Circulation

ORIGINAL RESEARCH ARTICLE

Uremic Toxin Indoxyl Sulfate Promotes Proinflammatory Macrophage Activation Via the Interplay of OATP2B1 and Dll4-Notch Signaling

Potential Mechanism for Accelerated Atherogenesis in Chronic Kidney Disease

Editorial, see p 97

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et al

BACKGROUND: Chronic kidney disease (CKD) increases cardiovascular risk. Underlying mechanisms, however, remain obscure. The uremic toxin indoxyl sulfate is an independent cardiovascular risk factor in CKD. We explored the potential impact of indoxyl sulfate on proinflammatory activation of macrophages and its underlying mechanisms.

METHODS: We examined in vitro the effects of clinically relevant concentrations of indoxyl sulfate on proinflammatory responses of macrophages and the roles of organic anion transporters and organic anion transporting polypeptides (OATPs). A systems approach, involving unbiased global proteomics, bioinformatics, and network analysis, then explored potential key pathways. To address the role of Delta-like 4 (Dll4) in indoxyl sulfate–induced macrophage activation and atherogenesis in CKD in vivo, we used 5/6 nephrectomy and Dll4 antibody in low-density lipoprotein receptor–deficient (Ldlr-/-) mice. To further determine the relative contribution of OATP2B1 or Dll4 to proinflammatory activation of macrophages and atherogenesis in vivo, we used siRNA delivered by macrophage-targeted lipid nanoparticles in mice.

RESULTS: We found that indoxyl sulfate–induced proinflammatory macrophage activation is mediated by its uptake through transporters, including OATP2B1, encoded by the SLCO2B1 gene. The global proteomics identified potential mechanisms, including Notch signaling and the ubiquitin-proteasome pathway, that mediate indoxyl sulfate-triggered proinflammatory macrophage activation. We chose the Notch pathway as an example of key candidates for validation of our target discovery platform and for further mechanistic studies. As predicted computationally, indoxyl sulfate triggered Notch signaling, which was preceded by the rapid induction of Dll4 protein. Dll4 induction may result from inhibition of the ubiquitin-proteasome pathway, via the deubiquitinating enzyme USP5. In mice, macrophage-targeted OATP2B1/Slco2b1 silencing and Dll4 antibody inhibited proinflammatory activation of peritoneal macrophages induced by indoxyl sulfate. In low-density lipoprotein receptor–deficient mice, Dll4 antibody abolished atherosclerotic lesion development accelerated in Ldlr-/- mice. Moreover, coadministration of indoxyl sulfate and OATP2B1/Slco2b1 or Dll4 siRNA encapsulated in macrophage-targeted lipid nanoparticles in Ldlr-/- mice suppressed lesion development.

CONCLUSIONS: These results suggest that novel crosstalk between OATP2B1 and Dll4-Notch signaling in macrophages mediates indoxyl sulfate–induced vascular inflammation in CKD.

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The full author list is available on p 94.

Key Words: atherosclerosis ■ chronic kidney diseases ■ indoxyl sulfate ■ inflammation ■ macrophage ■ Notch signaling

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Clinical Perspective

What Is New?

- Our study revealed a novel mechanism by which the uremic toxin indoxyl sulfate induces proinflammatory activation of macrophages and accelerates atherogenesis in chronic kidney disease (CKD).
- Membrane transport proteins including OATP2B1 mediate macrophage uptake of indoxyl sulfate.
- Indoxyl sulfate rapidly induces the Notch pathway ligand Dll4 in macrophages, seemingly via inhibiting the ubiquitin-proteasome pathway, and triggers Notch signaling.
- The blockade of Dll4 in vivo abrogated proinflammatory macrophage responses enhanced by indoxyl sulfate and retarded the accelerated progression of atherosclerosis and calcification in mice with experimental CKD.

What Are the Clinical Implications?

- The prevalence of CKD has been increasing globally because of the aging population and the diabetes mellitus pandemic.
- CKD increases the risk of cardiovascular disease. In particular, uremia is a nontraditional risk factor for cardiovascular disease.
- Clinical studies of current therapies including potent statins have failed to show beneficial effects on cardiovascular disease risk in advanced CKD patients.
- A new theory proposed in the present study may help to develop effective therapies against this major health threat.

Chronic kidney disease (CKD) is a global health threat, which increases risk of cardiovascular disease (CVD) morbidity and mortality.[1] The prevalence of atherosclerotic diseases including coronary artery disease, peripheral artery disease, and stroke is higher in CKD patients.[2] CKD accelerates atherosclerotic lesion progression in mice.[3] Epidemiological evidence indicates that progression of atherosclerosis in CKD cannot be completely explained by traditional CVD risk factors such as hypertension, diabetes mellitus, and dyslipidemia,[1] suggesting that nontraditional risk factors contribute to atherogenesis in CKD. The process of atherosclerosis is characterized by proinflammatory activation of macrophages. Despite its clinical impact, the mechanisms of macrophage activation in CKD, however, remain obscure.

CKD leads to the retention of uremic toxins that are normally excreted by the kidney,[4] causing end organ damage, which may thus contribute to CVD in CKD. Clinical evidence suggests that indoxyl sulfate, a uremic toxin metabolized from dietary tryptophan, is an independent risk factor for cardiovascular morbidity and mortality in CKD.[5] In cell culture experiments, indoxyl sulfate induces proinflammatory responses in endothelial cells[6] and reactive oxygen species production in the monocytic leukemia cell line THP-1.[7] Whether indoxyl sulfate activates primary macrophages and its underlying mechanisms, however, are unclear.

In tubular epithelial cells, indoxyl sulfate is excreted from blood to urine via organic anion transporters (OATs) and organic anion transporting polypeptides (OATPs).[8] Endothelial cells and smooth muscle cells express OATs.[8] However, no report has demonstrated the existence of OATs/OATPs and their functions in monocytes/macrophages. We previously reported that CKD accelerates macrophage activation in experimental atherosclerosis.[3] We demonstrated new triggering mechanisms for proinflammatory activation of human primary macrophages,[9–12] but how the CKD milieu activates macrophages is still not well understood.

In the present study, we identified OATs/OATPs in primary macrophages that mediate indoxyl sulfate uptake and proinflammatory responses. Proteomics and network analysis identified several pathways enriched in indoxyl sulfate-elicited macrophages, including Notch signaling and the ubiquitin-proteasome pathway. In vitro experiments revealed that OATP2B1-mediated indoxyl sulfate uptake triggers the Dll4-Notch signaling pathway, a molecular link that was previously unknown in any cell type and may involve the deubiquitinating enzyme USP5. In vivo evidence further demonstrated the role of OATP2B1 and Dll4 in vascular inflammation in CKD.

METHODS

Detailed methods of all procedures are provided in the online-only Data Supplement. The data, analytic methods, and study materials will be made available to other researchers on request for purposes of reproducing the results or replicating the procedure. The data are available through https://aikawalabs.bwh.harvard.edu/CKD.

Human peripheral blood mononuclear cells (PBMCs) cultured with 5% human serum underwent differentiation into macrophages in vitro. PBMCs were isolated from the buffy coat derived from deidentified healthy donors (Research Blood Components LLC, Boston, MA). The company recruited donors under a New England Institutional Review Board–approved protocol for the Collection of White Blood for Research Purposes (NEIRB#04–144). We had no access to the information about donors. Endotoxin-free indoxyl sulfate (Sigma-Aldrich) was used to stimulate human primary macrophages or the mouse macrophage-like cell line RAW264.7.

Proteomics of indoxyl sulfate-stimulated RAW264.7 cells involved tandem mass tagging sample preparation and liquid chromatography tandem mass spectrometry (LC-MS/MS) by the Orbitrap Fusion Lumos (Thermo Scientific). The MS/MS data were queried against the mouse UniProt database using the SEQUEST search algorithm, via the Proteome Discoverer Package (version 2.1, Thermo Scientific). The normalized tandem mass tagging reporter ion intensities were exported and subsequent quantitative analyses were done using R (version 3.3.2) and our original software for high-dimensional quantitative proteomics analysis.[13]

Pathway enrichment analysis used ConsensusPathDB.[14] Network analysis was performed using the NetworkX package v1.9 in Python v2.7.10. Disease genes were obtained from the DiseaseConnect[15] and MalaCards[16] databases. Network visualizations were made using Gephi v0.8.2.

All animal experiments were approved by the Brigham and Women's Hospital's Animal Welfare Assurance (protocol 2016N000219). Male low-density lipoprotein receptor–deficient ($Ldlr^{-/-}$) mice were fed a high-fat diet. To create 5/6 nephrectomy in $Ldlr^{-/-}$ mice (CKD mice), we used a well-established 2-step procedure of 5/6 nephrectomy, as previously used by us.[3,17] The procedure involved a seminephrectomy of the left kidney, followed by a right nephrectomy a week later. For the selective blockade of Dll4, we administered hamster antimouse Dll4 antibody (250 μg, IP) or control IgG twice a week. After 16 weeks, atherosclerotic lesions were evaluated. For in vivo silencing experiments, Slco2b1 siRNA, Dll4 siRNA, and control siRNA were encapsulated in macrophage-targeted lipid nanoparticles C12-200 and injected via tail vein (0.5 mg/kg twice a week), as previously described.[11,18] Aortic tissues and valve tissues were harvested, sectioned, and used for hematoxylin and eosin staining and immunohistochemistry.

Statistical analyses were performed using SPSS Statistics 22 (IBM) and GraphPad Prism 7.0 (GraphPad Software). The unpaired Student's *t* test was used for comparisons between 2 groups, or 1-way ANOVA followed by Tukey post hoc test or 2-way ANOVA followed by the Bonferroni test was used for comparisons of multiple groups. Data are expressed as mean±SEM for continuous variables. *P* values of <0.05 were considered statistically significant.

RESULTS

Indoxyl Sulfate Induces Proinflammatory Cytokines in Human Primary Macrophages In Vitro

Although a few studies suggested the effects of indoxyl sulfate in macrophage-like cancer cell lines,[7] its role in primary human macrophages has not been demonstrated. In a previous study, indoxyl sulfate reduced the cholesterol transporter ATP-binding cassette transporters G1 (ABCG1) expression in THP-1 cells.[7] Remarkably, macrophage-like cell lines and primary macrophages often respond differently to a stimulus. Indeed, we were not able to reproduce this result on ATP-binding cassette transporters G1 in human primary macrophages. Indoxyl sulfate did not suppress ABCG1 mRNA in human primary macrophages derived from PBMCs of 5 donors (Figure IA in the online-only Data Supplement, human primer designs for real-time PCR are listed in Table I in the online-only Data Supplement). Another study reported suppression of ATP-binding cassette transporters A1 (ABCA1) in mouse macrophages of CKD mice.[19] In our experiments, ABCA1 did not decrease in indoxyl sulfate–treated human primary macrophages. To establish unambiguous evidence, the present study thus used human primary macrophages in most in vitro experiments.

To test the hypothesis that indoxyl sulfate promotes vascular inflammation, we first examined the mRNA levels of proinflammatory (interleukin [IL]-1β, TNF-α and MCP-1) and anti-inflammatory molecules (MRC1, AMAC1, IL-10 and CD163) in PBMC-derived human primary macrophages in response to indoxyl sulfate (0.25–2.0 mmol/L; Figure 1A). Indoxyl sulfate induced the levels of IL-1β, TNF-α, and MCP-1 mRNA in a dose-dependent manner. But, indoxyl sulfate exerted no substantial effects on the mRNA levels of the anti-inflammatory molecules, other than AMAC1, which decreased at the highest concentration of indoxyl sulfate. A clinically relevant concentration of indoxyl sulfate in severe CKD (0.5–1.0 mmol/L)[4] increased the release of the IL-1β protein by macrophages (Figure 1B).

