Integrin αM promotes macrophage alternative M2 polarization in hyperuricemia-related chronic kidney disease

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Abstract

Background and purpose: Hyperuricemia is an essential risk factor in chronic kidney disease (CKD), while urate-lowering therapy to prevent or delay CKD progression is controversial. Alternatively activated macrophages in response to local microenvironment play diverse roles in kidney injury, repair, and fibrosis. Here, we aim to investigate whether and how macrophage ITGAM contributes to hyperuricemia-related CKD. Experimental approach: In vivo, we explored dynamic characteristics of renal tissue in hyperuricemia-related CKD. By incorporating mRNA and protein sequencing data, we analyzed gene expression profile, hub genes and potential pathways responsible for disease development, which was further confirmed using qPCR, western blotting, and immunofluorescent stainings. In vitro, we validated bioinformatic findings under different conditions of macrophages with interventions corresponding to core nodes in pathway. Key Results: Hyperuricemia-related CKD was characterized by the rise in serum uric acid, decline in renal function, macrophage alternative (M2) polarization, and kidney fibrosis. Integrated bioinformatic analyses revealed ITGAM as the potential core gene mediating disease progression which was associated with FAK/Akt1/β-catenin signaling. Notably, we confirmed the upregulated macrophage ITGAM, activated pathway, and macrophage M2 polarization in injured kidneys and macrophages. In vitro, we verified ITGAM/FAK/Akt1/β-catenin pathway participated in promoting macrophage M2 polarization through silencing Itgam and inhibiting FAK or Akt1 phosphorylation, where the expression of M2 phenotype macrophage markers and downstream molecules in pathway were down-regulated. Conclusion and implications: In hyperuricemia-related CKD, ITGAM promotes macrophage M2 polarization contributing to renal fibrosis through FAK/Akt1/β-catenin pathway. Targeting macrophage ITGAM might be a promising therapeutic approach for preventing or delaying CKD.

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What is already known

Prevalence of hyperuricemia has been sharply rising, however, it is controversial whether urate-lowering is effective to prevent or delay chronic kidney disease (CKD) progression.

What this study adds

Integrin ITGAM was found to be a hub gene promoting macrophage alternative polarization and contributing to renal fibrosis in hyperuricemia-related CKD through activating FAK/Akt1/β-catenin pathway

What is the clinical significance

Presenting integrin ITGAM with its related signaling pathway as potentially therapeutic strategy in preventing or delay CKD.

Abstract

Background and purpose:

Hyperuricemia is an essential risk factor in chronic kidney disease (CKD), while urate-lowering therapy to prevent or delay CKD progression is controversial. Alternatively activated macrophages in response to local microenvironment play diverse roles in kidney injury, repair, and fibrosis. Here, we aim to investigate whether and how macrophage ITGAM contributes to hyperuricemia-related CKD.

Experimental approach:

In vivo, we explored dynamic characteristics of renal tissue in hyperuricemia-related CKD. By incorporating mRNA and protein sequencing data, we analyzed gene expression profile, hub genes and potential pathways responsible for disease development, which was further confirmed using qPCR, western blotting, and immunofluorescent stainings. In vitro, we validated bioinformatic findings under different conditions of macrophages with interventions corresponding to core nodes in pathway.

Key Results:

Hyperuricemia-related CKD was characterized by the rise in serum uric acid, decline in renal function, macrophage alternative (M2) polarization, and kidney fibrosis. Integrated bioinformatic analyses revealed ITGAM as the potential core gene mediating disease progression which was associated with FAK/Akt1/β-catenin signaling. Notably, we confirmed the upregulated macrophage ITGAM, activated pathway, and macrophage M2 polarization in injured kidneys and Raw 264.7 macrophages. In vitro, we verified ITGAM/FAK/Akt1/β-catenin pathway participated in promoting macrophage M2 polarization through silencing Itgam and inhibiting FAK or Akt1 phosphorylation, where the expression of M2 phenotype macrophage markers and downstream molecules in pathway were down-regulated.

Conclusion and implications:

In hyperuricemia-related CKD, ITGAM promotes macrophage M2 polarization contributing to renal fibrosis through FAK/Akt1/β-catenin signaling pathway. Targeting macrophage ITGAM might be a promising

therapeutic approach for preventing or delaying CKD.

