

# Interleukin-36 $\alpha$ as a potential biomarker for renal tubular damage induced by dietary phosphate load

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## Keywords

dietary phosphate load; interleukin-36; phosphate excretion per nephron; renal tubular damage

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Excessive intake of phosphate has been known to induce renal tubular damage and interstitial inflammation, leading to acute kidney injury or chronic kidney disease in rodents and humans. However, sensitive and early biomarkers for phosphate-induced kidney damage remain to be identified. Our previous RNA sequencing analysis of renal gene expression identified interleukin-36 $\alpha$  (IL-36 $\alpha$ ) as a gene significantly upregulated by dietary phosphate load in mice. To determine the time course and dose dependency of renal IL-36 $\alpha$  expression induced by dietary phosphate load, we placed mice with or without uninephrectomy on a diet containing either 0.35%, 1.0%, 1.5%, or 2.0% inorganic phosphate for 10 days, 4 weeks, or 8 weeks and evaluated renal expression of IL-36 $\alpha$  and other markers of tubular damage and inflammation by quantitative RT-PCR, immunoblot analysis, and immunohistochemistry. We found that IL-36 $\alpha$  expression was induced in distal convoluted tubules and correlated with phosphate excretion per nephron. The increase in IL-36 $\alpha$  expression was simultaneous with but more robust in amplitude than the increase in tubular damage markers such as Osteopontin and neutrophil gelatinase-associated lipocalin, preceding the increase in expression of other inflammatory cytokines, including transforming growth factor- $\alpha$ , interleukin-1 $\beta$ , and transforming growth factor- $\beta$ 1. We conclude that IL-36 $\alpha$  serves as a marker that reflects the degree of phosphate load excreted per nephron and of associated kidney damage.

Phosphorus is an essential element of life both structurally and functionally [1]. It is a major constituent of the skeleton, cell membrane, and DNA/RNA in the form of calcium phosphate, phospholipids, and nucleic acids, respectively. It also participates in the regulation of energy metabolism and cell signaling in the form of

high-energy phosphate bonds and kinase-mediated protein phosphorylation. Although indispensable for life, phosphorus can be harmful when ingested excessively. In humans, administration of oral sodium phosphate tablets (OSP) for colon cleansing before colonoscopy occasionally results in acute kidney injury

## Abbreviations

AKI, Acute kidney injury; Ccr7, C-C chemokine receptor type 7; Cd11c, cluster of differentiation 11c; CKD, chronic kidney disease; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-1RAcP, interleukin-1 receptor accessory protein; IL-36 $\alpha$ , interleukin-36 $\alpha$ ; IL-36R, interleukin-36 receptor; IRI, ischemia–reperfusion injury; Kim-1, kidney injury molecule-1; Mcp-1, monocyte chemoattractant protein-1; NF $\kappa$ B, nuclear factor-kappa B; Ngal, neutrophil gelatinase-associated lipocalin; Nlrp3, nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3; OSP, oral sodium phosphate tablets; Tgf- $\beta$ 1, transforming growth factor- $\beta$ 1; Tnf- $\alpha$ , transforming growth factor- $\alpha$ ; UNx, uninephrectomy; UUU, unilateral ureteral obstruction.

(AKI) at the frequency of 1–4% with increased risk in the aged and in patients with chronic kidney disease (CKD) [2]. In rodents, high-phosphate diet has been often used in combination with uninephrectomy (UNx) and subtotal nephrectomy to accelerate and enhance kidney damage [3]. In response to increased phosphate intake, urinary phosphate excretion must be increased to maintain the phosphate homeostasis. This demand is met by increasing phosphate excretion per nephron, which was reported to correlate with renal tubular damage and interstitial inflammation [4]. This is consistent with the finding in humans that the risk for OSP-induced AKI was increased in the aged and in CKD patients whose functional nephron number is assumed to be low [2]. Thus, not only the amount of phosphate intake but also the number of functional nephrons determines the severity of kidney damage induced by dietary phosphate load. However, the mechanism by which the increase in phosphate excretion per nephron induces kidney damage has not been clarified.

To obtain a clue to understand the mechanism, we previously performed RNA sequencing analysis using kidneys from uninephrectomized mice fed the high-phosphate diet (2.0% inorganic phosphate) and from sham-operated mice fed the regular diet (0.35% inorganic phosphate) [5]. Besides expression of markers for renal tubular damage including Osteopontin, neutrophil gelatinase-associated lipocalin (Ngal), and kidney injury molecule-1 (Kim-1), expression of several inflammatory cytokines and chemokines was upregulated, suggesting that inflammation might play a critical role in the phosphate-induced kidney damage. In the present study, we report a unique expression profile of IL-36 $\alpha$  during chronic dietary phosphate load in mice and discuss the potential role that it may play in the pathophysiology and usefulness as a biomarker for phosphate-induced kidney damage.

## Materials and methods

### Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee from Jichi Medical University (approval number 18003-01). Male C57BL/6J mice at 8 weeks of age were subjected to either right UNx or sham operation (laparotomy alone) as previously described [5]. Four weeks after the surgery, the mice were placed on a diet containing 0.35%, 1.0%, 1.5%, or 2.0% inorganic phosphate for 10 days. Alternatively, male C57BL/6J mice at 12 weeks of age were placed on a diet containing 0.35% or 2.0% inorganic phosphate for 4 or