To ensure that potential endotoxin contamination in indoxyl sulfate did not affect our measurements, we used the limulus amebocyte lysate endotoxin assay. Endotoxin levels in our experiments were <0.005 EU/mL. Macrophage Toll-like receptors 2 and 4 (TLR2 and TLR4) recognize various bacterial substances, including lipopolysaccharides, and promote proinflammatory activation. To minimize the possibility that endotoxins stimulate the TLR pathway, we used siRNA for TLR2 or TLR4 under indoxyl sulfate (0.5 mmol/L) stimulation. mRNA levels of IL-1β, TNF-α, and MCP-1 under control siRNA, TLR2 siRNA, and TLR4 siRNA did not differ (Figure IIA in the online-only Data Supplement). The TLR4 antagonist TAK-242 also produced no effects on any of the mRNA levels (Figure IIB in the online-only Data Supplement). Furthermore, mass spectrometric analysis of 10 μmol/L indoxyl sulfate did not detect any significant signal other than that of indoxyl sulfate (Figure IIC in the online-only Data Supplement).

In addition, we investigated whether tryptophan, from which indoxyl sulfate is derived, could induce transcription of proinflammatory cytokines in macrophages (Figure 1C). We used tryptophan and indoxyl sulfate purchased from the same manufacturer (Sigma). Tryptophan did not induce proinflammatory cytokines in human primary macrophages.

Indoxyl sulfate is well known as a protein-bound toxin,[4] and thus this protein-bound form could represent a potential active form of the toxin. We examined whether indoxyl sulfate induces inflammation in medium with 4% albumin in the same concentration with human serum as previously reported.[20] Indoxyl sulfate induced proinflammatory cytokines in human primary macrophages in the albumin-containing medium (Figure 1D).

OATP2B1 Mediates the Cellular Uptake of Indoxyl Sulfate in Macrophages

OATs and OATPs contribute to the transcellular transport of uremic toxins in the kidney. However, no studies have reported their expression in macrophages. OATPs are encoded by genes in the SLCO superfamily. The cor-

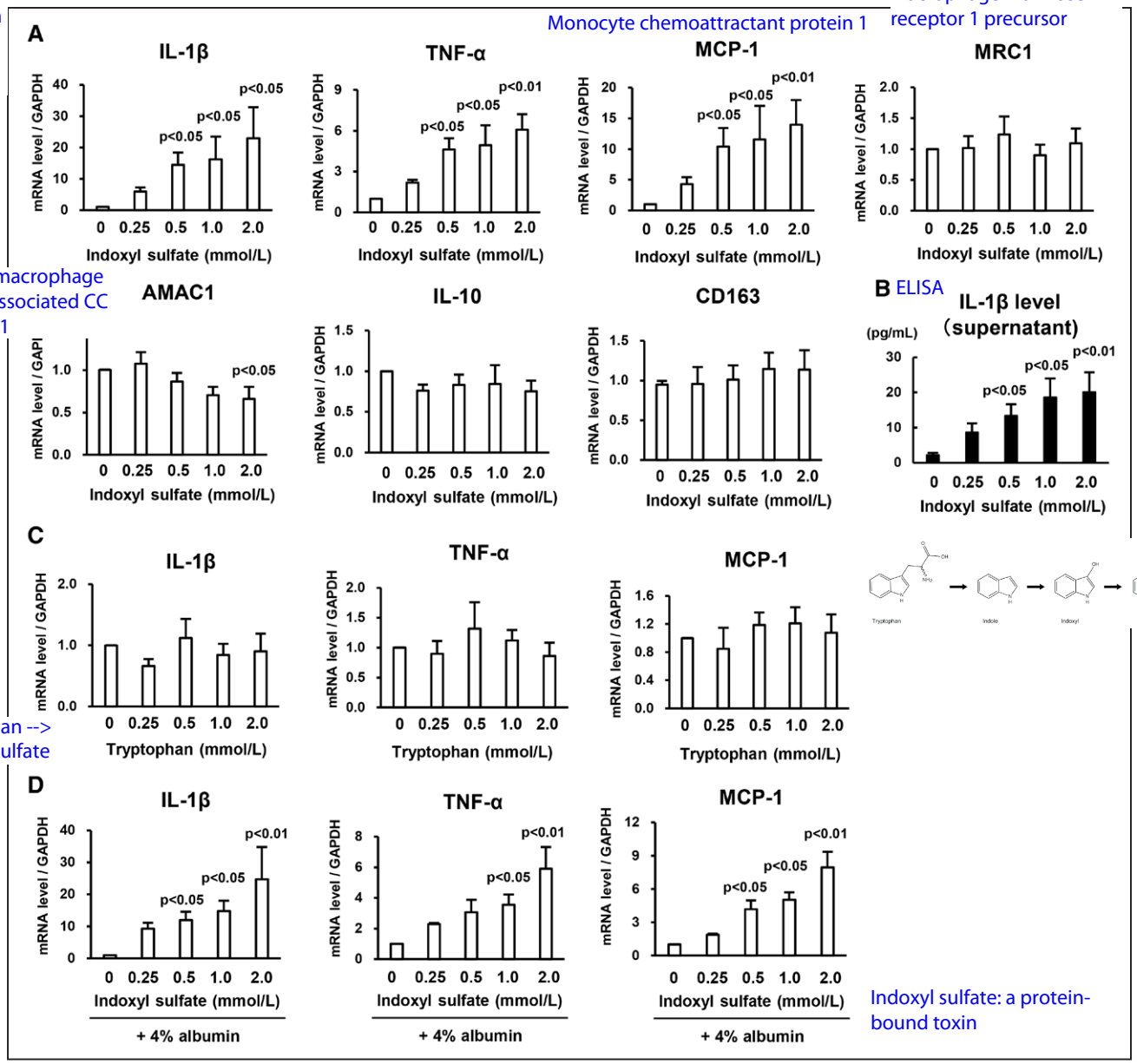
Inflammatory cytokines: IL-1, IL-12, and IL-18, tumor necrosis factor alpha (TNF-α), interferon gamma (IFNγ), and granulocyte-macrophage colony stimulating factor (GM-CSF).

proinflammatory cytokine

Monocyte chemoattractant protein 1

Macrophage mannose receptor 1 precursor

alternative macrophage activation-associated CC chemokine-1



Indoxyl sulfate: a protein-bound toxin

Figure 1. Indoxyl sulfate induces proinflammatory cytokine expression in human primary macrophages derived from peripheral blood mononuclear cells (PMBCs).

A, mRNA expression of IL-1β, TNF-α, MCP-1 MRC1, AMAC1, IL-10, and CD163 was measured in human primary macrophages after stimulation with indoxyl sulfate for 3 hours (9 different PBMC donors). **B**, IL-1β levels in supernatants of human primary macrophages were measured by ELISA after stimulation with indoxyl sulfate for 72 hours (9 donors). **C**, mRNA expression of IL-1β, TNF-α, and MCP-1 was measured in human primary macrophages after stimulation with tryptophan for 3 hours (6 donors). **D**, mRNA expression of IL-1β, TNF-α, and MCP-1 was measured in human primary macrophages after stimulation with indoxyl sulfate in medium with 4% albumin for 3 hours (8 donors). *P* value was calculated by 1-way ANOVA followed by Tukey test, based on a comparison with 0 mmol/L indoxyl sulfate. Error bars indicate ±SEM. AMACI indicates alternative macrophage activeion-associated CC-chemokine 1; CD163, cluster of differentiation 163; IL, interleukin; MCP, monocyte chemoattractant protein; MRC1, mannose receptor C-type 1; and TNF, tumor necrosis factor.

SD vs SEM?

Post hoc P value, like Bonferroni

responding gene symbols of OATPs (eg, OATP1A2 and OATP2B1) are SLCO followed by the same number-letter-number combination (eg, SLCO1A2 and SLCO2B1). OAT1-7, OAT10, and URAT1 are encoded by genes in the SLC22A family.

To investigate a potential novel mechanism for macrophage uptake of indoxyl sulfate, we first examined whether human primary macrophages express OATs or OATPs. We used the human proximal tubular cell line HK-2, derived from normal kidney, and the liver carcinoma cell line HepG2, as positive controls because renal tubular cells and hepatocytes express OATs and OATPs.[8,21] Human primary macrophages expressed mRNA of SLCO1A2 (OATP1A2), SLCO2B1 (OATP2B1), SLCO3A1 (OATP3A1), SLCO4A1 (OATP4A1), SLCO5A1 (OATP5A1), SLCO6A1 (OATP6A1), SLC22A10 (OAT5), SLC22A11 (OAT4), and SLC22A20 (OAT6) (Figure 2A, human and mouse primer designs for semiquantitative PCR are listed on Table II in the online-only Data Supplement).

