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Uremic toxin indoxyl sulfate induces trained immunity *via* the AhR-dependent arachidonic acid pathway in end-stage renal disease (ESRD)

Hee Young Kim, Yeon Jun Kang, Dong Hyun Kim, Jiyeon Jang, Su Jeong Lee, Gwanghun Kim, Hee Byung Koh, Ye Eun Ko, Hyun Mu Shin, Hajeong Lee, Tae-Hyun Yoo, Won-Woo Lee

Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul 03080, Republic of Korea • Institute of Infectious Diseases, Seoul National University College of Medicine, Seoul 03080, Republic of Korea • Laboratory of Autoimmunity and Inflammation (LAI), Department of Biomedical Sciences, and BK21Plus Biomedical Science Project, Seoul National University College of Medicine, Seoul 03080, Republic of Korea • Department of Biomedical Sciences, College of Medicine and BK21Plus Biomedical Science Project, Seoul National University College of Medicine, Seoul 03080, Republic of Korea • Department of Internal Medicine, College of Medicine, Yonsei University, Seoul, Republic of Korea • Wide River Institute of Immunology, Seoul National University, Hongcheon 25159, Republic of Korea • Division of Nephrology, Department of Internal Medicine, Seoul National University Hospital, Seoul 03080, Republic of Korea • Division of Nephrology, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 03722 Republic of Korea • Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine; Seoul National University Hospital Biomedical Research Institute, Seoul 03080, Republic of Korea

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Abstract

Trained immunity is the long-term functional reprogramming of innate immune cells, which results in altered responses toward a secondary challenge. Despite indoxyl sulfate (IS) being a potent stimulus associated with chronic kidney disease (CKD)-related inflammation, its impact on trained immunity has not been explored. Here, we demonstrate that IS induces trained immunity in monocytes *via* epigenetic and metabolic reprogramming, resulting in augmented cytokine production. Mechanistically, the aryl hydrocarbon receptor (AhR) contributes to IS-trained immunity by enhancing the expression of arachidonic acid (AA) metabolism-related genes such as Arachidonate 5-Lipoxygenase (ALOX5) and ALOX5 Activating Protein (ALOX5AP). Inhibition of AhR during IS training suppresses the induction of IS-trained immunity. Monocytes from end-stage renal disease (ESRD) patients have increased ALOX5 expression and after 6-day training, they exhibit enhanced TNF-α and IL-6 production to LPS. Furthermore, healthy control-derived monocytes trained with uremic sera from ESRD patients exhibit increased production of TNF-α and IL-6. Consistently, IS-trained mice and their splenic myeloid cells had increased production of TNF-α after *in vivo* and *ex vivo* LPS stimulation compared to that of control mice. These results provide insight into the role of IS in the induction of trained immunity, which is critical during inflammatory immune responses in CKD patients.

eLife assessment

The authors expand the concept of a new layer to training immunity, which is currently being highlighted by several colleagues in the field. The work provides **important** hints to understand end-stage renal disease. Overall, the rational approach leads to experimental results that are **solid**.

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Introduction

Over the last decade, a large body of evidence has demonstrated that innate cells can build up immunological memory resulting in enhanced responsiveness to subsequent stimulation, a phenomenon termed trained immunity (1). Compared with classical epitope-specific adaptive immunological memory based on an antigen-receptor, trained immunity of monocytes and macrophages is the long-term functional reprogramming elicited by an initial primary insult, mainly pathogen-associated molecular patterns (PAMPs), which leads to an altered response towards a subsequent, unrelated secondary insult after the return to a homeostatic state (2, 3). It has been well demonstrated that exposure of monocytes or macrophages to *Candida albicans*, fungal cell wall component β-glucan, or BCG (Bacille Calmette-Guérin) vaccine enhances their subsequent responses to unrelated pathogens or pathogen components such as lipopolysaccharide (LPS) (4, 5). The induction of trained immunity is associated with the interaction of epigenetic modifications and metabolic rewiring, which can last for prolonged periods of time (2, 3, 5–8). Mechanistically, certain metabolites derived from the upregulation of different metabolic pathways triggered by primary insult can influence enzymes involved in remodeling the epigenetic landscape of cells. This leads to specific changes in epigenetic histone markers, such as histone 3 lysine 4 trimethylation (H3K4me3) or histone 3 lysine 27 acetylation (H3K27ac), which regulate genes resulting in a more rapid and stronger response upon a subsequent, unrelated secondary insult (2–4, 7). In addition, it has been recently reported that long non-coding RNAs induce epigenetic reprogramming *via* the histone methyltransferase, MLL1. Subsequently, transcription factors such as Runx1 regulate the induction of proinflammatory cytokines following the secondary insult (9–11).

Many studies have provided evidence that trained immunity likely evolved as a beneficial process for non-specific protection from future secondary infections (3). However, it has also been suggested that augmented immune responses resulting from trained immunity is potentially relevant to deleterious outcomes in immune-mediated and chronic inflammatory diseases such as autoimmune diseases, allergy, and atherosclerosis (8, 10, 12–15). Thus, although most studies have focused on the ability of exogenous microbial insults to induce trained immunity, it is also conceivable that sterile inflammatory insults can evoke trained immunity. In support of this idea, oxLDL, lipoprotein a (Lpa), uric acid, hyperglycemia, and the Western diet have all been recently identified as endogenous sterile insults that induce trained immunity in human monocytes *via* epigenetic reprogramming (8, 10, 12, 13, 16). Thus, it is tempting to speculate that many endogenous insults that cause chronic inflammatory conditions may be involved in the induction of trained immunity in human monocytes and macrophages.

Chronic kidney disease (CKD) is recognized as a major non-communicable disease with increasing worldwide prevalence (17, 18). Loss of renal function in CKD patients causes the accumulation of over 100 uremic toxins, which are closely associated with cardiovascular risk and mortality due to their ability to generate oxidative stress and a proinflammatory cytokine milieu

(19). Reflecting this, cardiovascular disease (CVD) is a leading cause of death among patients with end-stage renal disease (ESRD) (20). Indoxyl sulfate (IS) is a major uremic toxin derived from dietary tryptophan *via* fermentation of gut microbiota (21). Since it is poorly cleared by hemodialysis, IS is one of the uremic toxins present at higher than normal concentrations in the serum of CKD patients (22, 23) and is associated with the progression of CKD and the development of CKD-related complications such as CVD (24). We and others have shown that IS promotes the production of proinflammatory cytokines such as TNF-α and IL-1β by monocytes and macrophages through aryl hydrocarbon receptor (AhR) signaling and organic anion transporting polypeptides 2B1 (OATP2B1)-Dll4-Notch Signaling (21, 25, 26), suggesting a role of IS as an endogenous inflammatory insult in monocytes and macrophages. Moreover, pretreatment with IS greatly increases TNF-α production by human macrophages in response to a low dose of LPS (25). Despite the function of IS as an endogenous inflammatory insult in monocytes and macrophages, little is known with regard to whether IS induces trained immunity. Thus, we investigated whether exposure to IS triggers trained immunity in an *in vitro* human monocyte model and an *in vivo* mouse model, as well as the mechanisms involved in IS-induced trained immunity. Our data show that IS triggers trained immunity in human monocytes/macrophages *via* AhR-dependent alteration of the arachidonic acid (AA) pathway, epigenetic modifications, and metabolic rewiring. Thus, this suggests IS plays a critical role in the initiation of inflammatory immune responses in patients with CKD.

Results

Indoxyl sulfate (IS) induces trained immunity in human monocytes

To explore whether exposure to IS is involved in the induction of trained immunity in human monocytes, an *in vitro* model of trained immunity was applied as previously reported by the Netea group (27). Freshly isolated human $CD14^+$ monocytes were preincubated for 24 hrs with or without IS and, after a subsequent 5-day culture in human serum, restimulated with lipopolysaccharide (LPS) or Pam3cys for final 24 hrs (**Fig. 1A**). Preincubation of monocytes with IS led to enhanced production of TNF-α, a major monocyte/macrophage-derived inflammatory cytokine, upon LPS stimulation. Since 10 ng/ml of LPS significantly increased both TNF-α and IL-6 secretion in IS-trained macrophages (**Fig. 1B**), we used this concentration of LPS in subsequent experiments. A clinically relevant concentration of IS in severe CKD has reported the range from 0.5 to 1.0 mmol/L (19). The preincubation effect of IS on cytokine production was observed at a concentration as low as 250 μM, which is the average IS concentration in patients with ESRD in our cohort (**Fig. 1C**) (25). Unlike IS, preincubation with other protein-bound uremic toxins (PBUTs), such as *p*-cresyl sulfate (PCS), Hippuric acid (HA), Indole 3-acetic acid (IAA) and kynurenic acid (KA), did not cause increased secretion of TNF-α or IL-6 in response to LPS stimulation (Fig. S1A). In addition, there was no obvious effect on cell viability following pre-incubation of macrophages with 1,000 μM of IS after a subsequent 5-day culture in human serum or after LPS stimulation (Fig. S1B). We also found that the enhanced cytokine production of IS-trained macrophages was not attributable to potassium derived from IS potassium salt (Fig. S1C). Moreover, the increased TNF-α and IL-6 production in IS-trained macrophages was not limited to LPS stimulation, as similar phenomena were observed following stimulation with Pam3cys, a TLR1/2 agonist (**Fig. 1D**). β-glucan-pretreated macrophages exhibit a prototypic feature of trained immunity, characterized by enhanced production of inflammatory cytokines upon restimulation with heterologous stimuli, LPS or Pam3cys (6, 27). As seen in Figure S1D, the level of TNF-α secreted by IS-trained macrophages was comparable with that secreted by β-glucan-trained macrophages, although β-glucan had a more potent effect on IL-6 production than did IS, suggesting that IS plays a role in the induction of trained immunity of human monocytes. Furthermore, alongside elevated TNF-α and IL-6 expressions, there was a notable increase in the mRNA expression of *IL-1β* and *MCP-1* (*CCL2*) observed in IS-trained macrophages, concomitant with a significant reduction in *IL-10*, a cytokine known for its anti-inflammatory properties, within

the same cellular context (**Fig. 1E**). Correspondingly, alterations in their protein levels mirrored the observed mRNA expressions (Fig. S1E). Circulating monocytes have been identified as a major immune cell subset that responds to IS in the serum of ESRD patients (21, 25). To examine whether uremic serum induces trained immunity of monocytes/macrophages, pooled sera from ESRD patients (184 ± 44 μM of average IS level) or from HCs were used to treat monocytes isolated from HCs for 24 hr at 30% (v/v), followed by training for 5 days (**Fig. 1F**). Training with pooled uremic serum of ESRD patients increased the production of TNF-α and IL-6 upon re-stimulation with LPS compared to monocytes treated with the pooled sera of HCs (**Fig. 1G-I**). In addition, expression of *IL-1β* and *MCP-1* mRNA was also augmented by training with the pooled uremic sera of ESRD patients (**Fig. 1I**). These results suggest that IS induces trained immunity in human monocytes, characterized by the increased expression of proinflammatory cytokines TNF-α and IL-6 and reduced expression of anti-inflammatory IL-10 in response to secondary TLR stimulation.

IS-induced trained immunity is regulated by metabolic rewiring

Metabolic rewiring is one of the most crucial processes regulating the trained immunity of monocytes and macrophages (33). Assessment of the metabolic profile of IS-trained macrophages on day 6 (prior to re-stimulation with LPS) showed that training with IS led to an enhanced extracellular acidification rate (ECAR) as a measure of lactate production, indicating increased glycolysis and glycolysis capacity (**Fig. 2A and B**). Moreover, basal and maximal respiration and ATP production gauged by the oxygen consumption rate (OCR) were also increased compared to that of non-trained cells (**Fig. 2C and D**). Enhanced glycolysis and glycolytic capacity in IS-trained cells remained higher even after re-stimulation with LPS (Fig. S2A-B), implying that the IS-training effect on metabolic rewiring is sustained regardless of the secondary stimulation. To further examine whether the metabolic rewiring by IS-trained cells is linked to the regulation of trained immunity, 2-deoxy-d-glucose (2-DG), a general inhibitor of glycolysis, was added to monocytes before training with IS. 2-DG completely inhibited the augmented production of TNF-α and IL-6 in IS-trained macrophages in response to re-stimulation with LPS (**Fig. 2E**). These data demonstrate that IS-trained immunity is linked to metabolic rewiring characterized by both enhanced glycolysis and augmented oxidative respiration.

Epigenetic modifications control IS-induced trained immunity

The induction of trained immunity relies on two key, closely intertwined mechanisms, epigenetic modification and metabolic rewiring of innate immune cells (2, 28, 29). We next sought to determine whether increased expression of TNF-α and IL-6 is a result of epigenetic changes. To this end, chromatin modification of histone 3 trimethylation of lysine 4 (H3K4me3) at the promoter sites of *TNFA* and *IL6* was analyzed. Chromatin immunoprecipitation (ChIP)-qPCR data illustrate that IS-trained macrophages exhibit enhanced H3K4me3 of *TNFA* and *IL6* promoters by day 6 after treatment with 1,000 μM of IS (**Fig. 3A and B**). This reflects what was previously demonstrated in trained innate immune cells (4, 22, 30). Moreover, IS-mediated enrichment of H3K4me3 was maintained even after secondary stimulation with LPS compared with non-trained cells (Fig. S3A-B). When IS-trained macrophages were pretreated with 5'-methylthioadenosine (MTA), a non-selective methyltransferase inhibitor, their production of TNF-α and IL-6 upon LPS stimulation was reversed to baseline (**Fig. 3C**), implying that IS-induced trained immunity is associated with epigenetic modification. To explore the potential regulation of IS-induced epigenetic modification by metabolic rewiring, we examined the enrichment of H3K4me3 at the promoters of *TNFA* and *IL6* subsequent to treatment with 2DG (**Fig. 3D**). Our findings suggest that metabolic rewiring influences epigenetic modification, implicating the participation of metabolites. Additionally, heightened enrichment of H3K4me3 at the promoter regions of *HK2* and *PFKP*, pivotal genes associated with glycolysis, was observed (Fig. S3C). To further elucidate epigenetic modifications in IS-induced trained immunity, we performed a whole-genome assessment of the histone marker H3K4me3 by ChIP-sequencing (ChIP-Seq) in IS-trained cells on day 6. Among 7,136 peaks, 59 differentially upregulated peaks and 316 downregulated

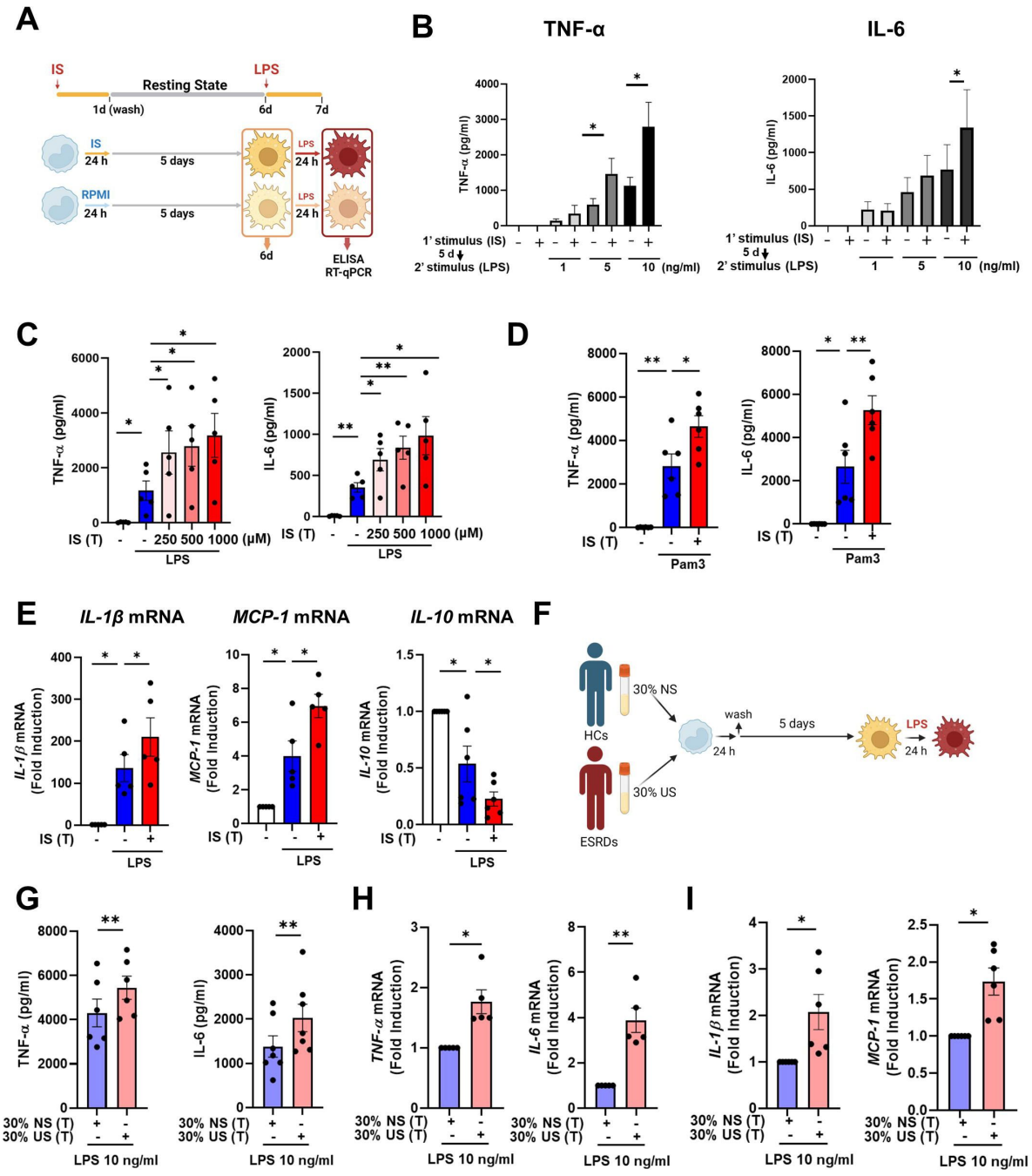

Figure 1.

IS induces trained immunity in human monocytes.

A. Schematic of *in vitro* experimental model for innate trained immunity. **B-C.** Human monocytes were treated with the indicated concentration of IS for 24 hr, followed by a subsequent 5-day culture in human serum. On day 6, the cells were restimulated with the indicated concentrations of LPS for 24 hr. TNF-α and IL-6 proteins levels were quantified by ELISA. **D.** After training with 1,000 μM IS, monocytes were restimulated with 10 μg/ml Pam3cys. TNF-α and IL-6 protein levels were quantified by ELISA. **E.** After training with 1,000 μM IS, monocytes were restimulated with 10 ng/ml LPS for 24 hr. The mRNA expression of *IL-1β*, *IL-10*, and *MCP-1* was analyzed by RT-qPCR. **F.** *In vitro* experimental scheme of uremic serum-induced trained immunity. **G-I**. The pooled normal serum (NS) from healthy controls (HCs) or uremic serum (US) from patients with ESRD were used for treatment of monocytes isolated from HCs for 24 hr at 30% (v/v) followed by resting for 5 days. After stimulation with LPS for 24 hr, TNF-α and IL-6 production were analyzed using ELISA (G) and RT-qPCR (H). After stimulation with LPS (10 ng/ml) for 24 hr, mRNA expression of *IL-1β* and *MCP-1* were determined by RT-qPCR (I). Bar graphs show the mean ± SEM. * = $p < 0.05$, and ** = $p < 0.01$ by two-tailed paired *t*-test.