Key words

Hyperuricemia; chronic kidney disease; integrin αM; macrophage M2 polarization.

Introduction

Prevalence of hyperuricemia has been rising in recent years due to unhealthy dietary patterns and lifestyles (Bakris et al., 2014; Chen-Xu et al., 2019). The kidneys, eliminating two-third uric acid in healthy individuals, are associated with new and progressive chronic kidney disease (CKD). Kidney impairment owing to hyperuricemia is mainly characterized by chronic interstitial nephritis, urate crystals (or stones), and kidney fibrosis(Sato et al., 2019; Wen et al., 2021). By far, as a modifiable metabolite, uric acid is a potential target to alleviate renal damages induced by hyperuricemia. But in the setting of CKD, it's controversial whether uric acid-lowering is an effective strategy to prevent or delay CKD progression(Oluwo & Scialla, 2021). Studies on exploring therapeutic targets that participate in hyperuricemia-related CKD are of great significance.

Integrin is the largest family of cell adhesion molecules and is involved in kidney development and diseases(Marek et al., 2014; Muller et al., 1997). Renal fibrosis could be induced by integrins through cell-matrix or cell-cell interactions (Pozzi & Zent, 2013). Integrins are αβ heterodimeric transmembrane glycoproteins and mainly divided into integrin β1, β2, and β3 families according to β subunits(Takada et al., 2007). As transmembrane receptors, integrins participates in cell proliferation, survival and migration, differentiation, and matrix homeostasis(Pozzi & Zent, 2013). Due to lack of enzymatic activity, integrins need to bind adaptor proteins for intracellular signal propagation, such as focal adhesion kinase (FAK), a key tyrosine kinase of intracellular signaling binding to a number of downstream molecules(Guan, 1997).

αMβ2, also known as macrophage antigen 1 (Mac-1), is the predominant leukocyte-specific β2 integrin abundantly expressed in monocytes/macrophages and dendritic cells(Martinez et al., 2020). The αMI-domain within αMβ2 mediates ligand binding and is responsible for substrate specificity, thus integrin αM mainly determined diverse functions of Mac-1(Podolnikova et al., 2015). Previous studies reported that integrin αM (ITGAM) overexpression was associated with macrophage infiltration and renal fibrosis(Dehnadi et al., 2017; Lange-Sperandio et al., 2006). Macrophages accumulate in injured kidneys and present as polarized M1 or M2 phenotype for pro-inflammatory or pro-fibrotic functions, respectively(Lee et al., 2020; Murray, 2017). Macrophage alternative (M2) polarization is considered as an essential feature of fibrosis(Feng et al., 2018). However, it's not well-elucidated whether ITGAM regulated macrophage M2 polarization in renal fibrosis and signaling pathways involved.

Here, integrin ITGAM is reported as the hub gene promoting macrophage M2 polarization in hyperuricemia-related CKD. We adopted integrated bioinformatic analysis and verified that ITGAM participated in kidney disease development through FAK/Akt1/β-catenin pathway. Our study reveals a mechanism in hyperuricemia-related kidney fibrosis that involves ITGAM expression and signaling related, and provides potential therapeutic targets to prevent or delay the progression of CKD.

Methods

Animal experimental design

Seven-week male mice (C57BL/6J, 22-25g) were purchased from Dossy Experimental Animal Co., Ltd (Chengdu, China). These mice were maintained in the controlled environment for 1 week (temperature at 20 ± 2 , humidity at 50–60%, and 12-hour light/dark cycle) and provided with food and water ad libitum. To study dynamic characteristic of uric acid-induced kidney injury, we randomly divided mice into 4 groups: day 0, day 7, day 14, and day 21 groups (n=6 per group). The mice in group day 7, 14 and 21 were administered with a mixture of adenine (0.16 g/kg) and potassium oxonate (2.4 g/kg) by oral gavage once daily, as previously described by our team(Pan et al., 2019; Ren et al., 2021). At day 0, 7, 14, and 21, each group anaesthetized with pentobarbital sodium (50 mg/kg, i.p) and sacrificed. Terminal blood were

immediately centrifuged and stored at -80 after collection. Kidney was divided and samples were processed and kept in 10% phosphate buffered formalin, 2.5% glutaraldehyde, or liquid nitrogen depending on purpose, and followed by quick delivery to storage. Animal research was performed in strict accordance with the Animal Research: Reporting of In Vivo Experiments guidelines(Percie du Sert et al., 2020). This study received approval granted by Animal Care and Use Ethics Committee of Sichuan University (Approval No. 20220303052).