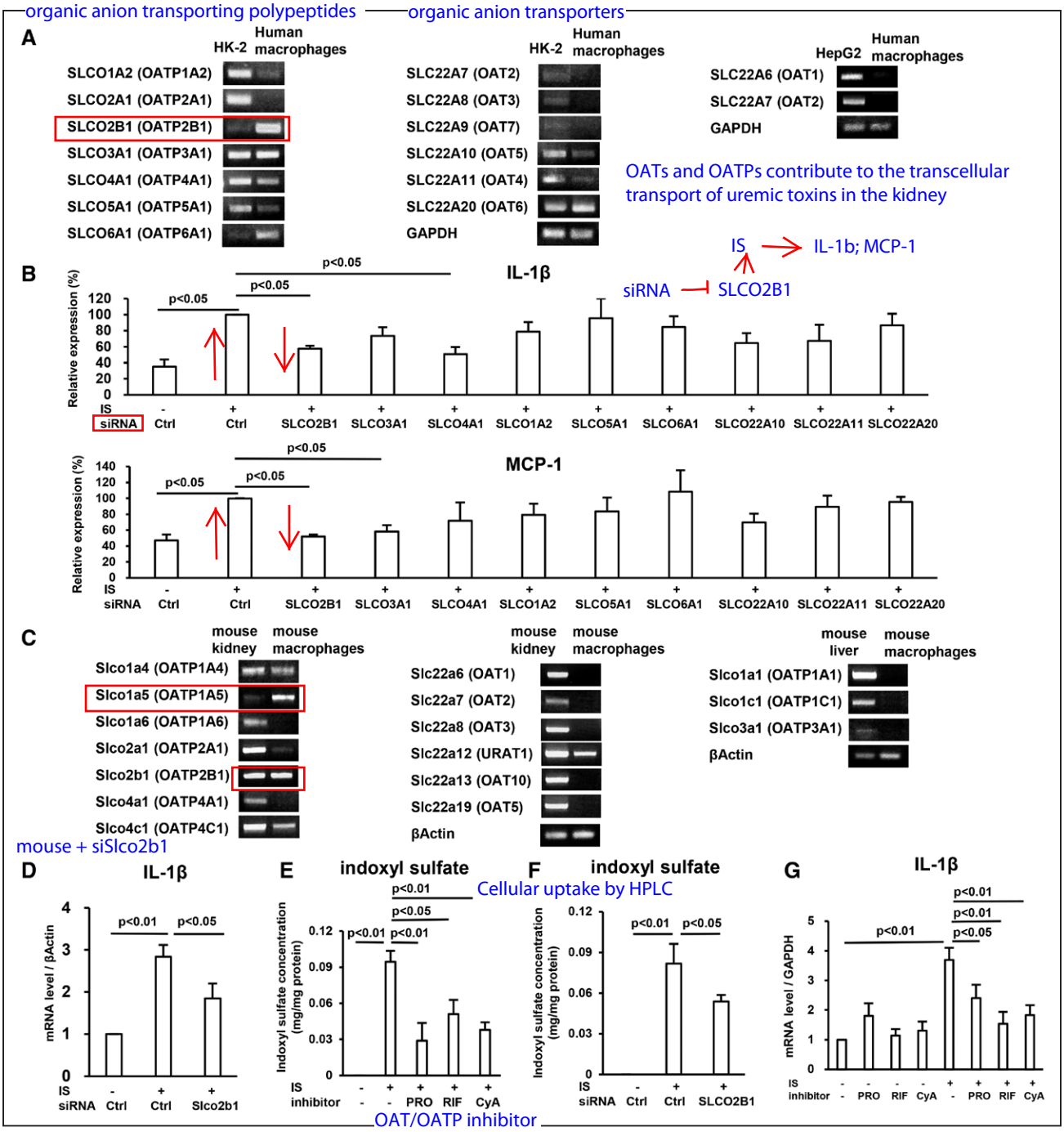


Figure 2. Macrophages express organic anion transporters (OATs) and organic anion transporting polypeptides (OATPs).
A, mRNA expression of OATs/OATPs was examined by polymerase chain reaction (PCR) in peripheral blood mononuclear cell (PMBC)–derived human primary macrophages. Protein names are noted in brackets. **B**, mRNA expression was measured in human primary macrophages after suppression by siRNA for 48 hours and stimulation with 0.5 mmol/L indoxyl sulfate (IS) for 3 hours (9 PBMC donors). **C**, mRNA expression of OATs/OATPs was examined by PCR in mouse peritoneal macrophages. Protein names are noted in brackets. **D**, mRNA expression was measured in mouse peritoneal macrophages after suppression by siRNA for 48 hours and stimulation with 0.5 mmol/L IS for 3 hours (n=6). **E**, Cellular uptake of IS in human primary macrophages was measured by high-performance liquid chromatography (HPLC) in the absence or presence of the OAT/OATP inhibitor 10 mmol/L probenecid (PRO), 100 µmol/L rifampicin (RIF), or 10 µmol/L cyclosporin A (CyA; 6 PBMC donors). **F**, Cellular uptake of IS in human primary macrophages was measured by HPLC in the presence of SLCO2B1 siRNA or control siRNA (6 donors). **G**, mRNA expression of IL-1β was measured after pretreatment with the OAT/OATP inhibitor 10 mmol/L probenecid (PRO), 100 µmol/L rifampicin (RIF), and 10 µmol/L cyclosporin A (CyA) for 30 minutes, and stimulation with 0.5 mmol/L IS for 3 hours (8 donors). *P* value was calculated by unpaired Student *t* test or 1-way ANOVA followed by Tukey test. Error bars indicate ±SEM. IL indicates interleukin; and MCP, monocyte chemoattractant protein.

To examine the causal role of any transporter(s) in indoxyl sulfate–triggered macrophage activation, we used siRNA silencing. At least a 70% reduction of the target mRNA was achieved with each siRNA (Figure IIIA in the online-only Data Supplement). siRNA of SLCO2B1, SLCO3A1, and SLCO4A1 suppressed the in-

duction of IL-1β and MCP-1 by indoxyl sulfate in human macrophages (Figure 2B).

We also identified the mRNA expression of Slco1a4 (OATP1A4), Slco1a5 (OATP1A5), Slco2a1 (OATP2A1), Slco2b1 (OATP2B1), Slco4c1 (OATP4C1), and Slc22a12 (URAT1) in mouse peritoneal macrophages (Figure 2C). Of note, human and mouse primary macrophages share the only transporter SLCO2B1 (OATP2B1) in our experiments (Figure 2A and 2C). As in the human cells, Slco2b1 siRNA suppressed indoxyl sulfate-induced IL-1β and MCP-1 expression in mouse macrophages (Figure 2D, Figure IIIB in the online-only Data Supplement, mouse primer designs for real-time PCR are listed in Table III in the online-only Data Supplement).

To further establish that OATP proteins indeed mediate the uptake of indoxyl sulfate by macrophages, we applied 3 OATP inhibitors: probenecid, rifampicin, and cyclosporine A.[21] Using high-performance liquid chromatography, we directly measured the concentration of indoxyl sulfate in human macrophages pretreated with these inhibitors. All 3 compounds produced statistically significant reduction of the indoxyl sulfate content in human primary macrophages (Figure 2E). Silencing of SLCO2B1 (OATP2B1) also suppressed the uptake of indoxyl sulfate in macrophages (Figure 2F, Figure IIIC in the online-only Data Supplement). OATP inhibitors further suppressed mRNA expression of IL-1β, TNF-α, and MCP-1 induced by indoxyl sulfate (Figure 2G, Figure IIID in the online-only Data Supplement). These experiments suggest that the SLCO2B1 gene product OATP2B1 mediates the uptake of indoxyl sulfate and promotes proinflammatory activation in primary macrophages.

Analytic Pipeline Reveals Notch Signaling as 1 of the Predominant Processes in Early-Responder Protein Clusters

We then took a systems approach to explore the mechanisms that mediate indoxyl sulfate–triggered proinflammatory macrophage activation in an unbiased fashion. Our initial step involved tandem mass tagging quantitative proteomics (see Methods section and Figure IVA in the online-only Data Supplement). For screening, we used the macrophage-like cell line RAW264.7 to avoid potential confounding donor-to-donor biological variation associated with primary cells. The key findings were later validated in human primary macrophages. We harvested unstimulated and indoxyl sulfate-stimulated RAW264.7 cells at 5 time points: 0, 15, and 30 minutes for early responses, and 1 and 3 hours for late responses, resulting in 4178 quantified proteins (≥2 unique peptides).

To identify the potential regulators, we then used an analytic workflow that involved clustering the quantified proteins based on their expression; defining the protein interaction network neighborhood around these clusters and calculating their disease associations; and performing pathway analysis on the clusters of interest (Figure IVB in the online-only Data Supplement, Methods). As the first step, we normalized the indoxyl sulfate-induced abundance profiles to their control counterparts and performed cluster analysis[13] to identify the predominant expression patterns. We focused on the 6 early-response (induced at 30 minutes) clusters that contained proteins with fold changes (FC) > 1.5 at 30 minutes with respect to the baseline (0 minutes; 130, 110, 125, 40, 18 and 2 proteins with FC > 1.5 for Cluster 5, 9, 12, 14, 15 and 19, respectively; Figure 3A, red outline).

To represent the protein interaction networks around the strongest signals within these 6 early-response clusters, we built networks consisting of the first neighbors of the FC >1.5 proteins for each cluster. We then calculated the network proximity of these networks to 44 human diseases and found that 4 clusters were particularly close to these diseases (28, 34, 37, and 40 diseases of 44 with an empirical P value < 0.05, for Clusters 5, 9, 12, and 14, respectively), indicating that these clusters may be centrally involved with disease pathogenesis including cardiometabolic disorders.

We used a network-based representation of pathways where the significantly enriched (FDR<0.05) pathways are connected to each other based on the number of shared proteins as reflected in the proteomics measurements. These pathway networks revealed distinct interconnected groups of pathways, providing us with a summary view of potential mechanisms related to indoxyl sulfate treatment. For example, Cluster 9 contained the pathway groups related to translation, DNA replication, glycolysis, chaperonin, membrane trafficking, endocytosis, and PDGF, as well as a group composed of Notch, Ubiquitin/proteasome, IL-1, and TNF signaling pathways (Figure 3B, Figure V in the online-only Data Supplement is provided for the high resolution version). In particular, Notch signaling was 1 of the most highly enriched pathways (FDR=0.010) in this pathway group. Cluster 14 also contained the pathways of TNFα, IL-1, proteasome, membrane trafficking, and endocytosis (data not shown). The network closeness analysis on the first neighbor network of FC > 1.5 proteins in Cluster 9 implicated significant associations with atherosclerosis and myocardial infarction (Figure 3C, Figure VI in the online-only Data Supplement is provided for the high resolution version).

Indoxyl Sulfate Activates Dll4-Notch Signaling in Human Primary Macrophages In Vitro

Our next step was to explore signaling mechanisms that link indoxyl sulfate to its downstream proinflammatory responses. We chose Notch signaling as an example of potential key regulators of macrophage activation by indoxyl sulfate. Based on the proteomics and pathway

mouse macrophage cell line RAW264.7 + IS

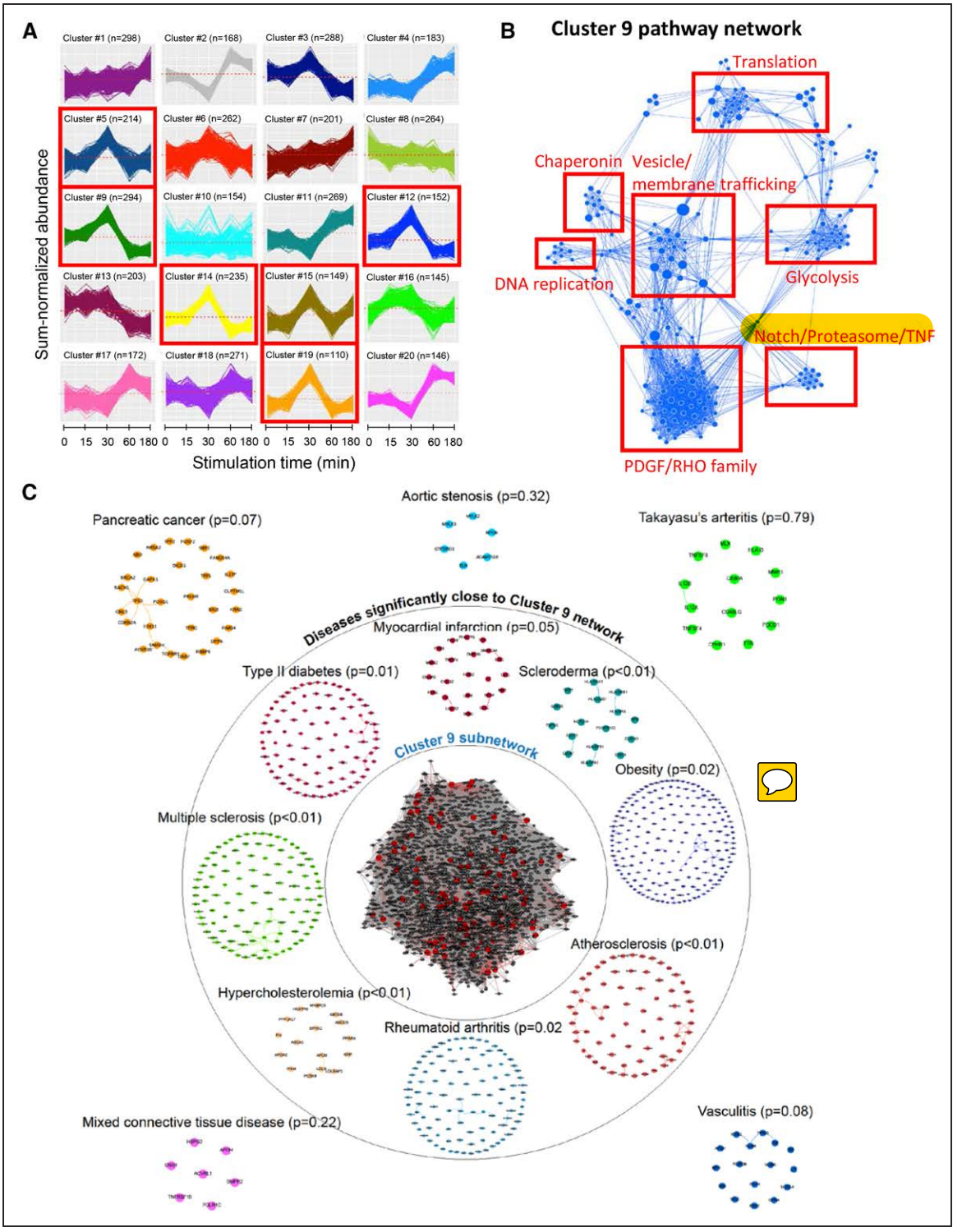


Figure 3. Global proteomics, pathway analysis and network analysis of the mouse macrophage cell line RAW264.7 stimulated with indoxyl sulfate.