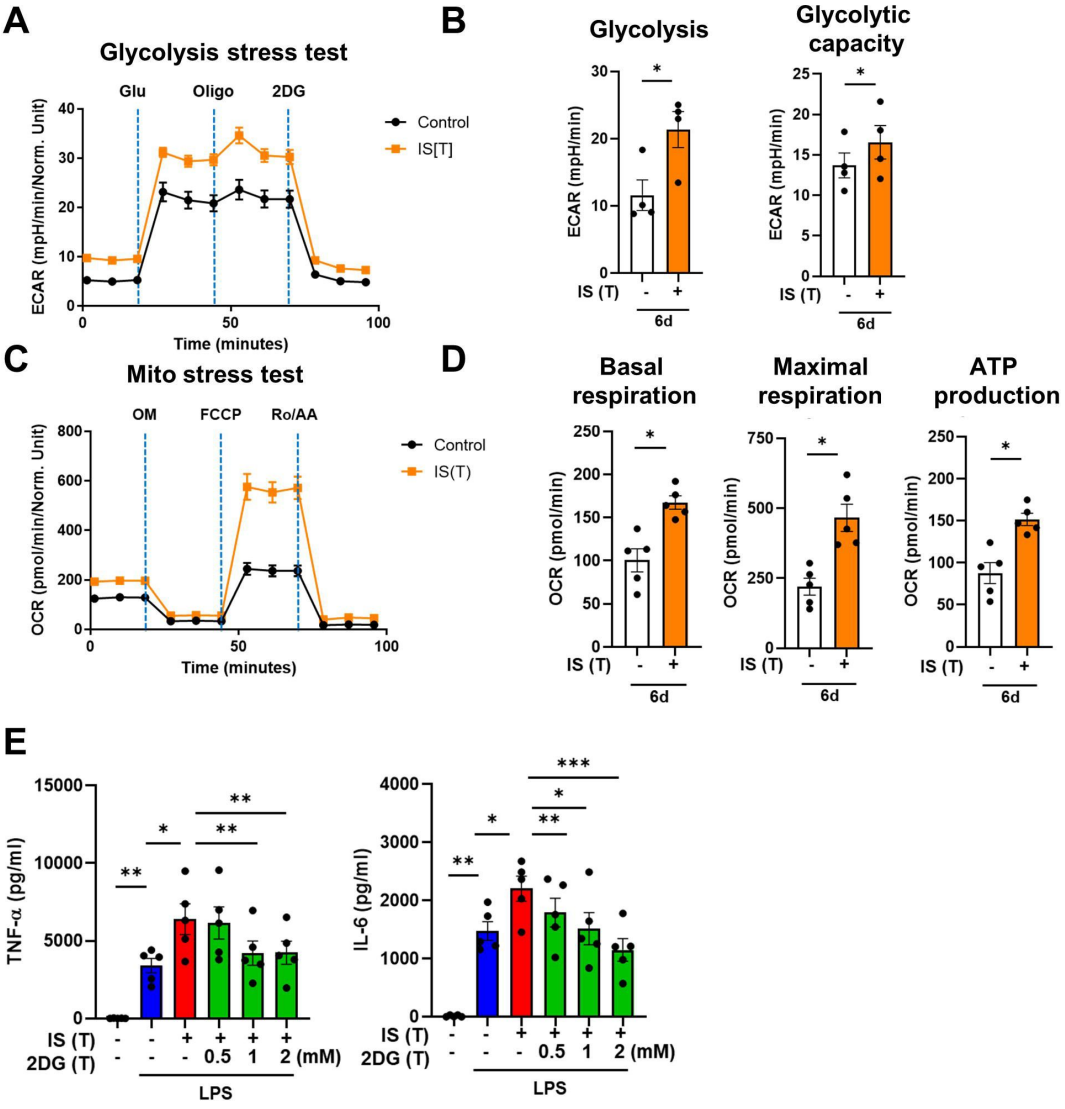

Figure 2.

IS-induced trained immunity is linked to metabolic rewiring.

Glycolysis and mitochondrial stress tests were conducted on IS (1,000 μM)-trained macrophages (n = 3~4) using the Seahorse XF-analyzer. **A.** ECAR (extracellular acidification rate) levels were measured after sequential treatment with glucose, oligomycin, and 2-DG. **B.** Cellular glycolysis and glycolytic capacity were analyzed. **C.** OCR (Oxygen consumption rate) levels were measured after sequential treatment with oligomycin, FCCP, and Rotenone/antimycin A (Ro/AA). **D.** Basal respiration, maximal respiration, and ATP production were analyzed. **E.** Monocytes were pretreated with 2DG, followed by IS-training for 6 days. Cells were restimulated with LPS for 24 hr and TNF-α and IL-6 in supernatants were quantified by ELISA (n = 5). Bar graphs show the mean ± SEM. *= $p < 0.05$, **= $p < 0.01$, and *** = $p< 0.001$ by two-tailed paired *t*-test.

peaks were detected in IS-trained cells (**Fig. 3E** and Table S1). To identify the biological processes affected in IS-mediated trained immunity, 59 upregulated peaks in IS-trained macrophages were analyzed through Gene Ontology (GO) analysis with Go biological process and the Reactome Gene Set. Activation of the innate immune response and positive regulation of the defense response were identified as major processes *via* Go biological process analysis. Further, genes involved in regulation of ornithine decarboxylase (ODC) and metabolism of polyamine were recognized as major gene sets *via* Reactome Gene Set analysis (**Fig. 3F**). A genome browser snapshot showing H3K4me3 binding illustrates that H3K4me3 is elevated at the promoters of important target genes associated with activation of the innate immune response, such as *IFI16* (interferon-gamma inducible protein 16), *XRCC5* (X-ray repair cross-complementing 5), and *PQBP1* (polyglutamine binding protein 1) and genes linked to the regulation of ornithine decarboxylase (ODC), such as *PSMA1* (proteasome 20S subunit alpha 1), *PSMA3* (proteasome 20S subunit alpha 3), and *OAZ3* (Ornithine Decarboxylase Antizyme 3, a protein that negatively regulates ODC activity) (**Fig. 3G**) (31). Additionally, differences in H3K4me3 enrichment patterns between the IS-training group and the control group were observed in *TNFA* and *IL6* (Fig. S3D-E). Our results show that epigenetic modification of innate immune response-related genes contributes to the induction of IS-trained immunity in human monocytes.

AhR, a potent endogenous receptor for IS, contributes to the induction of IS-trained immunity

Our previous study demonstrated that IS-induced TNF-α production in macrophages is regulated through a complex mechanism involving the interaction of NF-κB and SOCS2 with AhR (25). To explore the molecular mechanism underlying the regulation of IS-trained immunity, we investigated the role of AhR, a potent endogenous receptor for IS. Ligand-bound activated AhR is known to be immediately translocated into the nucleus and rapidly degraded (25, 32–34). Immunoblot analysis depicted in **Figure 4A** reveals persistent nuclear translocation of IS-mediated AhR even on day 6 (prior to re-stimulation with LPS), which was entirely inhibited by GNF351 treatment, an AhR antagonist, on day 6. Inhibition of AhR by GNF351 during IS training suppressed the increase in production of TNF-α and IL-6 following LPS restimulation on day 6 in IS-trained cells (**Fig. 4B and C**), implying that IS-mediated AhR activation may be involved in trained immunity. In addition to TNF-α and IL-6, enhancement of *IL-1β* and *MCP-1* mRNA expression in IS-trained cells was also completely inhibited, whereas decreased *IL-10* expression was completely reversed by GNF351 (**Fig. 4D**). To confirm the regulatory role of AhR in trained immunity, we tested whether 6-Formylindolo[3,2-b]carbazole (FICZ), a tryptophan-derived agonist of AhR, also induced trained immunity in human monocytes. FICZ-pretreated monocytes exhibited augmented expression of TNF-α and IL-6 in response to secondary stimulation with LPS compared to non-trained cells (Fig. S4A). Additionally, knockdown of AhR suppressed the expression of TNF-α and IL-6 in IS-trained cells (**Fig. 4E**), underscoring the significant role of ligand-bound activated AhR in the trained immunity of human monocytes. We next examined whether inhibition of AhR with GNF351 influences epigenetic modification and metabolic rewiring. Our ChIP-qPCR assay showed that enrichment of H3K4m3 on *TNFA* and *IL6* promoters in IS-trained macrophages was inhibited by GNF351 (**Fig. 4F**). Of note, assessment of the metabolic profile by measuring ECAR and OCR illustrates that GNF351 has no effect on metabolic rewiring, including enhanced glycolysis and mitochondrial respiration, in IS-trained cells on day 6 as depicted in **Figure 3** (Fig. S4B-C). This finding was corroborated by the immunoblotting data, which showed GNF351 had no inhibitory effect on IS-mediated enhancement of S6K activity, which is critical for inducing the aerobic glycolysis in human monocytes/macrophages (Fig. S4D). Our findings suggest that IS-activated AhR is involved in regulating epigenetic modifications of IS-trained macrophages.

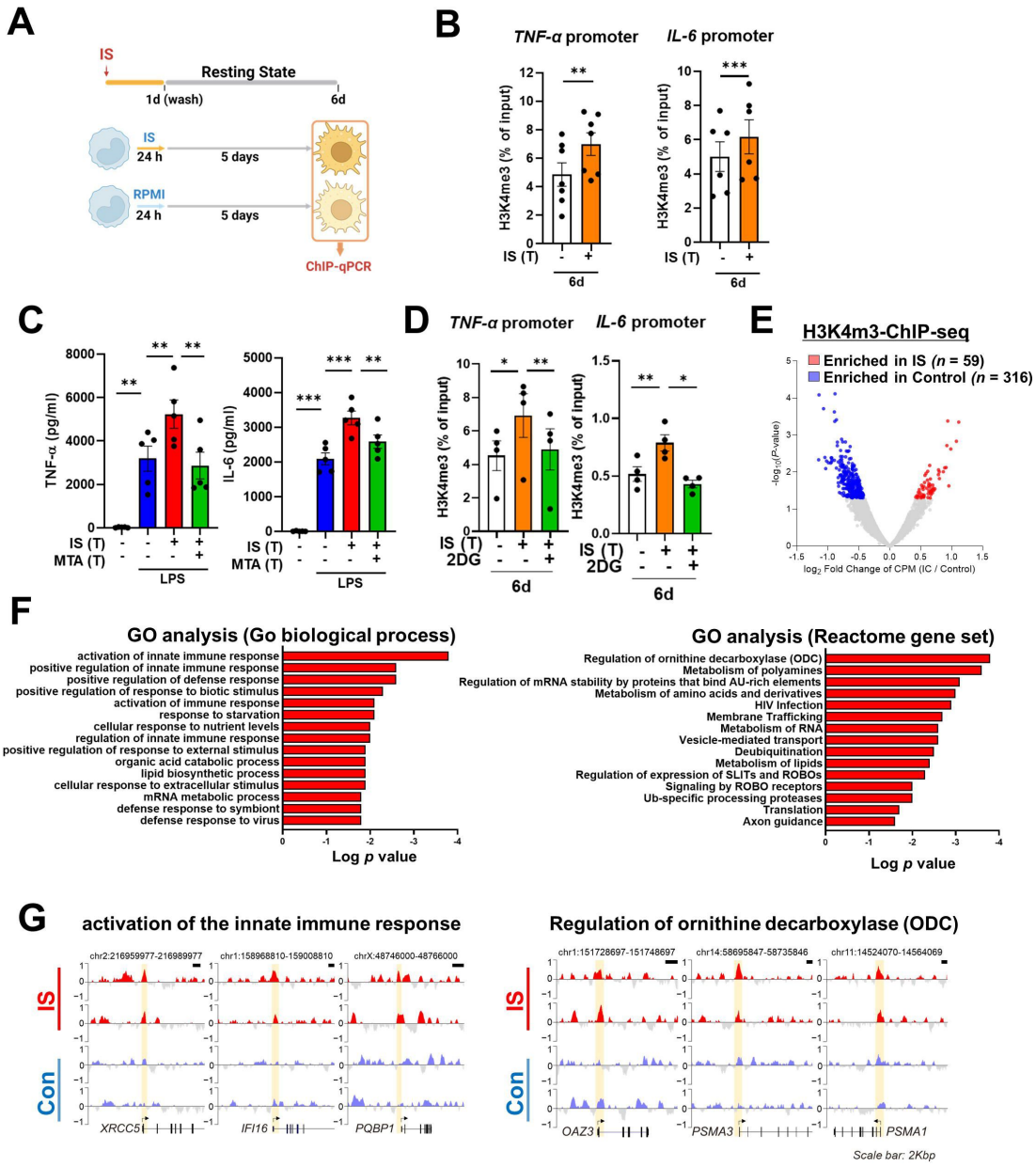

Figure 3.

IS-induced trained immunity is accomplished through epigenetic modification.

A. Experimental scheme of ChIP-qPCR for IS (1,000 μM)-trained macrophages. **B.** On day 6 after IS-training, cells were fixed with 1% formaldehyde, lysed, and sonicated. A ChIP assay was performed using anti-H3K4me3 antibody and enrichment of H3K4me3 in the promoter site of *TNFA* and *IL6* loci was quantified by qPCR. 1% input was used as a normalization control. **C.** Monocytes were pre-treated with 5′-methylthioadenosine (MTA, a non-selective methyltransferase inhibitor; 200 μM) and then were trained with IS for 6 days, followed by restimulation with LPS for 24 hrs. TNF-α and IL-6 proteins levels were quantified by ELISA. **D.** A ChIP assay was performed in IS-trained macrophages pre-treated with 2DG. 2% input was used as a normalization control. **E.** ChIP-Seq analysis was performed with anti-H3K4me3 antibody on chromatin isolated at day 6 from IS-trained and control macrophages. Enriched peaks in ChIP-Seq on H3K4me3 are shown as a volcano plot. (FC > 1.3, $p < 0.05$) **F.** Functional annotation of 59 upregulated Differentially regulated peaks (DRPs) on H3K4me3 in IS-trained macrophages were analyzed by Gene Ontology (Go) analysis with Go biological pathway and Reactome gene sets (FC > 1.3, $p < 0.05$). **G.** Screen shots of H3K4me3 modification in the promoter regions of *IFI16, XRCC5, PQBP1 PSMA1, PSMA3,* and *OAZ3*. *= $p < 0.05$, **= $p < 0.01$, and *** = $p < 0.001$ by two-tailed paired *t*-test.

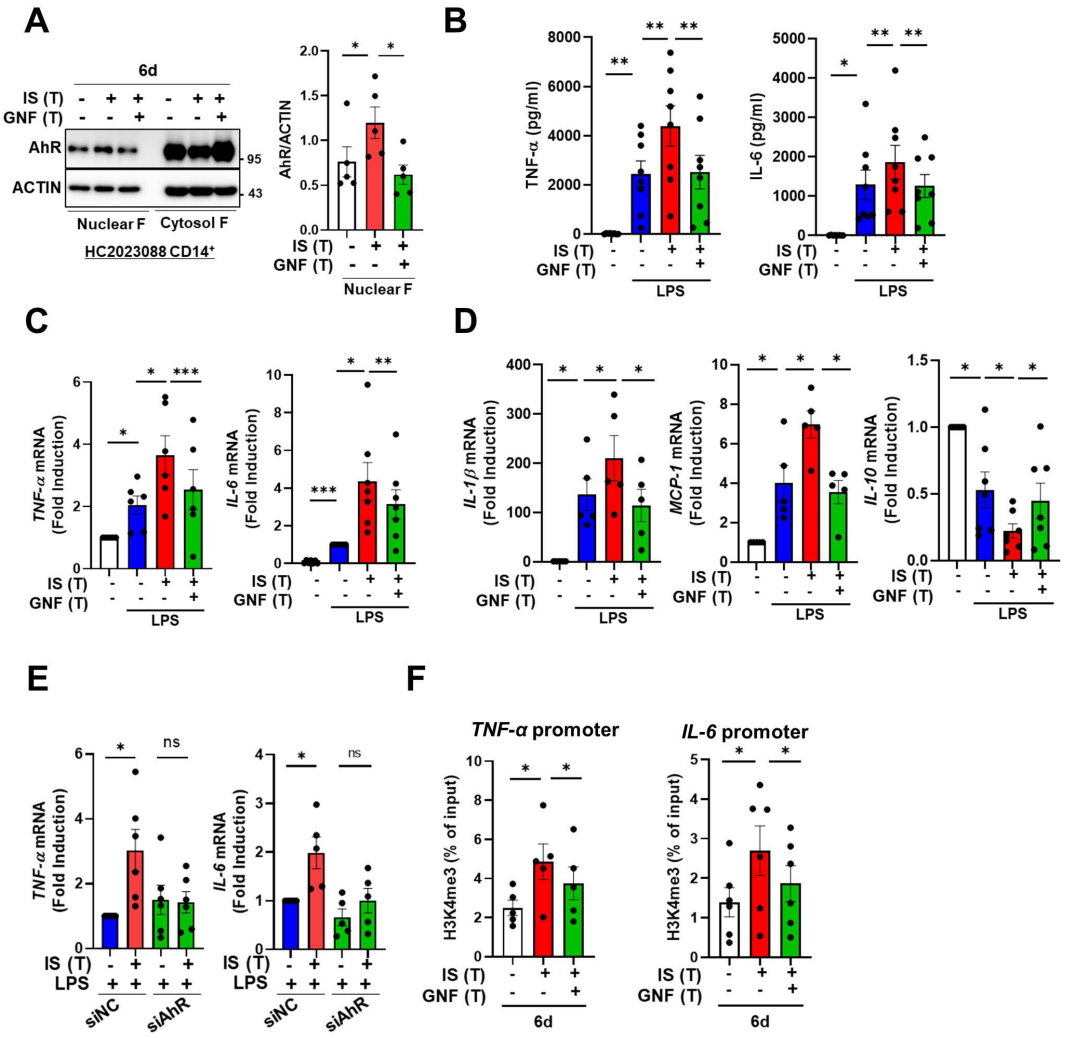

Figure 4.

IS-induced trained immunity is regulated by AhR.

Monocytes were pretreated with or without GNF351 (AhR antagonist; 10 μM) followed by IS (1,000 μM)-training for 6 days. **A.** On day 6, nuclear and cytosol fraction were prepared and immunoblotted for AhR protein. Band intensity in immunoblots was quantified by densitometry. β-ACTIN was used as a normalization control. **B-D.** On day 6, IS-trained cells with or without GNF351 were restimulated with LPS (10 ng/ml), for 24 hr. TNF-α and IL-6 in supernatants were quantified by ELISA (B). Expression of TNF-α and IL-6 (C) and *IL-1β*, *MCP-1,* and *IL-10* mRNA (D) was analyzed by RT-qPCR. **E.** Monocytes were transfected with siRNA targeting AhR (siAhR) or negative control (siNC) for 1 day, followed by stimulation with IS for 24 hours. After a resting period of 5 days, cells were re-stimulated with LPS for 24 hours. mRNA expression levels of *TNF*-α and *IL-6* were assessed using RT-qPCR. **F.** Enrichment of H3K4me3 on promoters of *TNFA* and *IL6* loci was assessed on day 6 after IS-training. 1% input was used as a normalization control. Bar graphs show the mean ± SEM. * = $p < 0.05$, **= $p < 0.01$, and *** = $p < 0.001$ by two-tailed paired *t*-test.

