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Glutathione Synthesis via the Cystine/Glutamate Transporter Promotes the Formation of Tertiary Lymphoid Structures in the Kidney

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Abstract:	Background: Tertiary lymphoid structure, an ectopic lymphoid tissue induced under chronic inflammation, develops in various kidney diseases and is associated with poor prognosis. The immune system requires metabolic resources to support immune function and lymphocyte proliferation. Hence, dramatic metabolic alterations presumably occur during the formation of tertiary lymphoid structure. However, it remains unclear whether metabolic remodeling occurs during this formation and its underlying mechanism. Methods: In a murine model of renal tertiary lymphoid structures, we used imaging mass spectrometry and metabolome analysis to investigate the metabolic pathway that characterizes tertiary lymphoid structures. We also performed in situ hybridization with immunofluorescence and pharmacological inhibition to explore the expression and function of the key molecules governing the pivotal metabolic pathway. We analyzed urine samples from mice and humans to explore the metabolites estimating the presence of renal tertiary lymphoid structures. Results: Significant glutathione accumulation and depletion of cysteine, which is essential for glutathione synthesis, was observed specifically within tertiary lymphoid structures. The kidneys with tertiary lymphoid structures exhibited higher glutathione concentrations than healthy kidneys. Tertiary lymphoid structures also showed significant accumulation of 4-HNE and 8-OHdG, markers of oxidative stress. Dendritic cells and fibroblasts within tertiary lymphoid structures expressed the cystine/glutamate transporter, that regulates glutathione synthesis, and supplied synthesized glutathione to lymphocytes, which lacked its expression. Pharmacological inhibition of the cystine/glutamate transporter prevented tertiary lymphoid structure formation in the kidney. Furthermore, enhanced glutathione synthesis within tertiary lymphoid structures was reflected in elevated urinary glutathione concentrations both in mice and humans, which effectively detected the presence of tertiary lymphoid structures in the kidney in IgA nephropathy patients. Conclusions: Glutathione significantly accumulated within tertiary lymphoid structures in the kidney. Inhibition of the cystine/glutamate transporter prevented the formation of tertiary lymphoid structures. Urinary glutathione served as a biomarker to detect tertiary lymphoid structures in the kidney.	
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Glutathione Synthesis via the Cystine/Glutamate Transporter Promotes the Formation of Tertiary Lymphoid Structures in the Kidney

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Abstract

Background: Tertiary lymphoid structure, an ectopic lymphoid tissue induced under chronic inflammation, develops in various kidney diseases and is associated with poor prognosis. The immune system requires metabolic resources to support immune function and lymphocyte proliferation. Hence, dramatic metabolic alterations presumably occur during the formation of tertiary lymphoid structure. However, it remains unclear whether metabolic remodeling occurs during this formation and its underlying mechanism.

Methods: In a murine model of renal tertiary lymphoid structures, we used imaging mass spectrometry and metabolome analysis to investigate the metabolic pathway that characterizes tertiary lymphoid structures. We also performed *in situ* hybridization with immunofluorescence and pharmacological inhibition to explore the expression and function of the key molecules governing the pivotal metabolic pathway. We analyzed urine samples from mice and humans to explore the metabolites estimating the presence of renal tertiary lymphoid structures.

Results: Significant glutathione accumulation and depletion of cysteine, which is essential for glutathione synthesis, was observed specifically within tertiary lymphoid structures. The kidneys with tertiary lymphoid structures exhibited higher glutathione concentrations than healthy kidneys. Tertiary lymphoid structures also showed significant accumulation of 4-HNE and 8-OHdG, markers of oxidative stress. Dendritic cells and fibroblasts within tertiary lymphoid structures expressed the cystine/glutamate transporter, that regulates glutathione synthesis, and supplied synthesized glutathione to lymphocytes, which lacked its expression. Pharmacological inhibition of the cystine/glutamate transporter prevented tertiary lymphoid structure formation in the kidney. Furthermore, enhanced glutathione synthesis within tertiary lymphoid structures was reflected in elevated urinary glutathione concentrations both in mice and humans, which effectively detected the presence of tertiary lymphoid structures in the kidney in IgA nephropathy patients.

Conclusions: Glutathione significantly accumulated within tertiary lymphoid structures in the kidney. Inhibition of the cystine/glutamate transporter prevented the formation of tertiary lymphoid structures. Urinary glutathione served as a biomarker to detect tertiary lymphoid structures in the kidney.

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Introduction

Tertiary lymphoid structures (also known as tertiary lymphoid tissues, tertiary lymphoid organs, or ectopic lymphoid tissues) are ectopic lymphoid structures that are induced in non-lymphoid organs by several stimuli, including autoimmunity, infection, and aging.¹ Tertiary lymphoid structures are mainly composed of lymphocytes and are supported, structurally and functionally, by fibroblasts with distinct phenotypes. Tertiary lymphoid structures serve as local sites of adaptive immune responses, in which vigorous lymphocyte proliferation and cytokine production occur. Tertiary lymphoid structures are formed in aged kidneys as well as in various kidney diseases, such as IgA nephropathy,² lupus nephritis,³ and kidney transplants.⁴ Recently, we reported that Tertiary lymphoid structures amplify inflammation by providing a microenvironment that allows intense interactions between kidney parenchymal and immune cells, leading to failed repair of the kidneys.^{5,6}

The immune system requires substantial metabolic resources for specific immune functions and lymphocyte proliferation. For example, glutathione is essential for the proliferation and activation of T cells,⁷ and interference of glutathione metabolism prevents normal immune responses.⁸ To meet the metabolic demand of immune cells, systemic metabolic remodeling is required.⁹ In the formation of tertiary lymphoid structures, because an enormous number of immune cells are ectopically recruited to non-lymphoid organs, it is speculated that dramatic metabolic remodeling occurs in immune cells in the organ. However, the mechanism of metabolic remodeling required for the formation of tertiary lymphoid structures remains unclear.

To elucidate this, we investigated the metabolic microenvironment within tertiary lymphoid structures in the kidney, utilizing imaging mass spectrometry and metabolome analysis. We hypothesized that specific metabolic pathways are significantly altered within tertiary lymphoid structures and play a crucial role in their formation.

Methods

Analysis of human specimens. All human specimens were analyzed after obtaining informed consent with the approval of the ethics committee at Kyoto University Hospital (Kyoto, Japan). 46 patients with IgA nephropathy at Kyoto University Hospital who underwent kidney biopsy between 2014 and 2018 were recruited (Table 1 and Supplemental Figure 1). Metabolome analysis of urine samples from the patients was conducted as described in the Supplemental Materials. For Figure 2, non-cancerous areas of nephrectomized kidney samples from patients with renal cell carcinoma were analyzed.

Imaging Mass Spectrometry. Matrix-assisted laser desorption/ionization-time of flight imaging mass spectrometry (MALDI- imaging mass spectrometry) was performed on murine kidneys as previously described.¹⁰ Image reconstruction was carried out using FlexImaging 4.1 and Image Quest 1.1 software. Full details are provided in the Supplemental Methods.

Study approval. All animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyoto20187), and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA). The sample sizes were determined based on preliminary experiments and previous studies using similar models. The chosen number of animals represents the minimum required to achieve reliable results while adhering to ethical standards and minimizing animal use in accordance with the ARRIVE checklists. All human specimens were procured and analyzed after obtaining written informed consent with the approval of the ethics committees of Graduate School of Medicine, Kyoto University (G562).