A, Model-based cluster analysis of indoxyl sulfate induced protein kinetics. The dashed red line indicates the theoretical trace for unchanged protein abundances. Red rectangles highlight early (30 minutes) induction clusters. **B**, The network of significantly enriched pathways (FDR < 0.05) of the Cluster 9 proteins. Node size indicates the significance of each pathway in –log(q-value) and the edge thickness represents the overlap between the genes of the two pathways they connect, measured in terms of the Jaccard index (see Methods). Groups of pathways, which share genes and therefore represent common biological processes, are highlighted with red rectangles. **C**, The network closeness of Cluster 9 first neighbors to various cardiovascular, malignant, metabolic and auto-immune diseases. The center network represents the first neighbor network of Cluster 9 consisting of the Cluster 9 proteins with FC>1.5 between 0 and 30 minutes (red nodes) and their first neighbors in the PPI network (grey nodes). The diseases inside the circle are those that are significantly close to the first neighbor network of Cluster 9 (empirical $P \leq 0.05$). The diseases outside the circle are those that are not significantly close to the first neighbor network of Cluster 9 (empirical $P > 0.05$). Disease modules are built by mapping the respective disease-related genes onto the PPI network.

analysis (Figure 3B), we tested the hypothesis that the Notch pathway participates in indoxyl sulfate-triggered proinflammatory activation of macrophages. Notch signaling involves ligands (Delta-like ligand [Dll]1, Dll3, Dll4, Jagged1, and Jagged2) and receptors (Notch1-4) and contributes to biological processes during development and disease mechanisms in adults.[22–24] We first examined the expression of Notch pathway components and the activation of this signaling mechanism in human primary macrophages. Indoxyl sulfate increased mRNA expression levels of Notch1 and prototypical Notch target genes (Hes1, Hey1, and Hey2), but did not change the expression of Dll1, Dll4, Jagged1, Jagged2, Notch2, Notch3, and Notch4 in human primary macrophages (Figure 4A and Figure VIIA in the online-only Data Supplement). Notch signaling requires cell-to-cell contact. On binding to a ligand (eg, Dll4), a receptor such as Notch1 undergoes proteolytic cleavage, transferring its intracellular domain (ICD) to the nucleus as a transcriptional regulator.[24] Accumulation of ICD thus indicates Notch activation. Western blots using the antibody that recognizes the neoepitope of cleaved Notch1 (Val1744)[25] showed that indoxyl sulfate increased the accumulation of Notch1-ICD within 3 hours in human primary macrophages (Figure 4B, Figure VIIB in the online-only Data Supplement). Indoxyl sulfate also increased the activity of a Notch-sensitive luciferase reporter gene that contains multiple binding sites for RBP-Jκ, the key transcription factor that Notch ICD activates (Figure VIIC in the online-only Data Supplement).

We previously demonstrated that Dll4-triggered Notch signaling promotes proinflammatory macrophage activation in vitro and in vivo.[9–11,24] We thus investigated the role this Notch ligand may play in Notch activation by indoxyl sulfate. Western blot analysis indicated that indoxyl sulfate induces Dll4 protein expression as early as 30 minutes, which precedes the accumulation of Notch1-ICD (Figure 4B). We then examined the kinetics of Dll4 protein induction. Consistent with the Western blotting data (Figure 4B), indoxyl sulfate rapidly increased Dll4 protein by 30 minutes as gauged by both flow cytometry (Figure 4C) and immunofluorescence staining (Figure 4D).

In Figure 4D, we noticed that Dll4 protein induction by indoxyl sulfate occurred heterogeneously. Inspired by this result, we performed the cluster analysis of single cell mass cytometry (CyTOF) of human primary macrophages[26] combined with SPADE algorithm using 6 clustering channels (Figure 4E). The resulting SPADE trees demonstrated the heterogeneity of control and indoxyl sulfate–elicited macrophages, which is in agreement with a recent understanding that macrophage heterogeneity is more multidimensional than a traditional theory of polarization,[27] and also consistent with our previous finding that interferon (IFN) γ–treated macrophages still contain several subpopulations.[12] In Figure 4E (left panel), Dll4 expression levels varied among clusters of control macrophages. In addition to the heterogeneity as gauged by baseline Dll4 expression in macrophages, their responses to indoxyl sulfate were also heterogeneous. Furthermore, indoxyl sulfate increased Dll4 protein expression in some clusters and nodes that coincide with the TNF-α-positive clusters (Figure 4E, middle panels), indicating that Dll4-expressing macrophage subpopulations are high responders to this uremic toxin and may have a more proinflammatory phenotype. We further examined potential links between Dll4 and anti-inflammatory molecules such as CD163. Patterns of macrophage hetetogeneity as determined by expression levels of Dll4 and CD163 differed (Figure 4E, left and right panels). The expression of CD163 did not substantially change by indoxyl sulfate either (Figure 4E, right panel). We therefore found no specific characteristics of macrophage clusters highly expressing anti-inflammatory CD163 in terms of their responses to this uremic toxin.

Indoxyl Sulfate Induced Inflammation Via OATP2B1-Dll4-Notch Signaling in Macrophages

To determine whether Notch signaling mediates the proinflammatory effects of indoxyl sulfate, we first used the γ-secretase inhibitor DAPT, a pan-Notch signaling inhibitor. DAPT suppressed the induction of IL-1β, TNF-α, and MCP-1 in indoxyl sulfate–elicited macrophages (Figure VIID in the online-only Data Supplement). To further examine relative contribution of Dll4 to macrophage activation by indoxyl sulfate, we specifically blocked Dll4. Dll4 siRNA and Dll4 neutralizing antibody reduced mRNA expression of IL-1β and MCP-1 induced by indoxyl sulfate in human primary macrophages (Figure 5A and 5B). These results indicate that Dll4-Notch signaling in part mediates indoxyl sulfate–induced proinflammatory responses in macrophages.

Moreover, we examined whether macrophage uptake of indoxyl sulfate by OATP2B1 indeed promotes Dll4-mediated macrophage activation. Silencing of SLCO2B1 (OATP2B1) by siRNA suppressed the induction of Dll4 protein (Figure 5C) and the Notch target gene Hes1 mRNA in indoxyl sulfate-treated human primary macrophages (Figure 5D). These results suggest that the novel interplay of OATP2B1 and Dll4-Notch mediates indoxyl sulfate–triggered proinflammatory activation in macrophages.

Indoxyl Sulfate Suppresses Dll4 Protein Degradation Via Inhibition of the Ubiquitin-Proteasome Pathway

We further aimed to determine a mechanism by which indoxyl sulfate rapidly increases Dll4 protein. As pre-

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Notch pathway components

prototypical target genes

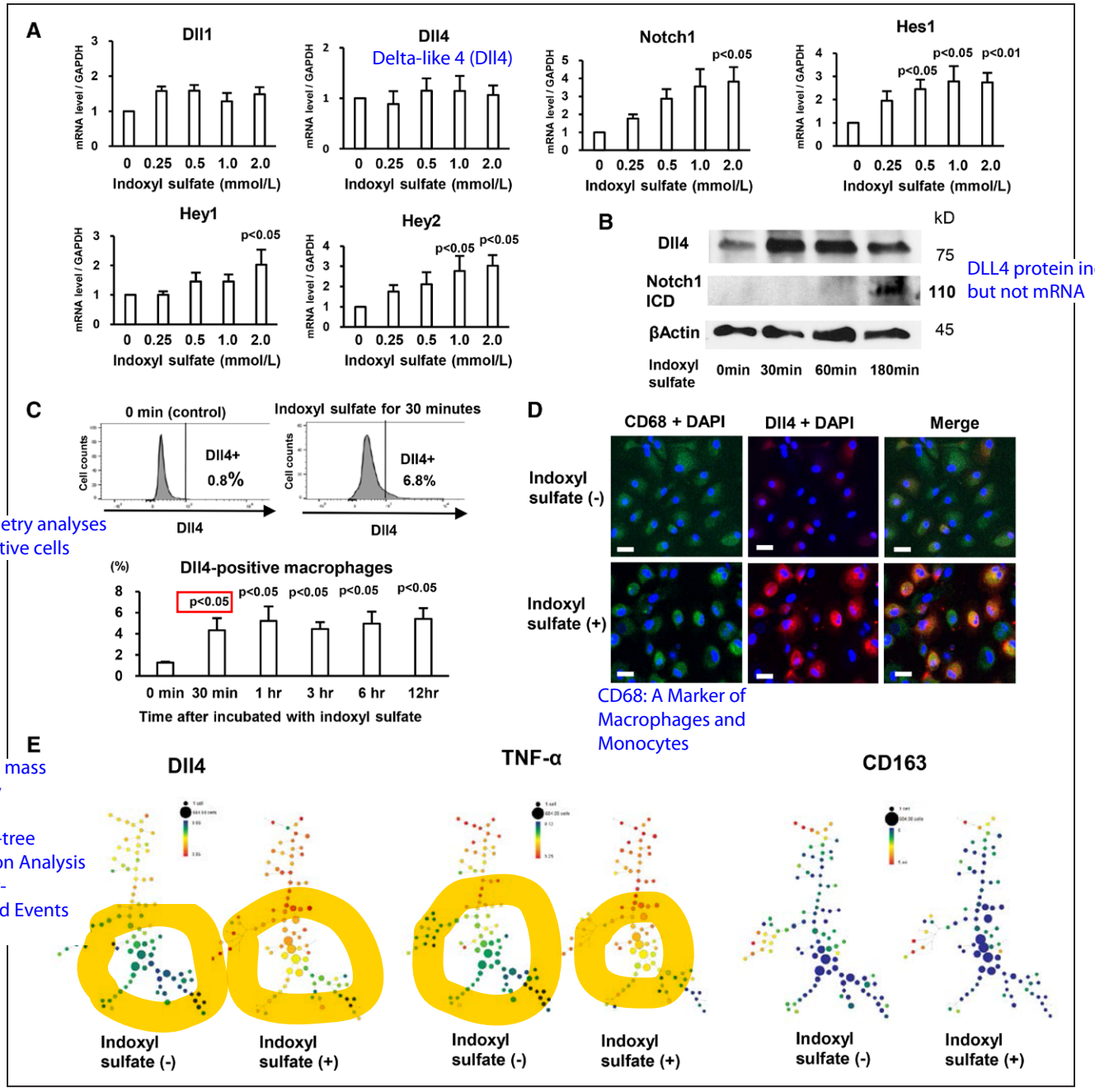
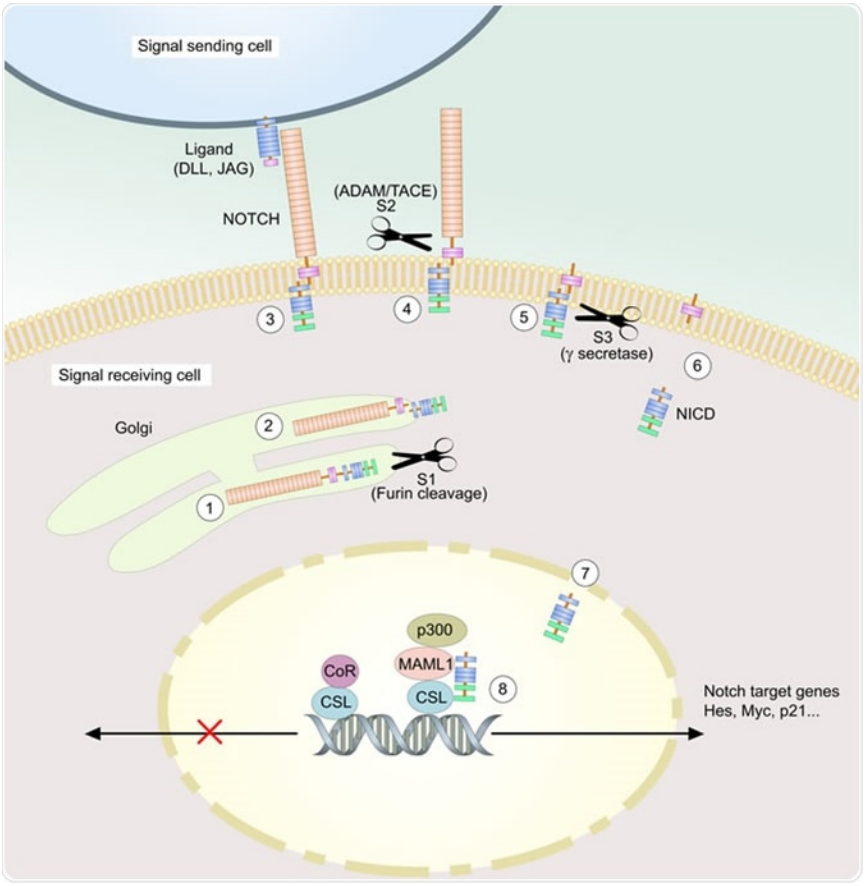
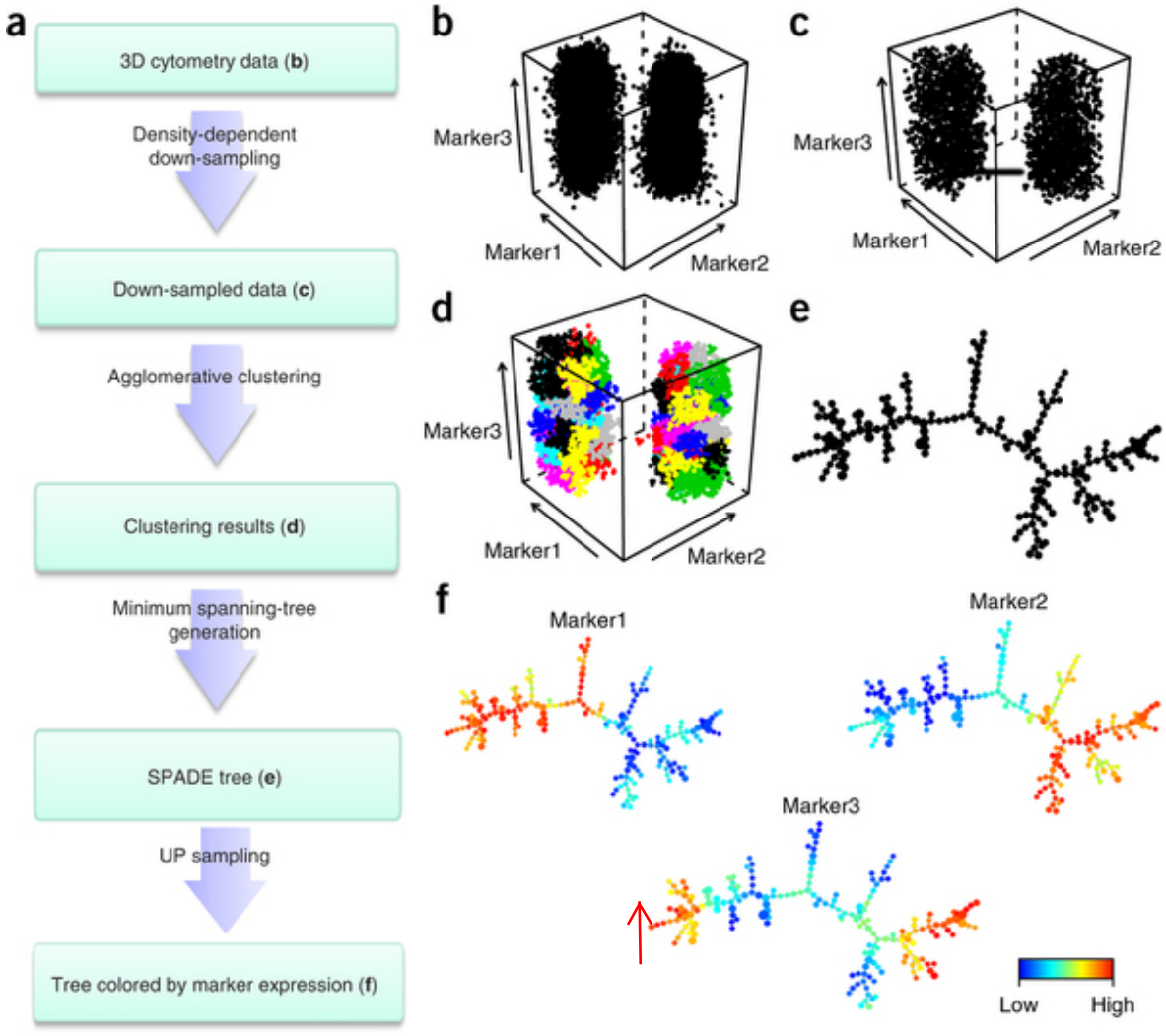