AhR-dependent induction of the arachidonic acid pathway is involved in IS-induced trained immunity

To explore which molecular mechanism is involved in the induction of IS-trained immunity, we performed RNA-sequencing (RNA-Seq) on day 6 (prior to restimulation with LPS) in IS-trained human macrophages. A total of 218 differentially expressed genes (DEGs), consisting of 71 upregulated and 147 downregulated genes, were identified in IS-trained macrophages compared to non-trained cells (**Fig. 5A** and S5A; FC ≥ ± 2, $p < 0.05$). Gene ontology (GO) analysis of these expression data using the Reactome Gene Set is displayed in **Figure 5B**. IS-trained macrophages had upregulated pathways including those involved in neutrophil degranulation, integrin cell surface interactions, extracellular matrix organization and arachidonic acid metabolism, whereas pathways associated with kinesins, cell cycle, and the Gα(i) signaling pathway were downregulated (**Fig. 5B**). Considering the key role of the arachidonic acid (AA) pathway in many inflammatory disorders, we decided to focus on this pathway in the induction of trained immunity by IS. Our findings were supported by Gene Set Enrichment Analysis (GSEA) using Molecular Signatures Database (MsigDB), in which genes related to AA metabolism were enriched in IS-trained macrophages compared to non-trained cells and more importantly, upregulated expression of these genes was inhibited by treatment with GNF351 as illustrated by heatmap analysis of major genes related to AA metabolism (**Fig. 5C and D** and Fig. S5F). Among AA metabolism pathways, the leukotriene metabolic process, but not the cyclooxygenase (COX) pathway, was primarily involved in the induction of IS-mediated trained immunity (Fig. S5B). Confirmatory RT-qPCR analysis on major AA metabolism-related genes was conducted using IS-trained macrophages obtained from independent, HCs (**Fig. 5E**). The mRNA expression of arachidonate 5-lipoxygenase (*ALOX5*: also known as *5-LOX* or *5-LO*) and arachidonate 5-lipoxygenase activating protein (*ALOX5AP*: also known as *FLAP*), the enzymes catalyzing AA into leukotrienes (a group of pro-inflammatory lipid mediators) (35–37), was higher in IS-trained macrophages than non-trained cells. In addition, the mRNA expression of *LTB4R1* (also known as *BLT1*), a high-affinity receptor for leukotriene B4 (LTB4), was also upregulated. The augmented expression of these AA metabolism-related genes was repressed by GNF351 pretreatment as shown by changes in expression of *CYP1B1*, a typical AhR target gene. Thus, this suggests that the IS-activated AhR pathway is involved in enhanced AA-metabolism in IS-induced trained immunity. Immunoblot analysis validated the upregulation of ALOX5 and ALOX5AP expression in IS-trained immunity, which was subsequently inhibited by GNF351 at the protein level (**Fig. 5F**). Furthermore, knockdown of AhR suppressed the IS-induced mRNA expression of *ALOX5*, *ALOX5AP*, and *LTB4R* on day 6 (**Fig. 5G**). Treatment with FICZ, an AhR agonist known to induce trained immunity (Fig. S4A), elicited increased expression of *ALOX5* and *ALOX5AP*, while treatment with KA, a major protein-bound uremic toxin that does not induce trained immunity (**Fig. 1A**), did not result in elevation of these genes, thereby implying the significant role of the AhR-AA pathway in IS-trained immunity (Fig. S5C).

We previously reported alterations in the transcriptome signature of *ex vivo* monocytes of ESRD patients (38). Comparison of the fold changes of RNA-Seq data in the present study and microarray data reported previously (GSE155326) revealed that the expression of *ALOX5* and *LTB4R1* is enhanced in IS-trained macrophages and *ex vivo* monocytes of ESRD patients (Fig. S5D). To further investigate the roles of the AA metabolism pathway in IS-trained immunity, zileuton, an ALOX5 inhibitor, and U75302, a BLT1 receptor inhibitor were used during the induction of trained immunity by IS (**Fig. 5H**, S5E-F). We found that IS-induced TNF-α and IL-6 production were largely suppressed by both zileuton and U75302 (**Fig. 5H**, S5F). Additionally, knockdown of ALOX5 inhibited IS-induced expression of TNF-α and IL-6 (Fig. S5H). In further exploration of the effects on epigenetic or metabolic reprogramming *via* the AA pathway, we conducted ChIP-qPCR assays and Western blot analyses following treatment with zileuton. Our results demonstrated that the enrichment of H3K4me3 on *TNFA* and *IL6* promoters in IS-trained macrophages was inhibited

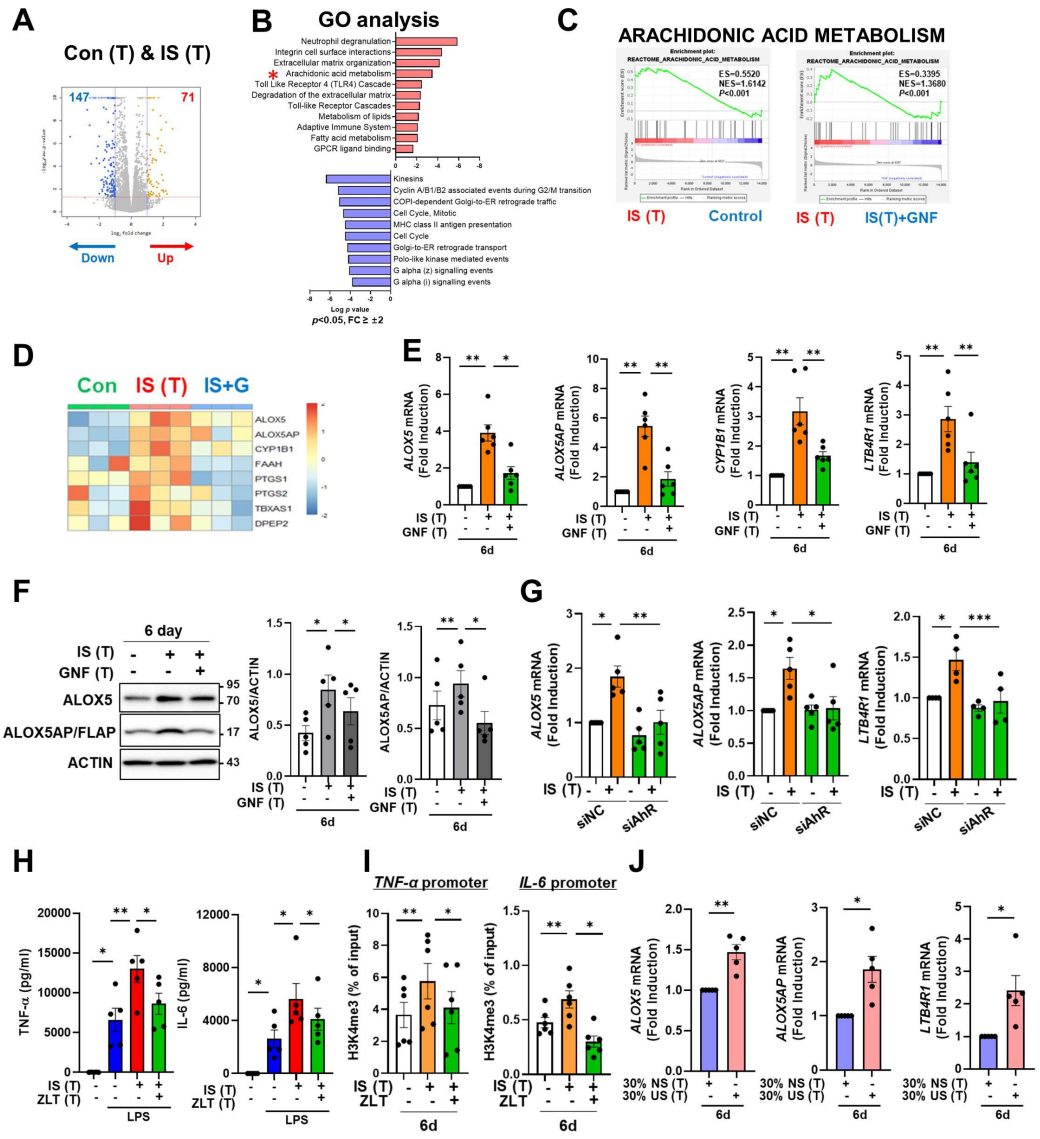

Figure 5.

AhR-dependent induction of the arachidonic acid pathway contributes to IS-induced trained immunity.

A. RNA-Seq analysis was performed on IS (1,000 μM)-trained monocytes. Volcano plots show differentially expressed genes between IS-trained and non-trained macrophages. **B.** Functional annotation of upregulated or downregulated genes (FC > ±2, $p < 0.05$) in IS-trained macrophages analyzed by Gene Ontology (GO) analysis with the Reactome Gene Set. **C-D.** GSEA (C) and heatmap (D) of genes related to the AA metabolism in IS-trained macrophages compared to non-trained cells or compared to IS-trained macrophages with GNF351 (10 μM) treatment were analyzed. **E-F.** On day 6 after IS-training with or without GNF351, expression of *CYP1B1*, *ALOX5*, *ALOX5AP*, and *LTB4R1* mRNAs were quantitated using RT-qPCR (E) and cell lysates were prepared and immunoblotted for ALOX5 and ALOX5AP proteins (F). Band intensity in immunoblots was quantified by densitometry. β-ACTIN was used as a normalization control. **G.** Monocytes were transfected with siRNA targeting AhR (siAhR) or negative control (siNC) for 1 day, followed by stimulation with IS for 24 hours. After a resting period of 5 days, mRNA expression level of each gene was assessed using RT-qPCR. **H.** Monocytes were pretreated with zileuton (ALOX5 inhibitor, 100 μM) and trained with IS for 6 days followed by restimulation with LPS (10 ng/ml) for 24 hr. TNF-α and IL-6 in supernatants were quantified by ELISA. **I.** A ChIP assay was performed in IS-trained macrophages pre-treated with zileuton. 2% input was used as a normalization control. **J.** The pooled normal serum (NS) from health controls (HCs) or uremic serum (US) from patients with ESRD were used to treat monocytes isolated from HCs for 24 hr at 30% (v/v) followed by resting for 5 days. Expression of *ALOX5*, *ALOX5AP*, and *LTB4R1* mRNAs were quantitated using RT-qPCR in trained macrophages with NS or US for 6 days. Bar graphs show the mean ± SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ by two-tailed paired *t*-test.

by zileuton, although phosphorylation of S6K remained unaffected (**Fig. 5I** and S5I). Thus, these findings suggest that AA metabolism plays a pivotal role in the induction of IS-trained immunity by serving as a crucial mediator between AhR signaling and epigenetic modification. We next tested whether training with uremic serum leads to increased expression of AA pathway-related genes within 6 days (prior to restimulation with LPS) as found in IS-trained macrophages. The expression of *ALOX5*, *ALOX5AP*, and *LTB4R1* mRNA was augmented by training with pooled uremic sera of ESRD patients compared with HCs (**Fig. 5J**), and this augmented expression was maintained after re-stimulation with LPS (Fig. S5J).

Histone-modifying enzymes such as lysine demethylase (KDM) and lysine methyltransferase (KMT) are linked to the induction of trained immunity by remodeling the epigenetic status of cells (3, 4). However, RNA-Seq data of IS-trained macrophages showed no obvious change in the expression profile of major histone-modifying enzymes (Fig. S6A). In agreement with this, mRNA expression of major histone modifying enzymes including *KDM5A*, *KDM5B*, *KDM5C*, *SETDB2*, *SETD7*, and *SETD3* were not changed in IS-trained macrophages on day 6 (Fig. S6B) (4, 39–41). Treatment with MTA, a non-selective methyltransferase inhibitor, partially inhibited expression of *ALOX5* and *ALOX5AP* mRNA (Fig. S6C), suggesting limited epigenetic regulation of the AA pathway (Fig. S6C). Ultimately, to validate the association between ChIP-Seq and RNA-Seq data, we employed Spearman's correlation for comparative analysis and conducted linear regression to ascertain the presence of a consistent global trend in RNA expression. Our findings unveiled a significant positive correlation, underscoring the consistent relationship between H3K4me3 enrichment and gene expression (Fig. S6D).

IS-induced trained immunity is validated by *ex vivo* and *in vivo models*

Since peripheral monocytes in ESRD patients are chronically exposed to uremic toxins like IS, we examined whether monocytes purified from ESRD patients before hemodialysis exhibit features of IS-trained macrophages. *Ex vivo* monocytes of ESRD patients had a higher production of TNF-α and mRNA expression of *IL-1β* and *MCP-1* after LPS stimulation than those of HCs (Fig. S7A-C). More importantly, monocytes of ESRD patients, which were trained by resting in the culture media for 6 days, significantly augmented production of TNF-α and IL-6 and expression of *IL-1β* mRNA upon LPS stimulation compared to those from HCs (**Fig. 6A-C**). Consistent with our findings (**Fig. 5**), the expression of ALOX5 in *ex vivo* monocytes of ESRD patients was significantly increased at the protein level compared with that of age-matched HCs (**Fig. 6D-F**). Moreover, monocyte-derived macrophages (MDMs) from ESRD patients also had higher expression of ALOX5 compared to MDMs of HCs (**Fig. 6G-H**), suggesting that IS in serum of ESRD patients contributes to the induction of trained immunity of monocytes/macrophages.

To examine the systemic *in vivo* effect of IS-trained immunity, we adopted a murine model in which IS was intraperitoneally injected daily for 5 days, followed by training for another 5 days and then re-stimulation with 5 mg/kg LPS for 75 min (**Fig. 6I**). The level of TNF-α in serum was increased in IS-trained mice compared to that of control mice (**Fig. 6J**). To further investigate the impact of IS-training on innate responses, splenic myeloid cells were isolated after 5 days of training (prior to injection with LPS) followed by *in vitro* stimulation with 10 ng/ml LPS for 24 h. The amount of TNF-α and IL-6 in the supernatant was augmented following culture with LPS-stimulated mouse splenic myeloid cells derived from IS-trained mice compared the control condition (**Fig. 6K**). Additionally, we observed upregulation of ALOX5 expression in *ex vivo* splenic myeloid cells of IS-treated mice compared to control mice (**Fig. 6L-M**), which was similar to what was observed in monocytes and macrophages from ESRD patients (**Fig. 6E-H**). Finally, treatment with zileuton, an ALOX5 inhibitor, inhibited the production of TNF-α and IL-6 in *ex vivo* splenic myeloid cells of IS-trained mice (**Fig. 6N**).

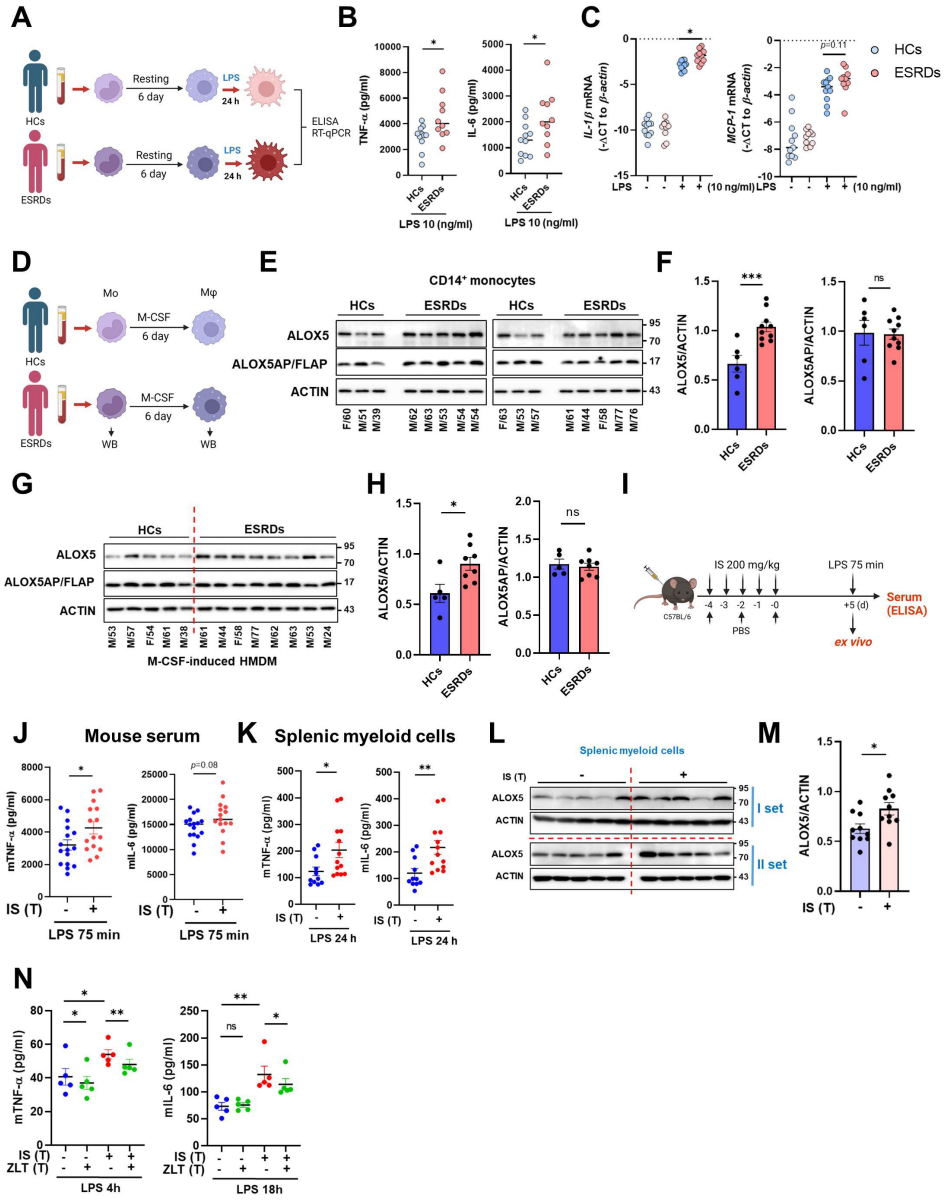

Figure 6.

Ex vivo and *in vivo* validation of IS-induced trained immunity.

A-C. CD14^{+} monocytes from ESRD patents and age-matched HCs were rested for 6 days and stimulated by LPS (10 ng/ml) for 24 hrs (A). TNF-α and IL-6 in supernatants were quantified by ELISA (B) and mRNA expression of *IL-1β* and *MCP-1* were quantitated using RT-qPCR (C). **D-G.** ALOX5 and ALOX5AP protein levels in monocytes of (E-F) and in M-CSF-derived HMDM (G-H) of ESRD patients and HCs were analyzed by immunoblot analysis. Band intensity in immunoblots was quantified by densitometry. β-ACTIN was used as a normalization control. **I.** C57BL/6 mice were injected daily with 200 mg/kg IS for 5 days and rested for another 5 days prior to LPS (5 mg/kg) treatment. Mice were sacrificed at 75 min post-LPS injection. **J.** TNF-α and IL-6 in serum were quantified by ELISA. **K.** Before LPS injection, IS-trained mice were sacrificed, and spleens were mechanically separated. Isolated splenic myeloid cells were treated *ex vivo* with LPS (10 ng/ml) for 24 hr and TNF-α and IL-6 in supernatants were quantified by ELISA. **L-M.** The level of ALOX5 protein in splenic myeloid cells isolated from IS-trained or control mice was analyzed by western blot. The graph shows the band intensity quantified by the densitometry (M). **N.** Isolated splenic myeloid cells were treated *ex vivo* with LPS (10 ng/ml), along with zileuton (100 μM). The levels of TNF-α and IL-6 in the supernatants were quantified using ELISA. The graphs show the median (B-C) or the mean ± SEM (F-N). *= $p < 0.05$, **= $p < 0.01$, and *** = $p < 0.001$ by unpaired non-parametric *t*-test or by two-tailed paired *t*-test between zileuton treatment group and no-treatment group (N).

Subsequently, we examined the impact of IS-trained immunity on mouse bone marrow-derived macrophages (BMDM). It was observed that BMDM from IS-trained mice did not exhibit heightened production of TNF-α and IL-6, and the expression level of ALOX5 in bone marrow progenitor cells remained unaltered when compared to non-trained cells, indicating that our acute IS-trained mice did not induce central trained immunity (Fig. S7D-E)(42, 43). Collectively, these findings offer compelling evidence for the involvement of IS and the induction of the AA pathway in the establishment of trained immunity in both monocytes and macrophages, both *ex vivo* and *in vivo*. Together, these data provide evidence for the role of IS and the induction of the AA pathway in the establishment of trained immunity of monocytes and macrophages both *ex vivo* and *in vivo*.

Discussion

Recent studies have reported that in addition to pathogenic stimuli, endogenous sterile inflammatory insults including ox-LDL, hyperglycemia and uric acid, also trigger trained immunity and contribute to chronic inflammation in cardiovascular diseases and gout (8, 10, 12, 16). In the present study, we provide evidence that IS, a major uremic toxin, provokes trained immunity in human monocytes/macrophage through epigenetic modification, metabolic rewiring, and AhR-dependent induction of the AA pathway, suggesting its important role in inflammatory immune responses in patients with CKD (**Fig. 7**).

CKD is associated with increased risk factors of cardiovascular disease (CVD) including traditional risk factors, such as hypertension, age and dyslipidemia, as well as non-traditional risk factors, such as oxidative stress and inflammation (23, 44). Further, recent cohort studies have shown that CKD is an independent risk factor for CVD (44). Uremia accompanying renal failure causes immune dysfunction, which is closely linked to the pathogenesis of CKD-related CVD (45). Among over 100 uremic toxins identified, IS is a prototypical protein-bound uremic toxin most likely to be participating in progressive pathophysiology of CVD including endothelial dysfunction, vascular calcification, and increased atherosclerosis (21, 26, 46, 47). Mounting evidence suggests that a prolonged hyperactivation of trained immunity is intimately related to the pathogenesis of atherosclerosis, the major contributor to cardiovascular diseases. Oxidized low-density lipoprotein (oxLDL), hyperglycemia, and the Western diet, all known to be associate with the progression of atherosclerosis, have been reported to induce trained immunity through epigenetic reprogramming (8, 10, 12). These findings suggest that IS plays a role as a typical endogenous inflammatory insult in activating monocytes and macrophages and modulating their responses. Given that IS is difficult to clear by hemodialysis, this toxin has a chronic effect on the immune system of patients. Nonetheless, little is known about the effects of IS on trained immunity.

