Renal CD14 expression correlates with the progression of cystic kidney disease

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Abstract

Monocyte and macrophage markers are among the most highly overexpressed genes in *cpk* mouse kidneys with severely progressive renal cystic disease. We now demonstrate that one of these markers, CD14, is abnormally transcribed, activated and shed in cystic kidneys. However, these abnormalities are not associated with an increased number of interstitial CD14-positive mononuclear cells. Instead, we show that most non-cystic and cystic renal tubular epithelia are CD14-positive and that CD14 can be produced even by distal nephron-derived principal cells. *Cd14* overexpression is significant in as early as 5-d old sporadically cystic *cpk* kidneys and it further increases during the disease progression. Similarly, in a *cpk* model with variable rates of cystic kidney disease progression, a (C57BL/6J-*cpk* × CAST/Ei)F1 intercross, *Cd14* expression positively correlates with kidney volume in 10-d old mice, exceeding the correlation of a gene encoding an established autosomal dominant polycystic kidney disease (ADPKD) marker, MCP-1 (r=0.94 vs. r=0.79; both p<0.001). Similarly, in a small group of ADPKD patients (n=16), baseline urinary CD14 levels (but not GFR) correlate with a two-year rate of total kidney volume change (overall r=0.43, p=0.09; for males r=0.74, p=0.02) suggesting potential utility of CD14 in predicting ADPKD outcomes.

Keywords

polycystic kidney disease; *cpk* mouse; innate immune response; CD14; biomarkers

INTRODUCTION

Polycystic kidney disease (PKD) is a major cause of end-stage renal disease in children and adults.[1] It affects over 600,000 people in the US and 12.5 million worldwide. Autosomal dominant PKD (ADPKD; MIM 173900; 173910) occurs in 1:400 to 1:1,000 individuals. ADPKD is caused by mutations in one of two genes, *PKD1* or *PKD2*.[2–5] Autosomal

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recessive PKD (RPKD; MIM 263200) occurs in 1:20,000 live births and is caused by defects in a single gene, *PKHD1*.[6, 7]

Innate immune system abnormalities are a dominant feature of both ADPKD and RPKD. In ADPKD, renal parenchyma is infiltrated by mononuclear cells,[8] perhaps in response to accelerated production of monocyte chemotactic protein 1 (MCP-1).[9, 10] We have observed monocytic infiltrates also in recessive PKD (RPKD).[11] Until recently, these PKD-related innate immune response abnormalities were thought to be associated with advanced stages of the disease progression and were not considered to have a substantial impact on clinical outcomes. Discovery of abnormal urinary excretion of MCP-1 in early disease stages in ADPKD patients and an animal model [9, 10] changed this paradigm by demonstrating that innate immunity is altered early in the disease pathogenesis. In addition, several recent lines of evidence suggest that immune responses directly promote cystogenesis, at least in animal models.[12–15]

To further elucidate the role of innate immune cells in PKD pathogenesis, we studied *cpk* mouse model of RPKD with variable rates of cystic kidney disease progression due to admixture of two genetic backgrounds.[16] In this model we have identified sixty monocyte/macrophage-associated markers that are over-expressed in kidneys from *cpk* mice with severely vs. mildly progressive cystic kidney disease.[11] An overexpression of macrophage markers associated with a wound healing- and fibrosis-promoting alternative activation pathway suggests that a PKD-associated mononuclear cell-like response contributes to the pathogenesis of interstitial fibrosis, a typical feature of advanced PKD. This hypothesis is consistent with the belief that interstitial inflammation is the leading cause of renal dysfunction in PKD.[17, 18]

The substantial magnitude of PKD-associated innate immune abnormalities was recently revealed by genome-wide transcription profiling studies. For example, in the *cpk* mouse model, genes encoding markers of macrophages, together with additional innate immune factors, represent the most highly over-expressed group of genes in a severely progressive cystic kidney disease.[11] Similar abnormalities were revealed by genome-wide expression profiling study of Han:SPRD-*Cy* rat kidneys that were harvested months before measurable changes in renal function.[19] The specific role of immunity in PKD pathogenesis is further suggested by cystogenesis-inhibiting effects of several immunosuppressive drugs (e.g., glucocorticoids, mycophenolate mofetil, and mTOR and TNFα inhibitors). [12–15]

In the current study, we characterize PKD-associated expression of CD14, a widely used marker of mature monocytes and macrophages and one of the most highly over-expressed genes in *cpk* mice with severely vs. mildly progressive cystic renal disease.[11] CD14 is a pattern recognition receptor[20] that operates in conjunction with Toll-like family of receptors (summarized in Kim *et al.*[21]) as a high affinity homing receptor for lipopolysacharide (LPS) playing a key role in LPS-induced immune response activation. However, it may also bind apoptotic cells and fungi. CD14 is highly expressed in mature monocytes and macrophages and to a lesser degree in a wide range of non-myeloid cell types including proximal tubular cells (summarized in Morrissey[22]). It is also found in milk, cerebrospinal fluid, serum and urine.

CD14 was originally described in mononuclear cells as a 55 kDa protein anchored to cell surface by linkage to glycosylphosphatidylinositol (GPI; mCD14).[23] However, after activation of these cells (e.g., by LPS), soluble CD14 can be released in large quantities either by protease-dependent shedding (as approximately a 48 kDa fragment) or by protease-independent release of a larger (approximately 55 kDa) protein that escaped

posttranslational modifications (including attachment of the GPI anchor)[24] (see schema; Figure 1).

In the current study, we present a comprehensive analysis of CD14 expression in kidneys of 10-d old *cpk* mice and its relationship to rates of renal cystic disease progression. We also characterize postnatal *Cd14* gene expression in *cpk* and wild type mice. Finally, we examine CD14 protein content in mouse and human cystic kidneys and explore CD14's potential as a putative marker for predicting rates of change in kidney volume in ADPKD.

RESULTS

Cd14 expression correlates with rates of renal cystic disease progression in *cpk* mice

We examined *Cd14* gene expression profiles of cystic kidneys from 10-d old mice selected among an F2 cohort of affected *cpk* mice (n=461) that were generated in an (C57BL/6J-*cpk*/ + × CAST)F1 intercross.[16] This intercross represents a model that provides variable rates of cystic kidney disease progression. Previously, we generated genome-wide renal expression analyses of the 7 most mildly affected and 7 most severely affected mice from this cross.[11] In the current study, we examined renal *Cd14* expression in the 7 most mildly affected mice, 8 mice selected evenly across phenotypic spectrum of renal cystic disease severity (defined by kidney length, weight and volume),[16] and an additional 7 unaffected mice. *Cd14* expression in these kidneys determined with quantitative TaqMan® assays correlated strongly with kidney volumes ((r=0.94, p<0.001; Figure 2a), resembling closely our initial Affymetrix 430 2.0 array-based *Cd14* expression analyses (data not shown). However, there was a gender difference in these correlations (r=0.95 and p<0.001 for males, r=0.74 and p=0.02 for females).

