The macrophage phenotype and inflammasome component NLRP3 contributes to nephrocalcinosis-related chronic kidney disease independent from IL-1–mediated tissue injury

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Primary/secondary hyperoxalurias involve nephrocalcinosis-related chronic kidney disease (CKD) leading to end-stage kidney disease. Mechanistically, intrarenal calcium oxalate crystal deposition is thought to elicit inflammation, tubular injury and atrophy, involving the NLRP3 inflammasome. Here, we found that mice deficient in NLRP3 and ASC adaptor protein failed to develop nephrocalcinosis, compromising conclusions on nephrocalcinosis-related CKD. In contrast, hyperoxaluric wild-type mice developed profound nephrocalcinosis. NLRP3 inhibition using the β-hydroxybutyrate precursor 1,3-butanediol protected such mice from nephrocalcinosis-related CKD. Interestingly, the IL-1 inhibitor anakinra had no such effect, suggesting IL-1-independent functions related CKD. Interestingly, the IL-1 inhibitor anakinra had no such effect, suggesting IL-1-independent functions related to nephrocalcinosis. NLRP3 inhibition using 1,3-butanediol protected mice from nephrocalcinosis. NLRP3 inhibition using 1,3-butanediol protected such mice from nephrocalcinosis-related CKD. Interestingly, the IL-1 inhibitor anakinra had no such effect, suggesting IL-1-independent functions related to nephrocalcinosis. NLRP3 inhibition using 1,3-butanediol protected mice from nephrocalcinosis.

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Accordingly, BHB-inhibited CaOx crystals induced activation of NLRP3 inflammasome in bone marrow–derived dendritic cells (Supplementary Figure S1). Furthermore, upon feeding C57BL/6N mice a 1,3-butanediol (1,3-B)–enriched diet, we observed increased plasma levels of the 1,3-B central metabolite—BHB (Figure 2a). These increased levels of BHB were sufficient to inhibit the activation of NLRP3 inflammasome in the kidneys of mice fed a high-oxalate diet (Figure 2b). The oxalate exposure induced a similar degree of nephrocalcinosis in the two treatment groups (Figure 3c and Supplementary Figure S2A). Moreover, 1,3-B treatment did not affect the nature and composition of renal CaOx crystal deposits (Figure 2d and Supplementary Figure S2B–E). Nevertheless, NLRP3 inhibition with 1,3-B reduced markers of renal excretory dysfunction, e.g., plasma creatinine levels, compared with the vehicle-treated group (Figure 2e). This preserved renal function was associated with less mRNA expression of the kidney injury marker (Kim)-1, reduced tubular injury, and reduced TUNEL positive tubular cells in the kidneys of mice receiving 1,3-B treatment (Figure 2f and Supplementary Figure S2F). Thus, starting from nephrocalcinosis of the same level of severity, NLRP3 inflammasome inhibition with 1,3-B attenuated the related CKD. In contrast to the experiments performed with Nlrp3- and Asc-deficient mice, these results clearly imply that NLRP3 plays a role in the progression of nephrocalcinosis-related CKD.

NLRP3 inhibition induces a shift in nephrocalcinosis-related macrophage infiltrates

Nephrocalcinosis-related progressive CKD is associated with increasing infiltration of macrophages into the renal interstitial compartment.\(^{12,17,19}\) By immunostaining, we observed that 1,3-B treatment reduced the number of F4/80+ intrarenal macrophage populations in mice with nephrocalcinosis (Figure 3a). Various macrophage phenotypes are associated with resolution of inflammation and tissue regeneration, and persistent injury and progression to tissue atrophy. Their heterogeneity is determined by the microenvironment.\(^{20–24}\) We, therefore, employed flow cytometry to understand the diversity of phenotypes among the infiltrating macrophages. We noted that nephrocalcinosis is associated with increased numbers of cluster of differentiation (CD)45+ leukocytes in oxalate-treated mice, compared with the control group (Figure 3b). However, 1,3-B treatment reduced the number of CD45+ leukocytes in mice with nephrocalcinosis. We identified infiltrating macrophages accordingly as CD45+F4/80+CD11b+\(^{22,26,27}\) (Figure 3c). Further phenotype analysis of macrophages revealed that these were M1-like pro-inflammatory (CD45+F4/80+CD11b+CD3CR1+CD206−) (Figure 3d) and M2a-like probiotic (CD45+F4/80+CD11b+CD3CR1+CD206+TGFβ+) macrophages\(^{22,26,27}\) (Figure 3e). The 1,3-B treatment inhibited NLRP3 and reduced the intrarenal numbers of M1- and M2a-like macrophages (Figure 3b–f). In contrast, 1,3-B massively increased the numbers of macrophages with an anti-inflammatory M2c-like phenotype (CD45+F4/80+CD11b+CD206+TGFβ−) (Figure 3f).