Biochemical measurements

We used fully clinical chemistry analyzer (Mindray BS-240) to test all the biochemical measurements as previously reported(Liu et al., 2021), including: serum uric acid (UA, μmol/L), serum creatinine (SCr, μmol/L), blood urea (mmol/L), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), total cholesterol (TC, mmol/L), serum triglycerides (TG, mmol/L), urine albumin (g/L), and urine creatinine (μmol/L).

Transcriptomics

The mRNA was sequenced by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). In brief, total RNA was extracted from the tissue using TRIzol® Reagent (Invitrogen, CA), followed by sample integrity, quality, and purity accordingly. Double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). After size selection and quantification, mRNA sequencing was performed using Illumina HiSeq xten/NovaSeq 6000 sequencer (2 × 150 bp read length). A dataset of clean reads aligned to reference genome was established for bioinformatic analysis.

Proteomics

Protein was sequenced by PTM-Biolab Co. Ltd (Shanghai, China). Kidney tissue containing 30 mg of protein was digested with trypsin. After digestion, eluates were desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. TMT labeling was performed according to the manufacturer's protocol for TMT kit/iTRAQ kit. The tryptic peptides were fractionated by high pH reverse-phase HPLC using Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). Samples were dried down and were stored at -20°C before LC-MS/MS measurement.

Fractions (0.1% formic acid) were subjected to NSI source followed by MS/MS in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z.

MS/MS data were identified by matching the raw data to the UniProtKB mouse database (version v06.06.14) using MaxQuant version 1.5.2.8 and its built-in Andromeda search engine for peak detection and quantification. Search parameters were set as follows: (i) full tryptic specificity was up to 4 missing cleavages; (ii) mass tolerance for precursor ions was 20 ppm in First search and 5 ppm in Main search; (iii) mass tolerance for fragment ions was 0.02 Da; (iv) carbamidomethyl on cysteine was set as fixed modification; and (v) acetylation modification and oxidation on Met were specified as variable modifications. FDR was adjusted to $< 1\%$ and minimum score for modified peptides was set > 40.

Bioinformatic analysis

All the omics datasets were based on unified gene symbol. For proteomic data, the highest interquartile range of transcript or peptide was chosen if multiple of them were mapped to the same gene. Differentially expressed genes (DEGs) were defined as FDR<0.05 and absolute value of fold change >1.5. The DEG dataset for further analysis consisted of those genes differentially expressed on both mRNA and protein levels. Gene ontology

(GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, and Ingenuity Pathway Analysis (IPA) were subsequently conducted to identify biological characteristics, biomarkers, and pathways related. We used molecular complex detection (MCODE) plugin from Cytoscape to analyze top clusters from the network of interactions between DEGs. We picked out the top 5 clusters and performed CentiScaPe to calculate centrality indexes and identify hub genes. Canonical pathway analysis in IPA led pathways based on these genes. Target gene was decided after comprehensively incorporating pathways and hub genes enriched. Finally, we selected DEGs positively correlated with targes gene and investigate potential pathways.

Kidney histology

Kidney samples were dehydrated with 10% phosphate buffered formalin followed by paraffin-embedding. Tissue sections of 4 μm were stained with periodic acid-Schiff (PAS) and Masson trichrome stain for morphology and fibrosis assessment, respectively. These sections were viewed using light microscopy and were semi-quantitatively estimated for renal tubular damage according to following scoring system: 0 for 0%, 0.5 for <10%, 1 for 10–25%, 2 for 26–50%, 3 for 51–75% and 4 for 76–100%. Severity of renal interstitial fibrosis was estimated according to the following four grades: 0% fibrosis (grade 0); 1-25% fibrosis (grade 1); 26-50% fibrosis (grade 2); and >50% fibrosis (grade 3) in interstitial area(Sethi et al., 2017).