Results

Tertiary lymphoid structures actively synthesized glutathione by consuming cystine and cysteine

Tertiary lymphoid structures were formed in aged injured kidneys after ischemia–reperfusion injury (IRI)¹¹ and were detected as aggregates of B and T cells that were supported by fibroblasts with distinct phenotype (Figure 1A and 1B). To visualize small-molecule metabolites localized within tertiary lymphoid structures, we used matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) imaging mass spectrometry and found significant accumulation of glutathione (reduced form: GSH, oxidized form: GSSG) within tertiary lymphoid structures (Figure 1C). Tandem (MS/MS) imaging mass spectrometry detected the strong glutathione signals from the site of lymphocyte accumulation to adjacent sites (Figure 1D). GSH accumulation within tertiary lymphoid structures was also confirmed by Desorption Electrospray Ionization (DESI)-MS/MS imaging (Supplemental Figure 2). Signal of cysteine, which is required for glutathione synthesis (Figure 1E), was selectively depleted within tertiary lymphoid structures in injured kidneys, whereas it was uniformly distributed in healthy kidneys (Figure 1F).

Quantitative metabolome analysis of various healthy tissues revealed that the kidneys contained much more cysteine and cystine (oxidized from cysteine) than the other organs (Figure 1G). In the kidney, cystine is reabsorbed by transporters consisting of heterodimers of rBAT and either b0AT or AGT1 (Figure 1H).^{12,13} Indeed, rBAT expression was detected exclusively in the proximal tubules, indicating active cystine reabsorption under physiological conditions (Figure 1I). These results suggest that the kidney is a cyst(e)ine-rich organ and that lymphocytes in tertiary lymphoid structures may actively consume cyst(e)ine to synthesize glutathione.

To comprehensively compare metabolite levels in the glutathione synthesis pathway (Figure 1E), we performed metabolome analysis of the kidney tissue. In accordance with the imaging mass spectrometry results, GSH and GSSG levels were significantly elevated in kidneys with tertiary lymphoid structures, accompanied by an increased GSSG/GSH ratio (Figure 1J). Cysteine-glycine (Cys-Gly), a breakdown product of extracellular glutathione (Figure 1E), was also elevated in kidneys with tertiary lymphoid structures, suggesting extracellular glutathione transport.¹⁴ In contrast, cystine, cysteine, and γ -glutamylcysteine, intermediate metabolites of the glutathione synthesis pathway

(Figure 1E), were decreased in the kidneys with tertiary lymphoid structures. In addition, NADPH was decreased in the kidneys with tertiary lymphoid structures, which is consumed in the reduction of cystine to cysteine and GSSG to GSH. NADPH is produced via the pentose phosphate pathway (PPP) (Figure 1E, Supplemental Figure 3A). PPP intermediate products such as 6-phosphogluconate, ribulose-5P, sedoheptulose-7P, and glyceraldehyde-3P were increased and glucose-6P was decreased (Supplemental Figure 3B). From these findings and the fact that glutathione is essential for lymphocyte proliferation and immune responses,^{7,8} we hypothesized that abundant cystine/cysteine in the kidneys forms a unique metabolic microenvironment that supports ectopic lymphocyte proliferation, leading to the formation of tertiary lymphoid structures.

Tertiary lymphoid structures represented a unique microenvironment with high oxidative stress

We further investigated the redox status of tertiary lymphoid structures by immunostaining for two established markers of oxidative stress, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE). 8-OHdG and 4-HNE highly accumulated in both murine and human tertiary lymphoid structures in the kidney (Figure 2). To emphasize the unique redox microenvironment of tertiary lymphoid structures, we analyzed oxidative stress levels in immune cell subpopulations from tertiary lymphoid structures, spleen, and peripheral blood using the CellROX oxidative stress detection reagent. The mean fluorescence intensity (MFI) of CellROX was significantly higher in immune cells from tertiary lymphoid structures than those from the spleen or peripheral blood (Supplemental Figure 4). Although we could not exclude the influence of different isolation procedures for each organ, along with the glutathione accumulation within tertiary lymphoid structures, these findings suggest that tertiary lymphoid structures in the kidney represent a unique microenvironment characterized by a dynamic equilibrium between oxidative stress and glutathione accumulation, which may play a pivotal role in regulating the formation of tertiary lymphoid structures and their function.

Dendritic cells and fibroblasts within tertiary lymphoid structures exclusively expressed the cystine/glutamate transporter, a key molecule in glutathione synthesis pathway

System x_c^- is the cystine/glutamate transporter that transports extracellular cystine into cells, and its transport activity is regulated by the light chain subunit encoded by *Slc7a11*.¹⁵ The rate-limiting step of glutathione synthesis is cystine import via the cystine/glutamate transporter (Figure 1E), and its expression is induced under oxidative stress and during cell proliferation.^{15,16} Because tertiary lymphoid structures are accompanied by high oxidative stress (Figure 2 and Supplemental Figure 4) and active cell proliferation,¹¹ we analyzed the expression of the cystine/glutamate transporter within tertiary lymphoid structures.

The expression of *Slc7a11* in the kidneys increased after IRI, reaching a peak at day 30 and 45, when mature tertiary lymphoid structures are formed (Figure 3A).¹⁷ The localization of the cystine/glutamate transporter could not be confirmed by commercially available antibodies because of their low sensitivity and specificity.^{18,19} Therefore, we used an RNAscope probe against *Slc7a11* and revealed its expression within tertiary lymphoid structures (Figure 3B). Interestingly, some *Slc7a11* signals co-localized with the *Itgax* RNAscope signal, a dendritic cell marker whose expression increased after IRI (Figure 3, C-E). Similarly, some *Slc7a11* RNAscope signals co-localized with p75 neurotrophin receptor (p75NTR), a marker of unique fibroblasts within tertiary lymphoid structures,¹¹ whose expression (gene name: *Ngfr*) also increased after IRI (Figure 3, F-I). Analysis using *P0-Cre/R26 tdTomato* mice, which lineage labels resident fibroblasts²⁰ as well as fibroblasts within tertiary lymphoid structures,¹¹ also showed that some *Slc7a11* signals co-localized with tdTomato-positive fibroblasts within tertiary lymphoid structures (Figure 3, J-L, arrowheads). Based on RNAscope analysis, approximately 24% (mean) of dendritic cells and 14% (mean) of fibroblasts expressed the cystine/glutamate transporter (Supplemental Figure 5).

Contrary to our initial expectations, the cystine/glutamate transporter was expressed in very limited cell populations within tertiary lymphoid structures; intercellular glutathione transport from these cells to lymphocytes was expected.^{14,21-24} Expression of *Abcc1*, which encodes MRP1 that is responsible for extracellular glutathione efflux (Figure 1E),²⁵ also increased after IRI (Figure 3M). MRP1 expression was shown to be localized to *Slc7a11*-positive dendritic cells (Figure 3, N-P) and

fibroblasts within tertiary lymphoid structures (Figure 3, Q-S). Analysis on P0-Cre/R26 tdTomato mice also showed that tdTomato-positive fibroblasts within tertiary lymphoid structures co-expressed *Slc7a11* and MRP1 (Figure 3, T-V, arrowheads). In snRNAseq dataset of mouse kidneys with tertiary lymphoid structures,⁵ *Slc7a11* was strongly expressed in dendritic cells and fibroblasts (Figure 3W), consistent with RNAscope findings. Taken together, these findings suggest that glutathione synthesis within tertiary lymphoid structures may be distinctly regulated, with dendritic cells and fibroblasts that express the cystine/glutamate transporter playing an important role.