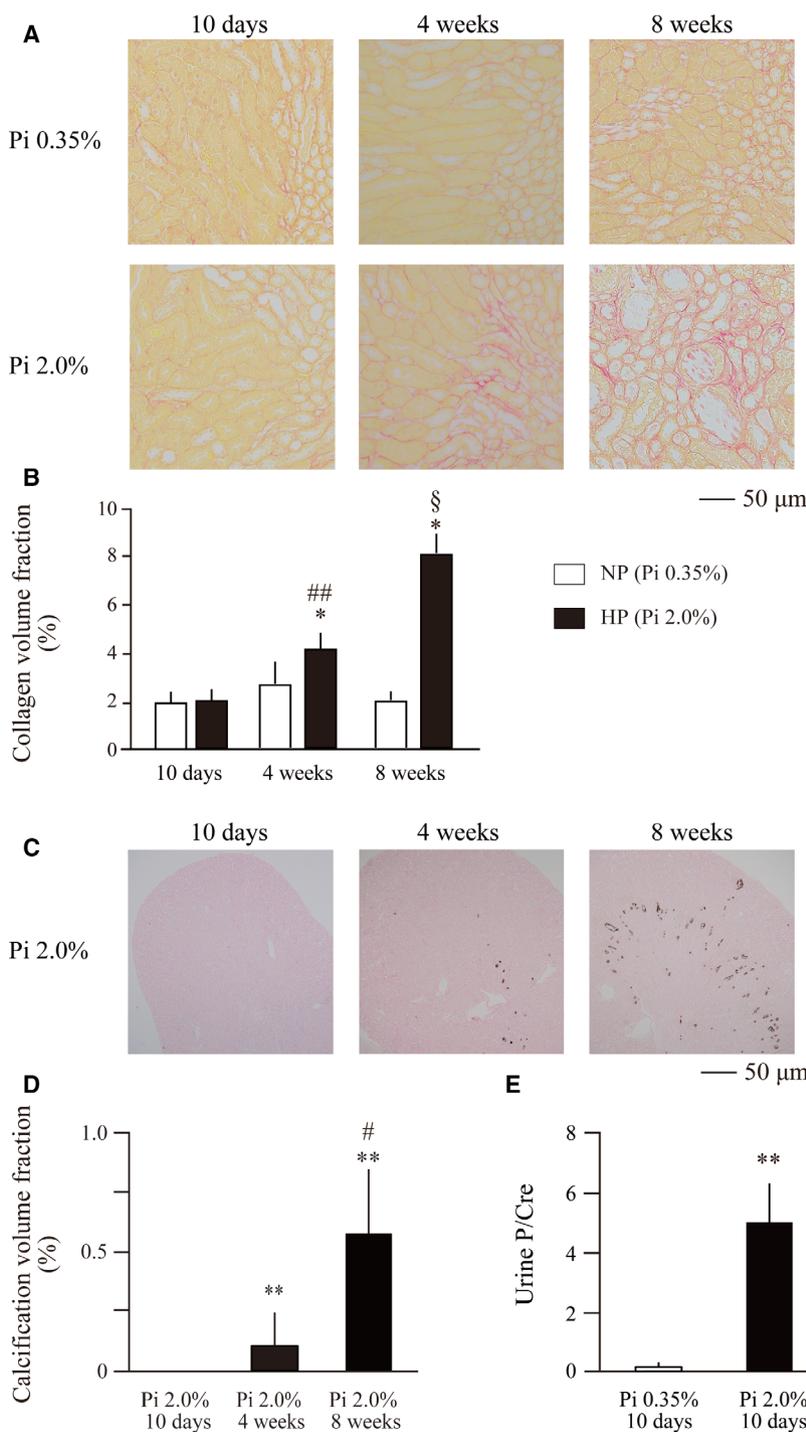
8 weeks. The mice were euthanized to harvest the left kidneys. A part of the kidney was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used for RNA and protein extraction. The rest of the kidney was subjected to histological and immunohistochemical analyses as described below.

### Histological and immunohistochemical analyses

After removing renal capsule, kidneys were fixed in 10% formalin solution overnight and then embedded in paraffin wax. Transverse sections of 3  $\mu\text{m}$  thickness were subjected to von Kossa staining to detect calcium phosphate deposits or immunohistochemical staining of IL-36 $\alpha$ , Klotho, F4/80, and Osteopontin protein. Briefly, the sections were deparaffinized in xylene and rehydrated in a graded EtOH series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water. For antigen retrieval, the sections were boiled in Target Retrieval Solution (Dako, Agilent Pathology Solutions, Santa Clara, CA, USA) for 15 min. The sections were blocked with PBS containing 0.2% (v/v) Triton X-100, 1% BSA, and 5% rabbit serum (IL-36 $\alpha$  and Klotho) or 5% goat serum (F4/80), or a blocking reagent included in Histofine Simple Stain Mouse MAX PO (Nichirei Corporation, Tokyo, Japan) (Osteopontin). After blocking, the sections were incubated with a goat polyclonal antibody against mouse IL-36 $\alpha$  (diluted 1 : 100 with PBS containing 1% BSA; AF2297; R&D Systems, Minneapolis, MN, USA), a goat polyclonal antibody against mouse Klotho (diluted 1 : 100 with PBS containing 1% BSA; BAF1819; R&D Systems), a rat monoclonal antibody against mouse F4/80 (diluted 1 : 100 with PBS containing 1% BSA; GTX26640; GeneTex, San Antonio, TX, USA), or a mouse monoclonal antibody against mouse Osteopontin (diluted 1 : 100 with PBS containing 1% BSA; sc-21742; Santa Cruz, Dallas, TX, USA) at  $4^{\circ}\text{C}$  overnight and then with Histofine Simple Stain Mouse MAX PO (Nichirei Corporation) at room temperature for 1 h. Immunoreactivity was visualized by incubation with the DAB substrate kit (Dako; Agilent Pathology Solutions). The sections were counterstained with hematoxylin. Images of the stained sections were digitized and analyzed using a microscope (BX-51; Olympus, Tokyo, Japan).