As observed using a common *in vitro* model of trained immunity established by Netea and other groups (**Fig. 1A**), $CD14^+$ monocytes, which are exposed for 24 h to the first insult with IS and rested for 5 days without IS, produced an augmented level of TNF-α and IL-6 and decreased level of IL-10 in response to an unrelated second insult with LPS or Pam3cys, which is a feature typical of trained immunity of monocytes (**Fig. 1B-E**). In contrast to TNF-α and IL-6, major proinflammatory cytokines, IL-10 exerts potent deactivating effects on macrophages and T cells, influencing various cellular processes in inflammatory diseases (48, 49). Additionally, it is noteworthy that IL-10-deficient macrophages exhibit an augmentation in the proinflammatory cytokine TNF-α (50, 51). Therefore, the reduced gene expression of IL-10 by IS-trained monocytes may contribute to the heightened expression of proinflammatory cytokines. Mechanistic studies have demonstrated that the induction of trained immunity is coordinated through the interplay of epigenetic modifications and metabolic rewiring, which is broadly characterized as prolonged changes in transcription programs and cell physiology that do not involve permanent genetic changes, such as the mutations and recombination events crucial for

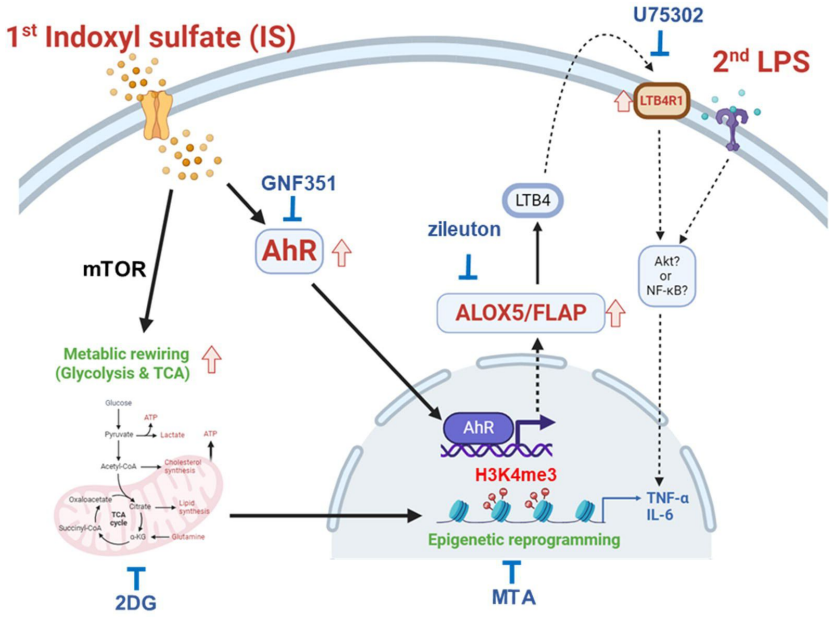

Figure 7.

Proposed mechanism of IS-induced trained immunity.

IS-induced trained immunity in human monocytes is mediated by epigenetic reprogramming and metabolic rewiring *via* histone modification H3K4m3 and increased glycolysis and mitochondrial respiration, respectively. Direct interaction of uremic toxin IS with the AhR in human monocytes activates AhR signaling pathways that are involved in enhanced expression of the arachidonic acid metabolism-related genes ALOX5, ALOX5AP, and LTB4R1 and augmented production of TNF-α and IL-6 upon stimulation with LPS as secondary stimulus *via* epigenetic regulation. A pivotal role of each pathway or molecule was confirmed by *in vitro* assay with inhibitors including GNF351 (an AhR antagonist), zileuton (an ALOX5 inhibitor), U75302 (a BLT1 receptor inhibitor), 2DG (a glycolysis inhibitor), and MTA (a non-selective methyltransferase inhibitor). Meanwhile, the AhR-independent mechanism contributes to metabolic rewiring, such as increased glycolysis in IS-trained macrophages, which leads to enhanced proinflammatory responses upon secondary stimulation.

adaptive immunity (2). In the present study, ChIP-Seq and real-time metabolic analysis show that the induction of IS-trained immunity in human monocytes is attributable to epigenetic modification and metabolic rewiring (**Fig. 2** and **3**). Consistent with previous findings (5, 12), trimethylation of histones at H3K4 on *TNFA* and *IL6* promoters was increased in IS-trained macrophages and maintained even after secondary stimulation with LPS (**Fig. 3B** and Fig. S3B). Furthermore, the production of TNF-α and IL-6 upon LPS stimulation was completely inhibited by pretreatment with 5'-methylthioadenosine (MTA), a methyltransferase inhibitor (**Fig. 3C**), demonstrating that IS-induced trained immunity is associated with epigenetic modification. Moreover, H3K4me3-ChIP-Seq data showed that IS-induced trained immunity accompanied by epigenetic reprogramming and H3K4me3 was enriched in genes related to activation of the innate immune responses as illustrated by gene ontology (GO) analysis (**Fig. 3E-G**).

We identified AhR as a critical mediator of IS-trained immunity in human monocytes (**Fig. 4**). AhR is a ligand-activated nuclear transcription factor (TF), which is activated by several exogenous compounds, such as benzo[a]pyrene environmental pollutants and 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), as well as by multiple endogenous ligands including tryptophan and indole metabolites(52). AhR plays a multifaceted role in modulating cellular mechanisms such as inflammation, cell growth, and antioxidant responses (53). AhR is expressed by various immune cells, and its signaling exerts integrative effects on the cellular environment and metabolism of the immune responses (54). However, little is known about the role of AhR in the induction of trained immunity.

In the present study, we show that IS-trained immune responses, characterized by the expression of proinflammatory cytokines and chemokines, were attenuated by GNF351, an AhR antagonist, or through the knockdown of AhR using siRNA (**Fig. 4B-E**). This inhibition was accompanied by repression of enriched H3K4me3 on *TNFA* and *IL6* promoters in IS-trained macrophages (**Fig. 4F**), indicating an AhR-dependent mechanism. However, increased glycolysis and mitochondria respiration in IS-trained macrophages were not suppressed by the blockade of AhR activation with GNF351, suggesting the AhR activation is not directly involved in metabolic rewiring in IS-trained immunity (Fig. S4B-D).

Metabolic rewiring, especially upregulation of aerobic glycolysis, is known as a major mechanism underlying the induction of trained immunity *via* regulation of epigenetic modification by metabolites, such as mevalonate and fumarate generated from this metabolic rewiring (55). Furthermore, in addition to establishing the bidirectional link between heightened glycolysis and activating histone modifications, as evidenced by the suppression of histone modification by 2DG, a glycolysis inhibitor, in IS-induced immunity (**Fig. 3D**), recent studies have demonstrated that immune priming long non-coding RNAs (IPLs) also induce epigenetic reprogramming by influencing 3D nuclear architecture(3, 56) and that RUNX1, a transcription factor, contributes to the induction of trained immunity by overexpression of RUNX1 target genes (10), suggesting that a variety of mechanism is involved in epigenetic modification in trained immunity.

Although inducers of trained immunity, such as β-glucan, BCG, uric acid, and oxLDL, initiate intracellular signaling and metabolic pathways, each *via* different receptors, the most common pathway is the Akt/mTOR/HIF1α-dependent induction of aerobic glycolysis (3, 15, 55). Our data also revealed that training with IS led to enhanced glycolysis, which is critical for the production of TNF-α and IL-6 upon LPS stimulation as confirmed by experiments with 2DG, a glycolysis inhibitor (**Fig. 2E**). Recent studies have shown that IS activates mTORC1 in a variety of cells such as epithelial cells, fibroblasts, and THP-1 cells mainly *via* the Organic Anion Transporters (OAT)/NADPH oxidase/ROS pathway, but not the AhR pathway (57). Our findings suggest that IS-trained macrophages acquire the characteristics of trained immunity by AhR-dependent and -independent mechanisms and enhances proinflammatory responses upon secondary stimulation. Thus, addressing the mechanism underlying AhR-independent metabolic rewiring in IS-trained macrophages will require further investigations.

A finding of particular interest in our study is that the induction of IS-induced trained immunity is dependent on the AhR-ALOX5/ALOX5AP axis, as depicted by RNA-Seq analysis and confirmatory *in vitro* analysis of mRNA and protein expression (**Fig. 5** and Fig. S5). ALOX5 and ALOX5AP are major rate-limiting enzymes associated with the arachidonic acid (AA) pathway, involved in the production of leukotrienes, proinflammatory lipid mediators derived from AA (36). Among leukotrienes, leukotriene B4 (LTB4), an extremely potent inflammatory mediator, binds to G protein-coupled protein, LTB4R, and enhances inflammatory responses by increased phagocytosis and activation of the signaling pathway for the production of cytokines (35, 37). Mechanistically, the LTB4-LTB4R signaling pathway induces the PI3K/Akt or NF-κB pathways (58, 59). AhR-dependent LTB4 production through enhanced ALOX5 expression in hepatocytes reportedly induces hepatotoxicity *via* neutrophil infiltration (60). The increase in ALOX5 activity and LTB4 expression has been reported in patients with ESRD (61–63) and ALOX5 mediates mitochondrial damage and apoptosis in mononuclear cells of ESRD patients (61). Thus, antagonists of ALOX5/ALOX5AP have been used for treatment in CKD (63). Consistent with these findings, peripheral monocytes derived from ESRD patients in our cohort have increased expression of ALOX5 and this increase was maintained after differentiation into macrophages with M-CSF (**Fig. 6E-H**). Considering that pretreatment with the uremic serum of ESRD patients elicits a change in gene expression and cytokine production as observed in IS-trained macrophages, it is likely that increased ALOX5 in monocytes and macrophages of ESRD patients is mediated by IS in uremic serum. Our previous studies have shown that IS is an important uremic toxin in the serum of ESRD patients that elicits proinflammatory responses of monocytes (21). Furthermore, the AhR-ALOX5-LTB4R1 pathway is involved in IS-induced trained immunity of patients with ESRD.

Recent studies have demonstrated that trained immunity of macrophages is implicated in the pathogenesis of CVD, such as atherosclerosis (10, 12, 13, 15). Considering a higher plasma level of IS in ESRD patients even after hemodialysis (22, 23), this is potentially difficult to reconcile with trained immunity in which there typically is a short exposure to the training stimulus followed by a period of rest (2, 3). However, when ESRD monocytes exposed to the IS in the circulation enter atherosclerotic plaques and differentiate into macrophages, they are no longer exposed to IS, because this is protein-bound and hence not expected to the present in the plaque microenvironment.

Murine models have been widely used to investigate the specific contribution of inducers of trained immunity and the underlying mechanisms to a long-term functional modification of innate cells *in vivo* (7, 41, 64). Mice trained with β-glucan enhance the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β by monocytes and macrophages in response to secondary microbial stimuli and subsequently obtain increased protection against various microbial infections (15, 55). Our *in vivo* and *ex vivo* mouse experiments for trained immunity demonstrate that IS-trained immunity has biological relevance (**Fig. 6I**). Previous studies have shown that intraperitoneal injection of exogenous IS into wild-type C57BL/6 mice leads to an increase of IS in the plasma until 3~6 hr post-injection despite its rapid excretion by the kidney. In this period, the expression of pro-inflammatory, pro-oxidant, and pro-apoptotic genes in peritoneal macrophages was upregulated (57, 65). Moreover, intraperitoneal injection of IS daily for 3 days elevates IS in the plasma of mice and activates the mTORC1 signaling pathway in the mouse kidney (57). This suggests that the IS-injected mouse model is suitable for investigating the mechanisms underlying IS-mediated immune responses. Our data show increased TNF-α in serum after injection of LPS (**Fig. 6J**). Moreover, ALOX5 protein expression was increased in splenic myeloid cells derived from IS-trained mice and *ex vivo* stimulation with LPS for 24 hr induced TNF-α and IL-6 expression in these splenic myeloid cells (**Fig. 6K-M**). Thus, this suggests a systemic induction of IS-trained immunity in the mouse model.

In conclusion, the current study provides new insight into the role of IS as an inducer of trained immunity as well as the underlying mechanisms in human monocytes/macrophages by investigating the effect of IS *in vivo* and *in vitro* using experimental models of trained immunity. Here, we demonstrate that IS, a major uremic toxin, induces trained immunity characterized by the increased proinflammatory TNF-α and IL-6 in human monocytes following secondary stimulation through epigenetic modification and metabolic rewiring. IS-mediated activation of AhR is involved in the induction of trained immunity through enhanced expression of arachidonic acid (AA) metabolism-related genes such as ALOX5 and ALOX5AP. Monocytes from patients with ESRD exhibit increased expression of ALOX5 and after 6-day resting, they exhibit enhanced TNF-α and IL-6 production to LPS. Furthermore, the uremic serum of ESDR patients causes HC-derived monocytes to increase the production of TNF-α and IL-6 upon LPS re-stimulation, implying IS-mediates trained immunity in patients. Supporting our *in vitro* findings, mice trained with IS and their splenic myeloid cells had increased production of TNF-α after *in vivo* and *ex vivo* LPS stimulation. These results suggest that IS plays an important role in the induction of trained immunity, which is critical in inflammatory immune responses in patients with CKD and thus, it holds potential as a therapeutic target.

Methods

Human monocyte preparation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation (Bicoll-Separating Solution; BIOCHROM Inc, Cambridge, UK). Monocytes were positively purified from PBMCs with anti-CD14 magnetic beads (Miltenyi Biotec Inc, Auburn, CA). For *in vitro* trained immunity experiments, purified monocytes were treated with IS for 24 h, followed by washing with pre-warmed PBS and incubation for another 5 days in RPMI medium supplemented with 10% human AB serum (HS, Sigma-Aldrich, St. Louis, MO), 100U/ml penicillin, and 100μg/ml streptomycin (Gibco, Grand Island, NY). On day 6, cells were re-stimulated with LPS or Pam3cys for 24 hr, and the supernatants and their lysates were collected and stored at -80°C until use. In some experiments, chemical inhibitors were used for a 1 hr pre-treatment at the indicated concentrations prior to the treatment with IS. To test the effect of uremic serum on the induction of trained immunity, $CD14^+$ monocytes purified from HC donors were seeded into 48-well plates and incubated for 24 hr at 30% (v/v) with the pooled uremic sera (US) from ESRD patients or the pooled normal sera (NS) from HCs, followed by washing with pre-warmed PBS and incubation for another 5 days. On day 6, cells were re-stimulated with LPS for 24 hr. To examine whether monocytes from ESRD patients inhibit features of IS-trained immunity, $CD14^+$ monocytes were purified from ESRD patients and age-matched HCs, followed by stimulation with LPS for 24 hrs. In addition, purified monocytes were seeded and rested for 6 days in RPMI medium supplemented with 10% human AB serum, 100U/ml penicillin, and 100μg/ml streptomycin to induce trained immunity. On day 6, the cells were stimulated with LPS for 24 hr. In some experiments, monocyte-derived macrophages (MDMs) were differentiated from purified $CD14^+$ monocytes from ESRD patients or age-matched HCs in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, BioWest, NuaillÉ, France), 50 ng/ml recombinant human M-CSF (PeproTech, Rocky Hill, NJ, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin. On day 6, MDMs were used for immunoblot analysis.

Chemicals and antibodies

Indoxyl sulfate (IS) potassium salt, GNF351, 5'-methylthioadenosine (MTA), 2-deoxy d-glucose (2DG), and zileuton were purchased from Sigma-Aldrich (Burlington, MA, USA). Lipopolysaccharides (LPS) from E. coli 0111: B4 were purchased from InvivoGen (San Diego, CA, USA) for *in vitro* experiments and Sigma-Aldrich for *in vivo* experiments. U-75302 was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Anti-AhR and anti-5-loxygenase (5-LOX)

antibodies (Ab) for immunoblot assay and anti-trimethyl H3K4 (H3K4me3) Ab for chromatin immunoprecipitation (ChIP) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-ALOX5AP (FLAP) antibody was obtained from Abcam Inc. (Cambridge, UK).

Enzyme-linked immunosorbent assay (ELISA)

The amounts of TNF-α and IL-6 in culture supernatants of LPS or Pam3cys-re-stimulated IS-trained macrophages were quantified using commercial human ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA). Optical density was measured using the Infinite M200 (Tecan, Männedorf, Switzerland).

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Quantitative RT-PCR

Total RNA was prepared using RNA purification kit (Macherey-Nagel GmbH & Co. KG, Germany), followed by cDNA synthesis (Bio-line, London, UK), and then real-time quantitative RT-PCR was performed with the CFX system (Bio-Rad, Hercules, CA) using the SensiFAST SYBR® Lo-ROX (Bio-line, London, UK). Sequences of primers used in this investigation are shown in Table S3. Normalization of gene expression levels against the expression of ACTINB using the comparative CT method (ΔΔCT) was used for quantification of gene expression.

ChIP-qPCR and ChIP-Seq

Cells were washed with Dulbecco's PBS and crosslinked for 5 min with 1% formaldehyde at room temperature (RT), followed by quenching with 100 mM glycine for 5 min. Cells were harvested with lysis buffer (50 mM HEPES, pH7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% Triton X-100) with protease inhibitors on ice for 10 min and were then washed with washing buffer (10 mM Tris-HCl, pH7.0, 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA) for 10 min. The lysates were resuspended and sonicated in sonication buffer (10 mM Tris-HCl, pH8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxylcholated and 0.5% N-laurolsarcosine) using a Bioruptor® (diagenode, Denville, NJ) with 30s on and 30s off on a high-power output for 25 cycles. After sonication, samples were centrifuged at 12,000 rpm for 10 min at 4°C and 1% sonicated cell extracts were saved as input. Cell extracts were incubated with protein A agarose loaded with the H3K4me3 Ab overnight at 4°C, and then Ab-bound agarose beads were washed twice with sonication buffer, once with sonication buffer with 500 mM NaCl, once with LiCl wash buffer (10 mM Tris-HCl, pH8.0. 1 mM EDTA, 250 mM LiCl and 1% NP-40), and once with TE with 50 mM NaCl. After washing, DNA was eluted in freshly prepared elution buffer (1% SDS and 0.1 M $NaHCO_3$). Cross-links were reversed by overnight incubation at 65°C with RNase A, followed by incubation with proteinase K for 1 hr at 60°C. DNA was purified with NucleoSpin™ gDNA Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Germany). For ChIP-qPCR assays, immunoprecipitated DNA was analyzed by quantitative real-time PCR and results were normalized against input DNA. The sequences of primers used for ChIP-qPCR are shown in Table S4 (5, 66).

For ChIP-seq experiments, purified DNA were prepared for DNA libraries using TruSeq DNA Sample Prep Kit according to Library Protocol TruSeq ChIP Sample Preparation Guide 15023092 Rev. B. Next, illumina sequencing were performed using NovaSeq 6000 S4 Reagent Kit according to sequencing protocol of NovaSeq 6000 System User Guide Document # 1000000019358 v02. Sequenced reads were trimmed using Trimmomatic software. Fragments were aligned to hg19 using Bowtie2 software. Aligned fragments of H3K4me3-ChIP samples were concatenated into a single file to generate consistent peak ranges between samples using the makeTagDirectory function of Homer Suite. For each sample, regions of H3K4me3 enrichment compared to the input sample were collected using callpeaks function in MACS3 software. H3K4me3-rich regions from the same group of different donors were compared to peaks in linked samples using the findoverlap function of the GenomicRange R-package, and 11,123 peaks were collected for further

analysis. For quantitative comparisons between IC-trained groups and controls, the number of fragments of each peak in BEDPE was collected using the coverage function of the BEDtools software. Then, the number of fragments in the peak was normalized to CPM and significance was compared using edgeR R-package. Finally, we selected 7,136 peaks with at least 15 CPM from the larger average group to exclude lowly H3K4me3 enriched peaks.