Pharmacological inhibition of the cystine/glutamate transporter suppressed the formation of tertiary lymphoid structures

Glutathione is essential for lymphocyte activation and proliferation,⁷ but lymphocytes lack *Slc7a11* expression and have a low cystine uptake capacity.^{26,27} Therefore, other cell types must support lymphocytes for glutathione synthesis. *In vitro*, dendritic cells and fibroblasts expressing *Slc7a11* contribute to extracellular redox regulation via glutathione synthesis, and maintain cysteine supply to *Slc7a11*-deficient cells, such as lymphocytes.^{14,21-24} However, it is unclear whether this mechanism contributes to the formation of tertiary lymphoid structures *in vivo*.

To investigate this, we pharmacologically inhibited the cystine/glutamate transporter in the model of tertiary lymphoid structures. Sulfasalazine, a potent inhibitor of the cystine/glutamate transporter, was administered daily from day 14 after IRI, when the formation of tertiary lymphoid structures was initiated (a preventive model; Figure 4A). Interestingly, the formation of tertiary lymphoid structures was significantly inhibited in the kidneys of sulfasalazine-treated mice (Figure 4, B-D). Sulfasalazine treatment also decreased the expression of *Cd4* (a T-cell marker) and *Cd19* (a B-cell marker), consistent with the suppression of the formation of tertiary lymphoid structures (Figure 4E). Furthermore, the expression of tertiary lymphoid structure-associated homeostatic chemokines and cytokine (*Cxcl13*, *Ccl19*, and *Ifng*) also decreased.

Although the formation of tertiary lymphoid structures was shown to correlate with kidney damage,¹⁷ *Havcr1* (the gene encoding Kim1) and *Colla1* mRNA expression was not attenuated by sulfasalazine treatment, despite the suppression of tertiary lymphoid structures (Figure 4F). Immunofluorescence of Kim1 and α SMA also showed that the degree of tubular injury and interstitial fibrosis was similar in both groups (Figure 4G). This could be attributed to sulfasalazine-induced nephrotoxicity, as sulfasalazine is known to induce crystalluria and accumulation of reactive oxygen species (ROS) in the kidney.^{28,29} Kim1 expression increased with higher doses of sulfasalazine (Supplemental Figure 6), suggesting a dose-dependent nephrotoxic effect. Similar to Kim1, desmin expression was observed in IRI-kidneys from both sulfasalazine- and vehicle-treated mice, and in contralateral kidneys its expression was more prominent in sulfasalazine-treated mice (Supplemental Figure 7). These results indicate that sulfasalazine induces tubulointerstitial injury, and that inhibition of the formation of tertiary lymphoid structures by sulfasalazine is not mediated by the attenuation of kidney damage.

Metabolome analysis confirmed that sulfasalazine treatment decreased glutathione and Cys-Gly levels in the kidney (Figure 4H). Although cyst(e)ine levels did not fully recover, presumably because of impaired cystine reabsorption due to sustained tubular injury by sulfasalazine,^{28,29} cystine levels tended to increase in the sulfasalazine group. This could be attributed to attenuated formation of tertiary lymphoid structures that consume cyst(e)ine to synthesize glutathione.

We also administered sulfasalazine daily from day 30, when tertiary lymphoid structures were already established, to day 45 post-IRI to assess whether it could revert established tertiary lymphoid structures (a treatment model; Supplemental Figure 8A). Although the effect was not as pronounced as in a preventive model in Figure 4, sulfasalazine treatment from day 30 partially reversed the formation of tertiary lymphoid structures (Supplemental Figure 8B-D), with a corresponding decrease in the expression of tertiary lymphoid structure-related molecules (Supplemental Figure 8E). In addition, we assessed the effect of sulfasalazine in unilateral ureteral obstruction (UUO) model, another established model of renal tertiary lymphoid structure formation (Supplemental Figure 9A). Although tertiary lymphoid structures were smaller in size compared to IRI model, they became evident by day 14 following UUO. The formation of tertiary lymphoid structures was significantly

inhibited in the kidneys of sulfasalazine-treated mice (Supplemental Figure 9B-D). The expression of *Cxcl13* and *Ifng* was reduced by sulfasalazine treatment, presumably because of the reduced size of tertiary lymphoid structures (Supplemental Figure 9E). Consistent with the findings in IRI model, GSH levels were reduced in the kidney of sulfasalazine-treated mice (Supplemental Figure 9F), presumably reflecting the attenuation of the formation of tertiary lymphoid structures. These findings indicate that sulfasalazine attenuates tertiary lymphoid structure formation in two independent models.

To further support the *in vivo* findings, we conducted *in vitro* co-culture experiments that mimic the microenvironment of tertiary lymphoid structures (Supplemental Figure 10A). A previous report confirmed intercellular glutathione exchange between dendritic cells and lymphocytes, and showed its inhibition by sulfasalazine;¹⁴ to complement this, we employed *Slc7a11*-specific siRNA (Supplemental Figure 10B). We found that intracellular glutathione levels in T cells co-cultured with *Slc7a11*-knockdown (KD) dendritic cells significantly decreased under both normal conditions and oxidative stress induced by tert-butyl hydroperoxide (TBHP) (Supplemental Figure 10, C-E). In contrast, T cells co-cultured with *Slc7a11*-KD fibroblasts did not show significant decrease in intracellular glutathione levels under normal conditions, but a decrease was observed under oxidative stress. Furthermore, the decrease of intracellular glutathione levels was also observed in T cells co-cultured with dendritic cells or fibroblasts pre-treated with sulfasalazine (Supplemental Figure 11). These findings suggest that intercellular glutathione exchange occurs between lymphocytes and dendritic cells or fibroblasts, and plays an important role under oxidative stress conditions, as seen in tertiary lymphoid structures.

The inhibition of the cystine/glutamate transporter hinders glutathione synthesis and induces ferroptosis, a regulated form of cell death characterized by excessive accumulation of lipid peroxides.^{30,31} Considering the significant reduction in tertiary lymphoid structures on day 30 post-IRI, we investigated oxidized lipids within the kidneys on day 21 post-IRI, a time point corresponding to active formation of tertiary lymphoid structures. The formation of tertiary lymphoid structures was suppressed in the kidneys of sulfasalazine-treated mice on day 21, although still observed (Figure 5A, 5B, Supplemental Figure 12). TUNEL-positive cells and 4-HNE accumulation were observed within

tertiary lymphoid structures in both sulfasalazine-treated and vehicle-treated mice, while cleaved caspase-3, an apoptosis marker, was not evident in either group (Figure 5C). TUNEL-positive cells within tertiary lymphoid structures increased in the sulfasalazine-treated group, while the number of Ki67-positive cells remained unchanged (Figure 5C, 5D). These findings indicate that non-apoptotic cell death might occur within tertiary lymphoid structures and further expedited by the inhibition of the cystine/glutamate transporter. The ratio of oxidized phosphatidylcholines (OxPCs) to non-oxidized phosphatidylcholines (PCs; the most abundant phospholipid in mammalian cells³²) was elevated in the kidneys of vehicle-treated mice (Figure 5E). Interestingly, OxPCs accumulated to a greater extent in the kidneys of sulfasalazine-treated mice. In particular, higher levels of PC(25:1;O) (1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine; PONPC) and PC(21:1;O) (1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine; POVPC), heavily toxic OxPCs that trigger ferroptosis,³³⁻³⁵ accumulated in sulfasalazine-treated kidneys after IRI. We also examined the expression of key ferroptosis-related genes, *Gpx4* and *Acsf4*. In the kidneys with tertiary lymphoid structures, *Gpx4* expression was modestly decreased while *Acsf4* expression was increased compared to controls, consistent with ferroptosis-associated changes (Supplemental Figure 13). Following sulfasalazine treatment, *Gpx4* expression showed a slight increase toward baseline levels, whereas *Acsf4* expression remained elevated. Although further validation is required, these results may suggest that the inhibition of the cystine/glutamate transporter could disrupt the equilibrium between cell proliferation and cell death (possibly ferroptosis) within tertiary lymphoid structures, leading to attenuation of tertiary lymphoid structures.