Western blot analysis

Total protein extracted from kidney tissue was loaded into 10–12% SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (0.2 μm, Bio-Rad Laboratories, Inc.), as previously described. Immunoblots were visualized using and quantified by ImageJ software. Primary antibodies included: α-tubulin (Abcam, ab179484, 1:1000, RRID: AB_2890906), GAPDH (Zen BioScience, 200306-7E4, 1:5000, RRID: AB_2722713), collagen-I (Abcam, ab270993, 1:1000), collagen-IV (Abcam, ab6586, 1:1000, RRID: AB_305584), fibronectin (Boster Biological Technology, ba1772, 1:1000), α-SMA (Abcam, ab7817, 1:1000, RRID: AB_262054), TGF-β (Abcam, ab92486, 1:1000, RRID: AB_10562492), IL-1β (Abcam, ab9722, 1:1000, RRID: AB_308765), IL-6 (Huabio, EM170414, 1:1000), TNF-α (Huabio, R1203-1, 1:1000), ITGAM (Abcam, ab8878, 1:1000, RRID: AB_306831), SRC (Huabio, ET1602-03, 1:1000), p-SRC (Huabio, ET1609-15, 1:1000), FAK (Huabio, ET1602-25, 1:1000), p-FAK (Huabio, RT1216, 1:1000), Akt1 (Huabio, ET1609-47, 1:1000), p-Akt1 (Huabio, ET1701-36, 1:1000), GSK-3β (Huabio, ET1607-71, 1:1000), p-GSK-3β (Huabio, ET1607-54, 1:1000), β-catenin (Huabio, EM0306, 1:1000). The density quantitative analysis was conducted by Image J software.

Immunofluorescence staining

Kidney samples were mounted with O.C.T. compound medium (Tissue-Tek) and stored at -80 °C. Frozen samples were cut into 4-μm sections followed by immediate fixation (10% PBS-buffered formalin), washing, and dehydration. Sections were blocked with 10% PBS-buffered horse serum for 1 hour at room temperature, incubated with diluted primary antibodies at 4 °C overnight, and exposure to second antibodies and DAPI. Images were captured using A1R MP+ multiphoton confocal microscope (Nikon) and AxioCam HRc digital camera (Carl Zeiss).

Quantitative real-time PCR analysis

Total RNA was extracted from kidney tissue using total RNA extraction Kit (BioTek, Winooski, VT, United States). Reverse transcription was performed using PrimeScript RT Reagent Kit (Takara Bio, Inc., Otsu, Japan) according to instruction. Reactions of PCR amplification were quantified using the iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) in a PCR system (CFX Connect; Bio-Rad, Hercules, CA, United States). Results were presented as relative mRNA levels to GAPDH.

Cell culture and treatments

The mouse macrophage cell line (Raw 264.7) and mouse kidney proximal tubular cell line (TCMK1) were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Raw 264.7

(RRID: CVCL_0493) and TCMK1 (RRID: CVCL_2772) cells were cultured in RPMI 1640 and DMEM/F12 media (both were supplemented with 10% FBS), respectively. To investigate whether tubular cell is the essential mediator of effect from uric acid on macrophage, we co-cultured Raw 264.7 and TCMK1 together by putting transwell inserts carrying TCMK1 to 6-well plates with Raw 264.7. Cells were exposure to uric acid at concentration of 800 μM, as previously reported(Ren et al., 2021). Based on different intervention purposed, before adding uric acid, cells were treated with Itgam siRNA (Genepharma Co., Ltd., Suzhou, China), FAK inhibitor (PF562271, Selleck Chemicals LLC, USA), and Akt inhibitor (MK-2206 2HCl, Selleck Chemicals LLC, USA) as instructed.

Statistical analysis

Results are presented as mean ± standard deviation based on independent experiments. Sample sizes estimation per group were calculated using "resource equation" and confirmed or confirmed by post hoc analyses in G*power 3 software, as previous described. Student's t-test was used for two-group comparison. One-way ANOVA and further Tukey test were used for multiple comparisons. A two-sided p value less than 0.05 was considered statistically significant and reported.