Figure 4. Indoxyl sulfate activates Dll4-Notch signaling.
A, mRNA expression of Notch pathway components (Dll1, Dll4, Notch1) and prototypical target genes (Hes1, Hey1 and Hey2) was measured in human primary macrophages after stimulation with indoxyl sulfate for 3 hours (9 peripheral blood mononuclear cell [PBMC] donors). **B**, The expression of Dll4 and the accumulation of Notch1 intracellular domain (ICD) was measured in human primary macrophages by Western blotting after stimulation with 0.5 mmol/L indoxyl sulfate. These blots from 1 donor represent 3 different PBMC donors. **C**, Flow cytometry analyses of Dll4-positive cells in human primary macrophages after stimulation with indoxyl sulfate (5 donors). *P* value was calculated by 1-way ANOVA followed by Tukey test, based on a comparison with 0 minute (min). Error bars indicate ±SEM. **D**, Double immunostaining (yellow) localized Dll4 (red) in CD68-positive (green) macrophages in human primary macrophages stimulated with 1 mmol/L indoxyl sulfate for 1 hour. Nuclei are visualized with DAPI. Scale bar, 50 μm. These data are representative of 3 different PBMC donors that produced similar results. **E**, SPADE clustering of CyTOF data of human macrophages. Each SPADE tree is a color heat map showing relative Dll4, TNF-α, or CD163 immunostaining of "nodes" or clusters of human macrophages after incubation with or without 1 mmol/L indoxyl sulfate for 48 hours. The SPADE heatmap shows heterogeneous expression patterns of Dll4, TNF-α, and CD163 in each condition. CD163 indicates cluster of differentiation 163; and TNF, tumor necrosis factor.

sented in Figure 4B, indoxyl sulfate increases Dll4 protein by 30 minutes in human primary macrophages. We then compared the kinetics of Dll4 protein and mRNA in indoxyl sulfate-treated macrophages. In the mouse macrophage-like cell line RAW264.7, indoxyl sulfate induced Dll4 protein expression within 5 to 15 minutes (Figure 6A, left panel) whereas Dll4 mRNA induction did not occur until 5 hours after incubated with indoxyl sulfate (Figure 6B, left panel). In human primary macrophages, Dll4 mRNA did not significantly increase over



researchers from Stanford University describe the use of Spanning-tree Progression Analysis of Density-normalized Events (SPADE), a density-based algorithm for visualizing single-cell data and enabling cellular hierarchy inference among subpopulations of similar cells.



(a) Flowchart for the use of SPADE on a simulated 3D flow cytometry data set. (b) 3D scatterplot of a simulated data set, which is constructed from four abundant subpopulations. (c) Density down-sampled version of b showing the same structure but with fewer cells. (d) Agglomerative hierarchical clustering on the down-sampled cells to generate cell clusters. (e) SPADE tree connecting all cell clusters. (f) SPADE tree color-coded according to the median expression intensities of markers of cells within each node, where each node is a cell cluster from d; each of the three trees represents the distribution of the different marker expression across all the cell clusters.

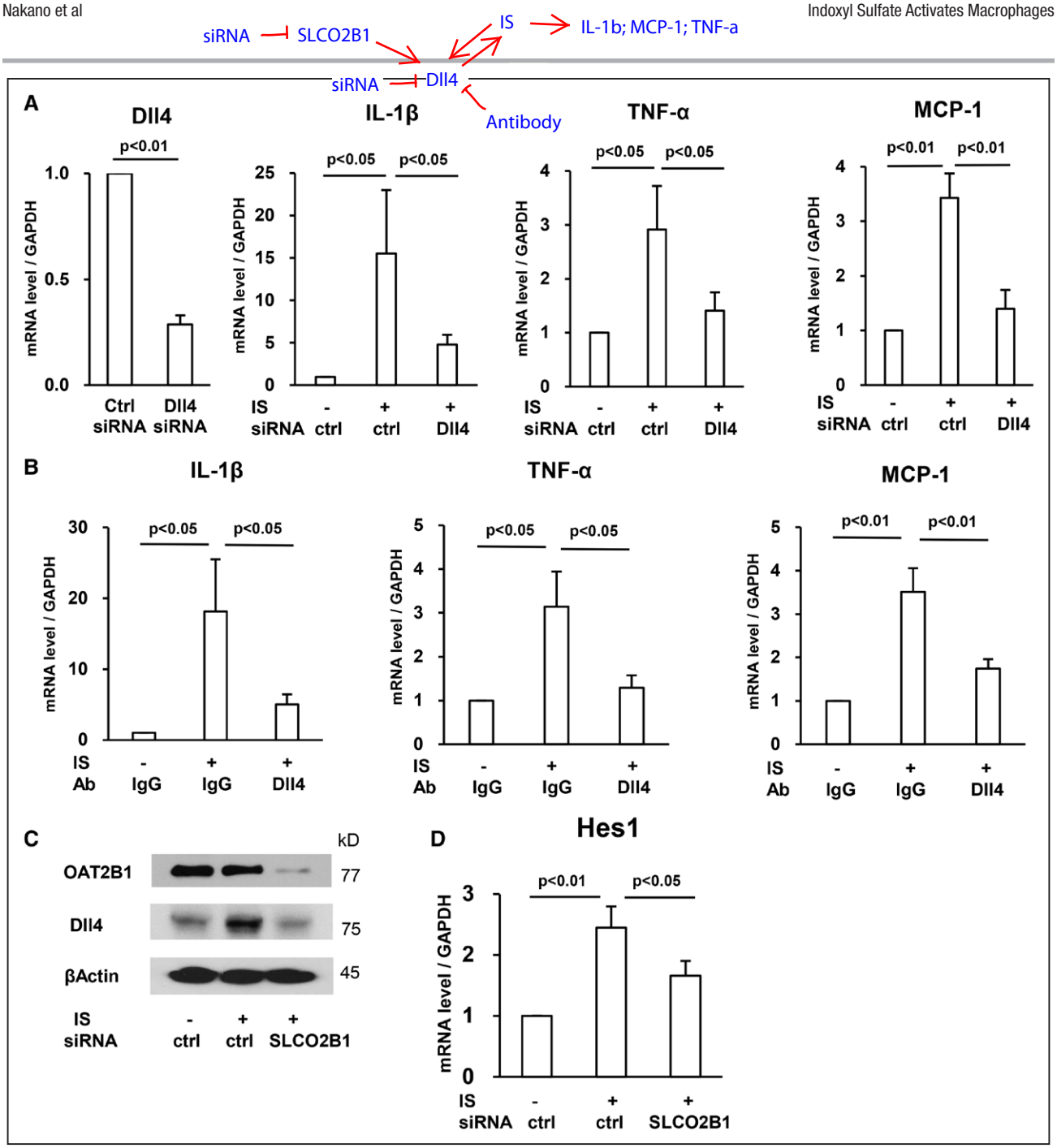
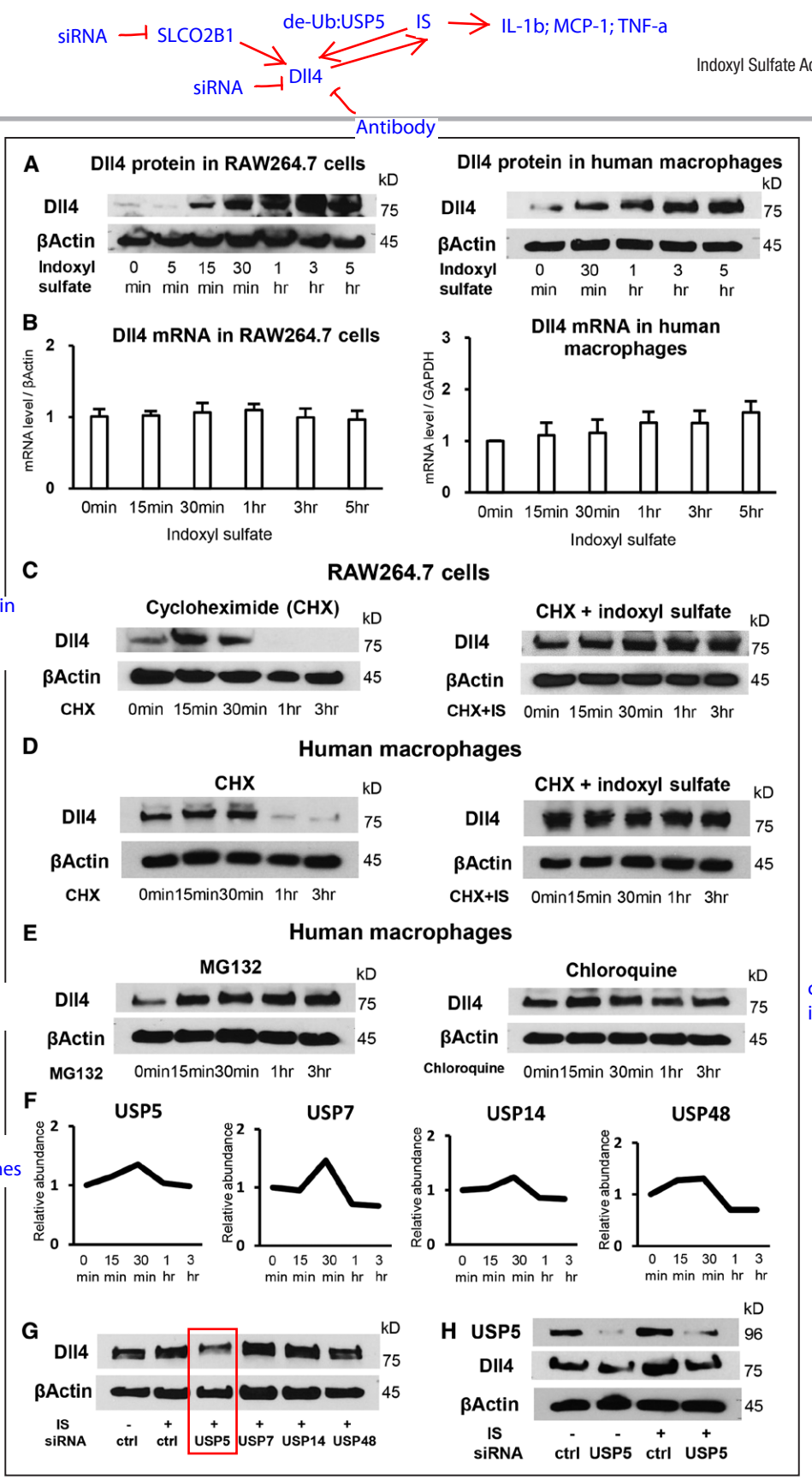