### Quantification of histological findings

Using computerized image analysis software (IMAGE PRO 9.32; Media Cybernetics, Rockville, MD, USA), we quantified the collagen volume fraction stained with Picro-Sirius Red and the calcification volume fraction stained with von Kossa in cortical tubulointerstitial area. We also quantified F4/80 expression in cortical tubules as the percentage of antibody-positive areas within a given microscope field in cortical tubulointerstitial areas without vessels or glomeruli. Data were expressed as the percentage of the total tubulointerstitial field area. In each sample, at least five

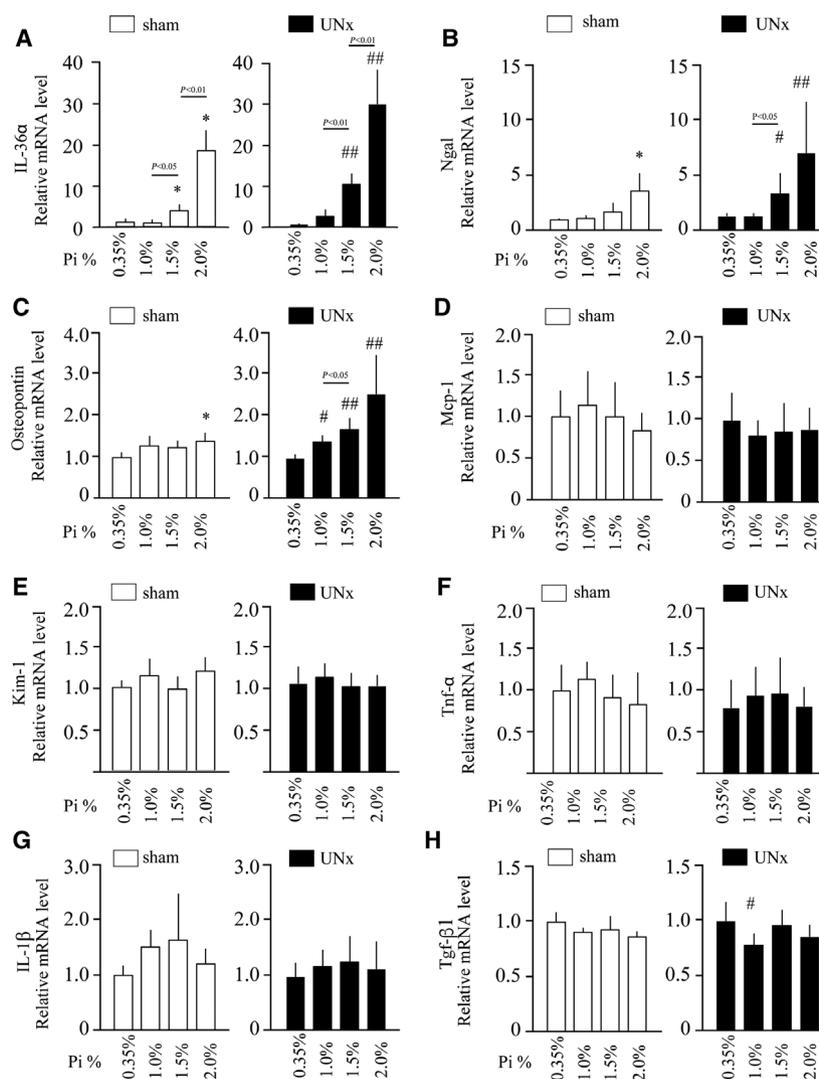


**Fig. 1.** Fibrosis and nephrocalcinosis induced by dietary phosphate load in mice. Wild-type mice at 12 weeks of age were placed on the regular diet (Pi 0.35%, NP) or high-phosphate diet (Pi 2.0%, HP) for 10 days ( $N = 8$  for Pi 0.35%,  $N = 8$  for Pi 2.0%), 4 weeks ( $N = 7$  for Pi 0.35%,  $N = 8$  for Pi 2.0%), and 8 weeks ( $N = 5$  for Pi 0.35%,  $N = 5$  for Pi 2.0%), respectively. (A) Sirius Red staining detected interstitial fibrosis in the kidneys. Bar = 50  $\mu$ m. (B) The area of the Sirius Red-positive regions was measured and expressed as a percentage of the total area using IMAGE PRO Version 9.32. The data are presented as mean  $\pm$  SD. \* $P < 0.05$  vs mice on the high-phosphate diet for 10 days, ## $P < 0.01$  vs mice on the high-phosphate diet for 10 days, and § $P < 0.05$  vs mice on the high-phosphate diet for 4 weeks by the Steel–Dwass test. (C) von Kossa staining of paraffin-embedded kidney sections in mice fed a high-phosphate diet (2.0% Pi) for 10 days ( $N = 8$ ), 4 weeks ( $N = 6$ ), and 8 weeks ( $N = 5$ ). Bar = 50  $\mu$ m. (D) The area of the von Kossa-positive regions was measured and expressed as a percentage of the total area using IMAGE PRO Version 9.32. The data are presented as mean  $\pm$  SD. \*\* $P < 0.01$  vs mice on the high-phosphate diet for 10 days and # $P < 0.05$  vs mice on the high-phosphate diet for 4 weeks by the Steel–Dwass test. (E) Urine phosphate concentration corrected with urine creatinine concentration in mice fed the high-phosphate diet (Pi 2.0%: solid bars) and normal phosphate diet (Pi 0.35%: empty bars) for 10 days ( $N = 8$  per each group). The data are presented as mean  $\pm$  SD. \*\* $P < 0.01$  vs mice on the normal phosphate diet for 10 days by the Steel–Dwass test.

randomly selected cortical tubulointerstitial fields (0.162 mm<sup>2</sup> per field) were assessed at  $\times 400$  magnification for quantification of collagen volume fraction and F4/80-positive areas, and a single kidney section (16.2 mm<sup>2</sup> per field) was assessed at  $\times 40$  magnification for quantification of calcification volume fraction. Quantification was performed in a blinded manner.

### Immunoblot analysis

The frozen kidneys were crushed by Cryo-Press (Microtec Co., LTD, Chiba, Japan). The tissue powder was homogenized in lysis buffer containing 25 mM Na-Hepes (pH 7.4), 100 mM NaCl, 0.75% (v/v) NP-40, 1 mM EDTA, and 1 $\times$  protease inhibitor cocktail (cOmplete; Roche, Mannheim,



Germany). After severing genomic DNA by sonication, the lysate containing 15  $\mu$ g protein was separated by SDS/PAGE and probed with the antibody against mouse IL-36 $\alpha$  (AF2297; R&D Systems) or actin (MAB1501, clone C4; Millipore, Darmstadt, Germany).