RESULTS

**Nlrp3- and Asc-deficient mice with hyperoxaluria do not develop nephrocalcinosis, and hence no oxalate nephropathy**

To confirm the published phenotype of Nlrp3-deficient mice upon induction of hyperoxaluria, and to extend these finding on the adaptor protein ASC, we fed Nlrp3- and Asc-deficient mice the same oxalate-rich and calcium-depleted diet previously reported to induce a robust chronic oxalate nephropathy in wild-type (WT) mice.\(^{2,17}\) In contrast, both mutant mouse strains lacked intrarenal CaOx deposits and consequently did not develop kidney disease despite significant oxaluria and calciumuria (Figure 1). The findings replicate the previous finding for Nlrp3-deficient mice and confirm the same phenotype for Asc deficiency. As Nlrp3- and Asc-deficient mice did not develop nephrocalcinosis, no conclusion can be drawn regarding a role of the NLRP3 inflammasome in the progression of nephrocalcinosis-related CKD.

**The NLRP3 inhibitor β-hydroxybutyrate attenuates nephrocalcinosis-related CKD**

To exclude any genetic interference of the mutant mouse strains with oxalate diet–induced nephrocalcinosis, we switched to a pharmacologic approach, to specifically modulate the NLRP3 inflammasome in chronic oxalate nephropathy. β-hydroxybutyrate (BHB) is a recently described NLRP3 inflammasome inhibitor that prevents K⁺ efflux and reduces ASC oligomerization and speck formation.\(^{18}\)

We hypothesized that NLRP3 is involved in nephrocalcinosis-related CKD and sought to confirm the phenotype of Nlrp3-deficient mice, as well as mice deficient for the second inflammasome component ASC in chronic oxalosis. In addition, we wanted to verify the role of IL-1β-dependent renal inflammation in this disease model. Finally, we speculate on a potential role of NLRP3 and ASC, independent of IL-1, in TGFR-mediated activation of fibroblasts, contributing to chronic tissue remodeling.

 compartment.\(^{7,10}\) For example, in acute oxalosis, crystals of CaOx directly activate the NLRP3 inflammasome in intrarenal dendritic cells, a process driving IL-1β–dependent intrarenal inflammation and tubule injury.\(^{11}\)

However, in chronic oxalate nephropathy, the published data are less clear. Nlrp3-deficient mice were found to be protected, but they did not have comparable nephrocalcinosis, so concluding that NLRP3 inflammasome/IL-1–related inflammation contributed to their CKD progression is problematic.\(^{5,12}\) Data on caspase-1-independent functions of NLRP3 and ASC, provide early signs that these may contribute to nephrocalcinosis-related CKD.\(^{13–16}\) For example, NLRP3 also regulates transforming growth factor beta (TGFβ) receptor (TGFR) signaling and its subsequent immunomodulatory effects, as in experimental systemic lupus erythematosus and lupus nephritis.\(^{13}\) In non-immune cell populations, such as tubular epithelial cells, the effect of NLRP3 on TGFR signaling contributes to tissue remodeling upon unilateral ureteral obstruction.\(^{14,15}\)

We noted that nephrocalcinosis is associated with the diversity of phenotypes among the infiltrating macrophages. We, therefore, employed flow cytometry to understand the diversity of phenotypes among the infiltrating macrophages. We noted that nephrocalcinosis is associated with increased numbers of cluster of differentiation (CD)45+ leukocytes in oxalate-treated mice, compared with the control group (Figure 3b). However, 1,3-B treatment reduced the number of CD45+ leukocytes in mice with nephrocalcinosis. We identified infiltrating macrophages accordingly as CD45+F4/80+CD11b+\(^{22,26,27}\) (Figure 3c). Further phenotype analysis of macrophages revealed that these were M1-like pro-inflammatory (CD45+F4/80+CD11b+CD3CR1+CD206−) (Figure 3d) and M2a-like probiotic (CD45+F4/80+CD11b+CD3CR1+CD206+TGFβ+) macrophages\(^{22,26,27}\) (Figure 3e). The 1,3-B treatment inhibited NLRP3 and reduced the intrarenal numbers of M1- and M2a-like macrophages (Figure 3b–f). In contrast, 1,3-B massively increased the numbers of macrophages with an anti-inflammatory M2c-like phenotype (CD45+F4/80+CD11b+CD206+TGFβ−) (Figure 3f).
Figure 1 | Nlrp3- and Asc-deficiency protects mice from nephrocalcinosis and chronic oxalate nephropathy. C57BL/6N wild-type (WT), Nlrp3-deficient (Nlrp3−/−), and Asc-deficient (Asc−/−) mice were fed either a high-oxalate or a control diet for 14 days. Pizzolato staining illustrating CaOx crystal deposition in the kidney (original magnification x25; a). Quantification of crystal deposition (b). Plasma creatinine levels (c). Periodic acid–Schiff (PAS) staining illustrating tubular injury (original magnification x200; d). Quantification of tubular injury (e). mRNA expression of kidney injury marker (Kim)-1 was performed on kidney RNA isolates (f). Data are given as mean ± SEM, from 6 to 7 mice in each group. *P < 0.05; **P < 0.01; ***P < 0.001. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Figure 2 | The NLRP3 inhibitor β-hydroxybutyrate attenuates nephrocalcinosis-related CKD. C57BL/6N mice were fed either a high-oxalate diet or a high-oxalate diet combined with 1,3-butanediol (1,3-B), for 14 days. Plasma β-hydroxybutyrate levels (a). Western blot for interleukin (IL)-1β and Caspase-1 (casp 1) from kidney tissues. β-actin was used as loading control (b). Pizzolato staining illustrating CaOx crystal deposition in the kidney (original magnification x25) and its quantification (c). X-ray diffraction analysis of mouse kidneys (d). Plasma creatinine levels (e). mRNA expression of kidney injury marker (Kim)-1 was performed on kidney RNA isolates (f). Periodic acid–Schiff (PAS) staining illustrating tubular injury (original magnification x200) and quantification of tubular injury (g). Data are given as mean ± SEM, from 6 to 7 mice in each group. n.s., not significant; **P < 0.01; ***P < 0.001. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Figure 3 | NLRP3 inhibition induces a phenotype shift in nephrocalcinosis-related macrophage infiltrates. C57BL/6N mice were fed either a high-oxalate diet or a high-oxalate diet combined with 1,3-butanediol (1,3-B), for 14 days. Immunostaining illustrating F4/80 macrophages and their quantification (a). Flow cytometric analysis of infiltrating CD45+ leukocytes, and absolute cell numbers (b–f). Gating strategy and absolute numbers of CD45+F4/80+CD11b+ macrophages in kidneys (c). Phenotypes of renal infiltrating macrophages identified (continued)
Treatment with 1,3-B inhibited NLRP3 and attenuated nephrocalcinosis-related CKD, in association with a shift from pro-inflammatory (M1-like) and profibrotic (M2a-like) macrophage infiltrates toward a predominant anti-inflammatory (M2c-like) macrophage phenotype.