Figure 5. Indoxyl sulfate–induced proinflammatory cytokine expression is mediated by SLCO2B1-Dll4-Notch signaling in human macrophages. **A**, mRNA expression was measured in human primary macrophages after pretreatment with Dll4 siRNA or control siRNA (ctrl) for 48 hours and stimulation with IS for 3 hours (8 peripheral blood mononuclear cell [PBMC] donors). **B**, mRNA expression was measured in human primary macrophages after pretreatment with Dll4 antibody (Ab) or control IgG for 24 hours and stimulation with IS for 3 hours (8 PBMC donors). **C**, The expression of Dll4 was measured by Western blotting after suppression by siRNA for 48 hours and stimulation with 1.0 mmol/L IS for 1 hour. These blots from one donor are representative of the 3 different PBMC donors. **D**, mRNA expression of the prototypical Notch target Hes1 was measured in human primary macrophages after suppression by siRNA for 48 hours and stimulation with 0.5 mmol/L IS for 3 hours (6 PBMC donors). *P* value was calculated by unpaired Student *t* test or 1-way ANOVA followed by Tukey test. Error bars indicate ±SEM.

time whereas Dll4 protein rapidly increased (Figure 6A and 6B, right panels).

The effects of indoxyl sulfate on endocytosis, recycling, and degradation of Dll4 protein may contribute to its rapid induction.[28] We first examined whether indoxyl sulfate inhibits the degradation process of Dll4 protein. We monitored protein levels of Dll4 in the presence of the translation inhibitor cycloheximide (CHX) in RAW264.7 cells (Figure 6C). In the presence of CHX, Dll4 protein was completely lost by 1 hour. In contrast, when CHX and indoxyl sulfate were applied together, Dll4 protein levels were sustained up to 3 hours, suggesting that Dll4 degradation is inhibited by indoxyl sulfate. Likewise, in human macrophages, CHX treatment resulted in a near complete loss of Dll4 signal by 3 hours of treatment; however, the signal was stabilized



Cycloheximide: a protein synthesis inhibitor in eukaryotes

IS increases the protein stability of DLL4

MG132: a proteasome inhibitor

chloroquine: lysosome inhibitor

USP5/7/14/48: deubiquitinating enzymes

RAW264.7

PBMC

Figure 6. Indoxyl sulfate suppresses the degradation of Dll4 protein in macrophages.
A, The expression of Dll4 protein was measured in RAW264.7 cells (left) and human primary macrophages (right) by Western blotting after stimulation with 1.0 mmol/L indoxyl sulfate. **B**, mRNA expression of Dll4 was measured in RAW264.7 cells (left, n=4 experiments) and human primary macrophages (right, n = 8 donors) after stimulation with 1 mmol/L indoxyl sulfate. **C**, Dll4 protein was measured in RAW264.7 cells treated with cycloheximide (CHX) with or without indoxyl sulfate (1 mmol/L). **D**, Dll4 protein was measured in human primary macrophages treated with CHX with or without indoxyl sulfate (IS; 1 mmol/L). **E**, Dll4 protein was measured in human primary macrophages pretreated with proteasome inhibitor MG132 (left) or lysosomal inhibitor chloroquine (right). **F**, Tandem mass tagging–derived indoxyl sulfate-induced protein abundance profiles for USP5, 7, 14, and 48 in RAW264.7 cells. **G** and **H**, Dll4 protein was measured in human primary macrophages by Western blotting after siRNA treatment for 48 hours and stimulation with 1.0 mmol/L IS for 1 hour. All Western blotting experiments are representative of either 3 different RAW264.7 experiments or 3 human peripheral blood mononuclear cell (PBMC) donors that produced similar results.

by the addition of indoxyl sulfate (Figure 6D). In addition, the proteasome inhibitor MG132 increased Dll4 protein (Figure 6E, left). On the other hand, the lysosome inhibitor chloroquine had no effect on Dll4 protein level (Figure 6E, right panel). These results indicate that indoxyl sulfate may suppress degradation of Dll4 protein via inhibition of ubiquitin-proteasome pathway, thereby causing a rapid induction of Dll4 protein levels.

We further explored other potential effects of indoxyl sulfate on Dll4 trafficking. Epsin1 mediates endocytosis of Dll4, however its expression did not change after stimulation with indoxyl sulfate (Western blotting, data not shown). Rab proteins (eg, Rab1, Rab14) mediate trafficking of Dll4. E3 ubiquitin-protein ligase MIB1 promotes endocytosis of Dll4.[29] Silencing of Rab1, Rab14, or MIB1, however, did not suppress inflammatory responses (eg, IL-1β, MCP-1) induced by indoxyl sulfate (data not shown).

Indoxyl Sulfate Increases USP5 and Suppresses Degradation of Dll4

To explore the mechanism for inhibition of ubiquitin-proteasome pathway, we reanalyzed our proteomics data in search of known ubiquitin proteasome pathway components. Recent reports demonstrated that deubiquitinating enzymes activate Notch signaling.[30] We found that, in our proteomics data, indoxyl sulfate indeed increased the abundance of deubiquitinating enzymes, USP5, USP7, USP14, and USP48 by 30 minutes (Figure 6F). We examined whether these deubiquitinating enzymes indeed contribute to the indoxyl sulfate-induced increase of Dll4. Silencing of USP5, but not USP7, USP14, and USP48, in human primary macrophages inhibited indoxyl sulfate-dependent increase of Dll4 (Figure 6G). This finding on USP5 was confirmed in a second experiment (Figure 6H). These results suggest that indoxyl sulfate increases deubiquitinating enzyme USP5 and suppresses degradation of Dll4 protein via inhibition of the ubiquitin proteasome pathway.

Macrophage-Targeted Slco2b1 Silencing Inhibits Macrophage Activation Induced by Indoxyl Sulfate In Vivo

To examine the proinflammatory role of indoxyl sulfate in vivo, we injected this uremic toxin (100 mg/kg per day) in C57BL/6 mice for 7 days as previously reported.[31] To determine the relative contribution of macrophage OATP2B1 for macrophage activation in vivo, we used macrophage-targeted lipid nanoparticles (C12-200) to deliver siRNA for the mouse OATP2B1-encoding gene Slco2b1 via tail vein injection.[11,32] We administered C12-200-Slco2b1 siRNA or C12-200-control siRNA to C57BL/6 mice, which were also injected with indoxyl sulfate intraperitoneally for 7 days. We isolated F4/80 (+) peritoneal macrophages. The efficacy of inhibition of Slco2b1 mRNA in macrophages in vivo was >70%. Systemic administration of macrophage-targeted Slco2b1 siRNA suppressed indoxyl sulfate–induced expression of IL-1β and MCP-1 in peritoneal macrophages (Figure VIIIA in the online-only Data Supplement).

Indoxyl Sulfate Induces Proinflammatory Cytokines Via Dll4-Notch Signaling in Mouse Macrophages In Vivo

We also administered Dll4 neutralizing antibody or isotype control IgG (250 μg, twice a week) to C57BL/6 mice which were also injected indoxyl sulfate intraperitoneally for 7 days. We collected peritoneal cells and then isolated macrophages using F4/80 magnetic beads. Indoxyl sulfate administration induced proinflammatory genes (eg, IL-1β, MCP-1) in peritoneal macrophages isolated from IgG control mice, which was abrogated by Dll4 blockade by the neutralizing antibody (Supplemental Figure VIIIB).

CKD Induces Proinflammatory Cytokines in Macrophages in Mice

To further examine macrophage activation in CKD, we performed 5/6 nephrectomy in fat-fed Ldlr$^{-/-}$ mice (CKD mice). First, we isolated splenic macrophages from CKD mice and measured proinflammatory gene expression. Expression levels of IL-1β, TNF-α, and MCP-1 were higher in CKD mice as compared with those in sham-operated mice (Figure IXA in the online-only Data Supplement). Some Notch signaling components tended to be higher in CKD mouse macrophages but there was no statistical significance (Figure IXA in the online-only Data Supplement).