Cd14 expression was more strongly correlated with kidney volume than expression of *Ccl2,* which showed moderately strong correlation with kidney volume (r=0.79, p<0.001; Figure 2b). *Ccl2* encodes MCP-1, the only surrogate marker of PKD progression extensively validated in ADPKD patients and an animal PKD model.[9, 10] Similar to the *Cd14* expression data, these analyses closely resembled Affymetrix 430 2.0 array data generated during our initial evaluation of *Ccl2* expression (data not shown).

Renal *Cd14* expression in *cpk* mice is abnormally increased in early postnatal period

The above renal *Cd14* gene expression data reflect a snapshot at 10-days of age across a spectrum of renal cystic phenotypes resulting from variable rates of disease progression. In the *cpk* model, these variable rates were induced by different admixtures of two distinct genetic backgrounds in the (C57BL/6J-*cpk*/+ × CAST)F1 intercross. To better characterize the temporal course of *Cd14* expression during cystic disease progression, we examined its expression in 0-, 5-, 10-, 15-, 20- and 25-day old (P0-P25) kidneys from *cpk* and wild-type mice with an exclusive C57BL/6J genetic background. Consistent with previous observations of developmental regulation of CD14 expression in humans,[25] these analyses revealed that renal *Cd14* expression in wild type mice is developmentally regulated. Specifically, in the wild type kidneys *Cd14* expression increased over 4-fold during the first 3 weeks of life (Figure 2c). In *cpk* kidneys, increase in *Cd14* expression was even more accelerated, leading to a significant (1.4-fold; p<0.05) difference between *cpk* and wild-type kidneys by 5-days of age (Figure 2c). The *Cd14* over-expression in *cpk* kidneys further increased with progressing age until P25, the last scored timepoint before presumable death due to uremia around 4 wks of age. In contrast, over-expression of *Ccl2*, an MCP-1 encoding gene, in *cpk* vs. wild type kidneys was less prominent and more variable (Figure 2d).

CD14+ mononuclear cell content is not significantly increased in *cpk* kidneys

Since monocytes and macrophages are major sources of CD14, we examined the numbers of these cells in 10-d old *cpk* kidneys. Staining with several anti-CD14 antibodies identified interstitial monocyte- and macrophage-like cells in both cystic *cpk* and wild-type kidneys. In contrast to *Cd14* overexpression in *cpk* kidneys, the number of strongly CD14-positive interstitial cells per mm^2 tissue (excluding cyst and tubular lumina) was not significantly increased in the cortex of *cpk* vs. wild-type kidneys, and it was significantly decreased in medulla of *cpk* vs. wild-type kidneys (Figure 3a–b). These data indicate that increased levels of *Cd14* gene expression in *cpk* kidneys cannot be attributed to an increased number of interstitial CD14-positive mononuclear cells.

CD14 can be produced by cystic and non-cystic renal tubular epithelia

In addition to monocytes and macrophages, CD14 can be expressed by non-myeloid cells such as endothelial cells and proximal tubular cells (summarized in Morissey[22]). To determine whether these non-myeloid cells participate in CD14-associated innate immune responses in PKD, we compared patterns of antigenic CD14 expression/deposition in kidneys from 10-d old *cpk* mice vs. wild-type littermates. Similar to previous studies demonstrating that proximal tubular cells can produce CD14,[22] our immunohistochemical analyses revealed varying intensities of CD14 protein staining that were detected in non-cystic tubular epithelia of wild-type and *cpk* kidneys (Figure 3a). In addition, cell membrane and cystoplasm of epithelial cell lining of most *cpk* cysts also stained with anti-CD14 antibody and the intensity of this staining was stronger in kidneys with more advanced disease (P15 and P20; Supplementary figure 1). This CD14 production by principal cells of collecting duct that form epithelial cell lining of most RPKD cysts has not been previously described. The capacity of these cyst-forming epithelial cells to produce CD14 protein is further supported by the presence of both *Cd14* mRNA and CD14 protein in principal cells of a SV40-immortalized internal medullary collecting duct cell line mIMCD-K2 (Figure 3c).

cpk kidneys contain increased levels of shedded CD14 fragments

To explore whether the PKD-associated increase in *Cd14* gene expression translates also to an increased generation and/or processing of the CD14 protein, we examined kidneys of 10-d old *cpk* and wild type mice by immunoblotting with anti-CD14 antibodies. In contrast to the gene expression profiling data, immunoblotting analyses showed decreased levels of total CD14 protein in *cpk* kidneys (Figure 4). However, the content of the 48 kDa CD14 variant that is generated by proteolytic shedding from immunologically activated or injured cells[26] was significantly increased in the *cpk* vs. wild type kidneys (Figure 4).

CD14 processing is abnormal in human RPKD kidneys

Informed by CD14 expression and processing abnormalities in the *cpk* mouse, we also characterized CD14 expression in human PKD kidneys. First, we tested the expression of antigenic CD14 in kidneys from ~21-wk old control and RPKD fetuses. Similar to the 10-d old *cpk* kidneys, the RPKD kidneys contained strongly CD14-positive interstitial cells. However, cytoplasmic staining of human fetal cystic and non-cystic renal tubular cells was more intense when compared to CD14-positive interstitial cells (Figure 5a, left panel). Immunoblotting analyses of RPKD kidneys vs. control fetal kidneys (Figure 5b, left panel) closely parallel the lower CD14 content in cystic *cpk* vs. wild-type kidneys. Similarly, immunoprecipitated urine from RPKD patients with mild to moderately advanced disease (CrCl 39–123 ml/min) showed the presence of both soluble CD14 forms (48 and 55 kDa, Figure 5c).

CD14 is abnormally processed and excreted in ADPKD

Consistent with previous reports of interstitial mononuclear cell infiltrates in ADPKD kidneys,[8] we observed the presence of CD14+ mononuclear cells in interstitium of end-stage ADPKD kidneys (Figure 5a, right panel). In contrast to RPKD, CD14 staining of tubular epithelia in ADPKD was more sporadic and epithelial lining of only a subset of renal cysts was formed by CD14-positive cells.