**IL-1 does not contribute to nephrocalcinosis-related CKD**
Activation of NLRP3 inflammasome leads to caspase-1–dependent maturation and secretion of IL-1β, which activates IL-1 receptor (IL-1R)/myeloid differentiation primary response 88 (MyD88)–dependent tissue inflammation and injury, as in acute oxalate nephropathy. To explore whether NLRP3 contributes to the progression of nephrocalcinosis-related CKD via secretion of IL-1β, we treated hyperoxaluric mice with the IL-1 inhibitor anakinra, the recombinant version of the intrinsic IL-1 receptor antagonist. We observed that like 1,3-B, anakinra did not affect diet-induced nephrocalcinosis per se (Figure 4a). Unexpectedly, anakinra had no effect on any of the aforementioned parameters of nephrocalcinosis-related CKD (Figure 4b–e). Moreover, anakinra did not change the phenotype of infiltrating macrophages in the kidney (Figure 4f). These results imply that NLRP3 and ASC contribute to the progression of nephrocalcinosis-related CKD independent of the inflammasome-driven maturation and secretion of IL-1β.

**NLRP3 inhibition suppresses nephrocalcinosis-related interstitial fibrosis**
Progressive nephrocalcinosis is characterized by diffuse interstitial fibrosis, a process widely thought to contribute to CKD progression. As NLRP3 inhibition with 1,3-B significantly reduced the numbers of intrarenal profibrotic CD45+ F4/80+ CD11b+ CX3CR1+ CD206+ TGFB+ macrophages, we analyzed the renal mRNA expression levels of the fibrosis markers, such as α-smooth muscle actin (α-SMA), fibronectin (FIB-1), and fibroblast-specific protein-1 (FSP-1) in mice with nephrocalcinosis (Figure 5a–c). The NLRP3 inhibition with 1,3-B significantly suppressed the intrarenal expression levels of these fibrosis markers (Figure 5a–c). Immunostaining showed a significant decrease in the areas positive for α-SMA, silver, or collagen 1α1 after NLRP3 inhibition with 1,3-B (Figure 5d–f). Furthermore, we employed magnetic resonance imaging (MRI) to quantify T1 and T2 relaxation times upon NLRP3 inhibition. We found that 1,3-B treatment reduced T1 and T2 times in the MRI analysis, compared with vehicle treatment, suggesting less intrarenal inflammation and renal fibrosis in mice with nephrocalcinosis (Figure 6). Thus, we conclude that treatment with the NLRP3 inhibitor 1,3-B reduces pro-inflammatory and fibrotic macrophage subsets and is associated with substantially reduced interstitial fibrosis in nephrocalcinosis-related CKD.

**NLRP3 augments TGFR signaling in fibroblasts, independent of the inflammasome complex in vitro**
NLRP3 has been reported to augment TGFR signaling in epithelial and dendritic cells. We speculated that NLRP3 may play a similar role in renal fibroblasts in renal fibrosis. We therefore investigated the role of NLRP3 in primary murine embryonic fibroblasts (pMEFs) and primary renal fibroblasts (Supplementary Figure S3A) isolated from WT and Nlrp3-deficient mice, with increasing doses of recombinant murine TGFB. The TGFB-induced proliferation was entirely absent in Nlrp3-deficient pMEFs (Figure 7a). Deficiency of NLRP3 also resulted in diminished TGFB– and platelet-derived growth factor (PDGF)–B–induced proliferation of Nlrp3-deficient renal fibroblasts (Supplementary Figure S3B and C). Deficiency of NLRP3 also led to diminished TGFB–induced pSmad-3 expression in renal fibroblasts (Supplementary Figure S3D and E). These data suggest a nonredundant role of NLRP3 on TGFR signaling in fibroblasts. The Toll-like receptor 4 agonist lipopolysaccharide (LPS) dramatically increased the protein expression of NLRP3 in pMEFs, a response obviously absent in Nlrp3-deficient cells (Figure 7b). Subsequently, TGFB stimulation increased the mRNA expression levels of numerous profibrotic genes, such as α-SMA, fibronectin, collagen1α1, and connective tissue growth factor (CTGF) in WT pMEFs, an effect entirely absent in Nlrp3-deficient pMEFs (Figure 7c–f). These data imply that NLRP3 is a nonredundant element for the TGFR signaling pathway, and in fibroblasts.