Urinary GSH was a candidate biomarker for tertiary lymphoid structures in mice and patients with IgA nephropathy

Next, we examined whether glutathione accumulation within tertiary lymphoid structures is reflected in urinary GSH concentrations. Indeed, urinary GSH levels increased over the course of the formation of tertiary lymphoid structures after IRI (Figure 6A). On the contrary, serum GSH levels did not differ significantly between mice with tertiary lymphoid structures and control mice

(Supplemental Figure 14), suggesting that increased urinary GSH concentrations are not simply a reflection of elevated serum GSH levels. Additionally, we compared urinary GSH levels in aged mice with tertiary lymphoid structures and in young mice after the same extent of IRI, which lack tertiary lymphoid structures in the kidney, as previously demonstrated.¹¹ Unlike aged mice, urinary GSH levels in young mice did not significantly increase, despite both groups experiencing the same extent of IRI (Figure 6B). This indicates that the upregulation of glutathione synthesis within tertiary lymphoid structures is possibly reflected by increased urinary GSH concentrations. Urinary GSH levels also tended to decrease following sulfasalazine treatment, possibly due to the reduction of tertiary lymphoid structures (Figure 4 and Figure 6C).

Next, we comprehensively investigated the metabolites in urine samples from 46 patients with IgA nephropathy (Table 1 and Supplemental Figure 1) by non-targeted metabolome analysis. The presence or absence of tertiary lymphoid structures in the kidney was confirmed by PAS and immunofluorescence staining (Figure 6D and 6E). Similar to murine tertiary lymphoid structures and human tertiary lymphoid structures in nephrectomized kidneys (Figure 2), 4-HNE and 8-OHdG accumulation was also observed within tertiary lymphoid structures of IgA nephropathy patients (Supplemental Figure 15). Multivariable analysis (partial least squares-discriminant analysis [PLS-DA]) revealed that urinary metabolite profiles differed depending on the presence or absence of tertiary lymphoid structures in the kidney, and GSH was one of the metabolites that exhibited the most significant alterations (Figure 6F and 6G). Indeed, urinary GSH levels were significantly elevated in patients with tertiary lymphoid structures in the kidney (Figure 6H). A multivariable logistic regression model was constructed to estimate the presence of tertiary lymphoid structures in the kidney with two covariables: urinary GSH levels and estimate glomerular filtration rate (eGFR) (Table 2). This model showed high accuracy in estimating the presence of tertiary lymphoid structures in the kidney and the receive operating characteristic (ROC) curve revealed a high area under the curve (AUC) value of 0.92 (Figure 6I). The optimal sensitivity and specificity were 86% and 83%, respectively. These results suggest that similar metabolic alterations occur in murine and human kidneys with tertiary lymphoid structures and that urinary GSH could be a noninvasive diagnostic biomarker to detect tertiary lymphoid structures in the kidney.

In addition, we analyzed urinary GSH levels in patients with tertiary lymphoid structures in the kidney at the time of diagnosis and one year later. Of the 18 patients with tertiary lymphoid structures in the kidney recruited in Figure 6, nine received steroid treatment and had urine samples available at both time points (Supplemental Table 1). While overall urinary GSH levels before and after the treatment did not show a significant difference (Supplemental Figure 16A), a significant decrease in urinary GSH levels was observed in seven patients who experienced more than a 50% reduction in urinary protein levels following steroid treatment (Supplemental Figure 16B). While we were unable to confirm the regression of tertiary lymphoid structures due to the lack of re-biopsy, together with the previous reports indicating that steroid treatment reduces the size of tertiary lymphoid structures¹⁷ and that the presence of tertiary lymphoid structures is correlated with increased proteinuria in IgA nephropathy patients,² these findings possibly suggest that urinary GSH levels may reflect the resolution of tertiary lymphoid structures in certain subpopulations of IgA nephropathy patients.

Discussion

There is a lack of knowledge about alterations of the metabolic system that supports the formation of tertiary lymphoid structures. In this study, we revealed that active glutathione synthesis contributes to the formation of tertiary lymphoid structures. We further demonstrated that an increase in urinary GSH concentrations during the formation of tertiary lymphoid structures is a common phenomenon in both mice and humans, and proposed the utility of urinary GSH as a biomarker to detect tertiary lymphoid structures in the kidney.

Lymphocyte proliferation and immune responses require vast metabolic resources, especially thiols such as cysteine and glutathione.^{7,8} Although the rate of glutathione synthesis depends on cysteine availability, cysteine is quickly oxidized to its dimer form cystine in the extracellular space (Figure 1E). Indeed, cysteine concentrations in serum and culture medium are very low compared to that of cystine.^{36,37} Therefore, cells that require glutathione for their survival and proliferation must import cystine from the extracellular environment via the cystine/glutamate transporter (encoded by *Slc7a11*). The expression of the cystine/glutamate transporter is induced in the context of high oxidative stress and cell proliferation,^{15,38,39} and its inhibition hinders glutathione synthesis leading to

ferroptosis.^{30,31,40,41} In immune cells, T cells do not express the cystine/glutamate transporter and depend instead on thiol supply from other cell types for their survival *in vitro*.^{14,24,26,42,43} However, it was unclear whether the intercellular glutathione exchange shown *in vitro* also occurs *in vivo*.

Dendritic cells and fibroblasts play important roles in the formation of tertiary lymphoid structures.^{11,44} Dendritic cells express the cystine/glutamate transporter, import cystine, and export synthesized glutathione to the extracellular space via MRP1.^{14,21,24} Thereby, dendritic cells maintain the surrounding environment in a reducing state and support lymphocyte proliferation.^{14,21} Fibroblasts also import cystine via the cystine/glutamate transporter and reduce it to cysteine, maintaining the pericellular environment in a reducing state *in vitro*.^{22,23} In this study, we found that dendritic cells and fibroblasts within tertiary lymphoid structures in the kidney exclusively express the cystine/glutamate transporter, and that glutathione supply from these cells to lymphocytes seems to be required for the formation of tertiary lymphoid structures and maintenance *in vivo*. Intercellular glutathione exchange between dendritic cells/fibroblasts and lymphocytes was also suggested by our co-culture experiment (Supplemental Figure 10, 11), although we acknowledge that our *in vitro* experiments do not fully recapitulate the *in vivo* phenomena. While the number of *Slc7a11*-expressing dendritic cells or fibroblasts within tertiary lymphoid structures is limited (Supplemental Figure 5), and the mechanism by which this small population contributes to the substantial glutathione accumulation within tertiary lymphoid structures (Figure 1) remains unclear, our findings suggest that *Slc7a11*-expressing dendritic cells and fibroblasts play a pivotal role in driving glutathione synthesis within the microenvironment of tertiary lymphoid structures.