### Quantitative RT-PCR (qPCR)

The frozen kidneys were homogenized in RNAiso Plus (Takara, Tokyo, Japan). The lysate was transferred to microcentrifuge tube and extracted with one-fifth volume of chloroform. RNA in the aqueous phase was precipitated with an equal volume of isopropanol, washed with 75% EtOH, and dissolved in RNase-free water. Reverse transcription of RNA (0.5  $\mu$ g) was carried out using ReverTra Ace qPCR RT Master Mix with gDNA Remover (FSQ-301; Toyobo, Osaka, Japan) according to the manufacturer's protocol. Quantitative RT-PCRs contained 12.5 ng of cDNA, 410 nM of each

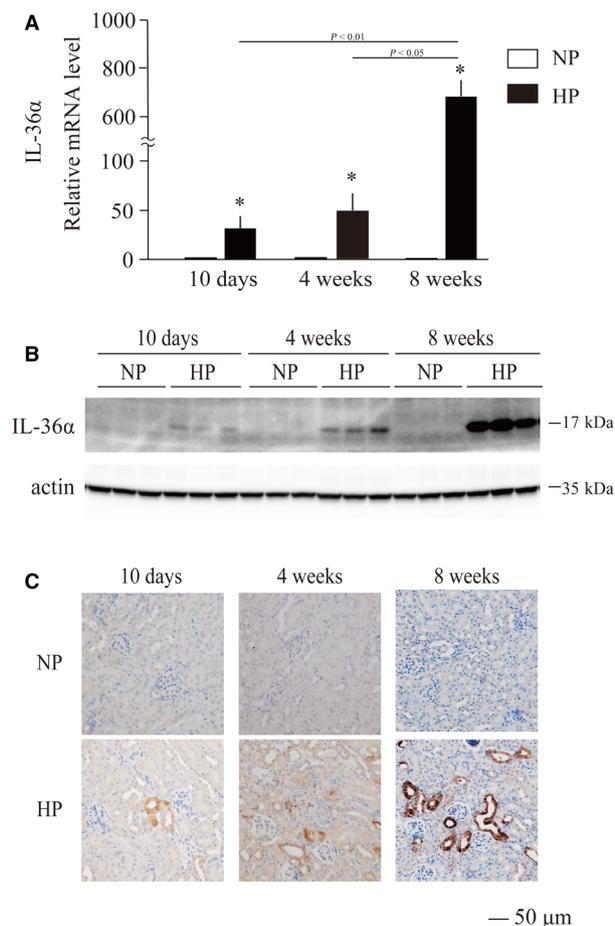
primer, and 6  $\mu$ L of SYBR Green PCR Master Mix (THUNDERBIRD SYBR qPCR Mix, QPS-201; Toyobo) in a total volume of 12  $\mu$ L. The reaction (95  $^{\circ}$ C for 1 min followed by 45 cycles of 95  $^{\circ}$ C for 10 s, 60  $^{\circ}$ C for 50 s) was performed on the Roche LC480 system. Relative mRNA levels were calculated by the comparative threshold cycle method using cyclophilin as an internal control. The forward (F) primers and reverse (R) primers used were as follows:

Cyclophilin, F: TGGAGAGCACCAAGACAGACA, R: TGCCGGAGTCGACAATGAT;

IL-36 $\alpha$ , F: CAGCATCACCTTCGCTTAGAC, R: AGTGTCCAGATATTGGCATGG;

nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (Nlrp3), F: CGAGACCTCTGGGAAAAAGCT, R: GCATACCATAGAGGAATGTGATGTACA;

Ngal, F: GAAATATGCACAGGTATCCTC, R: GTAA TTTTGAAGTATTGCTTGTTT;



**Fig. 3.** Effects of prolonged dietary phosphate load on renal IL-36 $\alpha$  expression. Wild-type mice at 12 weeks of age were placed on a regular diet containing 0.35% Pi (NP) or a high-phosphate diet containing 2.0% Pi (HP) for 10 days ( $N = 8$ ), 4 weeks ( $N = 6$ ), or 8 weeks ( $N = 5$ ). Kidneys were excised from mice fed a diet containing 0.35% and 2.0% inorganic phosphate for 10 days, 4 weeks, and 8 weeks. (A) Relative mRNA expression levels of IL-36 $\alpha$  in the kidney are indicated. All data are presented as mean  $\pm$  SD. \* $P < 0.01$  vs mice 0.35% Pi-fed mice, by Wilcoxon's rank-sum test. (B) Immunoblot analysis of the kidney lysates using the antibody against IL-36 $\alpha$  and  $\beta$ -actin as an internal control. (C) Immunohistochemical staining for IL-36 $\alpha$  protein in the kidney from mice fed the NP or HP for 10 days, 4 weeks, and 8 weeks. Bar = 50  $\mu$ m.

Osteopontin, F: TCCAAAGAGAGCCAGGAGAG, R: GGCTTTGGAAGCTTGCTTGAC;

monocyte chemoattractant protein-1 (Mcp-1), F: GGCTCA GCCAGATGCAGTTAAC, R: GCCTACTCATTGGGAT CATCTTG;

Kim-1, F: CTGGAATGGCACTGTGACATCC, R: GCAGATGCCAACATAGAAGCCC;

transforming growth factor- $\alpha$  (Tnf- $\alpha$ ), F: AAGCCTG TAGCCCACGTCGTA, R: GGCACCACTAGTTGGTT GTCTTTG;

interleukin-1 $\beta$  (IL-1 $\beta$ ), F: TGAAGTTGACGGACCC-CAAA, R: TGATGTGCTGCTGCGAGATT;

transforming growth factor- $\beta$ 1 (Tgf- $\beta$ 1), F: TTGCT TCAGCTCCACAGAGA, R: TGGTTGTAGAGGGCAA GGAC;

cluster of differentiation 11c (Cd11c), F: GTTTGTCT CAGACGGG, R: GCGGGTTCAAAGACGATGG; and

C-C chemokine receptor type 7 (Ccr7), F: CTCTTC AAGGACTTGGGCTG, R: CCTGGGAGAGGTCCTTG TAG.