To address whether inflammasome complex formation is required for NLRP3–TGFR signaling, we stimulated LPS–primed WT pMEFs with adenosine triphosphate (ATP), a known activator of the NLRP3 inflammasome complex. We found that concomitant LPS and ATP treatment of WT pMEFs did not change the expression of profibrotic genes upon TGFB stimulation (Figure 7c–f), suggesting that formation of the NLRP3 inflammasome complex is not required for the role of NLRP3 in TGFR signaling in fibroblasts. In addition, we observed that pMEFs do not secrete IL-1β in the supernatant upon LPS and ATP stimulation (Supplementary Figure S4), suggesting that they fail to form an NLRP3 inflammasome complex. Together, these data imply that NLRP3 augments the TGFR signaling pathway in murine fibroblasts and has a role in TGFB–driven fibroblast proliferation and extracellular matrix production.

**DISCUSSION**
We hypothesized that NLRP3 contributes to nephrocalcinosis-related CKD, possibly by activating the NLRP3 inflammasome

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**Figure 3** (continued) as M1-like pro-inflammatory (CD45+F4/80+CD11b+CX3CR1+CD206–), M2a-like profibrotic (CD45+F4/80+CD11b+CX3CR1+CD206+TGFB+), and anti-inflammatory M2c-like phenotype (CD45+F4/80+CD11b+CD206–TGFB–), with absolute cell numbers (d, f). Data are given as mean ± SEM, from 6 to 7 mice in each group. n.s., not significant. *P < 0.05; **P < 0.01; ***P < 0.001. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Figure 4 | Recombinant interleukin (IL)-1 receptor antagonist therapy does not modulate nephrocalcinosis-related chronic kidney disease. C57BL/6N mice were fed a high-oxalate diet with or without the IL-1R antagonist anakinra, for 14 days. Pizzolato staining illustrating CaOx crystal deposition (original magnification x25) and its quantification in the kidney (a). Periodic acid–Schiff (PAS) staining illustrating tubular injury (original magnification x200) and its quantification (b). Quantification of TUNEL positive tubular cells (c). mRNA expression of kidney injury marker (Kim)-1 was performed on kidney RNA isolates (d). Plasma creatinine levels (e). Flow cytometric analysis of infiltrating CD45+ leukocytes, CD45+F4/80+CD11b+ macrophages, M1-like (CD45+F4/80+CD11b+CX3CR1+CD206−), and M2-like profibrotic and anti-inflammatory (CD45+F4/80+CD11b+CX3CR1+CD206+) macrophage quantification in the kidney (f). n.s., nonsignificant. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
and IL-1β–related renal inflammation and injury. Using Nlrp3-deficient mice to investigate this issue would not have allowed reliable conclusions, owing to the lack of comparable nephrocalcinosis induction. Thus, we explored this question with further experiments, also considering IL-1–independent functions of NLRP3. Our experiments with Nlrp3- and Asc-deficient mice confirmed the lack of sufficient nephrocalcinosis. Therefore, we used additional experimental strategies. Our results suggest a nonredundant role of NLRP3 in nephrocalcinosis-related CKD, involving IL-1–independent effects, such as macrophage polarization and renal fibrogenesis. We used a murine model of oxalate-induced nephrocalcinosis identical to those used in previous studies by several groups.12,17,19 We observed that, similar to