Immunoblotting of human end-stage ADPKD kidneys resembled RPKD data with low CD14 content in cystic vs. normal control kidneys (Figure 5b, right panel). These data suggested that abnormal CD14 shedding in PKD kidneys is followed by its washout to urine or plasma. Consistent with this hypothesis, immunoprecipitated urine from ADPKD patients with mild to moderately advanced disease (CrCl 49–129 ml/min) showed the presence of both soluble CD14 forms (48 and 55 kDa, Figure 5c). The CD14 was present also in cyst fluid in approximately 10 to 100-fold higher concentrations than urinary CD14 levels (determined by ELISA).

CD14, a candidate predictor of ADPKD progression rates

Because *Cd14* gene expression in *cpk* kidneys correlated strongly with rates of renal cystic disease progression (Figure 2), we speculated that urinary levels of shed CD14 may also correlate with rates of disease progression in ADPKD. Quantitative ELISA-based analyses of CD14 levels in a small group of ADPKD patients (n=16; 9 Caucasian males and 7 females; average iothalamate glomerular filtration rate (GFR) 86 ml/min) support this hypothesis. Specifically, baseline urinary CD14 levels correlated with a two-year rate of PKD progression determined by a relative change (second year follow up to baseline ratio) in total kidney volume (TKV; overall r=0.43, p=0.09; for males only r=0.74, p=0.02; Figure 6) suggesting potential utility of urinary CD14 levels for predicting ADPKD outcomes. Adjustment for urinary creatinine decreased these correlations (overall r=0.26, p>0.20, for males r=0.55, p=0.12). In comparison, the correlation of the relative TKV change with the initial iothalamate GFR was not significant (r=(−0.22), p>0.20) in the studied group of patients.

In contrast to CD14, the correlation of urinary MCP1 levels with the rate of ADPKD progression was weak and did not reach statistical significance (overall r=0.19, for males only r=0.21, both p>0.20). Similarly, weaker correlations were obtained between *Ccl2* (vs. *Cd14*) expression and rates of cystic kidney disease progression in *cpk* kidneys (Figure 2).

Since serum represents a potential source of urinary CD14 or, aternatively, renal CD14 may leak to systemic circulation, we also evaluated serum CD14 levels in this ADPKD cohort. The overall correlation of logarithmically adjusted serum CD14 values with a two-year rate of PKD progression determined by a relative TKV change (r=0.43, p=0.09, n=17) resembled correlations of urine CD14 values with TKV change. However, these correlations did not appear to vary by gender (r=0.44 males, r=0.42 females). Correlations between unadjusted as well as adjusted CD14 urinary and serum values were weak and not significant (r<0.25, p>0.20, n=28). Together, these data point to urine and serum CD14 as potentially independent candidate markers of PKD progression. However, these observations require validation on a larger well-characterized ADPKD cohort.

DISCUSSION

We have recently demonstrated that genes encoding CD14 and other innate immune system factors are most highly over-expressed in age-matched *cpk* mice with severe vs. mild rates of renal cystic disease progression.[11] The current study confirmed and extended this initial observation.

Particularly intriguing is the finding of *Cd14* over-expression in 5-d old *cpk* (vs. unaffected) kidneys (Figure 2c) that occurred in very early stages of the disease. Similarity between this early *Cd14* expression pattern and that of *Ccl2*, a gene encoding the potent chemotactic factor MCP-1, which recruits mononuclear cells to sites of injury and inflammation (Figure 2d), suggests that the observed *Cd14* expression abnormalities in cystic kidneys reflect accelerated mononuclear cell recruitment due to increased MCP-1 expression. However, we did not observe significantly higher numbers of CD14+ mononuclear cells in cystic *cpk* kidneys. Instead, our data suggest that alternative CD14-producing (e.g., epithelial) cells are the major source of early CD14 expression abnormalities in cystic kidneys. Since renal epithelial cells from proximal tubules[22] and, as our data suggest, also from distal nephron segments, express CD14, it is likely that both CD14 and MCP-1 expression abnormalities in *cpk* kidneys reflect an independent response of renal tubular cells to the cystic disease-related injury and/or incomplete or aberrant tubular cell differentiation. The absence of early over-expression of genes encoding other macrophage markers (e.g., CD68 or CD163) in 5-d old *cpk* kidneys (data not shown) further supports this hypothesis. Interestingly, the *Cd14* and *Ccl2* expression abnormalities preceded significant changes in expression of *Lcn2* (data not shown), a gene encoding an acute renal tubular injury marker NGAL (summarized by Devarajan[27]). Together, these observations suggest that innate immune abnormalities represent one of the earliest responses exerted by renal tubular cells affected by a cystogenesis-promoting gene defect. It is conceivable that these innate immune responses alter susceptibility of renal tubular cells to cystogenic stimuli. A reduction of cystogenesis by inhibition of TNFα,[15] a downstream component of the CD14/TLR4 signaling pathway (summarized in Togbe *et al.* [28]), is consistent with this hypothesis. This paradigm also suggests that immunosuppressive rather than antiproliferative effects of sirolimus and mycophenolate mofetyl are responsible for cystogenesis inhibiting effects of these drugs.[13, 14]

Triggers of CD14 over-expression in PKD remain to be determined. We speculate that in RPKD, where the cystically dilated renal tubules do not form cysts that are isolated from the nephron, CD14 may be a marker of renal tubular cells' response to injury caused directly by the primary cystogenic defect. Alternatively, CD14 expression may reflect responses to renal epithelia injury caused by secondary or non-renal effects of cystogenic mutations (e.g., hypertension). Since several recent studies suggest that injury and/or renal tubular responses to injury are critical in cystogenesis (summarized in Verdeguer et al[29] and Li et al [15]), it is possible that CD14 levels may be associated with cystogenic potential or "nascent cyst formation". We hypothesize that similar concepts can be applied to ADPKD, but with additional levels of complexity due to different responses in cells that sustained the "second cystogenic hit" (reviewed by Pei[30]) and haploinsufficient ADPKD cells that may form microcysts after an injury.[31] Indeed, CD14 expression, processing and shedding may also reflect other effects of PKD on renal epithelia such as biomechanical effects of cyst expansion.