Enriched peaks were selected base on a *p*-value of 0.05 or less and log2 fold change of > 1.3. The selected enriched peaks were used for Go pathway analysis. Pathway analysis was conducted using Metascape web-based platform (67) and significant pathways were identified on the basis of Go biological process and Reactome gene sets. Significant pathways were selected with $p < 0.05$ and enrichment score (ES) > 1.5.

RNA-sequencing (RNA-Seq) and analysis

After RNA extraction, libraries for sequencing were prepared using the TruSeq Stranded mRNA LT Sample Prep Kit and sequencing were performed using NovaSeq 6000 System User Guide Document # 1000000019358 (Illumina). To analyze RNA-Seq data, trimmed reads were aligned to the human GRCh37 (NCBI_105.20190906). Gene expression profiling was performed using StringTie and then read count and FPKM (Fragment per Kilobase of transcript per Million mapped reads) were acquired. Differentially expressed genes (DEGs) were selected based on *p*-value of 0.05 or less. Selected data were applied to hierarchical cluster analysis to display basal and luminal differences and were further filtered according to gene expression levels with a log2 fold change of < -2 and > 2. DEGs were visualized using the R (ver. 4.1.1) and pheatmap package (ver. 1.0.8). For Gene Set Enrichment Analysis (GSEA), samples were categorized into distinct groups based on treatment conditions. All transcripts within annotated genes (~14,404 features in total) regarding expression values were uploaded to locally-installed GSEA software (ver. 4.2.3) (68). The analysis was performed by comparing the 'IS (T)' group against the 'Control' group to identify differentially enriched gene sets within the Reactome pathway database, particularly focusing on the arachidonic acid metabolism pathway. A similar comparison was made between the 'IS (T)' and 'IS (T)+GNF' groups to assess the effect of the GNF inhibitor. Outputs were filtered based on a nominal $p < 0.05$ and a normalized enrichment score (NES) > 1.3 to determine statistical significance. These thresholds were applied to ensure the robustness of the findings in the context of multiple hypothesis testing. The enrichment plots were generated to visualize the distribution of the gene sets and their enrichment scores. Lastly, pathway analyses were further substantiated using the Metascape web-based platform(67), with significant pathways identified using differentially expressed genes (DEGs) and selected based on a $p < 0.05$ and an enrichment score (ES) > 1.5.

Metabolic analysis

To profile the metabolic state of the cells, $CD14^+$ monocytes were seeded onto XFe24 cell culture plates (Seahorse Bioscience, Lexington, MA) with RPMI medium with 10% HS, followed by the induction of trained immunity for 6 days as described in **Fig. 1A**. Metabolic analysis on IS-trained macrophages was performed according to the manufacturer's instructions. For the glycolysis stress test, culture media was replaced with Seahorse XF Base media supplemented with 2 mM L-glutamine (pH7.4) and incubated for 1 hr in the non-CO2 incubator. Glucose (10 mM), oligomycin (2 μM), and 2-DG (50 mM, all from Sigma-Aldrich) were sequentially used to treat cells during real-time measurements of extracellular acidification rate (ECAR) using Seahorse XFe24 Analyzer (Seahorse Bioscience). For the mito stress test, cells were incubated with Seahorse XF Base media supplemented with 1 mM pyruvate, 2 mM L-glutamine, and 10 mM glucose (pH7.4) for 1 hr in the non-CO2 incubator. Oligomycin (1.5 μM), FCCP (2 μM), and rotenone/antimycin A (0.5 μM, all from Sigma-Aldrich) were sequentially used to treat cells during real-time measurements of oxygen consumption rate (OCR) using the Seahorse XFe24 Analyzer. Parameters of glycolysis stress test and mito stress test were calculated using Seahorse XF glycolysis or the mito stress test report generator program that was provided by the manufacturer.

Immunoblot analysis

Total proteins were prepared using radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM Na_2HPO_4, 0.5% sodium deoxycholate, 1% NP-40) containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Cell lysates were separated on an 8-12% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA), The membrane was incubated overnight at 4°C with primary Abs, such as anti-AhR, anti-ALOX5, and anti-ALOX5AP/FLAP, followed by incubation with peroxidase-conjugated secondary Abs for 1 h. The membranes were developed using the enhanced chemiluminescence (ECL) system.

WST (Water Soluble Tetrazolium Salt) assay

To test cell viability, IS-trained macrophages were re-stimulated with LPS for 24 hr. Culture media was changed with serum-free RPMI medium and the WST reagent (EZ-CYTOX, DoGenBio, Seoul, Korea) followed by incubation for 1-2 h. Measurement of the optical density value (450 nm) was performed by Infinite M200 (Tecan).

Mouse *in vivo* studies

For *in vivo* experiments, C57BL/6 mice (7-8 weeks) were injected intraperitoneally with 200 mg/kg IS in 100 μl PBS daily for 5 days. Another five days after IS injection, 5 mg/kg LPS (Sigma-Aldrich) were injected intraperitoneally 75 min prior to sacrifice. Whole blood was incubated at RT for 30 min and centrifuged at 3,000 g for 10 min at 4°C to collect mouse serum. The amount of TNF-α and IL-6 in serum was quantified using commercial mouse ELISA kits (Thermo Fisher Scientific). For *ex vivo* experiments using splenic myeloid cells, IS-trained mice were sacrificed and their spleens were aseptically collected. Single-cell splenic suspensions were prepared in PBS after passage through a 40 mm cell strainer. Splenocytes were seeded at 1 x 10^7 cells/well in 12-well plates. After incubation for 1 h, adherent cells were harvested for immunoblot analysis or stimulated with 10 ng/ml LPS for 24 hr. The amount of TNF-α and IL-6 in culture supernatants was quantified using commercial mouse ELISA kits (Thermo Fisher Scientific).

Statistical Analysis

A two-tailed paired or unpaired non-parametric t-test was performed to analyze data using Prism 8 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel 2013. *P* values of less than 0.05 were considered statistically significant.

Study approval

Study protocols were reviewed and approved by the IRB (institutional review board) of Seoul National University Hospital and Severance Hospital. Peripheral blood of ESRD patients and healthy controls (HCs) was drawn after obtaining written, informed consent. The methods were performed in accordance with the approved guidelines.

Author contributions

H. Y. Kim: conceived of the study, participated in its design and coordination, performed most of the experiments, data collection, and analysis, wrote manuscript and provided financial support. Y. J. Kang, D. H. Kim, J. Jang, S. J. Lee, and G. Kim: performed the experiments and data analysis. H. B. Koh and Y. E. Ko: performed patient sample collection, the experiments, and data analysis. H. M. Shin: participated in its design and performed data analysis. H. Lee and T.-H. Yoo: conceived of the study, participated in its design, collected patient samples and clinical information, and performed

data analysis. W-W.L.: conceived of the study, participated in its design and coordination, performed data analysis and writing of manuscript, and has full access to all the data in this study and financial support. All authors have read and approved the final manuscript.

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Editors

Reviewing Editor
Murim Choi
Seoul National University, Seoul, Korea, the Republic of

Senior Editor
Murim Choi
Seoul National University, Seoul, Korea, the Republic of

Reviewer #1 (Public Review):

In this study, Kim et al. investigated the mechanism by which uremic toxin indoxyl sulfate (IS) induces trained immunity, resulting in augmented pro-inflammatory cytokine production such as TNF and IL-6. The authors claim that IS treatment induced epigenetic and metabolic reprogramming, and the aryl hydrocarbon receptor (AhR)-mediated arachidonic acid pathway is required for establishing trained immunity in human monocytes. They also demonstrated that uremic sera from end-stage renal disease (ESRD) patients can generate trained immunity in healthy control-derived monocytes.

These are interesting results that introduce the important new concept of trained immunity and its importance in showing endogenous inflammatory stimuli-induced innate immune memory. Additional evidence proposing that IS plays a critical role in the initiation of inflammatory immune responses in patients with CKD is also interesting and a potential advance of the field.

Comments on the revised version:

In the revised manuscripts, the authors have addressed essentially almost all of the points raised by the reviewers and have revised the manuscript accordingly. The additional comments improved the manuscript and strengthened the overall impact of the paper.

https://doi.org/10.7554/eLife.87316.2.sa0

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *In this study, Kim et al. investigated the mechanism by which uremic toxin indoxyl sulfate (IS) induces trained immunity, resulting in augmented pro-inflammatory cytokine production such as TNF and IL6. The authors claim that IS treatment induced epigenetic and metabolic reprogramming, and the aryl hydrocarbon receptor (AhR)-mediated arachidonic acid pathway is required for establishing trained immunity in human monocytes. They also demonstrated that uremic sera from end-stage renal disease (ESRD) patients can generate trained immunity in healthy control-derived monocytes.*
>
> *These are interesting results that introduce the important new concept of trained immunity and its importance in showing endogenous inflammatory stimuli-induced innate immune memory. Additional evidence proposing that IS plays a critical role in the initiation of inflammatory immune responses in patients with CKD is also interesting and*

> *a potential advance of the field. This study is in large part well done, but some components of the study are still incomplete and additional efforts are required to nail down the main conclusions.*

Thank you very much for your positive feedback.

> *Specific comments:*
>
> *(1) Of greatest concern, there are concerns about the rigor of these experiments, whether the interpretation and conclusions are fully supported by the data. (1) Although many experiments have been sporadically conducted in many fields such as epigenetic, metabolic regulation, and AhR signaling, the causal relationship between each mechanism is not clear. (2) Throughout the manuscript, no distinction was made between the group treated with IS for 6 days and the group treated with the second LPS (addressed below). (3) Besides experiments using non-specific inhibitors, genetic experiments including siRNA or KO mice should be examined to strengthen and justify central suggestions.*

We are grateful for the invaluable constructive feedback provided.

(1) In response to the reviewer's feedback, we conducted additional experiments employing appropriate inhibitors to investigate the causal relationship among the AhR pathway, epigenetic modifications, and metabolic rewiring in IS-induced trained immunity. Notably, metabolic rewiring, particularly the upregulation of aerobic glycolysis via the mTORC1 signaling pathway, stands as a pivotal mechanism underlying the induction of trained immunity through the modulation of epigenetic modifications (Riksen NP et al. Figure 1). Initially, we assessed the enrichment of H3K4me3 at 6-day on promoters of TNFA and IL6 loci after treatment of zileuton, an inhibitor of ALOX5, and 2-DG, a glycolysis inhibitor. Additionally, we evaluated the alteration in the activity of S6K, a downstream molecule of mTORC1, following zileuton treatment. Our findings indicate that AhR-dependent arachidonic acid (AA) signaling induces epigenetic modifications, albeit without inducing metabolic rewiring, in IS-induced trained immunity (Author response image 1). However, IS stimulation promotes mTORC1-mediated glycolysis in an AhR-independent manner. Notably, inhibition of glycolysis with 2-DG impacts epigenetic modifications. We have updated Figure 7 of the revised manuscript to incorporate these additional experimental findings, elucidating the correlation between the diverse mechanisms implicated in IS-induced innate immune memory (Fig. 7 in the revised manuscript). These data have been integrated into the revised manuscript as Figure 3D and 5I, and supplementary Figure 5I.

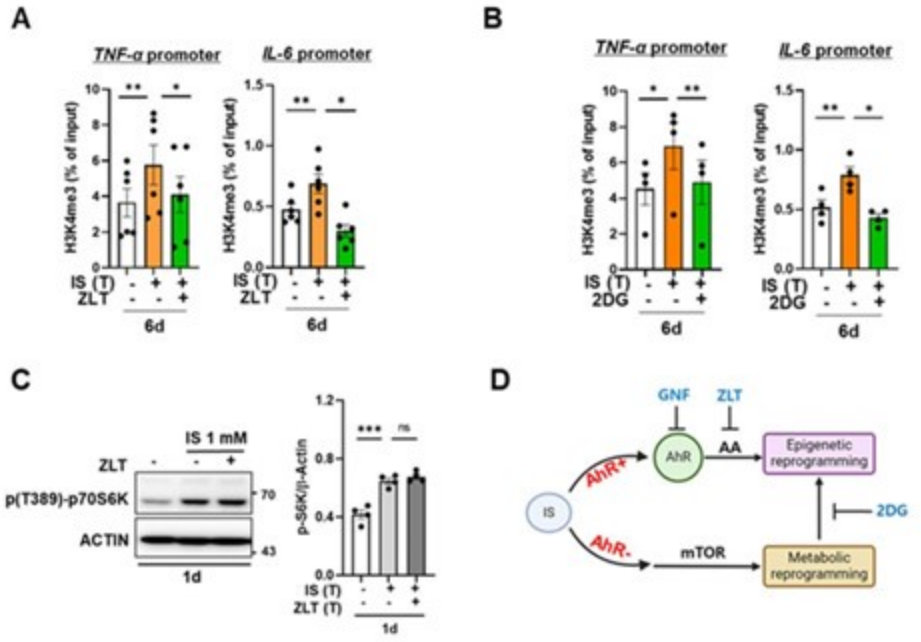
(2) We apologize for any confusion arising from the unclear description regarding the distinction between the group treated with IS for 6 days and the group subjected to secondary lipopolysaccharide (LPS) stimulation. It is imperative to clarify that induction of trained immunity necessitates 1 day of IS stimulation followed by 5 days of rest, rendering the 6th day sample representative of a trained state. Subsequent to this, a 24-hour LPS stimulation is applied, designating the 7th day sample as a secondary LPS-stimulated cell. This clarification is now explicitly indicated throughout the entirety of Figure 1A and Figure 3A in the revised manuscript.

(3) In accordance with your feedback, we performed siRNA knockdown of AhR and ALOX5 in primary human monocytes. AhR knockdown markedly attenuated the mRNA expression of TNF-α and IL-6, which are augmented in IS-trained macrophages. Similarly, knockdown of ALOX5 using ALOX5 siRNA abrogated the increase in TNF-α and IL-6 levels upon LPS stimulation in IS-trained macrophages (Author response image 2). Our experiments utilizing AhR siRNA corroborate the involvement of AhR in the expression of AA pathway-related molecules, such as ALOX5, ALOX5AP, and LTB4R1, in IS-induced trained immunity. These data

have been incorporated into the revised manuscript as Figure 4E and 5G, and supplementary Figure 5H.

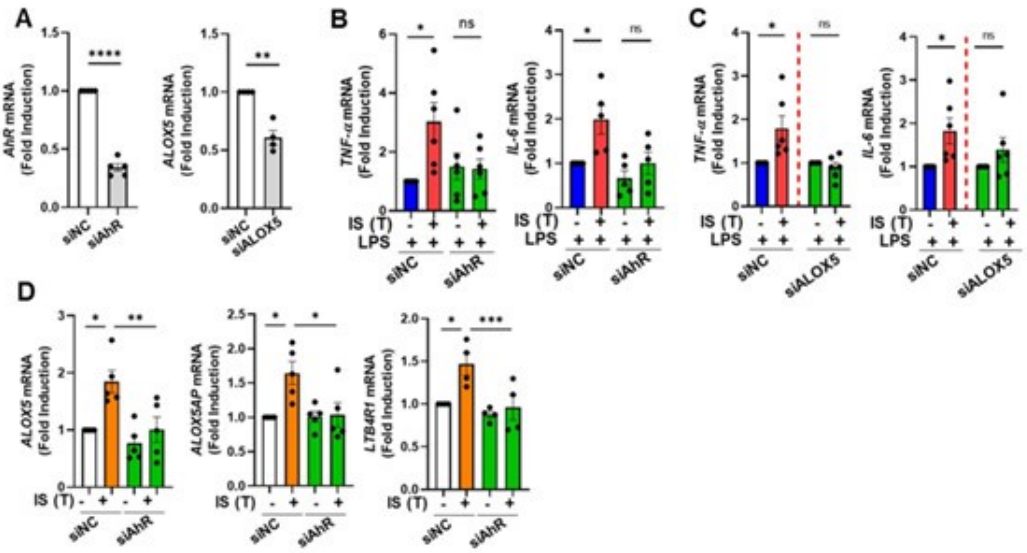
Author response image 1.

Epigenetic modification is regulated by arachidonic acid (AA) pathway and metabolic rewiring, but metabolic rewiring is not affected by the AA pathway. A-B. Monocytes were pre-treated with zileuton (ZLT), an inhibitor of ALOX5, or 2DG, a glycolysis inhibitor, followed by stimulation with IS for 24 hours. After a resting period of 5 days, the enrichment of H3K4me3 on the promoters of TNFA and IL6 loci was assessed. Normalization was performed using 2% input. C. Monocytes were pre-treated with zileuton (ZLT) and stimulated with IS for 24 hr. Cell lysates were immunoblotted for phosphorylated S6 Kinase, with β-actin serving as a normalization control. Band intensities in the immunoblots were quantified using densitometry. D, A schematic representation of the mechanistic framework underlying IS-trained immunity. Bar graphs show the mean ± SEM. * = p < 0.05, **= p < 0.01, and *** = p < 0.001 by two-tailed paired t-test.



Author response image 2.

Inhibition of IS-trained immunity by knockdown of AhR or ALOX5 in human monocytes. A-C. Human monocytes were transfected with siRNA targeting AhR (siAhR), ALOX5 (siALOX5), or negative control (siNC) for 1 day, followed by stimulation with IS for 24 hours. After a resting period of 5 days, cells were re-stimulated with LPS for 24 hours. mRNA expression levels of AhR and ALOX5 at 1 day after transfection, and TNF-α and IL-6 at 1 day after LPS treatment, were assessed using RT-qPCR. D. Human monocytes were transfected with AhR siRNA or negative control (NC) siRNA for 1 day, followed by stimulation with IS for 24 hours. After resting for 5 days, mRNA expression levels of ALOX5, ALOX5AP, and LTB4R1 were analyzed using RT-qPCR. Bar graphs show the mean ± SEM. * = p < 0.05, ** = p < 0.01, and *** = p < 0.001 by two-tailed paired t-test.



> *(2) The authors showed that IS-trained monocytes showed no change in TNF or IL-6, but increased the expression levels of TNF and IL-6 in response to the second LPS (Fig. 1B). This suggests that the different LPS responsiveness in IS-trained monocytes caused altered gene expression of TNF and IL6. However, the authors also showed that IS-trained monocytes without LPS stimulation showed increased levels of H3K4me3 at the TNF and IL-6 loci, as well as highly elevated ECAR and OCR, leading to no changes in TNF and IL-6. Therefore, it is unclear why or how the epigenetic and metabolic states of IS-trained monocytes induce different LPS responses. For example, increased H3K4me3 in HK2 and PFKP is important for metabolic rewiring, but why increased H3K4me3 in TNF and IL6 does not affect gene expression needs to be explained.*

We acknowledge the constructive critiques provided by the reviewer. While epigenetic modifications in the promoters of TNF-α, IL-6, HK2, and PFKP (Figure 3B and Supplementary Figure 3C in the revised manuscript), and metabolic rewiring (Figure 2A-D in the revised manuscript) were observed in IS-trained macrophages at 6 days prior to LPS stimulation, these macrophages do not exhibit an increase in TNF-α and IL-6 mRNA and protein levels before LPS stimulation. This lack of response is attributed to a 5-day resting period, allowing the macrophages to revert to a non-activated state, as depicted in Author response image 3 and 4. This phenomenon aligns with the concept of typical trained immunity.

Trained immunity is characterized by the long-term functional reprogramming of innate immune cells, which is evoked by various primary insults and which leads to an altered response towards a second challenge after the return to a non-activated state. Metabolic and epigenetic reprogramming events during the primary immune response persist partially even after the initial stimulus is removed. Upon a secondary challenge, trained innate immune cells exhibit a more robust and more prompt response than the initial response (Netea MG et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020 Jun;20(6):375-388).