Figure 5 | NLRP3 inhibition suppresses nephrocalcinosis-related interstitial fibrosis. C57BL/6N mice were fed either a high-oxalate diet or a high-oxalate diet combined with 1,3-butanediol (1,3-B), for 14 days. mRNA expression of α-smooth muscle actin (α-SMA) (a), fibronectin (FIB)-1 (b), and fibroblast specific protein (FSP)-1 (c) was performed on kidney RNA isolates. Immunohistochemistry images and quantification of α-SMA (d), silver (e), and collagen (col1α1) (f) was performed on kidney sections. Data are given as mean ± SEM, from 6 to 7 mice in each group. n.s., nonsignificant. **P < 0.01; ***P < 0.001. To optimize viewing of this image, please see the online version of this article at www.kidneyinternational.org.
Nlrp3-deficient mice, Asc-deficient mice failed to develop nephrocalcinosis and CKD, despite significant hyperoxaluria and calciuria. These results indicate the interference of NLRP3, ASC, and any other (hidden) genetic difference from WT mice used in this experiment, with CaOx crystal deposition in the kidney. To explore this possibility, we employed an independent pharmacologic approach, by feeding mice a ketogenic (1,3-B) diet to inhibit NLRP3. We observed that mice were protected from CKD, with no effect on nephrocalcinosis per se, thus confirming involvement of the NLRP3-ASC inflammasome complex in nephrocalcinosis-related CKD. Our results are in accordance with those of a previous study, which also reported that a small molecule, the NLRP3 inhibitor CP-456,773, protected mice from oxalate-induced CKD without affecting nephrocalcinosis.19 Furthermore, the NLRP3 inflammasome activation leads to IL-1β release, which sets up Myd88-dependent inflammation.9 In addition to inflammation, IL-1β has been reported to increase TGFβ production by synovial fibroblasts during fibrosis in arthritis.12 This response is counterbalanced by the intrinsic IL-1R antagonist (IL-1RA), a molecular mechanism explored for therapeutic purposes using recombinant IL-1RA.28 In contrast, we observed that IL-1R blockade did not protect mice from nephrocalcinosis-related fibrosis and CKD, thereby excluding a nonredundant role of IL-1 in the pathology of nephrocalcinosis-related CKD. In addition, we observed that NLRP3 inhibition, but not IL-1 inhibition, led to a shift of macrophages from a pro-inflammatory and profibrotic to an anti-inflammatory phenotype. Thus, our results clearly indicate IL-1-independent involvement of NLRP3 in the pathology of nephrocalcinosis-related CKD. This process may include IL-18, not addressed here, which is another immunoregulatory cytokine downstream of NLRP3.

**Figure 6 | Magnetic resonance imaging (MRI) of murine kidneys with nephrocalcinosis-related chronic kidney disease.** MRI was performed on kidneys of C57BL/6N mice fed either a high-oxalate diet or a high-oxalate diet combined with 1,3-butanediol (1,3-B), for 14 days. Representative images of T1 and T2 relaxation time of kidneys (a). Quantitative parameters of T1 relaxation time (b) and T2 relaxation time (c) showed significant differences between the groups in the cortex (C), corticomedullary region (CM), and medulla (M). Data are given as mean ± SEM, from 5 to 6 kidneys in each group. ***P < 0.001, versus vehicle. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
between the increase in NLRP3 expression in epithelial cells and epithelial-mesenchymal transition (EMT), and therefore, fibrosis.\textsuperscript{14,33} However, myofibroblasts are the main contributors to organ fibrosis, compared with other cell types. Only 5% of myofibroblasts are derived from EMT during fibrosis, whereas about 50% originate from proliferating tissue resident fibroblasts.\textsuperscript{30} Therefore, our in vitro studies focus on fibroblasts as effector cells in fibrosis, and we employed primary mouse embryonic and primary renal fibroblasts. Our results reveal a clear association between an increase in NLRP3 expression in fibroblasts and increased expression of markers of fibrosis. In addition, our results reveal that the NLRP3 inflammasome complex is indispensable for NLRP3–TGFβ signaling in fibroblasts, and therefore, organ fibrosis.

Figure 7 | NLRP3 augments transforming growth factor receptor (TGFR) signaling in fibroblasts independent of inflammasome complex in vitro. Murine embryonic fibroblasts (MEFs) from wild-type (WT) and Nlrp3-deficient mice were cultured in the presence of 10, 25, and 50 ng/ml recombinant murine transforming growth factor (rmTGFβ), for 24 hours. Cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (absorbance: 570 nm; a). Western blot for Nlrp3 and β-actin of lipopolysaccharide (LPS)-stimulated MEFs isolated from WT and Nlrp3-deficient (Nlrp–/–) mice (b). Real-time–quantitative polymerase chain reaction (RT-qPCN) of WT or Nlrp3–/–MEFs stimulated with either LPS, LPS + rmTGFβ, LPS + adenosine triphosphate (ATP), LPS + ATP + rmTGFβ, or left untreated (medium), for 6 hours, was quantified for mRNA expression of fibrosis markers α-smooth muscle actin (SMA) (c), fibronectin-1 (d), collagen (col)1α1 (e), and connective tissue growth factor (CTGF) (f). Data are mean ± SEM from 3 independent experiments. Data are given as mean ± SEM from 3 independent experiments. n.s., nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001, versus respective medium control. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Assessing the role of NLRP3 in the progression of nephrocalcinosis-related CKD with Nlrp3-deficient mice is difficult because, for yet unknown reasons, these mice do not develop sufficient nephrocalcinosis. A pharmacologic approach can circumvent this experimental problem. Two studies have both documented that small-molecule NLRP3 antagonists attenuate nephrocalcinosis-related CKD. Our data document this effect as being largely independent of NLRP3-driven IL-1 secretion. Our results suggest that alternative biological functions of NLRP3 apply, e.g., promoting pro-inflammatory or profibrotic macrophage phenotypes while suppressing anti-inflammatory macrophages. In addition, NLRP3 is a nonredundant mediator of TGFR signaling, in not only dendritic cells and tubular epithelial cells but also interstitial cells, which together promote CKD and renal fibrosis independent of IL-1-mediated renal inflammation. We conclude that NLRP3 is a promising therapeutic target to combat nephrocalcinosis-related CKD, e.g., primary hyperoxaluria, as well as other crystallopathies, and potentially other disease pathologies involving chronic tissue remodeling.