Sulfasalazine is a potent inhibitor of the cystine/glutamate transporter that inhibits glutathione synthesis and induces ferroptosis,^{30,31} and is regarded as a promising medication for cancer.^{16,41,45-47} Although there are several inhibitors of the cystine/glutamate transporter other than sulfasalazine, these drugs have limitations of low bioavailability and low selectivity to of the cystine/glutamate transporter *in vivo*.^{48,49} In our study, sulfasalazine administration inhibited the formation of tertiary lymphoid structures and attenuated the expression of tertiary lymphoid structure-related cytokines, with sustained tubular injury. Although these findings do not completely exclude the effect on kidney parenchymal cells or off-target effects, these results suggest that sulfasalazine prevents the formation

of tertiary lymphoid structures not by attenuating tubular injury but via its inhibitory effect on of the cystine/glutamate transporter specifically expressed within tertiary lymphoid structures. Although the direct application of sulfasalazine in patients with kidney diseases is difficult because of its nephrotoxicity,^{28,29} the inhibition of specific metabolic pathways of tertiary lymphoid structures may represent a new therapeutic approach against kidney diseases.

Tertiary lymphoid structures in the kidney are associated with aggravated histological inflammation and impaired kidney function, representing novel therapeutic targets in kidney diseases.⁶ We previously revealed that the stage and number of tertiary lymphoid structures correlate with the extent of tissue damage in both mice and humans, and prognosis of kidney transplants.^{4,17} Another group showed that the presence of tertiary lymphoid structures correlates with tissue damage and poor prognosis in patients with IgA nephropathy.² Therefore, early detection of tertiary lymphoid structures in the kidney could identify patients who would benefit from prompt treatment. As demonstrated in this study, urinary GSH levels accurately estimated the presence of tertiary lymphoid structures in the kidney in patients with IgA nephropathy. Thus, urinary GSH may have important clinical implications as a promising biomarker for identifying tertiary lymphoid structures in the kidney.

Our study has some limitations. First, we could not perform imaging mass spectrometry in human samples because of the need for unfixed, immediately frozen tissue samples. Second, RNAscope of *Slc7a11* in human kidney samples was unsuccessful, probably because of the sample fixation conditions. Third, since conditional *Slc7a11*-knockout mice were unavailable, our *in vivo* analysis relied on pharmacological inhibition of the cystine/glutamate transporter and cannot completely exclude off-target effects of sulfasalazine or the contribution of other cell types beyond dendritic cells and fibroblasts in glutathione synthesis.

Despite these limitations, our study revealed the unique metabolic microenvironment of tertiary lymphoid structures in the kidney (Supplemental Figure 17). Glutathione synthesis plays key roles in the formation of tertiary lymphoid structures, and a specific metabolic pathway in a limited cell population within tertiary lymphoid structures determines the outcome of the formation of tertiary lymphoid structures. Furthermore, elevated glutathione synthesis in tertiary lymphoid structures in the

kidney is reflected in urinary GSH concentration, suggesting the possibility of its clinical application as a novel biomarker to detect tertiary lymphoid structures in the kidney. Future studies will further elucidate the currently unrecognized metabolic landscape of tertiary lymphoid structures.

Acknowledgments

We thank Akiko Yamashita, Chihiro Ishizaki, Yoriko Setsuda, and Maki Ozone at Kyoto University Hospital Department of Nephrology for their excellent technical assistance. This work was partly supported by the World Premier International Research Center Initiative (WPI), MEXT, Japan. Part of this work appeared as an abstract at the annual meeting of the American Society of Nephrology.

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Supplemental Table 1: Changes in serum creatinine, eGFR, and urinary protein levels in IgA nephropathy patients at baseline and one year after kidney biopsy.

Supplemental Table 2: Primer sequences used for real-time PCR

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Figure Legends

Figure 1. Tertiary lymphoid structures in the kidney consume cyst(e)ine to synthesize glutathione.

(A) Experimental protocol for (B-D, F, J).

(B) Hematoxylin-eosin (HE) staining and immunofluorescence of B220 (green), CD3 ϵ (red), and DAPI (blue) of the kidneys 45 days after IRI.

(C) HE staining, immunofluorescence of B220 (green) and CD3 ϵ (red), and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) imaging mass spectrometry of GSH (m/z 306.0) and GSSG (m/z 611.1) in the kidneys 45 days after sham surgery and IRI. Sections used for imaging mass spectrometry were stained by HE after imaging mass spectrometry procedure, and their serial sections were analyzed by immunofluorescence.

(D) Tandem (MS/MS) imaging mass spectrometry of GSH and GSSG in the kidneys 45 days after IRI. Data were processed by the SCiLS lab software (Bruker Daltonics, Billerica, MA, USA). The color bar indicates peak intensity levels at m/z 306.0 (GSH) or 611.1 (GSSG), with red and blue representing high and low signals, respectively. The regions delineated by dotted squares were enlarged for detailed demonstration on the right.

(E) Scheme of intercellular glutathione metabolism.

(F) Orbitrap imaging mass spectrometry of cysteine and GSH in the kidneys 45 days after sham surgery and IRI. Kidney cortex and tertiary lymphoid structures are encircled by solid and dotted lines, respectively.

(G) Metabolome analysis of cysteine and cystine in the liver, brain, spleen, heart, skeletal muscle (Muscle), white adipose tissue (WAT), brown adipose tissue (BAT), and kidney ($n = 4$, each).

(H) Scheme of cystine transport mechanism on kidney tubules.

(I) Immunofluorescence of rBAT (red), LTL (green), and DAPI (blue) in the healthy kidney.

(J) Metabolome analysis of metabolites related to glutathione synthesis pathway and GSSG/GSH ratio in the kidneys 45 days after sham surgery and IRI ($n = 5$ in each group).

Values are means $\pm$ SE. Data were analyzed by (G) nonparametric multiple comparisons with Dunnett-type contrasts using the nparcomp R package with kidney as control and (J) Mann-Whitney

U test.

Scale bars: (B) 500 μ m, (C) 500 μ m, (D) 200 μ m, (F) 500 μ m, and (I) 50 μ m.