The specific consequences of increased renal CD14 expression, activation and shedding in PKD remain unknown. It is possible that intratubular CD14, as a potent stimulator of TNFα secretion,[32] promotes cystogenesis primarily by activating the cystogenic TNFα pathway. However, CD14 effects are likely complex. Since the shed CD14 is a known mediator of renal endotoxin-induced tubulointerstitial injury,[33] an endotoxin or other factors that bind to this pattern recognition receptor[20] may also lead to immune activation and/or injury of renal tubular cells in a complex process that resembles the activation of professional immune cells. We speculate that this "immune activation" of renal epithelial cells changes their susceptibility to cystogenic stimuli across developmental stages and that this activation can be triggered by numerous factors that lead to substantial renal distress. For example, it is possible that CD14 activation accelerates cystogenesis before response to PKD defects is

down-regulated at P12-14 (e.g., in an inducible orthologous *Pkd1* model[34]). However, immune stimuli may promote cystogenesis also in mature kidneys with increased susceptibility to PKD defects. Such cystogenesis-promoting effects were observed e.g., after renal ischemia-reperfusion injury[35] which, similarly to our PKD-related observations, leads to CD14 over-expression in renal tubular cells without increasing the number of infiltrating mononuclear cells.[22]

On a molecular level, as a major ligand for Toll-like receptor 4 (TLR4) CD14 may directly transactivate cystogenic pathways (reviewed in Torres and Harris[36]) modulated by TLR4 signaling, e.g., the Wnt pathway[37] or Rho and PI3K pathways (reviewed by Ruse and Knaus[38]). Altered TLR4 signaling may also affect the AP-1 signaling pathway (reviewed in Hu *et al.* [39]) that has been linked to cystogenesis in PKD1 defects.[40] Since TLR4 is expressed across the nephron including proximal tubules and collecting ducts (summarized in Good *et al*[41]), it is possible that renal tubular cell-derived CD14 may activate TLR4 locally in a paracrine (perhaps even autocrine) fashion. Alternatively, filtered or exosomal CD14[42] (that may be in part proximal nephron-derived) may exert more distal effects, e.g., by activating TLR4 on principal cells of collecting duct, a major cyst-forming cell type.

Additional evidence supporting the role of CD14/TLR4 signaling in PKD pathogenesis stems from the fact that the *Tlr4* gene is a candidate modifier of cystic kidney disease. It fulfills major modifier gene criteria established by the Complex Trait Consortium[43]: 1) it maps to 33 cM on chromosome 4 under the main quantitative trait locus for renal cystic and billiary phenotypes in *cpk* mice;[16] 2) its gene expression (data not shown) as well as the expression of its major ligand CD14 (Figure 2) correlates with the rates of cystic disease severity in *cpk* mice; 3) the *Tlr4* gene has C57BL/6J and CAST/Ei strain-specific haplotypes with several functionally relevant variants; 4) it is predominantly expressed in the newborn period (according to EST Profile Viewer; http://www.ncbi.nlm.nih.gov/unigene) a time period most relevant to the studied modifier gene effect;[16] 5) it is expressed by both renal tubular and biliary epithelial cells;[44, 45] and finally 6) TLR4 is a major regulator of cystogenic factor TNFα.[15] However, *Tlr4*'s modifier gene effects have to be validated by functional studies.

In the current study we observed strong correlations between *Cd14* expression and 10-d *cpk* kidney volumes that reflect rates of cystic disease progression in this model. To determine whether similar association between CD14 expression and the rate of disease progression exist also in ADPKD, we evaluated correlations between rates of cystogenesis (expressed as relative change in total kidney volume (TKV) over a two-year period) in a small group (n=16) of well-characterized patients selected from participants of the Emory ADPKD Cohort Study. Similar to studies that showed significant associations of unadjusted urine neutrophil gelatinase-associated lipocalin (NGAL) levels with high cyst growth rate in ADPKD[46] and faster doubling of serum creatinine in other chronic kidney disorders[47], our analyses presented in this manuscript revealed moderately strong correlations between unadjusted urinary CD14 levels and the rate of ADPKD (TKV) progression. However, our analyses are limited by small sample size that did not allow the evaluation of multiple covariate effects. Therefore, validation of these data has to be performed on a larger well-characterized cohort of ADPKD patients. Ideally, such analyses should also determine whether random or timed urinary CD14 excretion can be used as a short-term marker of disease activity that cannot be revealed by imaging studies due to too short follow up intervals. The use of CD14 as such a marker may allow a more accurate titration of supportive or future PKD specific therapies and may complement additional emerging markers of PKD progression, such as renal volume[48] or specific small peptides detected by capillary electrophoresis coupled to mass spectrometry (CE-MS).[49] CD14 may be especially useful as a marker if future therapies alter the CD14/TLR4 signaling pathway. In addition, it

remains to be determined whether functional polymorphisms within CD14 gene (e.g., its promoter)[50] influence specific PKD outcomes.

Finally, both *Cd14* mRNA expression in the studied mouse kidneys and urinary CD14 levels in ADPKD patients correlated better with rate of kidney volume change in males than in females. We speculate that these gender differences reflect distinct female-specific innate immune responses that include regulation of CD14 levels.[51] In addition, CD14 excretion in urine from females may be influenced by menstrual cycle similarly as excretion of other factors that activate components of CD14/TLR4 signaling (e.g., IL-1[52]).

In summary, we have performed the first comprehensive analysis characterizing CD14 expression in PKD. In the *cpk* mouse, a model of recessive PKD, we determined that abnormalities in *Cd14* gene expression represent an early postnatal event. In addition, in a model that provides variable rates of cystic kidney disease progression, *Cd14* expression positively and strongly correlated with the rate of cystogenesis reflected by kidney volumes of 10-d old *cpk* kidneys. We showed that similar to renal ischemia-reperfusion injury, CD14 expression abnormalities in *cpk* kidneys cannot be attributed to increased numbers of CD14+ monocytes or macrophages, but rather they reflect increased immunological activity of renal tubular cells. Results of CD14 studies in human kidneys from patients with RPKD closely resembled observations in *cpk* kidneys, in particular with respect to CD14 immunolocalization and renal CD14 content. Interestingly, we observed CD14 expression abnormalities also in ADPKD kidneys and our initial analyses indicate that CD14 is a candidate predictor of ADPKD progression.