MATERIALS AND METHODS

Animal studies
Male C57BL/6N mice, aged 6–8 weeks, were procured from Charles River Laboratories (Sulzfeld, Germany). Nlrp3- and Asf-deficient mice were provided by J. Tschopp (University of Lausanne, Lausanne, Switzerland) and V. Dixit (Genentech, San Francisco, CA) and backcrossed to the genetic background of WT mice for at least five generations. Mice were housed in groups of five in filter-top cages and had access to food and water ad libitum. Cages, nestlets, food, and water were sterilized by autoclaving before use. An oxalate-rich diet was prepared by adding 50 µmol/g sodium oxalate to a calcium-free standard diet (Sniff Spezialdiäten, Soest, Germany) as previously described.17 A BHB precursor, 1,3-B (20%) to a calcium-free standard diet (Ssniff Spezialdiäten, Soest, Germany) was prepared by adding 50 mol/g sodium oxalate. The diet was centrifuged at 10–50 ml of fresh Dulbecco’s Modified Eagle’s medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS), and 1% penicillin–streptomycin (PS). Cells were incubated at 37 °C and 5% CO2.

The media were changed every 48 hours to remove debris, and cell viability/proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (ThermoFisher Scientific, Darmstadt, Germany). Cells were subcultured upon confluence. The pMEFs were then stimulated with LPS (100 ng/ml; Sigma Aldrich), TGFβ (10, 25, or 50 ng/ml; R&D Systems, Wiesbaden, Germany) and ATP (5 mM; Sigma Aldrich). Primary renal fibroblasts were isolated from kidneys of WT and Nlrp3-deficient mice as described previously. Briefly, small sections of cortex were minced “cross-wise” and then transferred to gelatin-coated plates containing DMEM, 20% FCS, and 1% PS. Cells were incubated at 37 °C and 5% CO2. The media were supplemented with an additional 2 ml of DMEM/F12, 20% FCS, and 1% PS after 24 hours. The initial cell populations were established after 72 hours. Cells were characterized by immunofluorescence staining for α-SMA, cytokeratin, and E-cadherin (Supplementary Figure S3A). Cell proliferation was assessed using MTT cell proliferation assay (ThermoFisher Scientific, Darmstadt, Germany). Cells were subcultured upon confluence. Primary renal fibroblasts were then stimulated with TGFβ (50 ng/ml) for 30 minutes and harvested for protein isolation. Bone marrow–derived dendritic cells were established as described previously and treated with either vehicle or BHB (Sigma Aldrich) for 30 minutes before they were stimulated for 3 hours with 1 µg/ml LPS followed by 100 µg/ml CaOx crystals (Alfa Aesar, Karlsruhe, Germany) or 5 mM ATP (Sigma Aldrich) for 6 hours. Mouse tubular cells were generously provided by E.G. Neilson and were maintained in DMEM/F12, 10% FCS, 1% PS.

Assessment of renal injury
Kidney sections of 2 µm were stained with periodic acid–Schiff (PAS) reagent to assess renal injury, which was scored by assessing the percentage of necrotic tubules. Pizzolato’s staining visualized CaOx crystal deposition, which was quantified using ImageJ software (National Institutes of Health, Bethesda, MD) as described previously. F4/80+ macrophages (BioRad AbD Serotec, Kidlington, United Kingdom) were identified by immunostaining and analyzed by assessing the positively stained area in 15 high power fields (hpf) per section, using ImageJ software. Fibrotic areas were identified by immunostaining for 2-SMA (Dako, Hamburg, Germany), silver, and collagen 1α1. Quantification of the immunostaining was done using ImageJ software. An observer blinded to the experimental condition performed all assessments. A cell death detection (TUNEL) kit (Roche, Mannheim, Germany) was used to quantify dead cells. Plasma creatinine levels were measured using an enzymatic creatinine assay kit (Crystal Chem, Elk Grove Village, IL) per manufacturer protocol.

Cell culture studies
Primary murine embryonic fibroblasts (pMEFs) were isolated from 12.5 to 13 embryonic day pregnant WT and Nlrp3-deficient female mice. In brief, approximately 8–10 harvested embryos from these mice were placed in sterile phosphate buffered saline (PBS) and the bodies were separated from head, limbs, and all organs. The bodies were then transferred to a sufficient volume of trypsin/ethylenediaminetetraacetic acid (EDTA), where they were finely minced, using scissors, and incubated at 37 °C for 15 minutes. After incubation, the tissue was dissociated using a Pasteur pipette and then passed through the 100-µm filter. The filtrate was centrifuged at 1000 rpm for 5 minutes, and the pellet was resuspended in 10–50 ml of fresh Dulbecco’s Modified Eagle’s medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS), and 1% penicillin–streptomycin (PS). This was placed on 100-mm2 culture dishes and incubated at 37 °C and 5% CO2.

Kidney sections of 2 µm were collected at different time points after injection for RNA isolation, and the third part was kept in formalin to be used for histology analysis. All animal experiments were performed in accordance with the European law regarding protection of animal welfare and with approval by the local government authorities, Regierung von Oberbayern (reference number: 55.2-1-54-2532-189-2015).