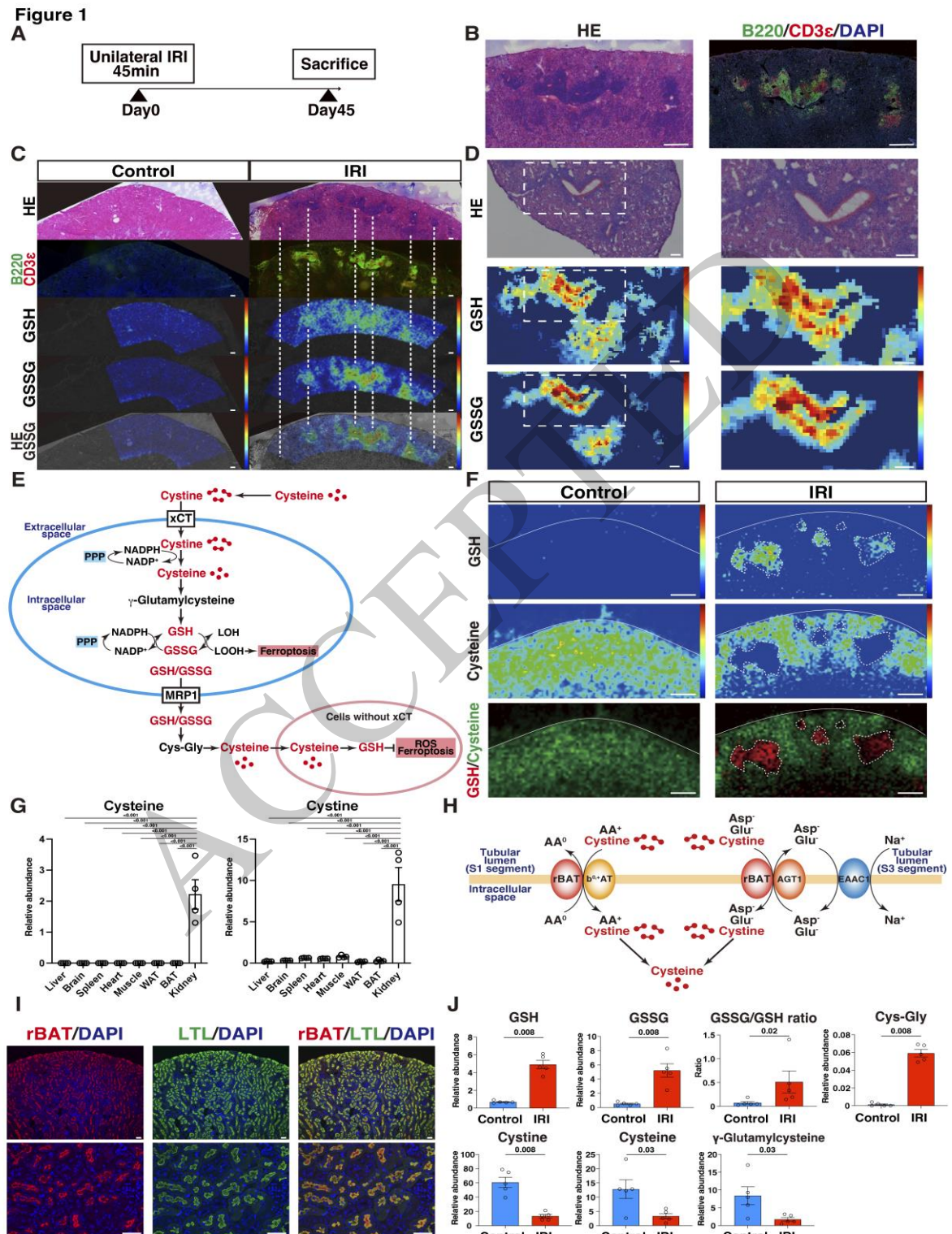


Figure 2. 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) accumulate within tertiary lymphoid structures in the kidney of both mice and humans.

Immunohistochemistry of 8-OHdG and 4-HNE in murine and human kidneys with tertiary lymphoid structures and control kidneys. Human nephrectomized kidney samples in which tertiary lymphoid structures were confirmed by immunostaining were used. 8-OHdG and 4-HNE were detected by alkaline phosphatase (red) and DAB (brown) staining, respectively. Sections were counterstained with eosin. Tertiary lymphoid structures are encircled by dotted lines. We investigated nephrectomized kidney samples from patients with renal cell carcinoma, focusing on the non-cancerous areas of the kidney where tertiary lymphoid structures were observed.

Scale bars: 50 μ m.

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Figure 2

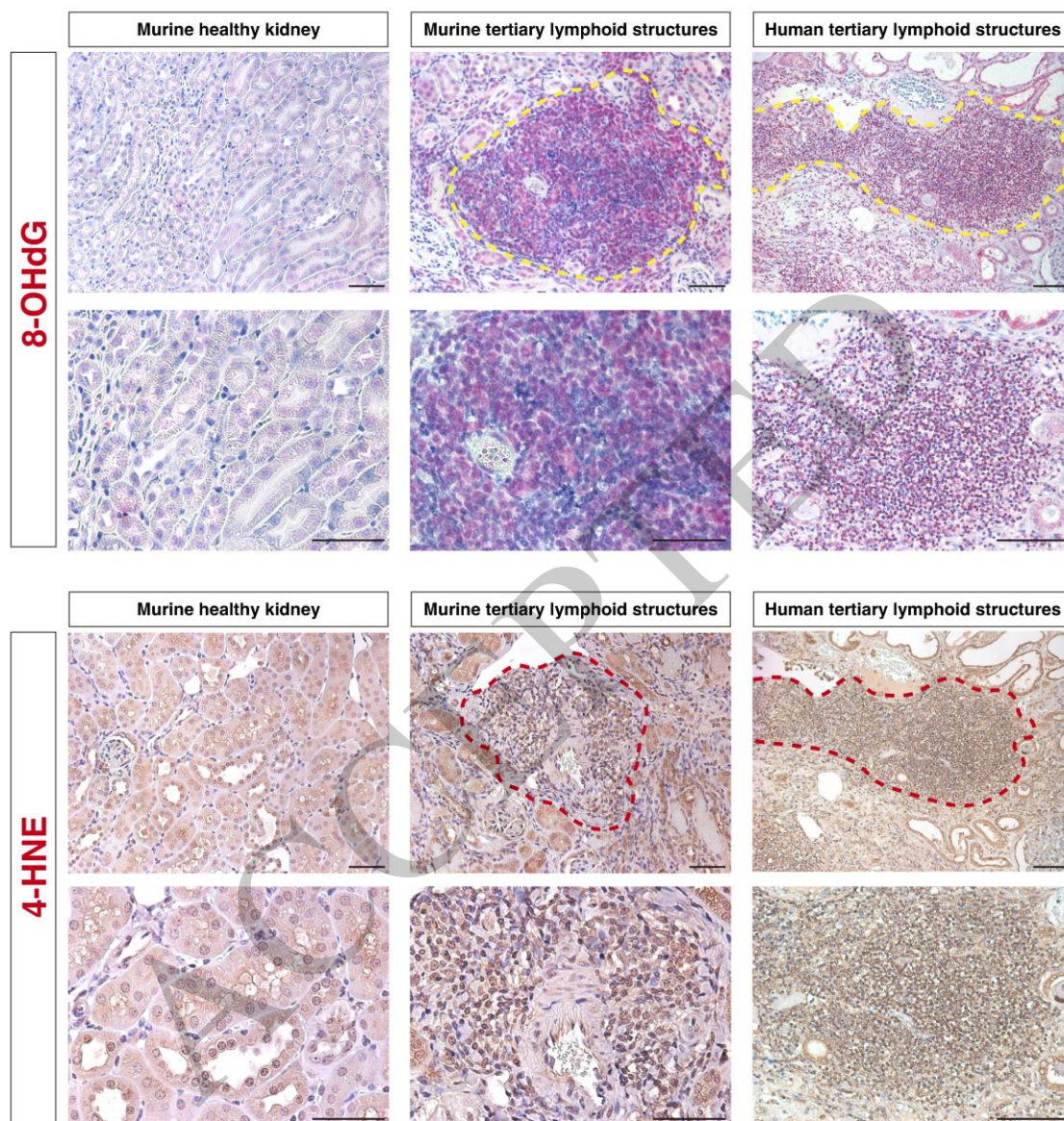


Figure 3. Selective expression of *Slc7a11* and MRP1 in dendritic cells and fibroblasts within tertiary lymphoid structures in the kidney.