MATERIALS AND METHODS

Mice

Details of the generation of the (C57BL/6J-*cpk*/+ × CAST)F1 intercross and subsequent identification of *cpk* mutants among the F2 mice using *Cys1[cpk]* allele-specific assay were previously described.[16, 53] All F2 mice were sacrificed 10 days after birth, their kidneys removed and their length, weight and volume recorded. One of the kidneys from each animal was snap-frozen and stored in liquid nitrogen, and the other was fixed in 10% buffered formalin for histological evaluation. Additional mice with C57BL/6J genetic background, either homozygous for the *cpk* mutation or their wild type (unaffected) littermates from the maintenance colony, were also sacrificed at postnatal days 0 to 25 and their kidneys harvested similarly to the above F2 mice. All protocols were approved by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee. The University of Alabama at Birmingham is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Gene expression profiling

We studied gene expression in mouse kidneys harvested from the (B6-*cpk*/+ × CAST)F1 intercross (7 with smallest kidney volume, 8 with kidney volumes evenly distributed across phenotypic spectrum of 461 *cpk* mice, and 7 unaffected controls). We also examined renal gene expression in *cpk* and wild type mice at postnatal day 0, 5, 10, 15, 20 and 25 (four biological replicas for each phenotype and timepoint). RNA and cDNA was prepared from whole kidneys as previously described.[11] Initial gene expression analyses were performed on mice generated in the intercross using Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix Inc., Santa Clara, CA) according to a previously described protocol.[11] All subsequent gene expression studies that are presented in this manuscript were performed with TaqMan® probes arranged into custom-designed low density arrays (Applied

Biosystems, Foster City, CA). TaqMan® assays relevant to this manuscript include: Mm00438094_g1 (*Cd14*), Mm00441243_g1 (*Ccl2*) and Mm00607939_s1 (*Actb;* a gene encoding beta-actin). C_T values were determined with 7000 SDS RQ software (version 1.1) and subsequently standardized using a C_T value for *Actb* as reference. *Actb* was recommended as a suitable reference gene for this model based on equivalence testing (a two one-sided t-test)[54] using the above Affymterix array data[11] and TaqMan® Mouse Endogenous Control Array (Applied Biosystems). The standardized C_T values were used to determine significance of studied associations.

Immunostaining and Immunoblotting

Formalin-fixed kidneys from affected *cpk* homozygous and unaffected wild type mice were paraffin embedded and cut into sections (3 and 5 μm). These were xylene-deparafinized, rehydrated, and stained using rabbit polyclonal anti-human CD14 (M-305) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and species-specific secondary antibody conjugated with biotin (Molecular Probes, Eugene, OR). Immunostaining was performed after blocking tissue sections for 30 min with PBS containing 1% bovine serum albumin (Sigma, St Louis, MO). Primary antibody diluted in blocking buffer was allowed to react with the tissues for 12-hours at 4°C, followed by four rinses with PBS. Immunohistochemical detection was performed with ABC complex/HRP and DAB chromogen (DAKO) after blocking with Avidin/Biotin blocking kit (Vector Laboratories, Burligame, CA). Stained tissue sections were analyzed with bright field microscopy using a Nikon E600 microscope equipped with a SPOT Insight digital camera (Diagnostic Instruments, Sterling Heights, MI). Renal CD14-positive cell counts were quantified by histomorphometry with Image Pro Plus v5.1 image analysis software (Media Cybernetics, Inc., Bethesda, MD). For each section and stain, six images each of cortex and medulla from different locations were selected systematically to minimize selection bias. For each image, CD14-positive cells were enumerated and the tissue area, excluding cyst and tubular lumina, were measured. Each CD14-positive cell was visually confirmed to assure exclusion of nonspecifically stained debris. Results were expressed as stained cells/mm^2 tissue. Statistical evaluations were performed with SPSS 11.5 statistical software (SPSS Inc.).

For immunoblotting, whole kidneys from three 10-d old *cpk* homozygotes with C57BL/6J genetic background, and kidneys from three age-matched wild type B6 mice were homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.7% Pepstatin and 1 tablet Complete Protein inhibitor per 50 ml (Roche Diagnostics, Penzberg, Germany). After 10-min centrifugation at $10,000 \times g$, the supernatant containing solubilized membranes as well as cytosolic material was denatured for 5 min at 95°C in 2X Laemmli reducing buffer, and was separated by 4–12% gradient SDS-PAGE (Invitrogen, Carlsbad, CA). The gel was electrophoretically transferred onto a Hybond-ECL membrane (Amersham, Piscataway, NJ) using a wet blotter (Invitrogen). The membrane was blocked with 5% nonfat milk in 1X PBS/0.05% Tween-20 for one hour at room temperature. Subsequent washing and dilutions were done with 1X PBS/0.05% Tween-20 at room temperature. The CD14 (M-305) antibody and anti-beta-actin antibody (Sigma) were detected after incubation with species-specific secondary antibodies conjugated with HRP (Molecular Probes). Activated chemiluminescence was captured on Hyperfilm-ECL (Amersham).

The expression of CD14 protein in mIMCD-K2 cell line of SV40 transformed internal medullary collecting duct cells (gift from Erik Schwiebert, University of Alabama at Birmingham) was validated with reverse-transcriptase PCR (RT-PCR) using intron-spanning primers that generate 258 bp and 375 bp products from cDNA and genomic DNA, respectively (5′-gcctttctcggagcctatct-3″ and 5′-tggcttcggatctgagaagt-3′).

Remnant human fetal RPKD and non-PKD kidneys were collected after obtaining informed consent according to a protocol approved by the Institutional Review Board of University of Alabama at Birmingham (UAB). This Board also approved the protocol for collection and analyses of remnant adult nephrectomized end-stage ADPKD kidneys, control kidneys and urine that were used in this study. The tissue processing, immunostaining and immunoblotting were performed in similar fashion as described above for mouse tissues.