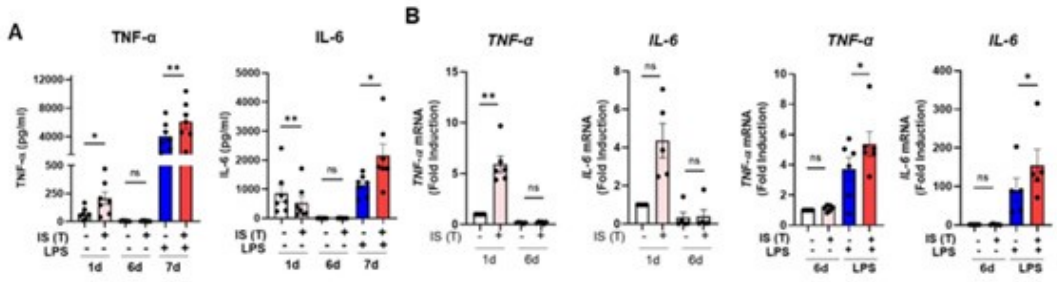
Numerous studies have demonstrated the observation of epigenetic modifications in the promoters of TNF-α and IL-6, and metabolic rewiring prior to LPS stimulation as a secondary challenge. However, cytokine production is contingent on LPS stimulation (Arts RJ et al. Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. Cell Metab. 2016 Dec 13;24(6):807-819; Arts RJW et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep. 2016 Dec

6;17(10):2562-2571; Ochando J et al. Trained immunity - basic concepts and contributions to immunopathology. Nat Rev Nephrol. 2023 Jan;19(1):23-37). The prolonged presence of higher levels of H3K4me3 on immune gene promoters, even after returning to baseline, is associated with open chromatin and results in a more rapid and stronger response, such as cytokine production, upon a secondary insult (Netea MG et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020 Jun;20(6):375-388).

The results in Figure 1B may be interpreted as indicating different LPS responsiveness in IStrained monocytes caused altered gene expression of TNF and IL-6. However, it is plausible that trained immune cells respond more robustly even to low concentrations of LPS. In fact, the aim of this experiment was to determine the appropriate LPS concentration.

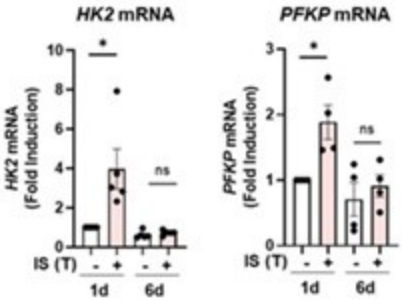
Author response image 3.

The changes in mRNA and protein level of TNF-α and IL-6 during induction of IS-trained immunity. Human monocytes were treated with or without IS (1 mM) for 24 hrs, succeeded by 5-day resting period to induce trained immunity. Cells were stimulated with LPS for 24 hrs. Protein and mRNA levels were assessed by ELISA and RT-qPCR, respectively. Bar graphs show the mean ± SEM. * = $p < 0.05$ and **= $p < 0.01$, by two-tailed paired t-test.



Author response image 4.

The changes in mRNA of HK2 and PFKP induced by IS during induction of IS-trained immunity. Human monocytes were treated with or without IS (1 mM) for 24 hrs, succeeded by 5-day resting period to induce trained immunity. mRNA levels were assessed by RT-qPCR. Bar graphs show the mean ± SEM. * = $p < 0.05$ by two-tailed paired ttest.



> *(3) The authors used human monocytes cultured in human serum without growth factors such as MCSF for 5-6 days. When we consider the short lifespan of monocytes (1-3 days), the authors need to explain the validity of the experimental model.*

We appreciate the reviewer's constructive critiques. As pointed out by the reviewer, human circulating CD14+ monocytes exhibit a relatively short lifespan (1-3 days) when cultured in

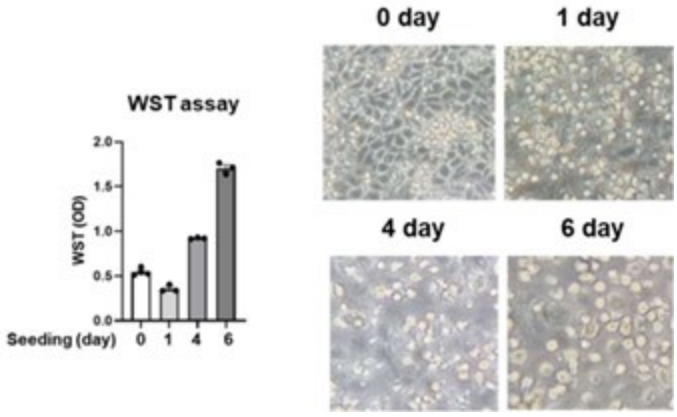
the absence of growth factors (Patel AA et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. J Exp Med. 2017 Jul 3;214(7):1913-1923). In this study, purified CD14+ monocytes were subjected to adherent culture for a duration of 7 days in RPMI1640 media supplemented with 10% human AB serum, a standard in vitro culture protocol widely employed in studies focusing on trained immunity (Domínguez-Andrés J et al. In vitro induction of trained immunity in adherent human monocytes. STAR Protoc. 2021 Feb 24;2(1):100365). In response to the reviewer's suggestions, we assessed cell viability on days 0, 1, 4, and 6, utilizing the WST assay. Despite a marginal reduction in cell viability observed at day 1, attributed to detachment from the culture plate, the cultured monocytes exhibited a notable enhancement in cell viability on days 4 and 6 when compared to days 0 or 1 (Author response image 5).

It has been demonstrated that the adhesion of human monocytes to a cell culture dish leads to their activation and induces the synthesis of substantial amounts of IL-1β mRNA as observed in monocytes adherent to extracellular matrix components such as fibronectin and collagen.

Morphologically, human adherent monocytes cultured with 10% human serum appear to undergo partial differentiation into macrophages by day 6, potentially explaining the observed lack of decrease in monocyte viability. Notably, Safi et al. have reported that adherent monocytes cultured with 10% human serum exhibit no significant difference in cell viability over a 7-day period when compared to cultures supplemented with growth factors such as M-CSF and IL-3 (Safi W et al. Differentiation of human CD14+ monocytes: an experimental investigation of the optimal culture medium and evidence of a lack of differentiation along the endothelial line. Exp Mol Med. 2016 Apr 15;48(4):e227).

Author response image 5.

Viability of human monocytes during the induction of trained immunity. Purified human monocytes were seeded on plates with RPIM1640 media supplemented with 10% human AB serum. Cell viability was assessed on days 0, 1, 4, and 6 utilizing the WST assay (Left panel). Cell morphology was examined under a light-inverted microscope at the indicated times (Right panel).

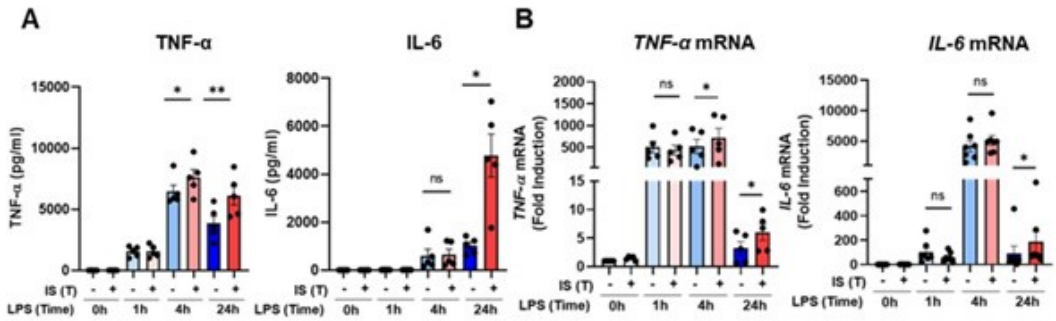


> *(4) The authors' ELISA results clearly showed increased levels of TNF and IL-6 proteins, but it is well established that LPS-induced gene expression of TNF and IL-6 in monocytes peaked within 1-4 hours and returned to baseline by 24 hours. Therefore, authors need to investigate gene expression at appropriate time points.*

We appreciate the valuable constructive feedback provided by the reviewer. As indicated by the reviewer, the LPS-induced gene expression of TNF-α and IL-6 in IS-trained monocytes exhibited a peak within the initial 1 to 4 hours, followed by a decrease by the 24-hour time point, as illustrated in Author response image 6. Nevertheless, the mRNA expression levels of TNFα and IL-6 were still elevated at the 24-hour mark. Furthermore, the protein levels of both TNFα and IL-6 apparently increased 24 hours after LPS stimulation. Due to technical constraints, sample collection had to be conducted at a single time point, and the 24-hour post-stimulation interval was deemed optimal for this purpose.

Author response image 6.

Kinetics of protein and mRNA expression of TNF-α and IL-6 after treatment of LPS as secondary insult in IS-trained monocytes. IS-trained cells were re-stimulated by LPS (10 ng/ml) for the indicated time. The supernatant and lysates were collected for ELISA assay and RT-qPCR analysis, respectively. Bar graphs show the mean ± SEM. * = p <0.05 and **= p < 0.01, by two-tailed paired t-test.



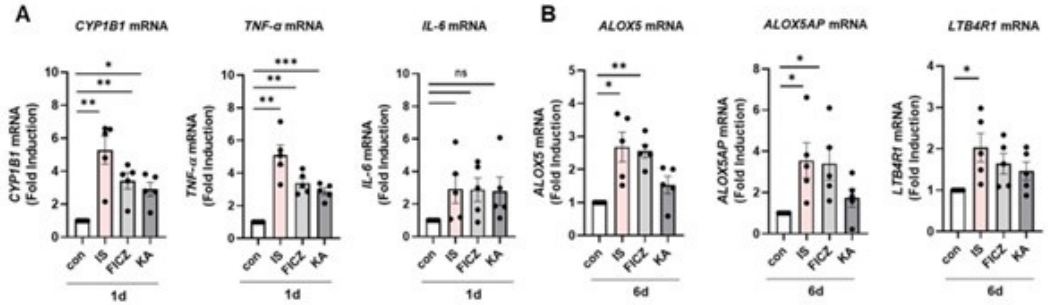
> *(5) It is a highly interesting finding that IS induces trained immunity via the AhR pathway. The authors also showed that the pretreatment of FICZ, an AhR agonist, was good enough to induce trained immunity in terms of the expression of TNF and IL-6. However, from this point of view, the authors need to discuss why trained immunity was not affected by kynurenic acid (KA), which is a well-known AhR ligand accumulated in CKD and has been reported to be involved in innate immune memory mechanisms (Fig. S1A).*

We appreciate the constructive criticism provided by the reviewer, and we comprehend the raised points. In our initial experiments, we hypothesized that kynurenic acid (KA), an aryl hydrocarbon receptor (AhR) ligand, might instigate trained immunity in monocytes, despite KA not being our primary target uremic toxin. However, our findings, as depicted in Fig. S1A, demonstrated that KA did not induce trained immunity. Notably, KA-treated monocytes exhibited induction of CYP1B1, an AhR-responsive gene, and elevated levels of TNF-α and IL-6 mRNA at 24 hours post-treatment, comparable to FICZ-treated monocytes. This observation underscores KA's role as an AhR ligand in human monocytes, as emphasized by the reviewer.

Of particular interest, proteins associated with the arachidonic acid pathway, such as ALOX5 and ALOX5AP - integral to the mechanisms underlying IS-induced trained immunity - did not exhibit an increase at day 6 following KA treatment, in contrast to the significant elevation observed with IS and FICZ treatments (Author response image 7). The rationale behind this disparity remains unknown, necessitating further investigation to elucidate the underlying factors. These data have been incorporated into the revised manuscript as Supplementary Figure 5C.

Author response image 7.

Divergent impact of AhR agonists, especially IS, FICZ, and KA on the AhR-ALOX5 pathway. Purified ytes underwent treatment with IS (1 mM), FICZ (100 nM), or KA (0.5 mM) for 1 day, followed by 5-day resting period to trained immunity. Activation of AhR through ligand binding was assessed by examining the induction of CYP1B1, an AhR ene, and cytokines one day post-treatment. The expression of genes related to the arachidonic acid pathway, such as ALOX5, 5AP, and LTB4R1, was analyzed via RT-qPCR six days after inducing trained immunity. Bar graphs show the mean ± SEM. * .05, **= p < 0.01, and ***= p < 0.001 by two-tailed paired t-test.



Indeed, it has been demonstrated that FICZ and TCDD, two high-affinity AhR ligands, exert opposite effects on T-cell differentiation, with TCDD inducing regulatory T cells and FICZ inducing Th17 cells. This dichotomy has been attributed to ligand-intrinsic differences in AhR activation (Ho PP et al. The aryl hydrocarbon receptor: a regulator of Th17 and Treg cell development in disease. Cell Res. 2008 Jun;18(6):605-8; Ehrlich AK et al. TCDD, FICZ, and Other High Affinity AhR Ligands Dose-Dependently Determine the Fate of CD4+ T Cell Differentiation. Toxicol Sci. 2018 Feb 1;161(2):310-320). These outcomes imply the involvement of an intricate interplay involving metabolic rewiring, epigenetic reprogramming, and the AhR-ALOX5 pathway in IS-induced trained immunity within monocytes.

> *(6) The authors need to clarify the role of IL-10 in IS-trained monocytes. IL-10, an anti-inflammatory cytokine that can be modulated by AhR, whose expression (Fig. 1E, Fig. 4D) may explain the inflammatory cytokine expression of IS-trained monocytes.*

We appreciate the reviewer's valuable comment, recognizing its significant importance. IL-10, characterized by potent anti-inflammatory attributes, assumes a pivotal role in constraining the host immune response against pathogens. This function serves to mitigate potential harm to the host and uphold normal tissue homeostasis. In the context of atherosclerosis (Mallat Z et al. Protective role of interleukin-10 in atherosclerosis. Circ Res. 1999 Oct 15;85(8):e17-24.) and kidney disease (Wei W et al. The role of IL-10 in kidney disease. Int Immunopharmacol. 2022 Jul;108:108917), IL-10 exerts potent deactivating effects on macrophages and T cells, influencing various cellular processes that could impact the development and stability of atherosclerotic plaques. Additionally, it is noteworthy that IL-10-deficient macrophages exhibit an augmentation in the proinflammatory cytokine TNF-α (Smallie T et al. IL-10 inhibits transcription elongation of the human TNF gene in primary macrophages. J Exp Med. 2010 Sep 27;207(10):2081-8; Couper KN et al. IL-10: the master regulator of immunity to infection. J Immunol. 2008 May 1;180(9):5771-7). As emphasized by the reviewer, the reduced gene expression of IL-10 by IS-trained monocytes may contribute to the heightened expression of proinflammatory cytokines. We have thoroughly addressed and

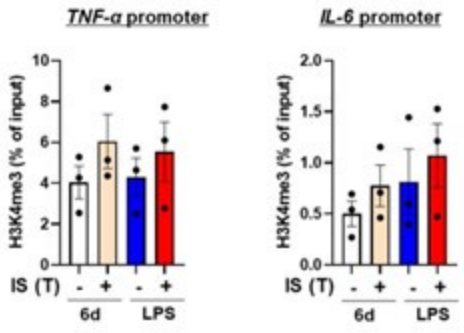
discussed this specific point in response to the reviewer's comment (Line 394-399 of page 18 in the revised manuscript).

> *(7) The authors need to show H3K4me3 levels in TNF and IL6 genes in all conditions in one figure. (Fig. 2B). Comparing Fig. 2B and Fig. S2B, H3K4me3 does not appear to be increased at all by LPS in the IL6 region.*

We are grateful for the constructive criticism provided by the reviewer. In response to the reviewer's comment, we endeavored to conduct an experiment demonstrating H3K4me3 enrichment on the promoters of TNF-α and IL-6 across all experimental conditions. However, due to limitations in the availability of purified human monocytes, we conducted an additional three independent experiments for ChIP-qPCR across all conditions. Despite encountering a notable variability among individuals, even within the healthy donor cohort, our results demonstrated an increase in H3K4me3 enrichment on the TNF-α and IL-6 promoters in IS-trained groups, irrespective of subsequent LPS treatment (Author response image 8).

Author response image 8.

Analysis of H3K4me3 enrichment on the promoters of TNFA and IL6 Loci in IS-trained macrophages. ChIP-qPCR was employed to assess the enrichment of H3K4me3 on the promoters of TNFA and IL6 loci before (day 6) and after LPS stimulation (day 7) in IS-trained macrophages. The normalization control utilized 2% input. Bar graphs show the mean ± SEM. The data presented are derived from three independent experiments utilizing samples from different donors.

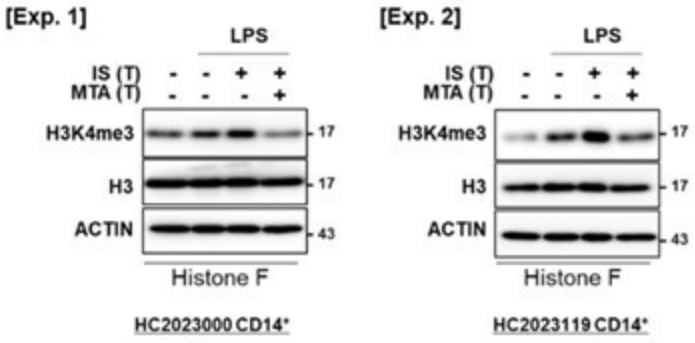


> *(8) The authors need to address the changes of H3K4me3 in the presence of MTA.*

We appreciate the constructive criticism provided by the reviewer. In response to the reviewer's feedback, we conducted an analysis of the changes in H3K4me3 in the presence of MTA, a general methyltransferase inhibitor, using identical conditions as depicted in Figure 2C of the original manuscript. Our findings revealed that MTA exerted inhibitory effects on the levels of H3K4me3, as isolated through the acid histone extraction method, which were otherwise increased by IS-training, as illustrated in Author response image 9.

Author response image 9.

The reduction of H3K4me3 by MTA treatment in IS-trained macrophages. IS-trained cells were restimulated by LPS (10 ng/ml) as a secondary challenge for 24 hrs, followed by isolation of histone and WB analysis for H3K4me3, Histone 3 (H3), and β-actin. The blot data from two independent experiments with different donors were shown.



> *(9) Interpretation of ChIP-seq results is not entirely convincing due to doubts about the quality of sequencing results. First, authors need to provide information on the quality of ChIP-seq data in reliable criteria such as Encode Pipeline. It should also provide representative tracks of H3K4me3 in the TNF and IL-6 genes (Fig. 2F). And in Fig. 2F, the author showed the H3K4me3 track of replicates, but the results between replicates were very different, so there are concerns about reproducibility. Finally, the authors need to show the correlation between ChIP-seq (Fig. 2) and RNA-seq (Fig. 5).*

We appreciate the constructive criticism provided by the reviewer.

As indicated by the reviewer, for evaluation of sample read quality, analysis was performed using the histone ChIP-seq standard from the ENCODE project, focusing on metrics such as read depth, PCR bottleneck coefficient (PBC)1, PBC2, and non-redundant fraction (NRF). Five of the total samples were displayed moderate bottleneck levels ($0.5 \leq PBC1 < 0.8$, $1 \leq PBC2 < 3$) with acceptable ($0.5 \leq NRF < 0.8$) complexity. One sample showed mild bottlenecks ($0.8 \leq PBC1 < 0.9$, $3 \leq PBC2 < 10$) with compliance ($0.8 \leq NRF < 0.9$) complexity. This quality metrics indicated ChIP-seq data quality meets at least the standards required for downstream analysis according to ENCODE project criteria (Author response image 10A).

To examine the differences in H3K4me3 enrichment patterns between two groups, we normalized the read counts around the TSS ±2 kb of human genes to CPM. Sequentially, we compared the average values of IS-treated macrophage compare to control and displayed in waterfall plots. In addition, we marked genes of interest in red including the phenotypes of IStrained macrophages (TNF and IL6), the activation of the innate immune responses (XRCC5, IFI16, PQBP1), and the regulation of ornithine decarboxylase (OAZ3, PSMA3, PSMA1) (Author response image 10B and C). Also, H3K4me3 peak tracks of TNF and IL6 loci and H3K4me3 enrichment pattern were added in supplementary Figure 3D and 3F in the revised manuscript.