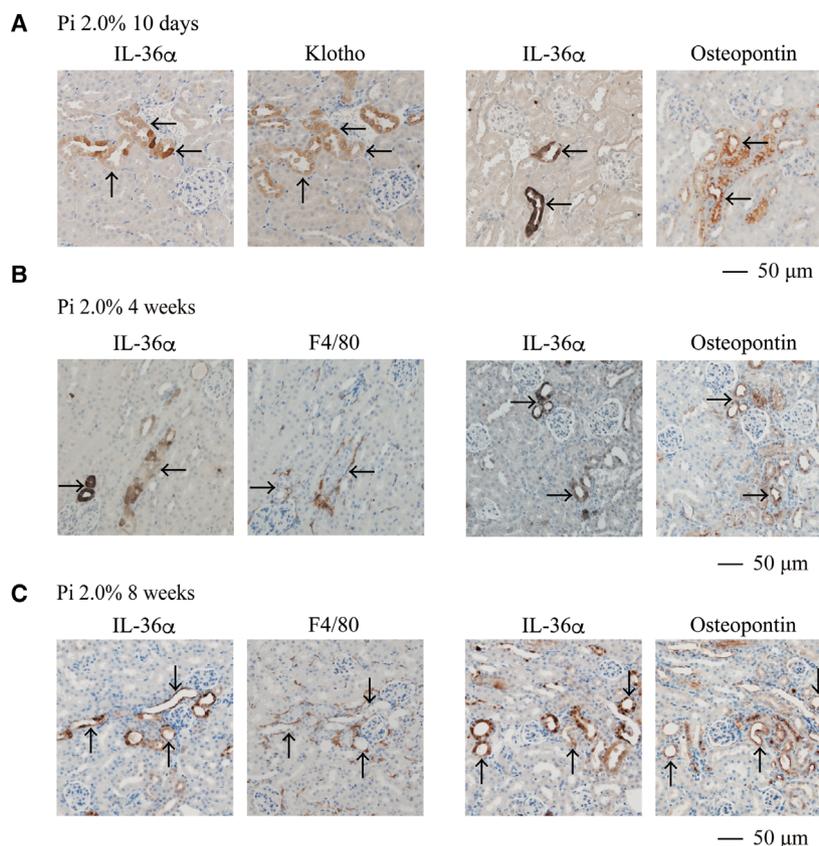
## Statistics

Data were analyzed using the Wilcoxon rank-sum test, or the Kruskal-Wallis test, followed by *post hoc* analysis with the Steel-Dwass test, where appropriate. A  $P$ -value  $< 0.05$  was considered statistically significant.

## Results

When normal adult mice were placed on a high-phosphate diet containing 2.0% inorganic phosphate, depositions of collagen and calcium phosphate became detectable within 4 weeks in renal tubules around the cortico-medullary junction (CMJ), which progressed to overt tubulointerstitial fibrosis and nephrocalcinosis within 8 weeks (Fig. 1A–D). Because an increase in urinary phosphate excretion was observed much earlier (Fig. 1E), we speculated that exposure of renal tubular cells to high-phosphate tubular fluid might have caused kidney damage prior to the development of tubulointerstitial fibrosis and nephrocalcinosis. To identify earlier changes that might have occurred before tubulointerstitial fibrosis and nephrocalcinosis became evident at the histological level, we evaluated renal expression of several markers for renal tubular damage (Osteopontin, Ngal, and Kim-1) and inflammation (Mcp-1, Tnf- $\alpha$ , IL-1 $\beta$ , Tgf- $\beta$ 1, and IL-36 $\alpha$ ) at 10 days after increasing phosphate excretion per nephron (Fig. 2).

We manipulated phosphate excretion per nephron by placing uninephrectomized mice and sham-operated mice on diet containing either 0.35%, 1.0%, 1.5%, or 2.0% inorganic phosphate for 10 days. Phosphate excretion per nephron in the uninephrectomized mice was supposed to be twofold higher than that in the sham-operated mice placed on the same diet. Quantitative RT-PCR (qPCR) analysis revealed significant upregulation of Ngal (Fig. 2B) and Osteopontin (Fig. 2C), but not Kim-1 (Fig. 2E), with an increase in the dietary phosphate content. Despite the evidence for renal tubular damage, histological examination failed to detect collagen and calcium