In Vivo Blockade of Dll4 Attenuates Atheroma Progression in CKD Mice

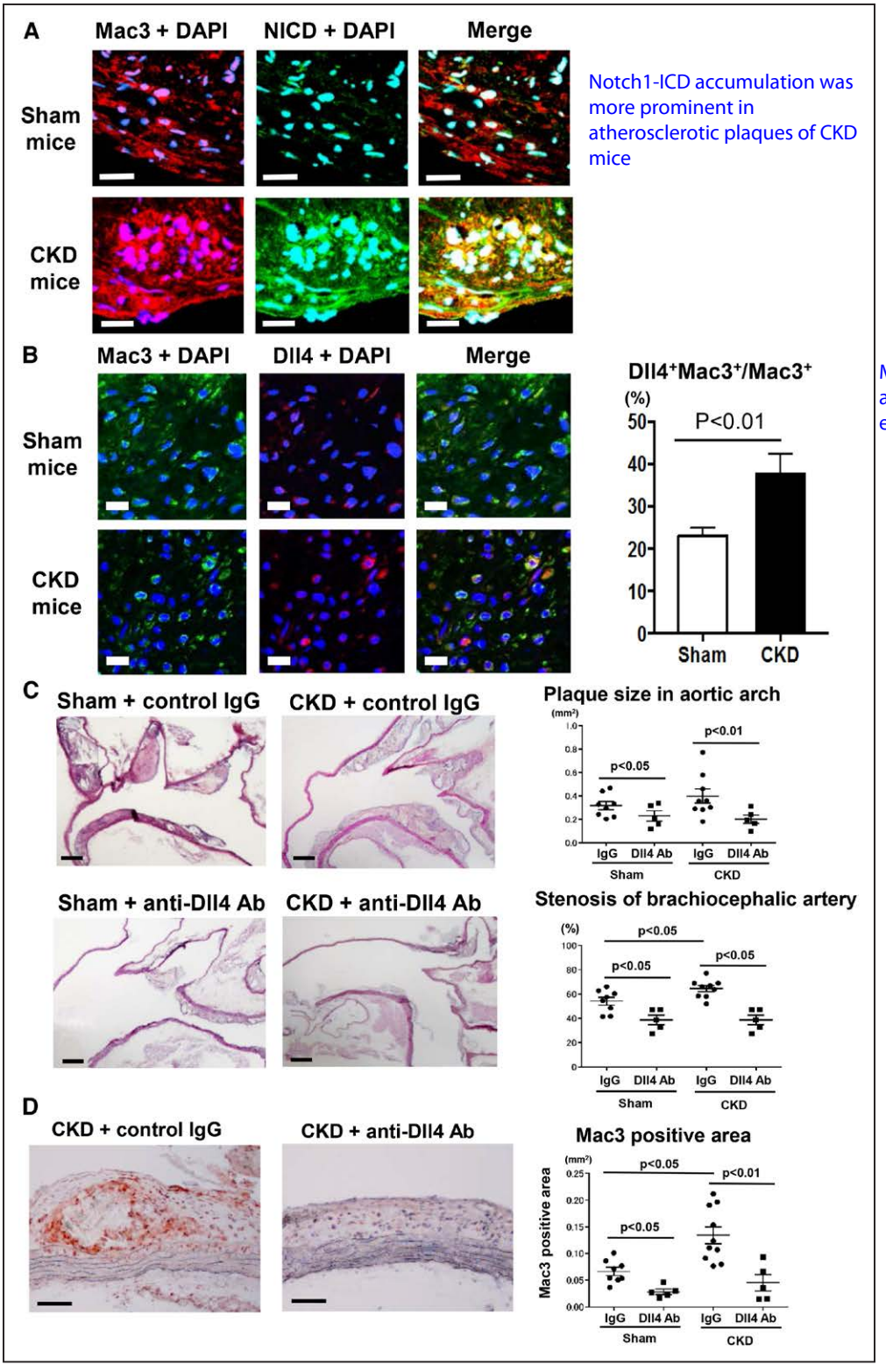
Moreover, we determined the role of Dll4-Notch signaling in atherogenesis in CKD. First, we examined activation of Notch signaling in CKD mice. Notch1-ICD accumulation was more prominent in atherosclerotic plaques of CKD mice as compared with control mice (Figure 7A). Double immunofluorescence indicated that many macrophages in CKD mouse lesions accumulated cleaved Notch1. We also evaluated macrophage expression of Dll4 in the aorta of CKD mice. In sham-operated mice, some plaque macrophages stained positively for Dll4 (Figure 7B), as we observed in in vitro experiments presented earlier in Figures 4D and 4E. In CKD mice, more macrophages in atherosclerotic lesions expressed Dll4. This tendency is also consistent with in vitro data.

ORIGINAL RESEARCH ARTICLE

Mac-3: macrophage marker; atherosclerotic plaque marker

Notch intercellular domain

CKD mice: 5/6 nephrectomy in fat-fed Ldlr−/− mice



Notch1-ICD accumulation was more prominent in atherosclerotic plaques of CKD mice

More macrophages in atherosclerotic lesions expressed Dll4

Ab ⊥ Dll4 ↓ Plaque

Figure 7. The effects of Dll4 blockade on atherogenesis in chronic kidney disease (CKD) mice.
A, In the aorta of CKD mice fed a HFD for 14 weeks, double immunostaining (yellow) localized Notch1 ICD (green) in Mac3-positive (red) macrophages. Fewer cells were double-positive in sham-operated mice. Nuclei are visualized with DAPI. The data represent 3 mice per group. Scale bar, 20 μm. **B**, Double immunostaining (yellow) localized Dll4 (red) in Mac3-positive (green) macrophages in atherosclerotic plaque. Nuclei are visualized with DAPI. Scale bar, 20 μm. The graph shows the percentage of Dll4+ macrophages to total macrophages (n=7 and 5). The average of 3 high power fields was used for the analysis. **C**, Hematoxylin and eosin (H&E) staining on longitudinal sections of the aortic arch (each n=5–9). **D**, Staining for Mac3 (macrophages) in the aorta (each n=5–9). Scale bar, 100 μm. *P* value was calculated by unpaired Student *t* test or 2-way ANOVA followed by the Bonferroni test. Error bars indicate ±SEM.

We then investigated the role of Dll4 in atheroma progression of CKD mice by administering Dll4 neutralizing antibody or isotype control IgG to fat-fed Ldlr$^{-/-}$ mice for 16 weeks and evaluated the atherosclerotic lesions (Supplemental Table 4). CKD induced by 5/6 nephrectomy accelerated the development of atherosclerotic plaques in the aortic arch and brachiocephalic arteries (Figure 7C). Dll4 blockade attenuated atherosclerotic lesion progression in CKD mice (Figure 7C), even though there was no significant difference between control IgG group and Dll4 antibody-treated group in serum biochemical characteristics including renal function, lipids and minerals, and blood pressure (Table IV in the online-only Data Supplement).

In Vivo Suppression of Dll4 Attenuates Inflammation and Calcific Potential in Atherosclerotic Plaques in CKD Mice

We then examined the role of Dll4 in vascular inflammation in CKD. Dll4 antibody administration tended to suppress the expression of proinflammatory molecules in the aorta (Supplemental Figure 9B). Particularly, 5/6 nephrectomy increased MCP-1 expression, which was abrogated by Dll4 blockade. CKD increased macrophage accumulation in aortic atherosclerotic plaques (Mac3, Figure 7D). Macrophage burden enhanced by 5/6 nephrectomy was suppressed by Dll4 antibody treatment.

Accelerated calcification in arteries and aortic valves represents cardiovascular disorders in CKD as we previously demonstrated.[3] CKD induced by 5/6 nephrectomy tended to increase alkaline phosphatase (ALP) activity, a marker of early-stage calcification, in the aorta, which decreased with Dll4 blockade (Figure IXC in the online-only Data Supplement). Molecular imaging of hydroxyapatite showed increased osteogenic activity in the aorta of CKD mice, as compared with sham-operated mice, which was abrogated by Dll4 antibody treatment (Figure IXD in the online-only Data Supplement). In addition, Dll4 suppression reduced ALP activity in aortic valve leaflets of CKD mice (Figure IXE in the online-only Data Supplement). A previous in vitro study revealed that indoxyl sulfate exerts inhibitory effects on the number and function of osteoclasts.[33] We thus examined whether Dll4 has the impact on macrophage differentiation into osteoclast-like cells (Figure X in the online-only Data Supplement). Dll4 antibody treatment did not change the expression of molecules associated with osteoclasts' functions such as cathepsin K and TRAP. Our data are consistent with a previous report that Dll4 does not regulate osteoclastic differentiation of macrophages.[34] We previously demonstrated that Dll4 antibody suppressed the inducers of osteoblastic differentiation Cbfa1 and BMP2,[10] indicating that such antiosteoblastogenesis effects of Dll4 blockade may inhibit calcification.

Relative Contribution of Macrophage OATP2B1/Slco2b1 and Dll4 to Indoxyl Sulfate-Triggered Atherogenesis

To further address mechanisms in depth, we attempted to examine the relative contribution of macrophage-derived OATP2B1 and Dll4 in accelerated atherogenesis in CKD, which is specifically induced by indoxyl sulfate. To accomplish this task, we coadministered indoxyl sulfate and siRNA for OATP2B1/Slco2b1 or Dll4 encapsulated in macrophage-targeted lipid nanoparticles C12-200. A previous study validated the efficacy of this method of in vivo siRNA delivery to macrophages.[32] We fed Ldlr$^{-/-}$ mice a high-fat diet for 12 weeks to develop atherosclerotic lesions. These mice did not undergo 5/6 nephrectomy. Instead, to dissect the role of OATP2B1 and Dll4 in indoxyl sulfate-induced macrophage activation, mice received this uremic toxin via intraperitoneal injection for 4 weeks before tissue harvesting. During the last 4 weeks, mice were also given Dll4 siRNA, Slco2b1 siRNA or control siRNA for 4 weeks. Figure IXF in the online-only Data Supplement demonstrates excellent in vivo silencing efficacy of Dll4 siRNA and Slco2b1 siRNA in mouse peritoneal macrophages. Indoxyl sulfate administration tended to increase the plaque size in the aortic arch and brachiocephalic artery stenosis (Figure 8A and 8B). Macrophage-targeted Dll4 or Slco2b1 siRNA abrogated indoxyl sulfate-enhanced plaque burden (Figure 8A and 8B) and vascular inflammation, as gauged by IL-1β mRNA levels in the aorta (Figure 8C). These results indicate that macrophage-derived Dll4 and OATP2B1/Slco2b1 indeed mediate indoxyl sulfate–induced atherogenesis.

DISCUSSION

In this study, we investigated the mechanisms by which indoxyl sulfate induces proinflammatory activation of human primary macrophages and accelerates atherogenesis. The key novel findings include: (1) indoxyl sulfate induces proinflammatory activation of macrophages in vitro and in vivo; (2) primary human and mouse macrophages express the OAT and OATP family transporters; (3) OATP2B1 expressed by both human and mouse macrophages mediates the uptake of indoxyl sulfate in vitro; (4) OATP2B1 mediates indoxyl sulfate-induced proinflammatory activation of macrophages in vitro and in vivo; (5) indoxyl sulfate rapidly induces Dll4 protein levels in macrophages in vitro, by suppressing degradation of Dll4 protein via the inhibitory effect of USP5 on the ubiquitin-proteasome pathway; (6) indoxyl sulfate triggers Notch signaling in vitro; (7) Dll4-Notch signaling mediates indoxyl sulfate-induced proinflammatory responses of macrophages in vitro and in vivo; (8) OATP2B1 mediates the rapid induction of Dll4 protein in indoxyl sulfate-elicited macrophages in vitro;