Materials

Details of all the reagents were listed where they appeared in text for the first time. Primer details are as follows: mouse GAPDH forward: 5'-CCCCCAATGTATCCGTTGTG-3', reverse: 5'-TAGCCCAGGATGCCCTTAGT-3'; mouse Itgam forward: 5'-GGAACCAGTGTGGTTGTTGC-3', reverse: 5'-GAGGTACTTGCAGGGGGATG-3'; mouse Icam-1 forward: 5'-CACGTGCTGTATGGTCCTCG-3', reverse: 5'-TAGGAGATGGGTTCCCCCAG-3'; mouse Rage forward: 5'-GGCATTCAGCTGTTGGTTGAG-3', reverse: 5'-CCTGATGCTGACAGGAGGGC-3'; mouse Gp1ba forward: 5'-TCCTCCAAAGGACTGTCTGTTC-3', reverse: 5'-GCTGTGGAGAAGGTACCCAG-3'; mouse Jam3 forward: 5'-TGACACGATCGGATTCAGCC-3', reverse: 5'-TTCTGGAACCTGGGATTGGC-3; mouse Fizz1 forward: 5'-CAGCTGATGGTCCCAGTGAAT-3', reverse: 5'-CAGTGGAGGGATAGTTAGCTGG-3'; mouse Ym1 forward: 5'-GAAGCTCTCCAGAAGCAATCCT-3', reverse: 5'-AGAAGAATTGCCAGACCTGTGA-3'; mouse Arg1 forward: 5'-GTAGACCCTGGGGAACACTAT-3', reverse: 5'-CTTCCTTCCCAGCAGGTAGC-3'; mouse Mr forward: 5'-GGCCAAGGTACTTCCAGGATT-3', reverse: 5'-CCCTGGCACAGCTCATACAT-3'.

Results

Progressive renal function decline and kidney fibrosis in hyperuricemia-related CKD

From day 0 to day 21, we set four time points and investigated dynamic characteristics of blood, urine, and kidney samples in hyperuricemia-related CKD mice (**Figure 1A**). Serum uric acid kept rising and reached peak at day 21. Similar trend to uric acid was observed in renal function measurements. Serum creatinine and urea went up quickly from day 0 to day 7, kept stable at high level at day 14, and then sharply increased to three and four times of baseline level respectively. Urine albumin-to-creatinine (UACR) rose rapidly and became 5-fold level at day 7 compared to day 0, and then slowly declined but still at high level at day 14 and 21 (**Figure 1B**). Histologic changes in PAS-staining sections indicated progressive tubular and glomerular injuries. Trichrome-staining sections presented the increasing deposition of collagen in tubular interstitial and glomerular mesangial compartments (**Figure 1C**). We further observed progressive inflammatory cell recruitment indicated by chemokine MCP-1, pro-inflammatory cytokine TNF-α, IL-6, IL-1β (**Figure 1D**), as well as fibrotic marker fibronectin, collagen-I, α-SMA (**Figure 1E**).

ITGAM is the hub gene potentially activating FAK pathway in hyperuricemia-related CKD

We combined mRNA and protein sequencing data and picked out those DEGs at both levels in kidneys of mice (**Figure 2A** &**Supplementary Figure 1A**). The details about digging out hub genes and pathways related are presented in **Supplementary Figure 1B.** In brief, we performed the enrichment analysis and canonical pathway analysis using up-regulated and down-regulated DEGs separately and presented functional characteristics accordingly (**Supplementary Figure 2**). After MCODE analysis, we picked out the top

5 clusters consisting of 334 DEGs (**Supplementary Figure 3**) followed by CentiScaPe analysis to obtain 49 hub genes. By matching the top enrichment pathways with hub genes, we found two most significantly enriched genes- Itgam and Itgb2, that encoded two subunits constituting the heterodimer Mac-1 (**Figure 2B**). The key functional role of Itgam was also highlighted by IPA biomarker analysis, with ITGAM ranking the 1st at both mRNA and protein levels. Itgam in day-21 group was up-regulated to 19-folds and 3.3-fold of that in day-0 group at mRNA and protein level, respectively (**Figure 2C**). To explore key pathways that were potentially regulated by ITGAM, we selected significant genes positively correlated with Itgam at mRNA and protein level, and performed the KEGG pathway enrichment analysis (**Figure 2D**). Since there is an abundance of literature on tyrosine kinases as major signal transducers by integrins(Berton & Lowell, 1999), we hypothesized that FAK signaling was activated by ITGAM in kidneys of hyperuricemia-related CKD mice (**Figure 2E**).