(A) *Slc7a11* mRNA levels in the kidneys at day 4, 14, 30, and 45 after sham surgery and ischemia-reperfusion injury (IRI) (n = 4 at each timepoint).

(B-D) *In situ* hybridization (ISH) with RNAscope of *Slc7a11* (red) and *Itgax* (light blue) combined with DAPI staining (blue) in the kidneys 45 days after IRI. The region delineated by dotted squares in (C) is enlarged for detailed demonstration in (D). White arrows indicate *Slc7a11*⁺*Itgax*⁺ cells.

(E) *Itgax* mRNA levels in the kidneys at day 4, 14, 30, and 45 after sham surgery and IRI (n = 4 at each timepoint).

(F-H) Combination of ISH (*Slc7a11* [red] and *Itgax* [light blue]) and immunofluorescence (p75NTR [green] and DAPI [blue]) in the kidneys 45 days after IRI. The regions delineated by dotted squares in (F) are enlarged for detailed demonstration in (G) and (H). White arrowheads represent *Slc7a11*⁺p75NTR⁺ cells.

(I) *Ngfr* (p75NTR) mRNA levels in the kidneys at day 4, 14, 30, and 45 after sham surgery and IRI (n = 4 at each timepoint).

(J-L) ISH of *Slc7a11* (green) combined with DAPI staining (blue) in P0-Cre/tdTomato mice 45 days after IRI (red: tdTomato signals in P0-lineage cells). The regions delineated by dotted squares in (J) are enlarged for detailed demonstration in (K) and (L). Artery is encircled by dotted line. White arrows and arrowheads indicate *Slc7a11*⁺tdTomato⁻ cells and *Slc7a11*⁺tdTomato⁺ cells, respectively.

(M) *Abcc1* (MRP1) mRNA levels in the kidneys at day 4, 14, 30, and 45 after sham surgery and IRI (n = 4 at each timepoint).

(N-P) Combination of ISH (*Slc7a11* [red] and *Itgax* [light blue]) and immunofluorescence (MRP1 [green] and DAPI [blue]) in the kidneys 45 days after IRI. The region delineated by dotted square in (N) is enlarged for detailed demonstration in (O) and (P);

(Q-S) A combination of ISH (*Slc7a11* [red]) and immunofluorescence (MRP1 [green], p75NTR [light blue], and DAPI [blue]) in the kidneys 45 days after IRI. The region delineated by dotted square in (Q) is enlarged for detailed demonstration in (R) and (S). White arrowheads represent *Slc7a11*⁺MRP1⁺p75NTR⁺ cells.

(T-V) A combination of ISH (*Slc7a11* [green]) and immunofluorescence (MRP1 [gray]) in P0-Cre/tdTomato mice 45 days after IRI (red: tdTomato signals in P0-lineage labeled cells). The regions delineated by dotted squares in (T) are enlarged for detailed demonstration in (U) and (V). White arrowheads indicate *Slc7a11*⁺MRP1⁺tdTomato⁺ cells.

(W) Dot plots displaying expression patterns of *Slc7a11* for each cluster by sn-RNAseq on kidneys 30 days after 45-minute IRI.⁵

(B, C, F, N, Q) The areas encircled by the dashed line indicate tertiary lymphoid structures.

Values are means ± SE. Data in (A, E, I, M) were analyzed by non-parametric trend test.

Scale bars (B-D, F-H, J-L, N-V): 10 μm.

Figure 3

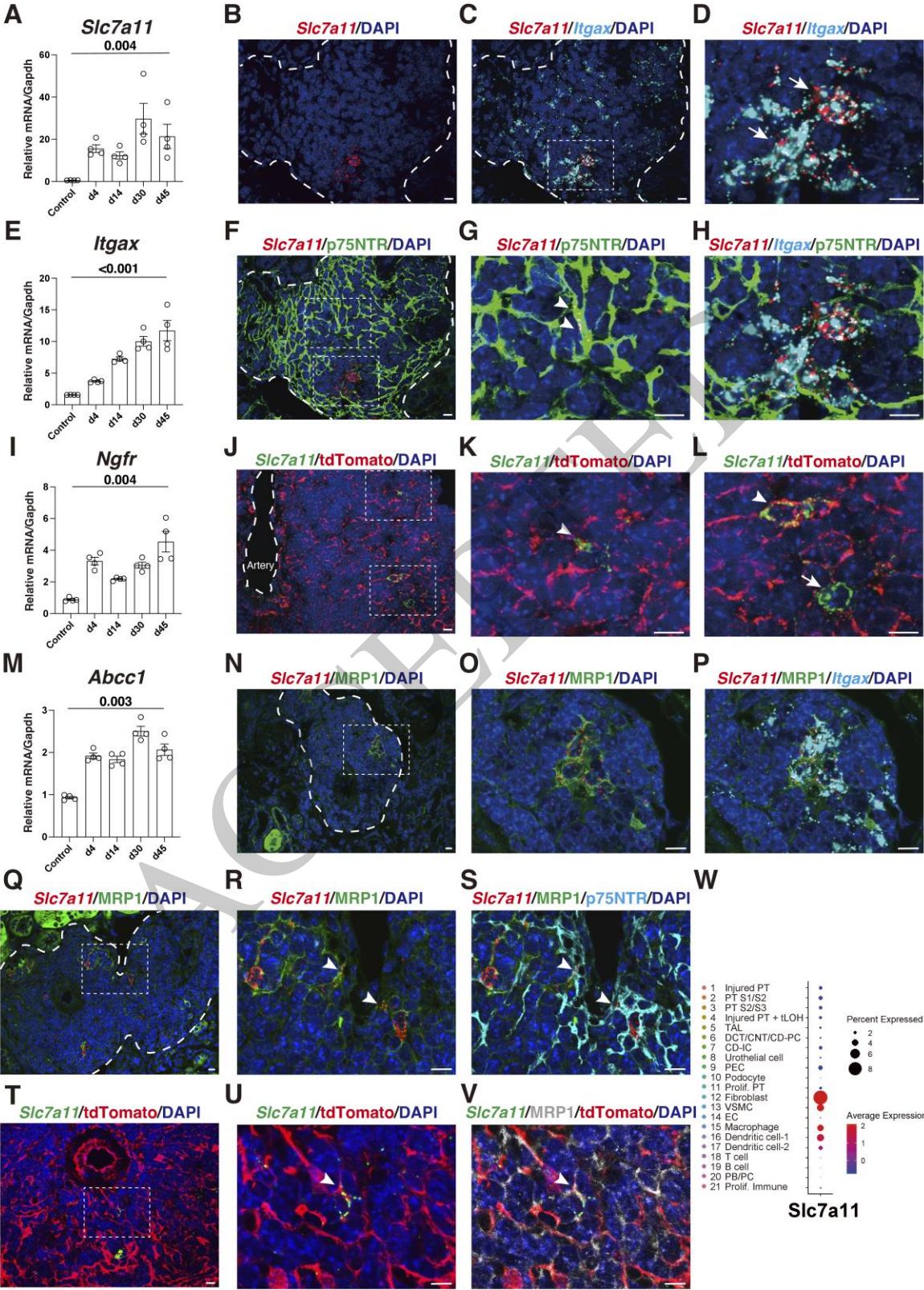


Figure 4. Inhibition of the cystine/glutamate transporter prevents the formation of tertiary lymphoid structures in aged injured kidney.

(A) Experimental protocol for (B-H).

(B) Representative images of the kidneys 30 days after IRI treated with sulfasalazine or vehicle from day14 in Periodic acid–Schiff (PAS) staining. Tertiary lymphoid structures are encircled by yellow lines.