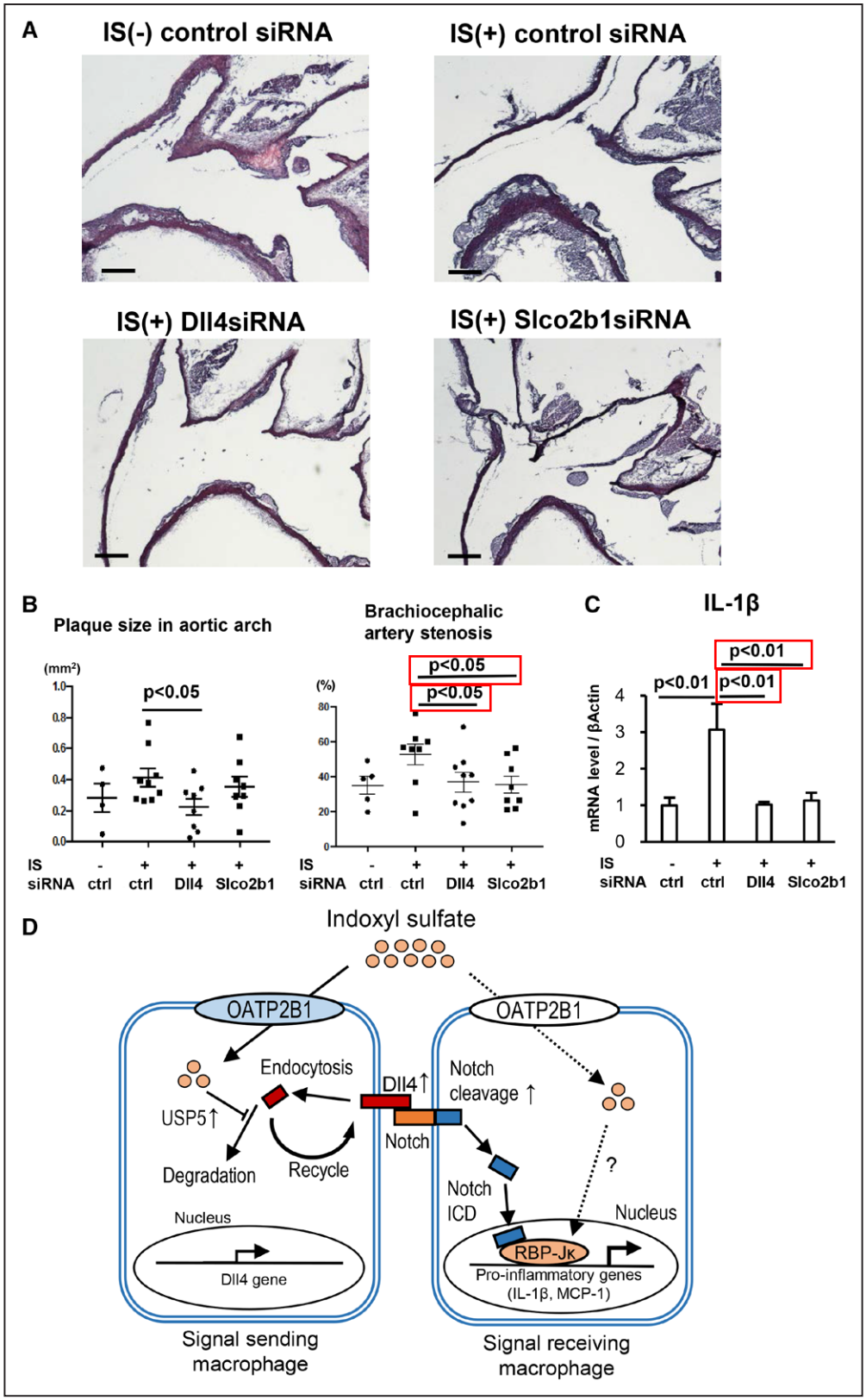


Figure 8. The effects of macrophage-targeted Dll4 or Slco2b1 blockade on atherogenesis in mice.
A, In the aorta of C57BL/6 mice fed a high-fat diet (HFD) for 12 weeks. After 8 weeks, indoxyl sulfate of 100 mg/kg/d or PBS was intraperitoneally injected to C57BL/6 mice for 4 weeks, and macrophage-targeted lipid nanoparticles (C12-200) containing control siRNA (Ctrl), Dll4 siRNA, or Slco2b1 siRNA were also injected via tail vein twice a week for 4 weeks. Hematoxylin and eosin (H&E) staining on longitudinal sections of the aortic arch. **B**, Plaque size in the aortic arch and stenosis rate of brachiocephalic artery (each n=5–9). **C**, The expression of IL-1β mRNA in aorta of mice (each n=5–9). *P* value was calculated by 1-way ANOVA followed by Tukey test. Error bars indicate ±SEM. **D**, The potential mechanism for proinflammatory activation of macrophages in chronic kidney disease (CKD) via crosstalk between OATP2B1 and the Dll4-Notch axis. Macrophages take up indoxyl sulfate via transporters such as OATP2B1. (*Continued*)

(9) in vivo administration of Dll4 neutralizing antibody attenuates macrophage accumulation, atherogenesis, and osteogenic activity in CKD mice; and (10) Dll4 and OATP2B1, expressed by macrophages, mediate indoxyl sulfate-triggered atherogenesis, as demonstrated by macrophage-targeted in vivo delivery of siRNA. Based on these findings, we propose a novel hypothesis on the role of the OATP2B1-Dll4-Notch axis (Figure 8D). We acknowledge, however, that other mechanisms may also participate in indoxyl sulfate-triggered macrophage activation.

Clinical studies have failed to show the effects of current therapies including potent statins on their beneficial effects on advanced CKD patients.[35] Uremic toxins contribute to the pathogenesis of CVD.[1] Among them, indoxyl sulfate is a major uremic toxin and serves as an independent predictor of cardiovascular risk in CKD patients.[5] An oral carbonic absorbent AST-120 adsorbs uremic toxins and precursors, including indole, excretes them into the feces, and has the potential to reduce the serum level of indoxyl sulfate.[36,37] Clinical trials of AST-120, however, demonstrate no beneficial effects on CKD progression and mortality.[37,38] The low reduction rate of serum indoxyl sulfate by AST-120 (10% to 23%) in humans may account for these failed studies.[36,37] The development of alternative therapies that suppress cellular responses to indoxyl sulfate is thus needed to reduce cardiovascular risk in CKD patients.

Chronic inflammation contributes to the pathogenesis of vascular diseases.[39] Macrophages play key roles in the development of atherosclerosis and the onset of its acute thrombotic complications.[40] Various metabolic insults such as dyslipidemia promote proinflammatory activation of macrophages.[40] Even with potent statins, many patients remain unsaved. Our laboratory has thus explored the mechanisms for macrophage activation beyond conventional risks.[9,10,12] Although previous studies reported monocyte/macrophage activation in CKD patients,[41] underlying mechanisms remain obscure. In the present study, we demonstrated in vitro and in vivo that indoxyl sulfate induces proinflammatory activation of macrophages, which is mediated by Dll4-Notch signaling, a molecular link previously unknown in any cell types. Better understanding the mechanisms of macrophage activation in the CKD milieu may help to develop much-needed new therapeutic strategies for the prevention of CVD in CKD. The in vivo experiments in the present study used male mice only. We therefore do not claim that new mechanisms proposed in this report also apply to females or that potential therapies developed based on our work may be effective in both men and women. Addressing these questions requires additional studies.

OATs/OATPs expressed in tubular epithelial cells transport uremic toxins including indoxyl sulfate.[8] OATs/OATPs excrete indoxyl sulfate from blood to urine. We discovered that macrophages express several OATs/OATPs. Our novel finding further indicates that the SLCO2B1 gene product OATP2B1 mediates the uptake of indoxyl sulfate by human and mouse macrophages in vitro. To demonstrate that this transporter indeed mediates indoxyl sulfate-triggered macrophage activation, we systemically administered in mice macrophage-targeted lipid nanoparticles containing Slco2b1 siRNA. This treatment suppressed proinflammatory responses of peritoneal macrophages elicited by indoxyl sulfate. These results suggest that OATP2B1 contributes to accelerated inflammation in CKD.

Dll4-Notch signaling promotes proinflammatory activation of macrophages and accelerates the development of cardiometabolic disorders, including atherosclerosis, vein graft disease, vascular calcification, type 2 diabetes mellitus, and fatty liver, as reported by us.[9–11] These studies, however, did not identify specific factors that induce Dll4 in these cardiometabolic organs. The role of Dll4-Notch in vascular inflammation in CKD also remains unreported. The present study associated indoxyl sulfate and Dll4-Notch axis. To assess a causal link, we suppressed Dll4-Notch signaling in macrophages in the uremic milieu. The blockade of Dll4 abrogated proinflammatory macrophage responses enhanced by indoxyl sulfate and retarded the progression of atherosclerosis and calcification in CKD mice. Our results suggest that indoxyl sulfate amplifies macrophage activation via Dll4-Notch signaling, leading to the progression of atherosclerosis.

In the present study, we have not focused on specific molecular mechanisms downstream of the Dll4-Notch pathway, such as nuclear factor-κB (NF-κB), that promote indoxyl sulfate-induced proinflammatory responses of macrophages. We previously demonstrated in vitro that Dll4-triggerd Notch signaling activates proinflammatory pathways such as ERK1/2, Akt, and NF-κB in human primary macrophages.[9] We showed in vivo that Dll4 suppression in metabolically-challenged mice can reduce NF-κB activation in the aorta and adipose tissue.[10] Our studies, including the present report, also provided in vitro and in vivo evidence that Dll4 regulates the expression of the NF-κB gene IL-1β.[10,18] Such proinflammatory factors as NF-κB may thus contribute to the proinflammatory gene expression in macrophages induced by indoxyl sulfate. A recent study by Wakamatsu et al[42] demonstrated that, although indoxyl sulfate activates NF-κB and IL-1β mRNA expression in the macrophage-like cell line THP-1, it fails to activate the NLRP3 inflammasome. These results indicate that indoxyl sul-

Figure 8 Continued. Dll4 protein usually undergoes endocytosis and recycling. Indoxyl sulfate may inhibits Dll4 degradation via USP5 and increase its recycling. The enhanced interaction of Dll4 and a receptor (eg, Notch1) may thus accelerate Notch signaling, inducing the expression of proinflammatory genes. Although our in vitro and in vivo data suggest the role of the Dll4-Notch pathway in indoxyl sulfate macrophage activation, other mechanisms may also contribute.

fate may not be sufficient to activate the secondary signals leading to inflammasome activation in THP-1 cells. The effects of indoxyl sulfate on the inflammasome in primary human macrophages and particularly in the vasculature may be addressed in the future studies.

Aryl hydrocarbon receptor (AhR), which is known as the dioxin receptor, may be an endogenous effector for indoxyl sulfate.[43] Previous reports showed AhR that mediates indoxyl sulfate-induced E-selectin expression in endothelial cells,[44] and tissue factor production in smooth muscle cells.[45] Furthermore, several studies demonstrated the relationship between AhR and Notch signaling. AhR activation induces Notch1 and Notch2 in lymphoid cells.[46] The AhR pathway is activated in patients and mice with CKD.[47] AhR activation may thus be an additional mechanism for the deleterious cardiovascular effects observed in CKD. Addressing crosstalk between Dll4-Notch and AhR in the context of indoxyl sulfate-triggered macrophage activation, however, requires further investigations.

Uremia is a nontraditional risk for atherosclerosis and CVD. Although the present study has focused on a specific mechanism involving indoxyl sulfate-triggered macrophage activation mediated by OATP2B1 and Dll4-Notch signaling, we recognize the multifactorial nature of accelerated atherogenesis in CKD. Protein carbamylation increases in CKD patients and accelerates atherosclerosis.[1] Higher levels of phosphate, parathyroid hormone, and fibroblast growth factor 23 associate with the development of atherosclerotic lesions and calcification.[48] Oxidative stress is higher in CKD and increases cardiovascular risk.[1] Circulating levels of asymmetrical dimethylarginine, an endogenous inhibitor of nitric oxide synthase, may cause endothelial dysfunction and accelerate atherosclerosis.[1] In the large pathogenic network, the relative contribution of indoxyl sulfate to atherogenesis in CKD remains to be further addressed.

The present study further explored underlying mechanisms in depth. Indoxyl sulfate rapidly induced Dll4 expression in macrophages via inhibition of its degradation. Recent reports suggest that the ubiquitin-proteasome system relates to the Notch signaling pathway and endocytosis of Notch receptors and ligands, including E3 ubiquitin ligases and deubiquitinating enzymes.[49] Our results show that indoxyl sulfate increased deubiquitinating enzyme USP5, which in turn increased Dll4 protein via inhibition of the ubiquitin proteasome pathway. Another group also reported that indoxyl sulfate inhibited ubiquitination and degradation of tissue factor in smooth muscle cells and increased a thrombotic risk.[50] Therefore, indoxyl sulfate has a function to inhibit ubiquitination and degradation of some proteins. Such effects of this major uremic toxin are attractive subjects of future studies.

In summary, we demonstrate several lines of novel evidence that indoxyl sulfate induces macrophage activation via the crosstalk between OATP2B1 and the Dll4-Notch axis. Despite their clinical impact, no effective therapies are currently available for the prevention or treatment of cardiovascular complications of CKD. Novel mechanisms for accelerated vascular inflammation in CKD revealed in the present study may provide molecular bases for the development of new therapeutic strategies against this global health threat.

ARTICLE INFORMATION

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Disclosures

None.

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