Macrophage ITGAM is highly expressed with alternative M2 polarization in hyperuricemia-related CKD

Based on findings from integrated bioinformatic analysis, we firstly verified the expression and location of ITGAM in kidneys of hyperuricemia-related CKD mice. Compared to Day 0, ITGAM expression significantly increased on Day 7, 14, and 21 with a trend continuous growth at both of mRNA and protein levels (**Figure 3A**). Considering ITGAM mainly but not only expressed in macrophage, we investigated the location of ITGAM in kidney tissue and found it highly co-located with macrophage marker F4/80 in tubulointerstitial and glomerular mesangial space (**Figure 3B**). Furthermore, FAK signaling was significantly activated, indicated by the increased phosphorylation of FAK and Akt1, the decreased phosphorylation of GSK-3β, and the increased expression of β-catenin (**Figure 3C**). Phosphorylated FAK and β-catenin mainly expressed in tubulointerstitial space (**Figure 3D**), supporting the hypothesis that activation of FAK signaling might be related to macrophage ITGAM and they co-worked together mediating macrophage polarization in hyperuricemia-related CKD. Both of proteomic and proteomic data revealed activated macrophage M2 polarization in kidney tissue on Day 21, clarified by greatly the increased mRNA expression of Arg1 and Mr (**Figure 3E**). We further performed qPCR to validate this finding and observed robust increase in Arg1 and Mr, followed by mild increase in Fizz1 (**Figure 3F**).

Uric acid directly induced macrophage M2 polarization and strongly activated ITGAM and FAK signaling

We performed in vitro experiments to explore how uric acid functions on macrophage. Since uric acid at high concentration causes tubular damages(Johnson et al., 2018; Milanesi et al., 2019), we designed two in vitro models: model1, using uric acid to stimulate Raw 264.7 macrophage; and model 2, using uric acid to stimulate the co-culture environment of Raw 264.7+proximal tubular cell TCMK1. Macrophages were collected after 24 hours and 48 hours of stimulation (**Figure 4A**). The ITGAM mRNA expression in model 1 quickly increased to peak after 24 hours, and ITGAM in model 1 was higher or equal to that in model 2 at time points of 24 and 48 hours, respectively (**Figure 4B**). Although not highly consistence in M2 polarization markers, two models both presented obvious M2 polarization especially after 48 hours (**Figure 4C**). ITGAM and downstream FAK signaling were activated after 24 hours of uric acid stimulation. As indicated by KEGG enrichment, we confirmed the higher phosphorylation of FAK and Akt1, lower phosphorylation of GSK-3β, and less degraded β-catenin (**Figure 4D**). Evidence above indicated that uric acid strongly activated ITGAM/FAK signaling in macrophage and M2 polarization, regardless of existence of tubular epithelial cells.

ITGAM induced macrophage M2 polarization through activating FAK/Akt1/β-catenin signaling

The mechanism that ITGAM mediated M2 polarization in macrophage was verified by silencing Itgam mRNA, and inhibiting FAK and Akt1 phosphorylation (**Figure 5A**). As shown by pre-experiment, we picked the Itgam siRNA with the highest knockdown efficiency (mean difference = 77.6%, **Supplementary Figure 3**). After knocking down Itgam, we observed the decreased phosphorylation of FAK and Akt1,

and reversed the upregulation of phosphorylated GSK-3β, together with downregulation of β-catenin protein (**Figure 5B**). Meanwhile, M2 polarization was greatly attenuated after Itgam knockdown, indicated by significant decrease in Arg1, Mr, Fizz1, and Ym1 mRNA expression (**Figure 5C**). Similar effects were found after FAK and Akt1 intervention. After inhibiting phosphorylation of these two proteins, the downstream signaling was significantly suppressed together with reduced M2 polarization (**Figures 5D-G**).

Discussion

Macrophages could promote renal fibrosis, a major driver of progression to end-stage kidney disease, and M2 macrophages is strongly associated with kidney fibrosis in both human and experimental diseases. In this study, we report that macrophage ITGAM contributes to kidney fibrosis in hyperuricemia-related CKD. Mechanistically, ITGAM promotes macrophage M2 polarization through activating FAK/Akt1/β-catenin pathway.