CD14 quantification in human serum and urine

Serum and urine from patients enrolled in the Emory ADPKD Cohort Study were collected and analyzed according to protocols approved by Institutional Review Boards at Emory University and University of Alabama at Birmingham. CD14 levels were determined using a CD14 ELISA assay (R&D Systems, Minneapolis, MN), according to manufacturer's protocol. Absorbances of technical duplicates were determined using Spectra Microplate Reader (Molecular Devices, Sunnyvale, CA). Statistical analyses were performed with SPSS 11.5 statistical software package (SPSS Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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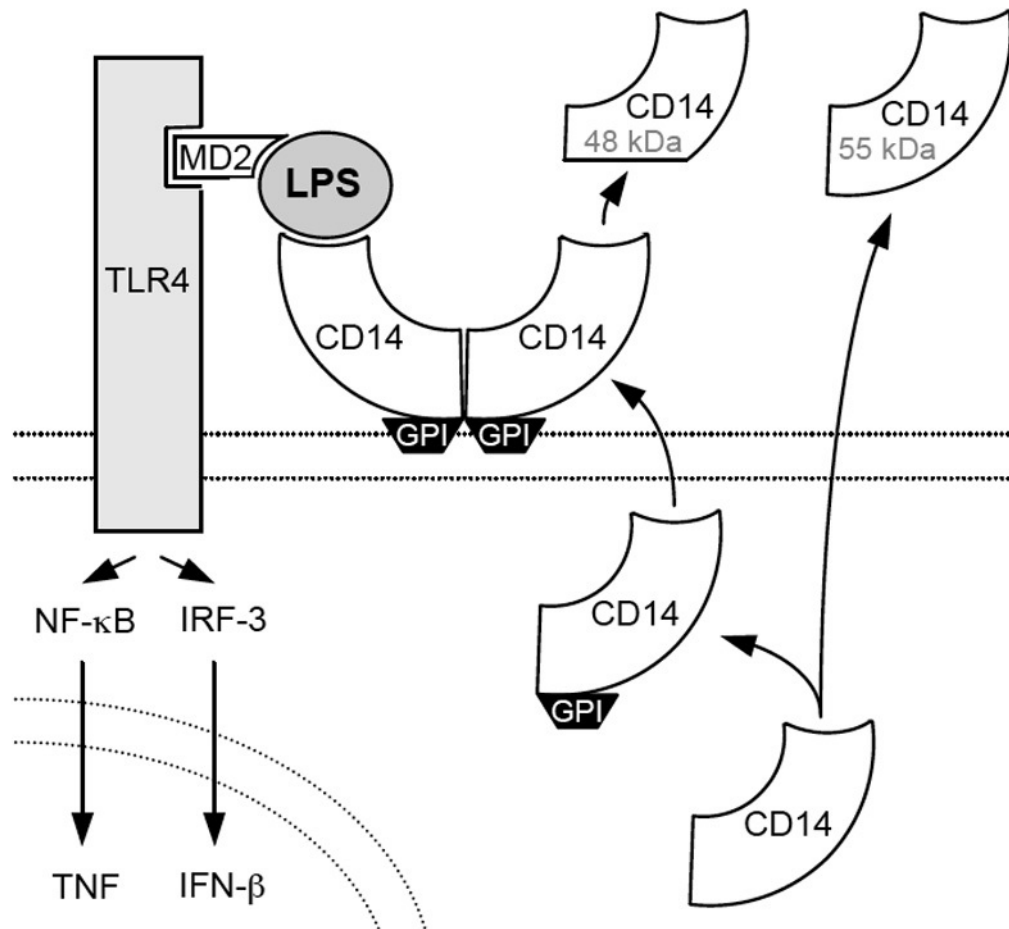
Figure 1. Schema of CD14-mediated immune response activation and CD14 shedding
CD14 is a high affinity lipopolysacharide (LPS) receptor that, in conjunction with Toll-like family receptors (e.g., TLR4), activates LPS-induced immune responses. CD14 is anchored to cell surfaces by a linkage to glycosylphosphatidylinositol (GPI). However, activated cells may release CD14 by proteinase-dependent (48 kDa) or proteinase-independent shedding (55 kDa protein).
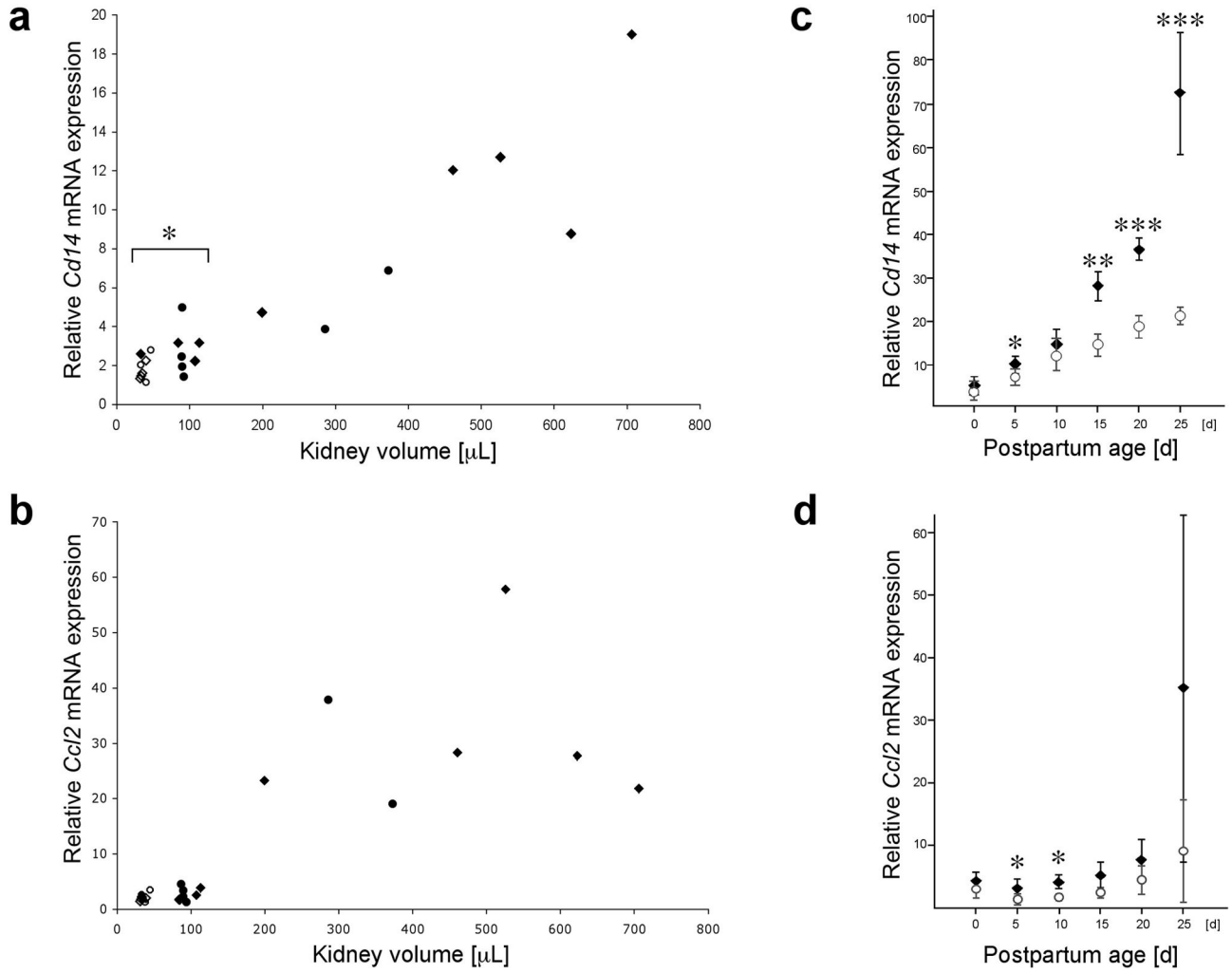
Figure 2. Correlation between *Cd14* expression and cystic kidney disease severity in *cpk* mice
Panel a shows a strong correlation of *Cd14* expression with kidney volumes in 10-d old mice generated in (C57BL/6J-*cpk* × CAST/Ei)F1 intercrosses (r=0.94, p<0.001). Black symbols designate affected *cpk* mice, white symbols correspond to unaffected littermates, diamonds represent males and circles females. This panel also demonstrates that *Cd14* expression is significantly increased even in the most mildly affected *cpk* kidneys vs. unaffected littermate kidneys (p<0.05; bracketed data points). **Panel b** demonstrates the outcomes of similar analyses for *Ccl2*, a gene encoding a surrogate PKD progression marker MCP-1. The correlation of *Ccl2* expression and kidney volumes was moderately strong (r=0.79, p<0.001). However, the