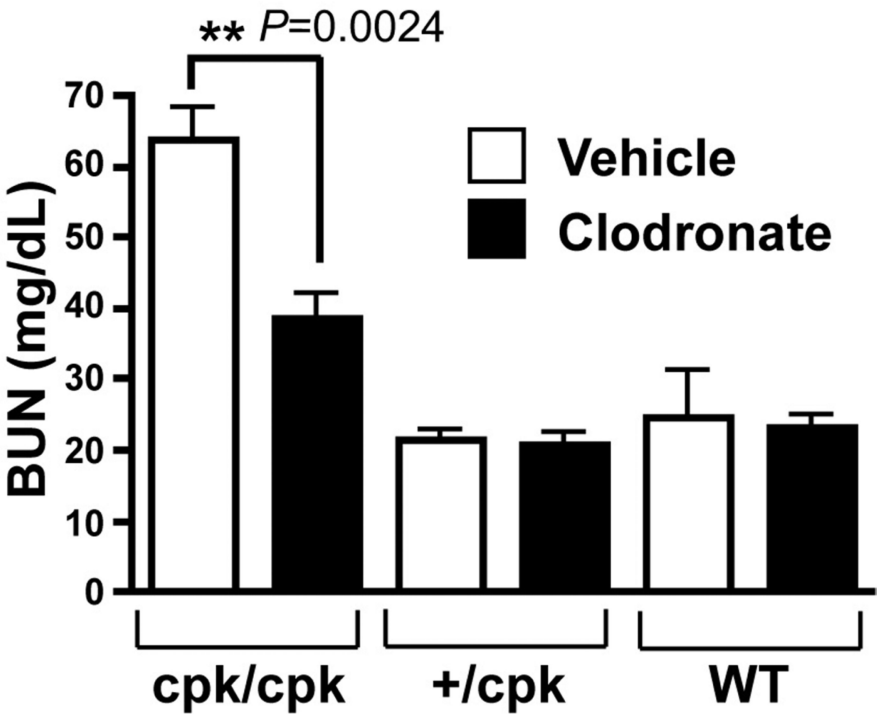

Figure 9. Clodronate treatment of cpk/cpk mice improves kidney function

Serum BUN concentration was measured in PN10 mice of the indicated genotypes (n=3-6 mice/condition) treated with either vehicle or clodronate, as indicated.