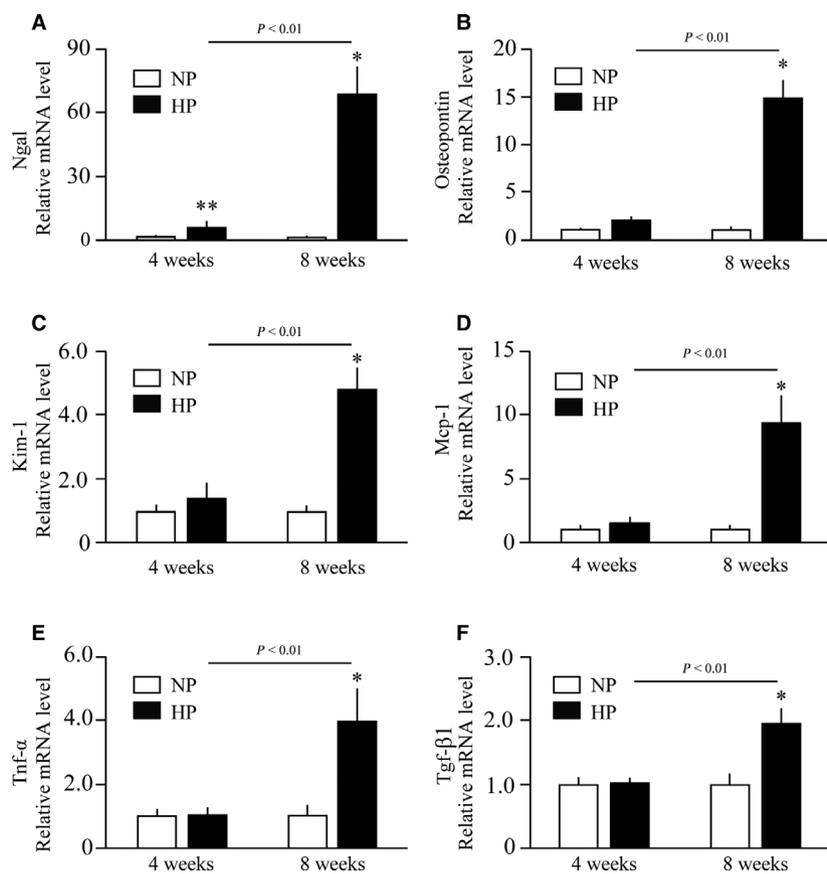


**Fig. 4.** Localization of IL-36 $\alpha$  expression induced by phosphate load. Wild-type mice at 12 weeks of age were placed on a high-phosphate diet containing 2.0% Pi for 10 days (A), 4 weeks (B), and 8 weeks (C). Serial paraffin sections of the kidneys were subjected to immunohistochemical staining using antibodies against IL-36 $\alpha$ , Klotho, Osteopontin, and F4/80. (A) Klotho-positive tubules expressed IL-36 $\alpha$ . Osteopontin-positive tubules expressed IL-36 $\alpha$ . (B, C) IL-36 $\alpha$ -positive tubules expressed Osteopontin and were surrounded by F4/80-positive cells. Arrows indicate identical renal tubule in serial sections. Bar = 50  $\mu$ m.

phosphate deposits within 10 days after the phosphate load, indicating that renal tubular damage at the molecular level had started much earlier than development of tubulointerstitial fibrosis and nephrocalcinosis at the histological level. Notably, uninephrectomized mice showed higher Osteopontin and Ngal mRNA levels than sham-operated mice placed on the same diet (1.5% and 2.0% inorganic phosphate). This finding is consistent with the previous observation that phosphate excretion per nephron correlated with renal tubular damage [4]. In contrast to the markers for tubular damage, those for inflammation were not upregulated at this time point (Fig. 2D,F,G,H) except IL-36 $\alpha$  (Fig. 2A). Renal IL-36 $\alpha$  expression was undetectable in mice fed the regular diet (0.35% inorganic phosphate) but significantly upregulated within 10 days after starting dietary phosphate load in a dose-dependent manner. Like Osteopontin and Ngal, IL-36 $\alpha$  mRNA levels were higher in uninephrectomized mice than those in sham-operated mice fed the same diet (1.5% and 2.0% inorganic phosphate).

Renal expression of IL-36 $\alpha$  was further increased after prolonged dietary phosphate load. Normal adult mice placed on high-phosphate diet containing 2.0%

inorganic phosphate had 47.9-fold and 697.8-fold higher IL-36 $\alpha$  mRNA levels within 4 and 8 weeks, respectively, than mice placed on a regular diet containing 0.35% inorganic phosphate (Fig. 3A). Consistent with the mRNA levels, IL-36 $\alpha$  protein was detectable by immunoblot analysis in the kidney lysates from mice fed the high-phosphate diet for 4 and 8 weeks, but not in those from mice fed the regular diet (Fig. 3B). In immunohistochemical analysis, strong IL-36 $\alpha$  expression was observed specifically in some renal tubules in the cortex (Fig. 3C), which did not colocalize with calcium phosphate depositions that appeared mainly in the CMJ but colocalized with Klotho, which is expressed in distal convoluted tubules [6] (Fig. 4A, arrows). IL-36 $\alpha$ -positive tubular cells also expressed Osteopontin, indicating that IL-36 $\alpha$  is expressed in injured renal tubules. After prolonged dietary phosphate load (4 and 8 weeks), Klotho expression was downregulated and difficult to detect by immunohistochemistry (data not shown). However, we confirmed consistent colocalization of IL-36 $\alpha$  and Osteopontin in renal tubular cells throughout the observation period (Fig. 4, arrows). Although F4/80-positive macrophages were infiltrated around the IL-36 $\alpha$ -positive tubules (Fig. 4B,C), they were negative



**Fig. 5.** Effects of prolonged dietary phosphate load on renal expression of markers for renal tubular damage and inflammation. Wild-type mice at 12 weeks of age were placed on a regular diet containing 0.35% Pi (NP) or 2.0% Pi (HP) for 4 weeks ( $N = 6$  per group) or 8 weeks ( $N = 5$  per group). Relative mRNA levels of Ngal (A), Osteopontin (B), Kim-1 (C), Mcp-1 (D), tumor necrosis factor- $\alpha$  (Tnf- $\alpha$ ) (E), and Tgf- $\beta$ 1 (F) are indicated. All data are presented as mean  $\pm$  SD. \* $P < 0.05$  vs mice fed 0.35%Pi for 8 weeks, \*\* $P < 0.01$  vs mice fed 0.35%Pi for 4 weeks by Wilcoxon's rank-sum test.

for IL-36 $\alpha$ , suggesting that IL-36 $\alpha$  expression may be basically restricted to damaged renal tubules.