ITGAM, also known as CD11b, was commonly used as a monocyte/macrophage surface marker(Ross & Lambris, 1982; Wolf et al., 2018). Gradually, ITGAM diverse functions were reflected by its rapid confirmational change that increased affinity for its more than 40 ligands, including ICAM-1(Dunne et al., 2002), fibrinogen(Altieri et al., 1990), fibronectin(Kanse et al., 2004), GPIbα(Ehlers et al., 2003), RAGE(Chavakis et al., 2003), JAM-c(Lange-Sperandio et al., 2006), and others(Fan & Ley, 2015). We examined main ligands frequently reported, and found ICAM-1 and fibronectin were significantly upregulated (**Figure 1E** and **Supplementary Figure 4A-B**), which indicated potential role of ITGAM in cell-ECM and cell-cell interactions in kidneys of hyperuricemia-related CKD. Further studies are needed to clarify how ITGAM mediates cross-talk between macrophage and other cells or compartments in kidney tissue. ITGAM was reported to be involved in various immune responses but with bidirectional effects(Rosetti & Mayadas, 2016). Negative regulation of ITGAM could be observed in systemic lupus erythematous and acute infectious diseases(Hu et al., 2016; Villanueva et al., 2022). On the contrary, ITGAM positively regulated chronic inflammatory diseases(Zirlik et al., 2007). Lange-Sperandio et al reported that high expression of Mac-1 and its ligands ICAM-1 and JAM-3 in murine unilateral ureteric obstruction (UUO) model, and knockout of Mac-1 greatly attenuated kidney fibrosis(Lange-Sperandio et al., 2006). Taking our results together, the functional role of ITGAM in contributing to kidney fibrosis through promoting M2 polarization could be inferred.

Both of integrin α and β subunits are single membrane-spanning segments with short cytoplasmic tails, thus need to interact with downstream tyrosine kinases to enable "outside-in" signal transduction(Kornberg et al., 1991; Takada et al., 2007). Binding to ligands (e.g extracellular matrix) induces integrins clustering at focal adhesions and connecting to intracellular molecules. FAK is one of the earliest identified central integrin signaling. The activated FAK undergoes autophosphorylation and then stimulates difference molecules corresponding to various pathways, including Src, Akt, Grb7, and others(Guan, 2010). Numerous studies reported FAK/Akt pathway participated in chronic inflammation and fibrosis, such as atherosclerosis(Yamaura et al., 2019), and lung and liver fibrosis(Gimenez et al., 2017; Lv et al., 2018). Of notes, we additionally observed the change in Src phosphorylation and found p-Src were both attenuated after silencing Itgam and inhibiting p-FAK. Our finding indicated that Src participated in ITGAM/FAK signaling and might act as the downstream molecular. It's to be further explored whether Src together with FAK stimulates Akt1, or acts through other pathways, such as Stat3 and Erk (Bolos et al., 2010; Jang et al., 2022; Pan et al., 2019). β-catenin activation, as the canonical pathway of Wnt signaling, is associated with macrophage M2 polarization in tumor malignant behaviors and renal fibrosis(Feng et al., 2018; Yang et al., 2018). Several studies have shown that Akt activation and phosphorylation of GSK-3β led to β-catenin stabilization. Accumulated β-catenin in cytosol then translocates into nucleus and stimulates transcription of target genes that participates in tissue fibrosis(MacDonald et al., 2009; Niehrs, 2012).

To our best acknowledgement, this is the first paper investigating the role of integrin and how it regulates macrophage M2 polarization in kidneys of hyperuricemia-related CKD. Hyperuricemia-related, especially hyperuricemia-induced CKD is relatively less studied, due to long time and difficulty when modeling. We firstly fed mice with potassium oxonate only at high dose (4.8g/kg) for up to 14 weeks with the purpose of establishing uric acid-induced CKD, but only found moderately increased serum uric acid (1.5-2 times of

baseline) and almost unchanged kidney tissue histology. Urate oxidase-knockout mouse is an optimal option, but low survival greatly limited experiment feasibility and reproducibility(Lu et al., 2018; Zhao et al., 2022). Not only signal pathway ITGAM regulates, how ITGAM mediates cell-cell or ECM-cell cross-talk is also potential mechanism of great meaning to be further explored.

In summary, macrophage integrin ITGAM might play the vital role in mediating kidney fibrosis in hyperuricemia-related CKD through activating its downstream pathway FAK/Akt1/β-catenin and promoting macrophage M2 polarization. This finding provides new insight into the prevention and treatment of kidney fibrosis induced by uric acid, and acts as potential therapeutic target, although more well-designed studies are needed to verify further mechanisms.

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Conflict of interest

The authors declare no competing interests.