Primary renal fibroblasts were then stimulated with TGFβ (50 ng/ml) for 30 minutes and harvested for protein isolation. Bone marrow–derived dendritic cells were established as described previously11 and treated with either vehicle or BHB (Sigma Aldrich) for 30 minutes before they were stimulated for 3 hours with 1 µg/ml LPS followed by 100 µg/ml CaOx crystals (Alfa Aesar, Karlsruhe, Germany) or 5 mM ATP (Sigma Aldrich) for 6 hours. Mouse tubular cells were generously provided by E.G. Neilson and were maintained in DMEM/F12, 10% FCS, 1% PS.
RNA preparation and real-time quantitative reverse transcriptase - polymerase chain reaction

An RNA extraction kit (Qiagen, Düsseldorf, Germany) was used to isolate total RNA from kidneys as well as in vitro cells using manufacturer instructions. RNA quality was assessed using agarose gels before transcription into cDNA using reverse transcriptase (Superscript II; Invitrogen, Carlsbad, CA). Real-time reverse transcriptase–polymerase chain reaction was performed using SYBR-Green polymerase chain reaction master mix, analyzed with a Light Cycler 480 (Roche, Mannheim, Germany). All gene expression values were normalized using 18s rRNA as a housekeeping gene. All primers used for amplification were purchased from Metabion (Martinsried, Germany) and are listed in Table 1.

Protein isolations and immunoblots

Proteins from cells and kidney tissues were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer, and protein concentration was determined using a Bradford assay. For western blot analysis, 50 µg of proteins were mixed with x4 sodium dodecyl sulfate loading buffer and denatured at 95°C for 5 minutes. Protein separation was performed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was incubated with 5% nonfat milk in the tris buffer for 1 hour at room temperature to block nonspecific binding. The membrane was then incubated overnight at 4°C with primary antibodies for NLRP3, pSmad-3, β-actin (all Cell Signalling, Danvers, MA), IL-1β (R&D Systems, Wiesbaden, Germany), and caspase-1 (Adipogen, San Diego, CA), followed by incubation with secondary anti-rabbit IgG labeled with HRP. Immunostained bands were detected using a chemiluminescence kit (ECL Kit, GE Healthcare, Amersham, United Kingdom).

Flow cytometry analysis

Kidneys were harvested from mice and then digested in a digestions buffer (collagenase/DNase1 solution) for 40 minutes at 37°C. Digested tissue was washed through a 70-µm filter and washed with PBS. For isolating leukocytes, a NycoDenz solution (Axis-Shield, Oslo, Norway) was used to separate CaOx crystals and tissue from renal immune cells. Single cell suspensions were then washed with wash buffer (0.1% bovine serum albumin, 0.01% sodium azide in PBS) and FcR blocked with anti-mouse CD16/32 (2.4G2) for 5 minutes. After blocking, cells were stained with fluorescent anti-bodies for the surface markers PE/Cy5 anti-mouse CD45, V450 anti-mouse CD11b, APC anti-mouse CD206, and PE-anti-mouse CX3CR1 (all antibodies purchased from BioLegend, Fell, Germany) for 30 minutes at 4°C in the dark. Following incubation, cells were washed and centrifuged, and GolgiPlug (BD Biosciences, Heidelberg, Germany) was added for 15 minutes to avoid release of intracellular cytokines. Cells were then washed, resuspended in cell fixaion/permeabilization buffer for an additional 15 minutes, and washed in permwash buffer. Intracellular antibody for PE/Cy7 anti-mouse TGFβ1 was added to the cell suspension for 40 minutes at 4°C. After incubation, cells were washed with PBS and reconstituted in fresh wash buffer. Flow cytometry analysis was performed on the FACS Canto II (BD Biosciences, Heidelberg, Germany), and data were analyzed using the software FlowJo 8.7 (Tree Star, Ashland, OR). For determining the absolute number of cells/µl, AccuCheck counting beads (Invitrogen, Carlsbad, CA) were used, and the absolute cell counts were calculated according to manufacturer instructions.

X-ray diffraction

The analysis of the mouse kidneys by X-ray powder diffraction was performed with the Rigaku Oxford Diffraction (formerly Agilent; www.rigaku-oxford.com) SuperNova A S2 (Dual) Diffractometer with an Atlas S2 CCD detector using the Mova (molybdenum, MoKα: λ = 0.71073 Å) X-ray source. A part of the pulverized kidneys was applied on a MicroMount (M1-L19-A2) (MTeGen, Ithaca, NY), with perflurorinated oil, and X-rayed at a temperature of T = 100 K.

Magnetic resonance imaging

Kidneys harvested from mice (n = 5 in each group) were processed in 2% agarose and placed in a whole-body coil for mice of a dedicated small animal ultra-high-field MR scanner (ClinScan 7 Tesla; both from Bruker, Ettlingen, Germany). Standard sequences for morphology and mapping of T1 and T2 relaxation times (Siemens, Erlangen, Germany) were performed on kidneys in a sagittal orientation. The mapping of relaxation times allows for specific magnetic properties of tissues to be quantified. For example, an increase in the T1 relaxation time may be associated with fibrosis, whereas prolonged T2 relaxation times are found in inflammation.17 For post-processing of images, three regions of interest were the cortex, corticomедullary region, and medulla, respectively, to determine T1 and T2 relaxation times (OsiRx, Bernex, Switzerland, open-source software).