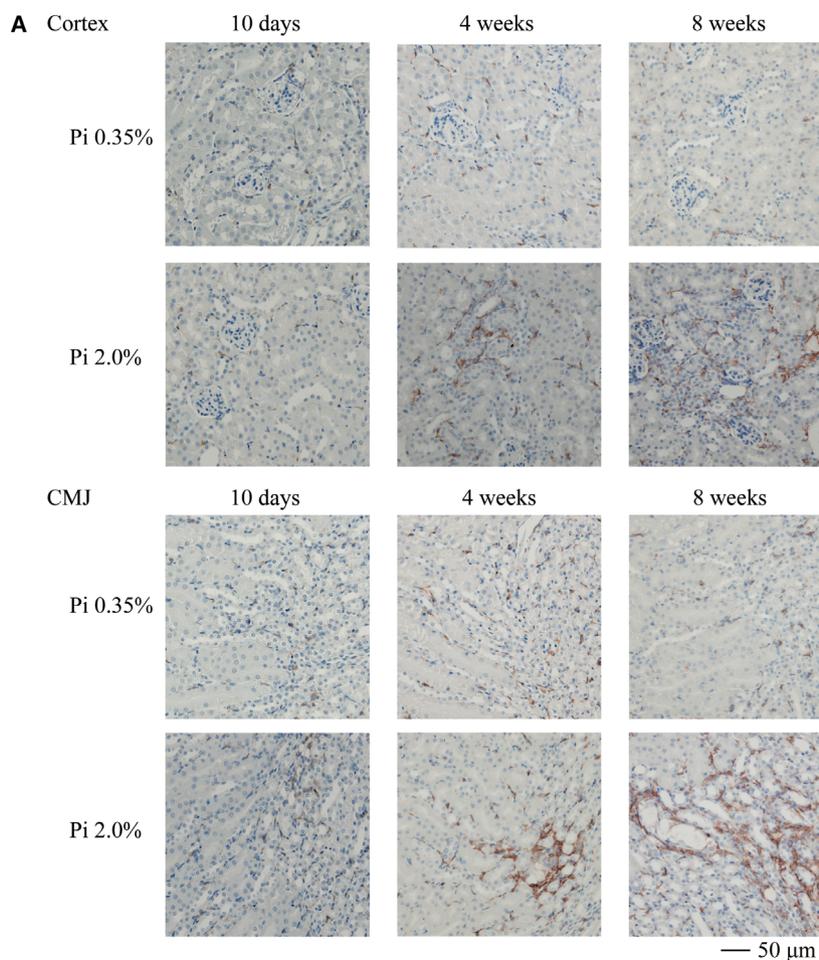
Besides IL-36 $\alpha$ , feeding of the high-phosphate diet for 4 weeks slightly raised the mRNA levels of Ngal (Fig. 5). Interstitial infiltration of F4/80-positive macrophages was observed in the cortex and CMJ, which was exacerbated with an increase in the period of dietary phosphate load (Fig. 6). After 8 weeks of high-phosphate diet feeding, expression of all the markers for inflammation and renal tubular damage so far examined was significantly increased (Fig. 5). Of note, expression of Nlrp3 and IL-1 $\beta$  was also induced (Fig. 7A,B), which are downstream targets of the nuclear factor-kappa B (NF $\kappa$ B) signaling activated by IL-36 $\alpha$  [7]. Furthermore, mRNA levels of Cd11c and Ccr7 expressed in dendritic cells [8] were increased (Fig. 7C,D), indicating dendritic cell infiltration that was reported to be induced by IL-36 $\alpha$  [9].

## Discussion

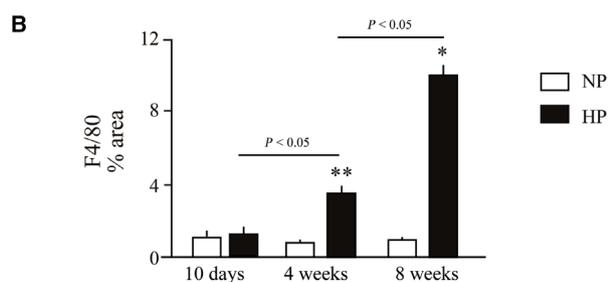
The present study identified IL-36 $\alpha$  as a robust and early marker for kidney damage induced by increased phosphate excretion per nephron. IL-36 $\alpha$  is a recently identified pro-inflammatory cytokine that belongs to

the IL-1 family and reported to contribute to the pathophysiology of several disorders associated with inflammation, including psoriasis, chronic obstructive pulmonary disease, and obesity [10,11]. Like other cytokines, IL-36 $\alpha$  is expressed primarily in immunocompetent cells, but is induced in renal tubular cells in patients and rodent models with AKI and CKD [9,12]. In mice, unilateral ureteral obstruction (UUO), ischemia-reperfusion injury (IRI), glomerulonephritis, and diabetic nephropathy were reported to induce expression of IL-36 $\alpha$  in renal tubular cells [9,13,14]. In humans, urine levels of IL-36 $\alpha$  were reported to increase in patients with AKI [12] and CKD [9]. These findings suggest that IL-36 $\alpha$  may serve as a biomarker for various types of kidney damage.

It is possible to argue that calcium phosphate precipitates might cause tubular obstruction, which potentially contributed to the tubular damage and thus increased IL-36 $\alpha$  expression. In fact, tubular dilatation was observed in mice fed the high-phosphate diet for 4 weeks or longer (Figs 1A and 3C). However, this does not explain the early phase of IL-36 $\alpha$  induction that occurred before calcium phosphate precipitations became detectable at the histological level at 4 weeks of phosphate load. These findings suggest that