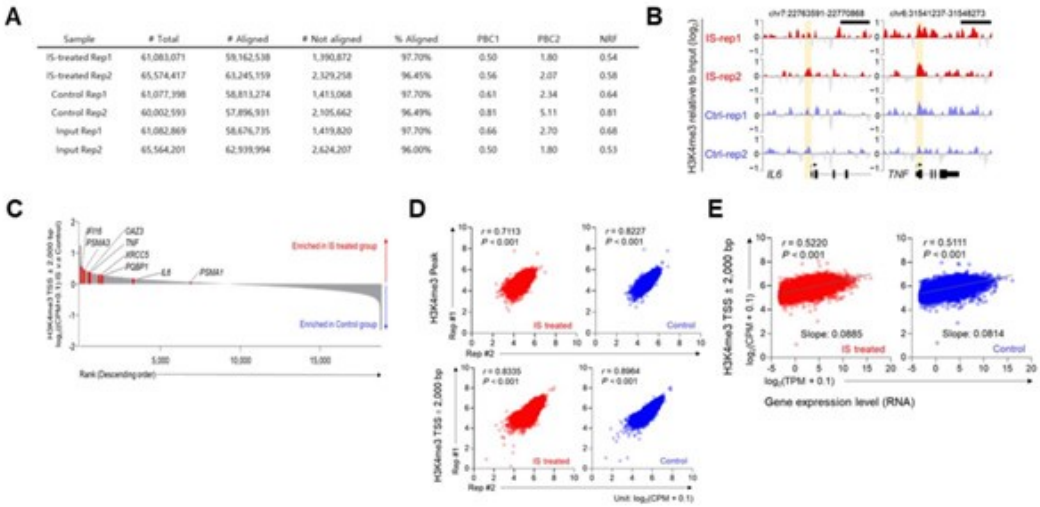
Next, to evaluate the consistency among replicates within a group, we analyzed enrichment values, expressed as Counts per Million (CPM) using edgeR R-package, by applying Spearman's correlation coefficients. we analyzed two sets included total 7,136 H3K4me3 peak sets, as described in Figure 3E in the revised manuscript and 2 kbp around transcription start sites (TSS) from hg19 human genomes. The resulting Spearman's correlation coefficients and associated P-values demonstrated a concordance between replicates, confirming reproducibility and consistent performance (Author response image 10D).

Finally, the correlation between gene expression and H3K4me3 enrichment around transcription start sites (TSS) has been reported in previous research (Reshetnikov VV et al. Data of correlation analysis between the density of H3K4me3 in promoters of genes and gene expression: Data from RNA-seq and ChIP-seq analyses of the murine prefrontal cortex. Data Brief. 2020 Oct 2;33:106365). To verify this association in our study, we applied Spearman's

correlation for comparative analysis and conducted linear regression to determine if a consistent global trend in RNA expression existed. In our analysis, count values from regions extending 2 kbp around the TSSs in H3K4me3 ChIP-seq data were converted to Counts per Million (CPM) using edgeR R-package. These were then contrasted with the Transcripts Per Million (TPM) values of genes. Our results revealed a significant positive correlation, reinforcing the consistent relationship between H3K4me3 enrichment and gene expression (Author response image 10E and Supplementary Fig. 6D in revised manuscripts).

Author response image 10.

The information on quality of ChIP-seq data and correlation between ChIP-seq and RNA-seq. A, information on quality of ChIP-seq data. B, H3K4me3 peak of promoter region on TNFA and IL6. C, The differences in H3K4me3 enrichment patterns between control group and IS-training group. D, The consistency among replicates within a group. E, Correlation between ChIP-seq and RNA-seq in IS-induced trained immunity.

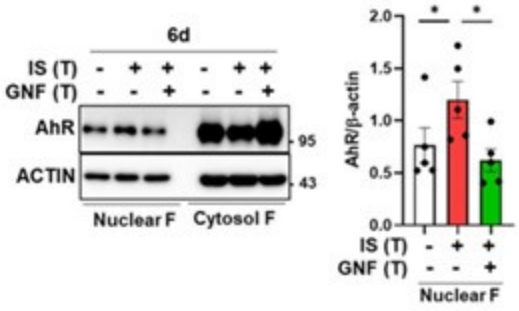


> *(10) AhR changes in the cell nucleus should be provided (Fig. 4A).*

We appreciate the constructive feedback from the reviewer. In response to the reviewer's suggestions, we investigated the nuclear translocation of AhR on 6 days after the induction of ISmediated trained immunity, as illustrated in Author response image 11. For this purpose, the lysate from IS-trained monocytes was fractionated into the nucleus and cytosol, and AhR protein was subsequently immunoblotted. The results depicted in Figure X demonstrate that IS-trained monocytes exhibited a higher level of AhR protein in the nucleus compared to non-trained monocytes. Notably, the nuclear translocation of AhR was significantly attenuated in IS-trained monocytes treated with GNF351. These findings imply that the activation of AhR, facilitated by the binding of IS, persisted partially up to 6 days, indicating that IS-mediated degradation of AhR was not fully recovered even on day 6 after the induction of IS training. Consequently, we have replaced Figure 4A in the revised manuscript.

Author response image 11.

The activation of AhR, facilitated by IS binding, is persisted partially up to 6 days during induction of trained immunity. The lysate of IS-trained cells treated with or without GNF351, were separated into nuclear and cytosol fraction, followed by WB analysis for AhR protein (Left panel). Band intensity in immunoblots was quantified by densitometry (Right panel). β-actin was used as a normalization control. Bar graphs show the mean ± SEM. * = $p < 0.05$, by two-tailed paired t-test.

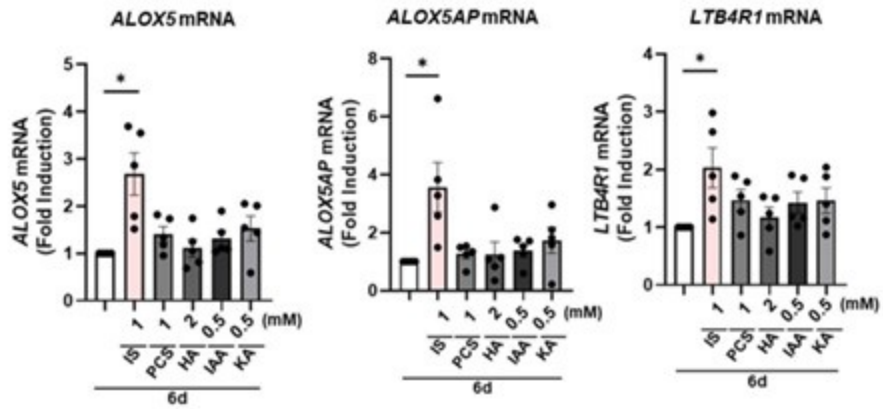


> *(11) Do other protein-bound uremic toxins (PBUTs), such as PCS, HA, IAA, and KA, change the mRNA expression of ALOX5, ALOX5AP, and LTB4R1? In the absence of genetic studies, it is difficult to be certain of the ALOX5-related mechanism claimed by the authors.*

We are grateful for the constructive criticism provided by the reviewer. In response to the reviewer's comment, we investigated whether uremic toxins, specifically PBUTs such as PCS, HA, IAA, and KA, induce changes in the mRNA expression of ALOX5, ALOX5AP, and LTB4R1 in trained monocytes. Intriguingly, the examination revealed no discernible induction in the mRNA expression of these genes by PBUTs, with the exception of IS, as depicted in Author response image 12 of the letter. These findings once again underscore the implication of the AhR-ALOX5 pathway in the induction of trained immunity in monocytes by IS.

Author response image 12.

No obvious impact of PBUTs except IS on the expression of arachidonic acid pathway-related genes on 6 days after treatment with PBUTs. Purified monocytes were treated with several PBUTs including IS, PCS, HA, IAA, and KA for 24 hrs., following by 5-day resting period to induce trained immunity. The mRNA expression of ALOX5, ALOX5AP, and LTB4R1 were quantified using RT-qPCR. Bar graphs show the mean ± SEM. * = $p < 0.05$, by two-tailed paired t-test.



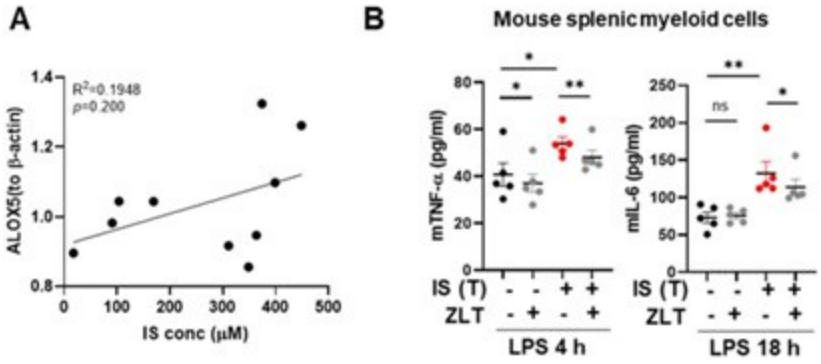
> *(12) Fig.6 is based on the correlated expression of inflammatory genes or AA pathway genes. It does not clarify any mechanisms the authors claimed in the previous figures.*

We express our sincere appreciation for the constructive criticism provided by the reviewer, and we have taken careful note of the points raised. In response to the reviewer's feedback, we adopted two distinct approaches utilizing samples obtained from ESRD patients and IS-trained mice. Initially, we investigated the correlation between ALOX5 protein expression in

monocytes and IS concentration in the plasma of ESRD patients presented in Figure 6E of the original manuscript. Despite the limited number of samples, our analysis revealed a nonsignificant correlation between IS concentration and ALOX5 expression; however, it demonstrated a positive trend (Author response image 13A). Subsequently, we examined the potential inhibitory effects of zileuton, an ALOX5 inhibitor, on the production of TNF-α and IL-6 in LPSstimulated splenic myeloid cells derived from IS-trained mice. Our findings indicate that zileuton significantly inhibits the production of TNF-α and IL-6 induced by LPS in splenic myeloid cells from IS-trained mice (Author response image 13B). These data were added in Figure 6N of the revised manuscript (Line 350-354 of page 16 in the revised manuscript).

Author response image 13.

Assessment of the correlation between ALOX5 and the concentration of IS in ESRD patients, and investigation of ALOX5 effects in mouse splenic myeloid cells in IS-trained mice. A. Examination of the correlation between ALOX5 protein expression in monocytes and IS concentration in the plasma of ESRD patients. B. C57BL/6 mice were administered daily injections of 200 mg/kg IS for 5 days, followed by a resting period of another 5 days. Subsequently, IS-trained mice were sacrificed, and spleens were mechanically dissociated. Isolated splenic myeloid cells were subjected to ex vivo treatment with LPS (10 ng/ml), along with zileuton (100 μM). The levels of TNF-α and IL-6 in the supernatants were quantified using ELISA. The graphs show the mean ± SEM. * = p < 0.05, by two-tailed paired t-test between zileuton treatment group and no-treatment group.



> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Minor corrections to the figures*
>
> *(1) No indicators for the control group in Fig. 1B.*

We thank you for the reviewer's comment. According to the reviewer's comment, the control group was indicated with (-).

> *(2) The same paper is listed twice in the references section. (No. 19 and 28)*

We thank you for the reviewer's comment. We deleted the reference No. 28.

> ***Reviewer #2 (Public Review):***
>
> *Manuscript entitled "Uremic toxin indoxyl sulfate (IS) induces trained immunity via the AhR-dependent arachidonic acid pathway in ESRD" presented some interesting findings. The manuscript strengths included use of H3K4me3-CHIP-Seq, AhR antagonist, IS treated cell RNA-Seq, ALOX5 inhibitor, MTA inhibitor to determine the roles of IS-AhR in trained immunity related to ESRD inflammation and trained immunity.*

Thank you very much for your positive feedback.

> ***Reviewer #2 (Recommendations For The Authors)****:*
>
> *However, the manuscript needs to be improved by fixing the following concerns.*
>
> *There are concerns:*
>
> *(1) The experiments in Figs. 1G, 1H and 1I need to have AhR siRNA, and siRNA control to demonstrate that the results in uremic toxins-containing serum-treated experiments were related to IS;*

We extend our gratitude to the reviewer for their invaluable comment, acknowledging its significant relevance to our study. In accordance with the reviewer's suggestion, we endeavored to conduct additional experiments utilizing AhR siRNA to elucidate the direct impact of IS present in the serum of end-stage renal disease (ESRD) patients on the induction of IS-mediated trained immunity.

Regrettably, owing to limitations in the availability of monocytes post-siRNA transfection, we were unable to establish a direct relationship between the observed outcomes in experiments utilizing uremic toxins-containing serum and IS in AhR siRNA knockdown monocytes. However, treatment with GNF351, an AhR antagonist, resulted in the inhibition of TNF-α production in trained monocytes exposed to uremic toxins-containing serum (Author response image 14).

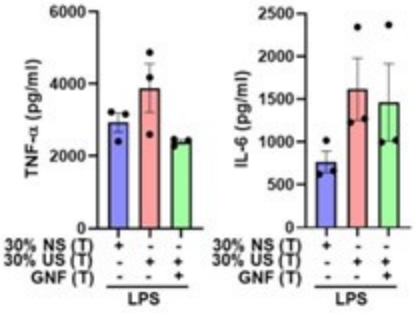
In our previous studies, we have already reported that uremic serum-induced TNF-α production in human monocytes is dependent on the AhR pathway, using GNF351 (Kim HY et al. Indoxyl sulfate (IS)-mediated immune dysfunction provokes endothelial damage in patients with end-stage renal disease (ESRD). Sci Rep. 2017 Jun 8;7(1):3057). Additionally, we have provided evidence demonstrating an augmentation in the activity of the AhR pathway within monocytes derived from ESRD patients, indicative of a significant reduction in AhR protein levels (Kim HY et al. Indoxyl sulfate-induced TNF-α is regulated by crosstalk between the aryl hydrocarbon receptor, NF-κB, and SOCS2 in human macrophages. FASEB J. 2019 Oct;33(10):10844-10858). It is noteworthy that other major protein-bound uremic toxins (PBUTs), such as PCS, HA, IAA, and KA, failed to induce trained immunity in human monocytes (Supplementary Figure 1A in the revised manuscript). Nevertheless, knockdown of AhR via siRNA effectively impeded the induction of IS-mediated trained immunity in human monocytes (Figure 4E in the revised manuscript).

Taken collectively, our findings suggest a critical role for IS present in the serum of ESRD patients in the induction of trained immunity in human monocytes.

Author response image 14.

Inhibition of uremic serum (US)-induced trained immunity by AhR antagonist, GNF351. Monocytes were pre-treated with or without GNF351 (AhR antagonist; 10 μM) for 1 hour, followed by treatment with pooled normal serum (NS) or uremic serum (US) at a concentration of 30% (v/v) for 24 hours. After a resting period of 5 days, cells were stimulated

with LPS for 24 hours. The production of TNF-α and IL-6 in the supernatants was quantified using ELISA. The data presented are derived from three independent experiments utilizing samples from different donors.



> *(2) Fig. 3 needs to be moved as Fig. 2*

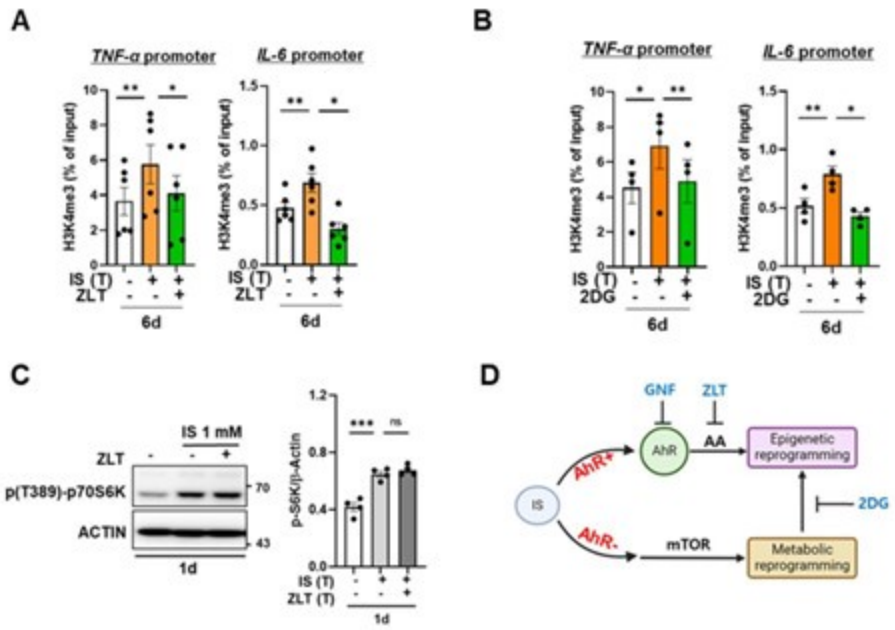
We express appreciation for the constructive suggestion provided by the reviewer. In response to the reviewer's comment, the sequence of Figure 3 and Figure 2 was adjusted in the revised manuscript.

> *(3, 4) The connection between bioenergetic metabolism pathways and H3K4me3 was missing; The connection between bioenergetic metabolism pathways and ALOX5 was missing;*

We appreciate the reviewer’s constructive criticism and fully understood the reviewer's points. In response to the reviewer's feedback, we conducted additional experiments employing appropriate inhibitors to elucidate the interrelation between bioenergetic metabolism and H3K4me3 and between bioenergetic metabolism and ALOX5. Initially, we assessed the enrichment of H3K4me3 at 6-day on promoters of TNFA and IL6 loci after treatment of 2-DG, a glycolysis inhibitor. Additionally, we evaluated the alteration in the activity of S6K, a downstream molecule of mTORC1, following treatment with zileuton, an inhibitor of ALOX5. Our findings indicate that AhR-dependent arachidonic acid (AA) signaling induces epigenetic modifications, albeit without inducing metabolic rewiring, in IS-induced trained immunity (Author response image 15). However, IS stimulation promotes mTORC1-mediated glycolysis in an AhR-independent manner. Notably, inhibition of glycolysis with 2-DG impacts epigenetic modifications. We have updated Figure 7 of the revised manuscript to incorporate these additional experimental findings, elucidating the correlation between the diverse mechanisms implicated in IS-induced innate immune memory (Fig. 7 in the revised manuscript).

Author response image 15.

Epigenetic modification is regulated by arachidonic acid (AA) pathway and metabolic rewiring, but metabolic rewiring is not affected by the AA pathway. A-B. Monocytes were pre-treated with zileuton (ZLT), an inhibitor of ALOX5, or 2DG, a glycolysis inhibitor, followed by stimulation with IS for 24 hours. After a resting period of 5 days, the enrichment of H3K4me3 on the promoters of TNFA and IL6 loci was assessed. Normalization was performed using 2% input. C. Monocytes were pre-treated with ziluton (ZLT) and stimulated with IS for 24 hr. Cell lysates were immunoblotted for phosphorylated S6 Kinase, with β-actin serving as a normalization control. Band intensities in the immunoblots were quantified using densitometry. D, A schematic representation of the mechanistic framework underlying IS-trained immunity. Bar graphs show the mean ± SEM. * = $p < 0.05$, **= $p < 0.01$, and *** = $p < 0.001$ by two-tailed paired t-test.

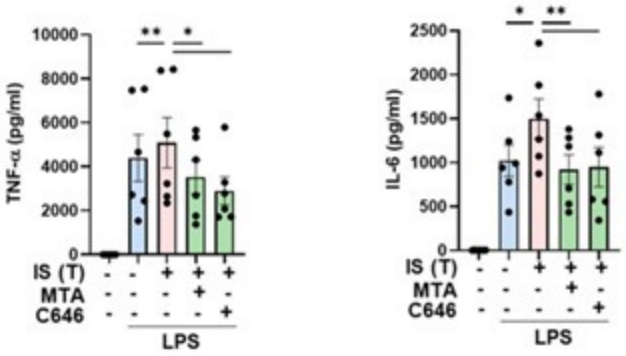


> *(5) It was unclear whether histone acetylations such as H3K27acetylation and H3K14 acetylation are involved in IS-induced epigenetic reprogramming or IS-induced trained immunity is highly histone methylation-specific.*

We appreciate the constructive comment provided by the reviewer. As highlighted by the reviewer, alterations in epigenetic histone markers, specifically H3K4me3 or H3K27ac, have been recognized as the underlying molecular mechanism in trained immunity. Due to limitations in the availability of trained cells, this study primarily focused on histone methylation. In response to the reviewer's inquiry, we briefly investigated the impact of histone acetylation using C646, a histone acetyltransferase inhibitor, on IS-induced trained immunity (Author response image 16). Our experiments revealed that C646 treatment effectively hinders the production of TNF-α and IL-6 by IS-trained monocytes in response to LPS stimulation, comparable to the effects observed with MTA (5'methylthioadenosine), a non-selective methyltransferase inhibitor. This suggests that histone acetylation also contributes to the epigenetic modifications associated with IS-induced trained immunity. We sincerely appreciate the valuable input from the reviewer.