difference in *Ccl2* expression levels between unaffected and mildly affected *cpk* kidneys was not significant. **Panels c** and **d** show relative renal *Cd14* and *Ccl2* expression in early postnatal period. Black diamonds represent mean gene expression values for specific time-points in affected *cpk* mice, white circles correspond to mean gene expression in unaffected littermates, bars indicate 1SD. Asterisks mark statistically significant differences between data for kidneys from *cpk* mice and unaffected littermates (* p<0.05, ** p<0.01, *** p<0.001).
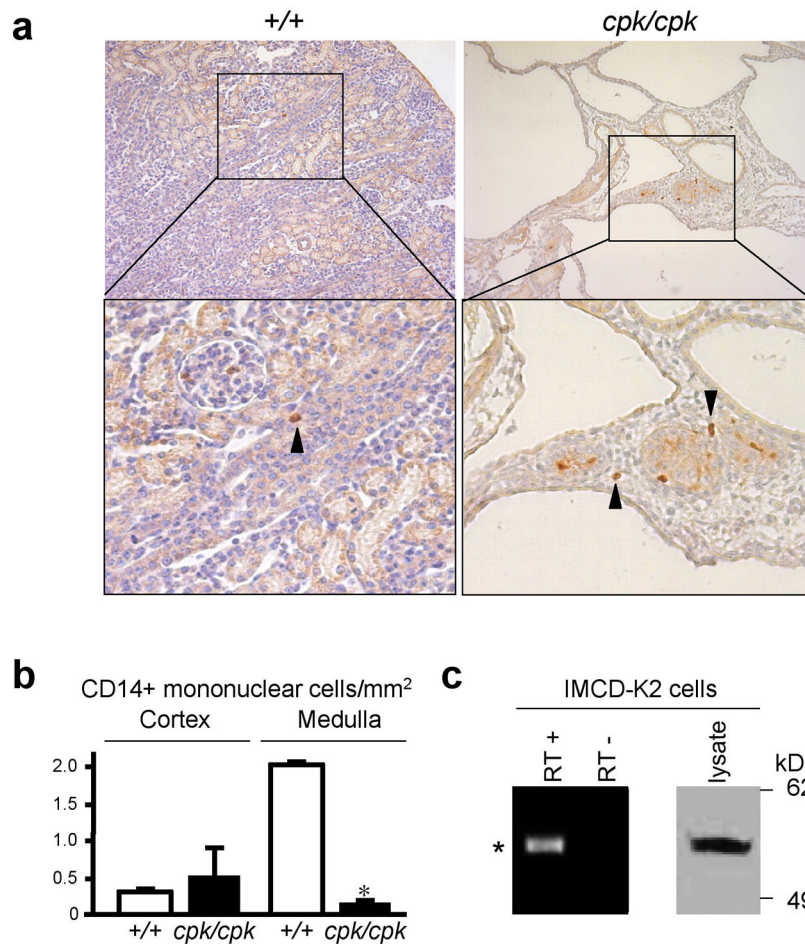
Figure 3. CD14-positive mononuclear cell content is not significantly increased in *cpk* kidneys
Panel a shows a representative micrograph of an anti-CD14 antibody stained section of wild type (+/+) 10-d old kidney showing few strongly positive CD14 cells in interstitium (presumably mature monocytes and macrophages; marked by arrows) and weaker staining in tubular cells. In 10-d old *cpk* kidneys there is a similar weak staining of both non-cystic and cystic renal epithelial cells as well as strong staining of CD14-positive interstitial cells. **Panel b** shows that the content of strongly stained CD14-positive mononuclear cells in interstitium is not significantly increased in *cpk* kidneys. **Panel c** demonstrates the CD14 producing capacity of principal cells from inner medullary collecting duct cell line mIMCD-K2 (IMCD); RT-PCR with *Cd14*-derived primers produced a cDNA-specific 258 bp product (asterisk), anti-CD14 immunoblotting of IMCD lysates showed a 55 kDa protein that is present in non-activated CD14-producing cells.
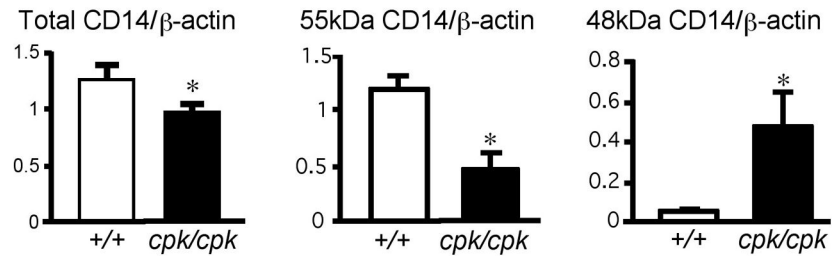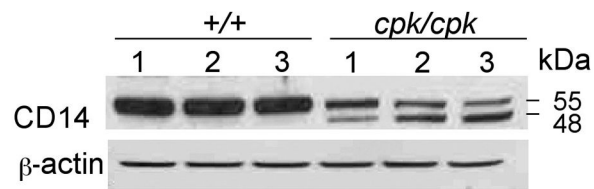
Figure 4. CD14 producing cells are abnormally activated in *cpk* kidneys
A representative immunoblot of kidney lysates from 10-d old *cpk* (*cpk/cpk*) and wild type (+/+) mice demonstrates that while the total CD14 content is reduced in the *cpk* kidneys, these kidneys contain increased amounts of the 48 kDa CD14 fragment that is generated by shedding of membrane-bound CD14 from activated cells.