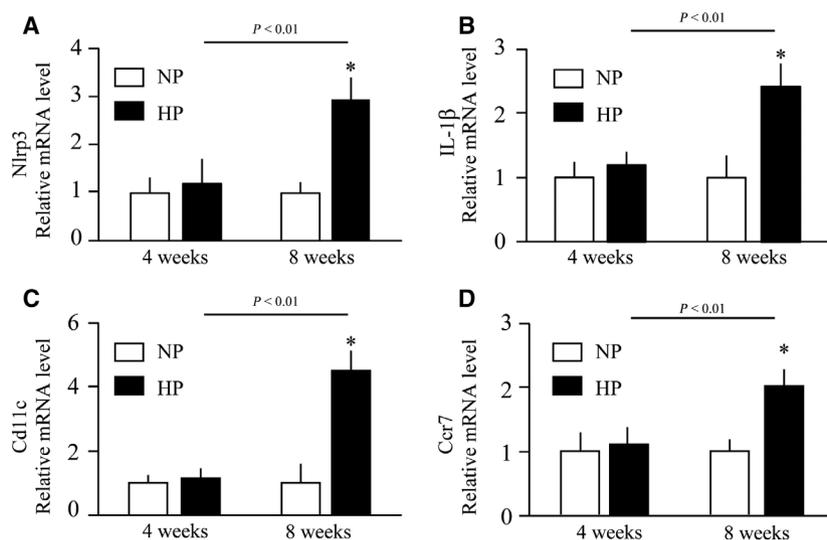
**Fig. 6.** Interstitial macrophage infiltration induced by dietary phosphate load. Wild-type mice at 12 weeks of age were placed on a regular diet (Pi 0.35%) or high-phosphate diet (Pi 2.0%) for 10 days, 4 weeks, or 8 weeks. (A) Paraffin sections of the kidneys were subjected to immunohistochemical staining using antibodies against F4/80. Interstitial infiltration of macrophages was observed in the cortex and CMJ in mice fed the high-phosphate diet for 4 and 8 weeks. Bar = 50  $\mu$ m. (B) The area of the F4/80-positive regions was measured and expressed as a percentage of the total area using IMAGE PRO Version 9.32. Empty and solid bars represent mice fed the regular diet (Pi 0.35%) or high-phosphate diet (Pi 2.0%), respectively, for 10 days ( $N = 8$  for Pi 0.35%,  $N = 8$  for Pi 2.0%), 4 weeks ( $N = 6$  for Pi 0.35%,  $N = 5$  for Pi 2.0%), and 8 weeks ( $N = 5$  for Pi 0.35%,  $N = 5$  for Pi 2.0%). The data are presented as mean  $\pm$  SD. The positive area was larger in mice fed the high-phosphate diet when compared with mice on the regular diet for 4 and 8 weeks. \* $P < 0.05$  vs mice fed 0.35%Pi for 8 weeks and \*\* $P < 0.01$  vs mice fed 0.35%Pi for 4 weeks, by Wilcoxon's rank-sum test.



phosphate itself, rather than tubular obstruction caused by calcium phosphate precipitations, may play a pathogenic role at least in the early phase during the course of tubular damage progression caused by dietary phosphate load, although the mechanism remains to be determined.

Osteopontin, Ngai, and Kim-1 were used as markers for renal tubular damage in this study. Osteopontin is a secreted phosphoglycoprotein with multiple functions, including recruitment of macrophages [15] and inhibition of calcium phosphate nucleation and aggregation [16]. Osteopontin appears protective against

calcification, because mice lacking Osteopontin were prone to nephrocalcinosis and stone formation [16]. Thus, induction of Osteopontin expression by dietary phosphate load can be regarded as a defense mechanism against nephrocalcinosis. Ngai is an early and robust marker for renal tubular damage reported to rise in urine about 1000-fold within hours in a mouse IRI model [17] and about 20-fold in patients after cardiac surgery [18]. Although induction of Ngai expression by dietary phosphate load occurred within 10 days and coincided with induction of IL-36 $\alpha$  (Fig. 2A,B), the maximum induction observed in the



**Fig. 7.** Effects of prolonged dietary phosphate load on renal expression of markers for renal inflammasome and dendritic cells. Wild-type mice at 12 weeks of age were placed on a regular diet containing 0.35% Pi (NP) or 2.0% Pi (HP) for 4 weeks ( $N = 6$  per group) or 8 weeks ( $N = 5$  per group). Relative mRNA levels of Nod-like receptor protein 3 (Nlrp3) (A), IL-1 $\beta$  (B), Cd11c (C), and Ccr7 (D) are indicated. All data are presented as mean  $\pm$  SD. \* $P < 0.05$  vs mice fed 0.35% Pi for 4 weeks by Wilcoxon's rank-sum test.

uninephrectomized mice fed 2.0% phosphate diet was no more than sevenfold and less potent than the 18.6-fold increase in IL-36 $\alpha$  expression. Kim-1 was less robust than Ngal, because induction of Kim-1 was not clear within 10 days even in the uninephrectomized mice fed the 2.0% phosphate diet (Fig. 2E), and reached 1.46-fold and 4.83-fold at most within 4 and 8 weeks, respectively (Fig. 5C). Taken together, IL-36 $\alpha$  may be potentially advantageous to Osteopontin, Ngal, and Kim-1 as a marker for kidney damage induced by increased phosphate excretion per nephron.

IL-36 $\alpha$  may serve not merely as a marker for kidney damage but play a critical role in the pathogenesis. Binding of IL-36 $\alpha$  to IL-36 receptor (IL-36R) recruits IL-1 receptor accessory protein (IL-1RAcP) to form the ternary complex composed of IL-36 $\alpha$ , IL-36R, and IL-1RAcP and activate NF $\kappa$ B, mitogen-activated protein kinase [19], and NLRP3 inflammasome, which promotes maturation and secretion of other inflammatory cytokines including IL-1 $\beta$  and IL-18 [9]. Chi *et al.* [9] reported that IL-36 $\alpha$  activated NLRP3 inflammasome in the UUO kidney. In addition, they showed that IL-36 $\alpha$  triggered dendritic cell-induced T-cell proliferation and Th17 differentiation and that ablation of the IL-36R gene or administration of an IL-36R antagonist alleviated the UUO-induced kidney damage [9]. Thus, a similar mechanism may be applicable to the kidney damage induced by increased phosphate excretion per nephron as well. In fact, a significant increase in Nlrp3, IL-1 $\beta$ , and dendritic cell markers (Cd11c and Ccr7) was observed within 8 weeks after starting dietary phosphate load (Fig. 7). Further studies are necessary to verify this hypothesis, including dietary phosphate load in IL-36R-deficient mice.

## Conclusions

IL-36 $\alpha$  serves as a marker that reflects the degree of phosphate load excreted per nephron and of associated kidney damage.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

YH and HK developed the study concept. YH, HK, KS, and YI carried out experiments and analyzed the results under the supervision of ST and MK. MK obtained funding and drafted the manuscript. All authors have read and approved the manuscript.

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