Author response image 16.

The role of histone acetylation in epigenetic modifications in IS-induced trained immunity. Monocytes were pretreated with MTA (methylthioadenosine, methyltransferase inhibitor) or C646 (histone acetyltransferase p300 inhibitor), followed treatment with IS 1 mM for 24 hrs. After resting for 5 days, trained cells were re-stimulated by LPS 10 ng/ml as secondary insult. TNF-α and IL-6 in supernatants were quantified by ELISA. Bar graphs show the mean ± SEM. * = $p < 0.05$ and **= $p < 0.01$ by two-tailed paired t-test.



> ***Reviewer #3 (Public Review):***
>
> *The manuscript entitled, "Uremic toxin indoxyl sulfate induces trained immunity via the AhRdependent arachidonic acid pathway in ESRD" demonstrates that indoxyl sulfate (IS) induces trained immunity in monocytes via epigenetic and metabolic reprogramming, resulting in augmented cytokine production. The authors conducted well-designed experiments to show that the aryl hydrocarbon receptor (AhR) contributes to IS-trained immunity by enhancing the expression of arachidonic acid (AA) metabolism-related genes such as arachidonate 5-lipoxygenase (ALOX5) and ALOX5 activating protein (ALOX5AP). Overall, this is a very interesting study that highlights that IS mediated trained immunity may have deleterious outcomes in augmented immune responses to the secondary insult in ESRD. Key findings would help to understand accelerated inflammation in CKD or RSRD.*

We greatly appreciate your positive feedback.

> ***Reviewer #3 (Recommendations for The Authors):***
>
> *This reviewer, however, has the following concerns.*
>
> *Major comments:*
>
> *(1) Figure 1B: IS is known to induce the expression of TNF-α and IL-6. This reviewer wonders why these molecules were not detected in the IS (+) LPS (-) condition.*

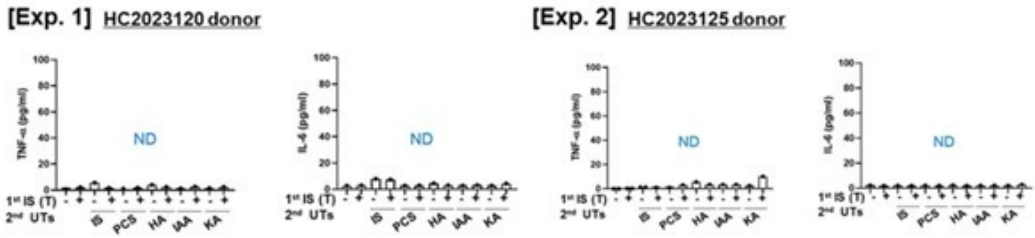
We appreciate the constructive comment provided by the reviewer. In our prior investigation, it was observed that the expression of TNF-α and IL-6 was induced 24 hours after IS treatment in human monocytes and macrophages (Couper KN et al. IL-10: the master regulator of immunity to infection. J Immunol. 2008 May 1;180(9):5771-7). In adherence to the trained immunity protocol, the medium was replaced at the 24 hours post-IS treatment to eliminate IS, with a subsequent change after a 5-day resting period. Probably, TNF-α and IL-6 are accumulated and detected in the IS (+) LPS (-) culture supernatant if the media was not changed at these specific time points. Our primary objective, however, was to ascertain the role of IS in the induction of trained immunity, prompting an investigation into whether IS contributes to an increase in the production of TNF-α and IL-6 in response to LPS stimulation as a secondary insult.

> *(2) 1' stimulus is IS followed by 2' stimulus LPS/Pam3. It would be interesting to know what the immune profile is when other uremic toxin is used for secondary insult, this would be more relevant in clinical context of ESRD.*

The reviewer's insightful comment is greatly appreciated. To address their feedback, IStrained macrophages were subjected to additional stimulation using protein-bound uremic toxins (PBUTs) as a secondary challenge. As illustrated in Letter figure 17, the examined uremic toxins, namely p-cresyl sulfate (PCS), Hippuric acid (HA), Indole 3-acetic acid (IAA), and kynurenic acid (KA), failed to elicit the production of proinflammatory cytokines, specifically TNF-α and IL-6, by IS-trained monocytes.

Author response image 17.

No obvious effect of protein-bound uremic toxin (PBUTs) as secondary insults on the production of proinflammatory cytokines in IS-trained monocytes. IS-trained monocytes were re-stimulated with several PBUTs, such as IS (1 mM), PCS (1 mM), HA (2 mM), IAA. (0.5 mM), and KA (0.5 mM) as a secondary challenge for 24 hrs. TNF-α and IL-6 in supernatants were quantified by ELISA. The data from two independent experiments with different donors were shown. ND indicates 'not detected'.

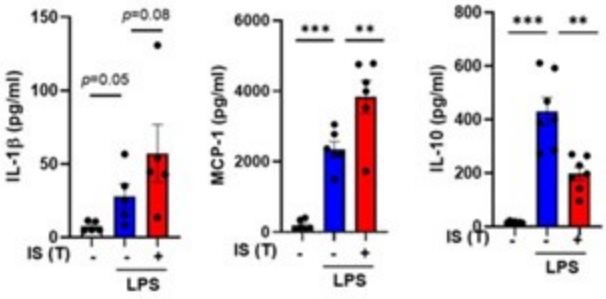


> *(3) The authors need to explain a rationale why RNA and protein data used different markers.*

We appreciate the constructive input provided by the reviewer. Given that TNF-α and IL6 represent prototypical cytokines synthesized by trained monocytes in humans, we conducted a comprehensive analysis of their mRNA and protein levels. In human macrophages, the release of active IL-1β necessitates a second priming event, such as the presence of ATP. Consequently, we posited that assessing the mRNA levels of IL-1β would suffice to demonstrate the induction of trained immunity in our experimental protocol. Nevertheless, in response to the reviewer's comment, we proceeded to assess the protein levels of IL-1β, IL-10, and MCP-1 as illustrated in Author response image 189. These data have been incorporated into the revised manuscript as supplementary Figure 1E.

Author response image 18.

Modulation of cytokine levels in IS-trained macrophages in response to secondary stimulation with LPS. Human monocytes were stimulated with the IS for 24 hr, followed by resting period for 5 days. On day 6, the cells were re-stimulated with LPS for 24 hr. The levels of each cytokine in the supernatants were quantified using ELISA. Bar graphs show the mean ± SEM. ** = $p < 0.01$ and ***= $p < 0.001$ by two-tailed paired t-test.



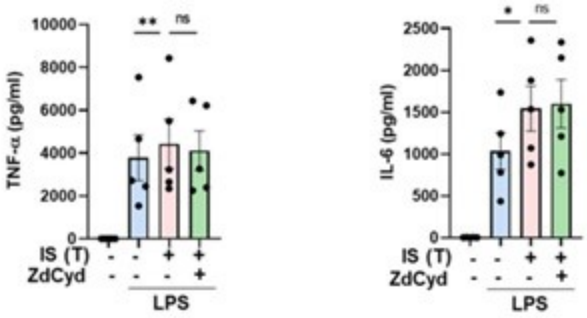
> *(4) Epigenetic modification primarily involves histone modification and DNA methylation. The authors presented convincing data on histone modification (Figure 2), but did not provide any insights in the promoter DNA methylation status.*

We express our gratitude to the reviewer for providing valuable comments, which highlight a crucial aspect of our study. Despite the well-established primary role of DNA methylation in epigenetic modifications, recent suggestions propose that histone modifications, particularly H3K4me3 or H3K27ac, play a predominant role in the induction of trained immunity. In this context, our primary inquiry was focused on determining whether IS, as an endogenous insult, induces trained immunity in monocytes, and if so, whether IS-trained immunity is mediated through metabolic and epigenetic modifications - recognized as the major mechanisms underlying the generation of trained immunity. It is imperative to note that our study's primary objective did not encompass the identification of various epigenetic changes. In response to the reviewer's inquiry, we conducted a brief examination of the impact of DNA methylation using ZdCyd (5-aza-2'-deoxycytidine), a DNA methylation inhibitor, on IS-induced trained immunity. Our experimental findings indicate that ZdCyd treatment exerts no discernible effect on the production of TNF-α and IL-6 by IS-trained monocytes upon stimulation with LPS, as illustrated in Author response image 19. However, a recent study has shed light on the role of DNA methylation in BCG vaccine-induced trained immunity in human monocytes (Bannister S et al. Neonatal BCG vaccination is associated with a long-term DNA methylation signature in circulating monocytes. Sci Adv. 2022 Aug 5;8(31):eabn4002). Consequently, further investigations utilizing DNA methylation sequencing are warranted to elucidate whether DNA methylation is implicated in the induction of IS-trained immunity.

Author response image 19.

The effect of DNA methylation on IS-induced trained immunity. Monocytes were pretreated with ZdCyd (5-aza-2'-deoxycytidine, DNA methylation inhibitor), followed by treatment with IS 1 mM for 24 hrs. After resting for 5 days, cells were re-stimulated by LPS 10 ng/ml as secondary insult. TNF-α and IL-6 in supernatants were quantified by

ELISA. Bar graphs show the mean ± SEM. * = $p < 0.05$ and **= $p < 0.01$ by two-tailed paired t-test.

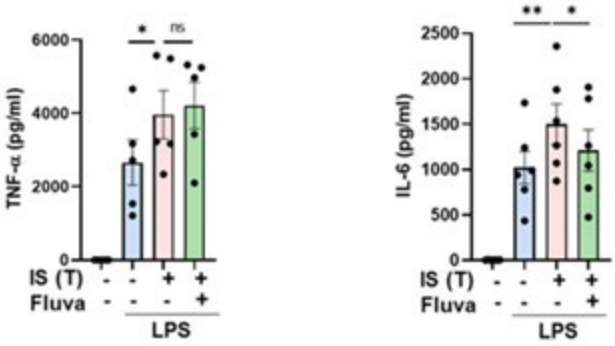


> *(5) Metabolic rewiring in trained immunity cells undergo metabolic changes which involved intertwined pathways of glucose and cholesterol metabolism. The authors presented nice data on glucose pathway (Figure 3) but failed to show any changes related to cholesterol metabolism.*

We express our gratitude to the reviewer for providing valuable comments, which underscore a noteworthy observation. In the current investigation, our primary emphasis has been on glycolytic reprogramming, recognized as a principal mechanism for inducing trained immunity in monocytes. This focus stems from preliminary experiments wherein Fluvastatin, a cholesterol synthesis inhibitor, demonstrated no discernible impact on TNF-α production by IS-trained monocytes, as illustrated in Author response image 20. Intriguingly, Fluvastatin treatment exhibited a partial inhibitory effect on the production of IL-6 by IS-trained monocytes. Subsequent investigations are imperative to elucidate the role of cholesterol metabolism in the induction of IS-trained immunity.

Author response image 20.

The effect of cholesterol metabolism on IS-induced trained immunity. Monocytes were pretreated with Fluvastatin (cholesterol synthesis inhibitor, HMG-CoA reductase inhibitor), followed treatment with IS 1 mM for 24 hrs. After resting for 5 days, cells were re-stimulated by LPS 10 ng/ml as secondary insult. TNF-α and IL-6 in supernatants were quantified by ELISA. Bar graphs show the mean ± SEM. * = $p < 0.05$ and **= $p < 0.01$ by two-tailed paired t-test.



> *(6) Trained immunity involves neutrophils in addition to monocyte/macrophages. It is evident from the RNAseq data that neutrophil degranulation (Figure 5B) is the top enriched pathway. This reviewer wonders why the authors did not perform any assays on neutrophils.*

We appreciate the reviewer for valuable comment. IS represents a major uremic toxin that accumulates in the serum of patients with chronic kidney disease (CKD), correlating with CKD progression and the onset of CKD-related complications, including cardiovascular diseases (CVD). Our prior investigations have demonstrated that IS promotes the production of TNF-α and IL-1β by human monocytes and macrophages. Additionally, macrophages pre-treated with IS exhibit a significant augmentation in TNF-α production when exposed to a low dose of lipopolysaccharide (LPS). Considering the pivotal role of proinflammatory macrophages and TNF-α, a principal cardiotoxic cytokine, in CVD pathogenesis, our focus in this study has primarily focused on elucidating the trained immunity of monocytes/macrophages. Consequently, all experiments were meticulously conducted using highly purified monocytes and monocytederived macrophages derived from both healthy controls and end-stage renal disease (ESRD) patients. The reviewer's observation regarding the potential involvement of neutrophils in trained immunity has been duly noted. Subsequent investigations will be imperative to explore the conceivable role of IS-trained neutrophils in the pathogenesis of CVD. Once again, we appreciate the reviewer for their valuable comment.

> *(7) Figure 5C (GSEA plots): This reviewer is not sure if one can present the plots assigned with groups (eg. IS(T) vs Control). More details are required in the Methods related to this.*

We apologize for any ambiguity resulting from the previously unclear description of methods concerning Gene Set Enrichment Analysis (GSEA) plots. To provide clarification, additional details pertaining to this aspect have been explained upon in the revised manuscript's Methods section.

> *(8) In vivo data (Figure 6 I-M): Instead of serum profile and whole set of spleen myeloid cells, it would be interesting to see changes of markers on peritoneal macrophages or bone marrow-derived macrophages since the in vitro findings are on monocyte-derived macrophages.*

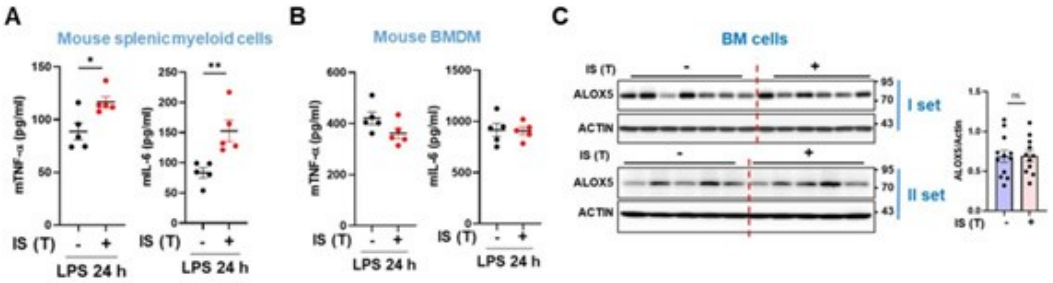
We appreciate comment and the insightful suggestion provided by the reviewer. In response to the reviewer's feedback, we conducted additional in vivo experiments to examine the production of TNF-α and IL-6 in bone marrow-derived macrophages (BMDMs) derived from IStrained mice. Upon LPS stimulation, we observed an increase in the production of TNF-α and IL-6 in spleen myeloid cells from IS-trained mice. However, no such increase in these cytokines was noted in BMDMs derived from the same mice (Author response image 22, A and B). In fact, we already observed that that the expression of ALOX5 was not elevated in BM cells derived from IS-trained mice presented in Figure 6L and M of the original manuscript (Author response image 22C).

Recent studies have indicated that trained immunity can be induced in circulating immune cells, such as monocytes or resident macrophages (peripheral trained immunity), as well as in hematopoietic stem and progenitor cells (HSPCs) within the bone marrow (central trained immunity) (Kaufmann E et al. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell. 2018 Jan 11;172(1-2):176-190.e19; Riksen NP et al. Trained immunity in atherosclerotic cardiovascular disease. Nat Rev Cardiol. 2023 Dec;20(12):799-811). It is plausible that central trained immunity in BM progenitor cells may not be elicited in our mouse model, which is relatively acute in nature. Further investigations are warranted to explore the role of IS in inducing central trained immunity, utilizing appropriate chronic disease models.

We have included this additional data as supplementary figures in the revised manuscript (Suppl. Fig. 7, D and E, and line 355-362 of page 16 in the revised manuscript).

Author response image 21.

Absence of trained immunity in bone marrow derived macrophages (BMDMs) derived from IStrained mice. A-B, IS was intraperitoneally injected daily for 5 days, followed by training for another 5 days. Isolated BM progenitor cells and spleen myeloid cells were differentiated or treated with LPS for 24 hr. The supernatants were collected for ELISA. C, The level of ALOX5 protein in BM cells isolated from IS-trained or control mice was analyzed by western blot. The graph illustrates the band intensity quantified by densitometry. Bar graphs show the mean ± SEM. * = $p < 0.05$ and **= $p < 0.01$, by unpaired t-test.



> *(9) Figure 7: There are no data on signaling pathway(s) that links IS and epigenetic changes, the authors therefore may want to add "?" to the proposed mechanism.*

We extend our sincere appreciation to the reviewer for providing valuable feedback. In light of the constructive comments provided by three reviewers, we have undertaken a series of additional experiments. These efforts have enabled us to propose a more elucidating schematic representation of the proposed mechanism, free of any ambiguous elements (Figure 7 in the revised manuscript). We are grateful for your insightful input.

> *(10) Demographic data (Table S2): ESRD patients have co-morbidities including diabetes (33% of subjects), CAD (28%). How did the authors factor out the co-morbidities in the overall context of their findings?*

We express gratitude to the reviewer for providing valuable comments, particularly on a noteworthy and significant aspect. The investigation employed an End-Stage Renal Disease (ESRD) Cohort involving approximately 60 subjects undergoing maintenance hemodialysis at Severance Hospital in Seoul, Korea. The subset of participants subjected to analysis consisted of stable individuals who provided informed consent and had not undergone hospitalization for reasons related to infection or acute events within the preceding three months.

> *(11) There are no data on the purity of IS.*

According to the reviewer's suggestion, we have included information regarding the purity (99%) of IS in the Methods section.

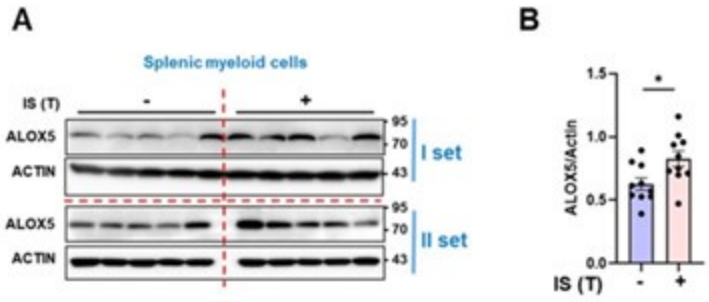
> *(12) Figure 6L: Immunoblot on b-actin were merged. This reviewer wonders how the authors analyzed these blots.*

We express gratitude for the constructive criticism provided by the reviewer, and we acknowledge and comprehend the concerns raised. In response to the reviewer's comments, a reanalysis of the ALOX5 expression level in Figure 6M was conducted, employing

immunoblot analysis on β-actin, as depicted in Figure 6L, with a short exposure time (Author response image 22).

Author response image 22.

ALOX5 protein exhibited an elevation in splenic myeloid cells obtained from IS-trained mice.



> *(13) qPCR data throughout the manuscript have control group with no error bar. The authors may not set all controls arbitrarily equal to 1 (Example Figure 1H and I). Data should be normalized in a test standard way. The average of a single datapoint may be scaled to 1, but variation must remain within the control groups.*

We express gratitude to the reviewer for their valuable feedback, acknowledging a comprehensive understanding of their perspectives. Our qPCR assays predominantly investigated the impact of various treatments on the expression of specific target genes (e.g., TNF-α, IL-6, Alox5) within monocytes/macrophages obtained from the same donors.

Subsequently, normalization of gene expression levels occurred relative to ACTINB expression, followed by relative fold-increase determination using the comparative CT method (ΔΔCT).

Statistical significance was assessed through a two-tailed paired analysis in these instances. Additionally, a substantial portion of the qPCR data was validated at the protein level through ELISA and immunoblotting techniques.

> *Minor Comments:*
>
> *(1) Molecular weight markers are missing in immunoblots throughout the manuscript.*

According to the reviewer's comment, molecular weight markers are added into immunoblots

> *(2) ESRD should be spelled out in the title.*

According to the reviewer's comment, we spelled out ESRD in the title.

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