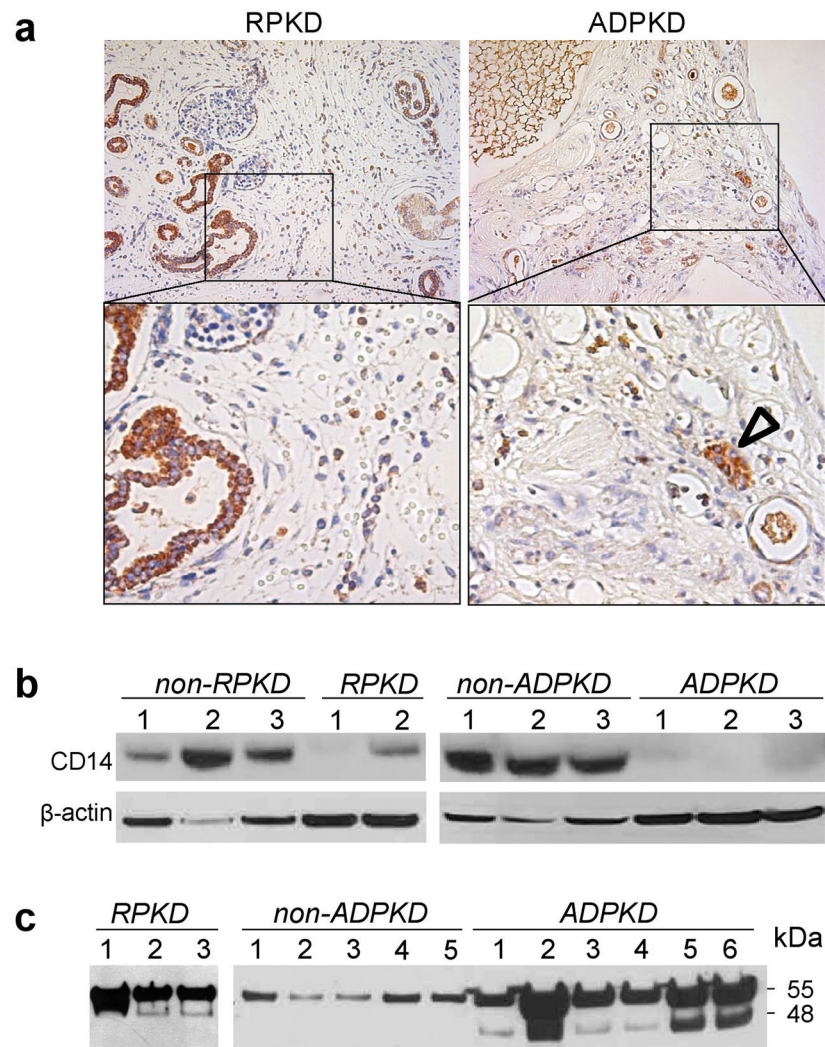
Figure 5. CD14 expression in human RPKD and ADPKD
Panel a shows representative micrographs of anti-CD14 antibody stained sections of fetal
RPKD and adult ADPKD kidneys. CD14-positive interstitial cells (presumed mature
monocytes and macrophages) are present in both RPKD and ADPKD kidneys. Cystic as
well as non-cystic epithelial cells were CD14-positive, resembling staining of *cpk* mouse
kidneys. However, staining of renal tubular cells in fetal RPKD kidneys was comparable in
strength to CD14-positive interstitial cells. CD14 content in cell membrane and cytoplasm
closely resembled known CD14 distribution in macrophages. Although not frequent, a
similar pattern of CD14 expression was observed also in some non-cystic tubular cells (an
arrow) and epithelial cell lining in end-stage ADPKD kidneys. The CD14-like staining
pattern was absent in corresponding mouse and human tissues stained with pre-immune sera
and/or control antibodies. **Panel b** shows that similar to *cpk* mouse kidneys, CD14 content is
decreased in lysates from RPKD and ADPKD kidneys. A possible explanation for the PKD-
associated reduction in renal CD14 content is its shedding by activated cells. After their
activation, these cells shed a GPI-anchor attached CD14 from cell membrane in the form of
its 48 kDa CD14 fragment as well as yet unprocessed 55 kDa CD14 protein that are both
present in urine from RPKD and ADPKD patients (**panel c**). Column numbers (**panels b**
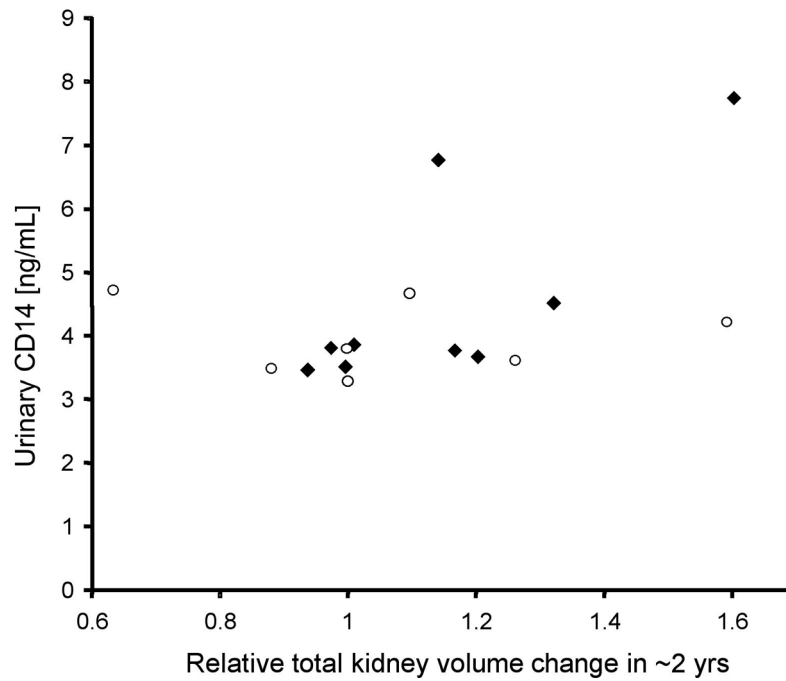and **c**) designate sample assignment of unique individuals.

Figure 6. CD14 is a candidate predictor of ADPKD progression
Urinary CD14 levels correlate with rates of two-year total kidney volume (TKV) change in
ADPKD patients. While the overall correlation was marginal (r=0.43, p=0.09), it was
moderately strong in males (designated by black diamonds; r=0.74, p=0.02). The correlation
of the TKV change and GFR determined by iothalamate was not significant (r=(−0.22),
p>0.20).