Table 1 | Primer sequences

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
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</thead>
<tbody>
<tr>
<td>Kidney injury marker (KIM)-1</td>
<td>Forward 5'-TCAGCTGGAAGTGCACAA-3', Reverse 5'-ACATGTTCAGCTTTGTGGACC-3'</td>
</tr>
<tr>
<td>Fibronectin-1</td>
<td>Forward 5'-GGATGGCACTGTCACCTC-3', Reverse 5'-ACTGGATGGGGTGGGAAT-3'</td>
</tr>
<tr>
<td>Fibroblast-specific protein (FSP)-1</td>
<td>Forward 5'-CAGCACCCTTCTCCTTGG-3', Reverse 5'-TAGGCCATTGTGTATGCAGC-3'</td>
</tr>
<tr>
<td>Collagen1/α1</td>
<td>Forward 5'-ACATGCTGCTTGTGTGGACC-3', Reverse 5'-TAGGCCATTGTGTATGCAGC-3'</td>
</tr>
<tr>
<td>α- smooth muscle actin (SMA)</td>
<td>Forward 5'-ACTGGGACGACATGGAAAAG-3', Reverse 5'-TCAGCTGGAAGTGCACAA-3'</td>
</tr>
<tr>
<td>Connective tissue growth factor (CTGF)</td>
<td>Forward 5'-CCGCAGAATCATGGCCCTGTA-3', Reverse 5'-CAAGTTTGTGGCCACACACAG-3'</td>
</tr>
<tr>
<td>Cluster of differentiation (CD)44</td>
<td>Forward 5'-AGCAGGCTGATACATTCAA-3', Reverse 5'-CAAGTTTGTGGCCACACACAG-3'</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Forward 5'-GCTTGGCTATAGGACTGAGTGTC-3', Reverse 5'-CCTTAGCATCAGGCCTTCTATG-3'</td>
</tr>
<tr>
<td>18s rRNA</td>
<td>Forward 5'-GCAATTATTCCCCATGAACG-3', Reverse 5'-AGGGCCTCACTAAACCATCC-3'</td>
</tr>
</tbody>
</table>
Statistical analysis
Data are presented as mean ± SEM. A comparison of groups was performed using an unpaired Student’s t-test and either one-way analysis of variance (ANOVA) with post hoc Bonferroni's correction, or two-way ANOVA for multiple comparisons. A value of \( P < 0.05 \) was considered to indicate statistical significance.

DISCLOSURE
All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Bone marrow–derived dendritic cells were pretreated with either vehicle or β-hydroxybutyrate (BHB; 20 mM) for 30 minutes, before being primed with lipopolysaccharide (LPS; 1 mg/ml) for 3 hours, followed by CaOx (100 µg/ml) or adenosine triphosphate (ATP; 5 mM) stimulations. Six hours later, supernatants were collected and interleukin (IL)-1β levels were measured by enzyme-linked immunosorbent assay (ELISA). Data are expressed as mean ± SEM from three independent experiments.

Figure S2. C57BL/6N mice were fed either a high-oxalate diet or a high-oxalate diet combined with 1,3-butanediol (1,3-B) for 14 days. Quantification of CaOx crystal deposition in kidneys assessed by Pizolatto staining at days 0, 3, 7, and 14 is shown (A). Mouse kidneys were analyzed by X-ray diffraction at day 14. Note that the crystals deposited in kidneys are neither CaOx dihydrate (A) nor CaOx trihydrate (B). mRNA expressions of cluster of differentiation (CD)44 (D) and osteopontin (E) in mouse tubular cells stimulated for 6 hours with CaOx crystals (300 mg/ml) isolated from kidneys of either vehicle or 1,3-B-treated mice are shown. Tubular cell death was quantified using TUNEL staining (F).

Figure S3. Primary renal fibroblasts from wild type (WT) and Nlrp3-deficient (Nlrp3−/−) mice were isolated as described in the Materials and Methods section. Cells were characterized by immunofluorescence staining for α-smooth muscle actin (α-SMA), cytokeratin, and E-cadherin (A). Cell proliferation was analyzed by MTT assay 24 hours after culturing in the presence of 10, 25, and 50 ng/ml of recombinant murine transforming growth factor beta (rTGFβ); B) or platelet-derived growth factor (rPDGF)-B (C). Cells were treated with TGFβ (50 ng/ml) for 30 minutes, and expression of pSmad3-3 was determined by western blot analysis. β-actin was used as a loading control (D). Quantification of western blots was performed by densitometry (E).

Figure S4. Murine embryonic fibroblasts (MEFs) from wild type (WT) and Nlrp3-deficient (Nlrp3−/−) mice and bone marrow–derived dendritic cells (BMDCs) from WT mice were primed with lipopolysaccharide (LPS; 1 µg/ml) for 3 hours, followed by adenosine triphosphate (ATP; 5 mM) stimulations. Six hours later, supernatants were collected and interleukin (IL)-1β levels were measured by enzyme-linked immunosorbent assay (ELISA). Supplementary material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES