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Figure Legends

Figure 1 Progressive renal dysfunction and kidney fibrosis in hyperuricemia-related CKD mice. (A) Brief instruction on model establishment and sample collection; (B) Biochemical measurements from day 0 to day 21, including serum uric acid, creatinine, urea, and urine albumin-to-creatinine ratio; (C) Periodic acid-Schiff (PAS) and Masson trichrome staining of renal tissue. PAS stain indicates progressively tubular atrophy and glomerulosclerosis. Trichrome stain indicates progressively interstitial collagen deposition.

Tubular injuries and interstitial fibrosis were quantified and presented; (D-E) From day 0 to day 21, renal tissue is characterized with progressively exacerbated inflammation and fibrosis. n=6; ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Figure 2 Bioinformatic analyses revealed ITGAM as the hub gene associated with downstream FAK pathway. (A) Sketch map of how we incorporated mRNA and protein sequencing data to select core genes and pathways related. In brief, we found out 1,337 DEGs both at mRNA and protein levels and further adopted hub gene analyses (MCODE followed by CentiScape in Cytoscape) to acquire simplified 49 genes. Finally, we ranked the top genes by degrees of enrichment to pathways. (B) There are 6 of 49 hub genes corresponding to top canonical pathways conducted by IPA. Itgam and Itgb2, as two heterodimers of Mac-1, ranked the 1st. Itgam on day 21, at mRNA and protein level, was upregulated to 19 and 3.3 times of day 0. (D) KEGG results based on genes significantly positively correlated with Itgam at both mRNA and protein levels; (E) Schematic diagram of ITGAM and related pathway in macrophage after exposure to uric acid.

Figure 3 ITGAM expression and macrophage M2 polarization in vivo. (A) ITGAM was increasingly upregulated at mRNA and protein levels; (B) ITGAM co-locates with macrophage marker F4/80; (C) ITGAM/FAK/Akt1/β-catenin pathway was activated in vivo (day 21 vs day 0); (D) Location of two core molecules in pathway, including p-FAK and β-catenin; (E-F) M2 polarization was activated in hyperuricemia-related CKD as revealed by omics data and PCR quantification in vivo. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Figure 4 Two experimental models in vitro validated overexpression of ITGAM, pathway activation, and M2 polarization of macrophages. (A) study design of uric acid at 800 μM stimulating Raw 264.7 *vs.* Raw 264.7 +TCMK1; (B) Raw 264.7 alone, under stimulation of uric acid, greatly overexpresses ITGAM and the expression levels in two models at 48 hours are not significantly different. (C) Either Raw 264.7 or co-culture system presented obvious activation of macrophage M2 activation after uric acid exposure. (D) Two models at different time points shows activation of ITGAM/FAK/Akt/β-catenin pathway. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Φιγυρε 5 Ιντερεντιονς οφ ΙΤΓΑΜ, ΦΑΚ, ανδ Ακτ εριφιεδ τηε παρτιςιπατιον οφ πατηωαψ ΙΤΓΑΜ/ΦΑΚ/Ακτ/β-ςατενιν ιν μαςροπηαγε Μ2 πολαριζατιον ιν ηψπερυριςεμια-ρελατεδ ˆΚΔ. (A) Design of ITGAM and pathway intervention; (B-C) Itgam silencing significantly inhibited pathway and attenuated M2 polarization; (D-E) Inhibition of FAK greatly inhibited downstream pathway and macrophage M2 polarization; (F-G) Inhibition of Akt downregulated β-catenin and macrophage M2 polarization. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Supplementary Materials

Supplementary Figure 1 Differentially expressed gene (DEG) selection and enrichment analysis. (A) DEG selection criteria and details of up-regulated and down-regulated DEGs; (B) Logistics and approach details in selecting target genes and pathways; (C) GO enrichment analysis based on DEGs.

Supplementary Figure 2 Hug gene selection and biomarker analysis. (A) Top 5 clusters of 334 DEGs after MCODE analysis in Cytoscape; (B-C) Incorporating biomarker analysis in IPA and CytoHubba hug gene selection showed ITGAM ranked the 1st at both of mRNA and protein levels.

Supplementary Figure 3 Details of Itgam siRNA efficiency and selection.

Supplementary Figure 4 Ligands of ITGAM in vitro. (A) qPCR results indicated Icam-1 and Rage two ligands were upregulated at mRNA level; (B) Western blotting showed only ICAM-1 was upregulated.