(C) Cumulative sizes of tertiary lymphoid structures per kidney cortex area (n = 6 in each group).

(D) Immunofluorescence of B220 (green), CD3 ϵ (red), and DAPI (blue) and p75NTR (green), CD11c (red), and DAPI (blue) in the kidneys 30 days after IRI treated with sulfasalazine or vehicle from day14.

(E) *Cd4*, *Cd19*, *Cxcl13*, *Ccl19*, and *Ifng* mRNA levels in the IRI kidneys (Control: kidneys 30 days after sham operation, n = 5; Vehicle: kidneys 30 days after IRI treated by vehicle from day14, n = 6; Sulfasalazine: the kidneys 30 days after IRI treated with sulfasalazine 400 mg/kg from day 14, n = 6).

(F) *Havcr1* and *Colla1* mRNA levels in the IRI kidneys (Control: kidneys 30 days after sham operation, n = 5; Vehicle: kidneys 30 days after IRI treated by vehicle from day14, n = 6; Sulfasalazine: the kidneys 30 days after IRI treated with sulfasalazine 400 mg/kg from day 14, n = 6).

(G) Immunofluorescence of Kim1 (green), α SMA (red), and DAPI (blue) of the IRI kidneys treated with either vehicle or sulfasalazine.

(H) Metabolome analysis of metabolites related to glutathione synthesis pathway (n = 4 or 5 in each group).

Values are means $\pm$ SE. Data were analyzed by (C) Mann-Whitney U test and (E, F, H) Steel test with the vehicle-treated group as control.

Scale bars: (B) 300 μ m; (D, G) 50 μ m.

Figure 4

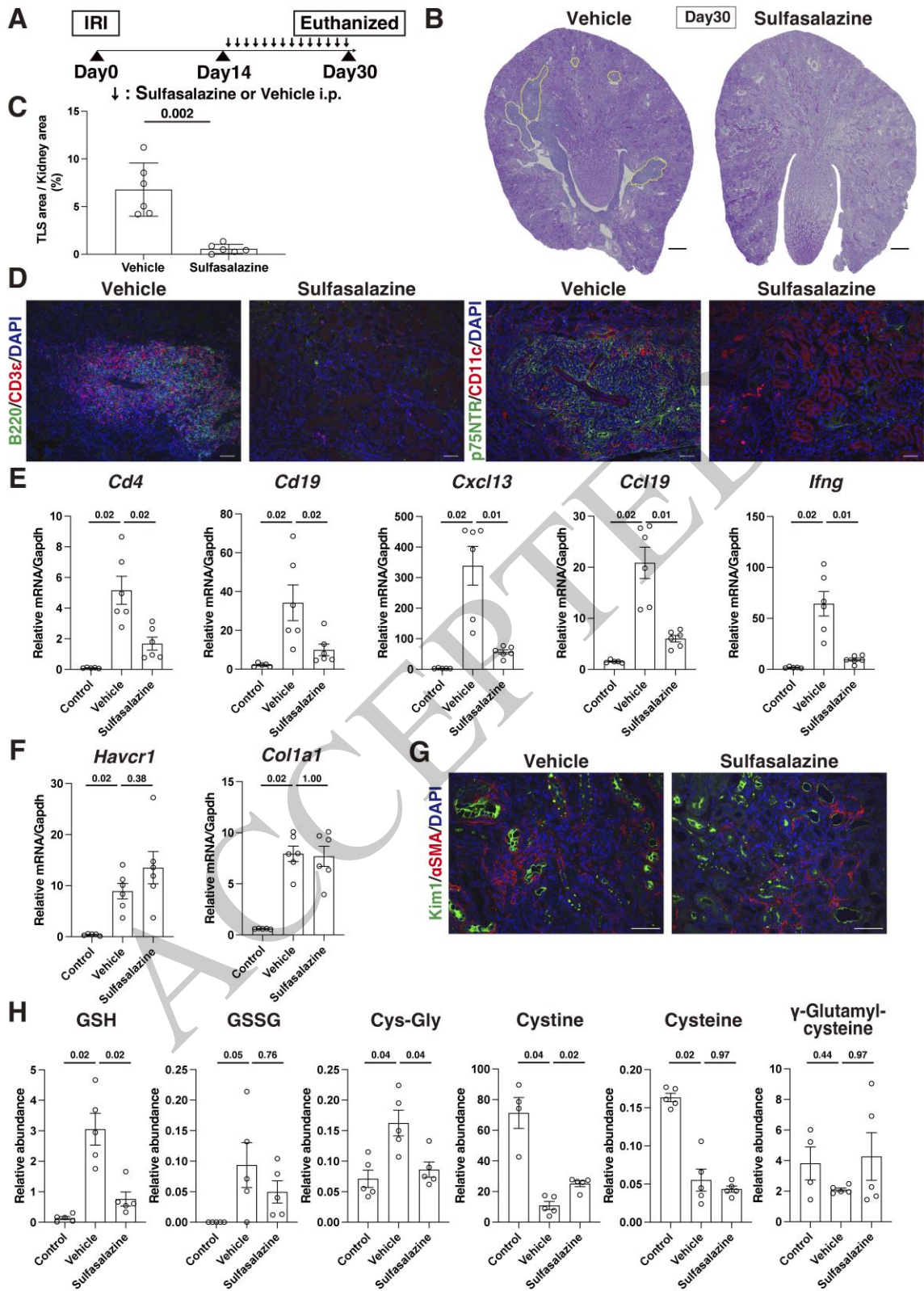


Figure 5. Oxidized lipids that trigger ferroptosis accumulate in sulfasalazine-treated kidneys after IRI.

(A) Representative images of the kidneys 21 days after IRI treated with sulfasalazine or vehicle from day 14 in PAS staining. Sulfasalazine or vehicle was administered in the same protocol as in Figure 4A. Tertiary lymphoid structures are encircled by the yellow lines.

(B) Quantification of the size of tertiary lymphoid structures in the kidneys at day 14 (n = 5), day 21 (n = 5), and day 30 after IRI (n = 6 in each group).

(C) TUNEL staining (red) with DAPI staining (blue), immunohistochemistry of 4-HNE and cleaved caspase 3, and immunofluorescence for Ki67. Samples of the healthy spleens and kidneys of the cisplatin nephropathy model were used as positive controls for TUNEL staining and immunohistochemistry of cleaved caspase 3, respectively. 4-HNE and cleaved caspase 3 were detected by DAB staining;

(D) Numbers of TUNEL-positive cells and Ki67-positive cells within tertiary lymphoid structures per the size of tertiary lymphoid structures in the kidneys treated with sulfasalazine or vehicle 21 days after IRI.

(E) Ratio of oxidized phosphatidylcholines (OxPCs) per PCs in the kidneys treated with sulfasalazine or vehicle 21 days after IRI. Contralateral kidneys in each group were used as control (n = 5 in each group). PC(25:1;O), PC(21:1;O), and PC(20:1;O) are oxidized form of linoleic acid in PC(34:2), arachidonic acid in PC(36:4), and docosahexaenoic acid in PC(38:6), respectively.

Values are means $\pm$ SE. Data were analyzed by (B) multiple Mann-Whitney U test, (D) Mann-Whitney U test, and (E) 2-way ANOVA after aligned rank transform.

Scale bars: (A) 300 μ m; (C) 50 μ m.

Figure 5

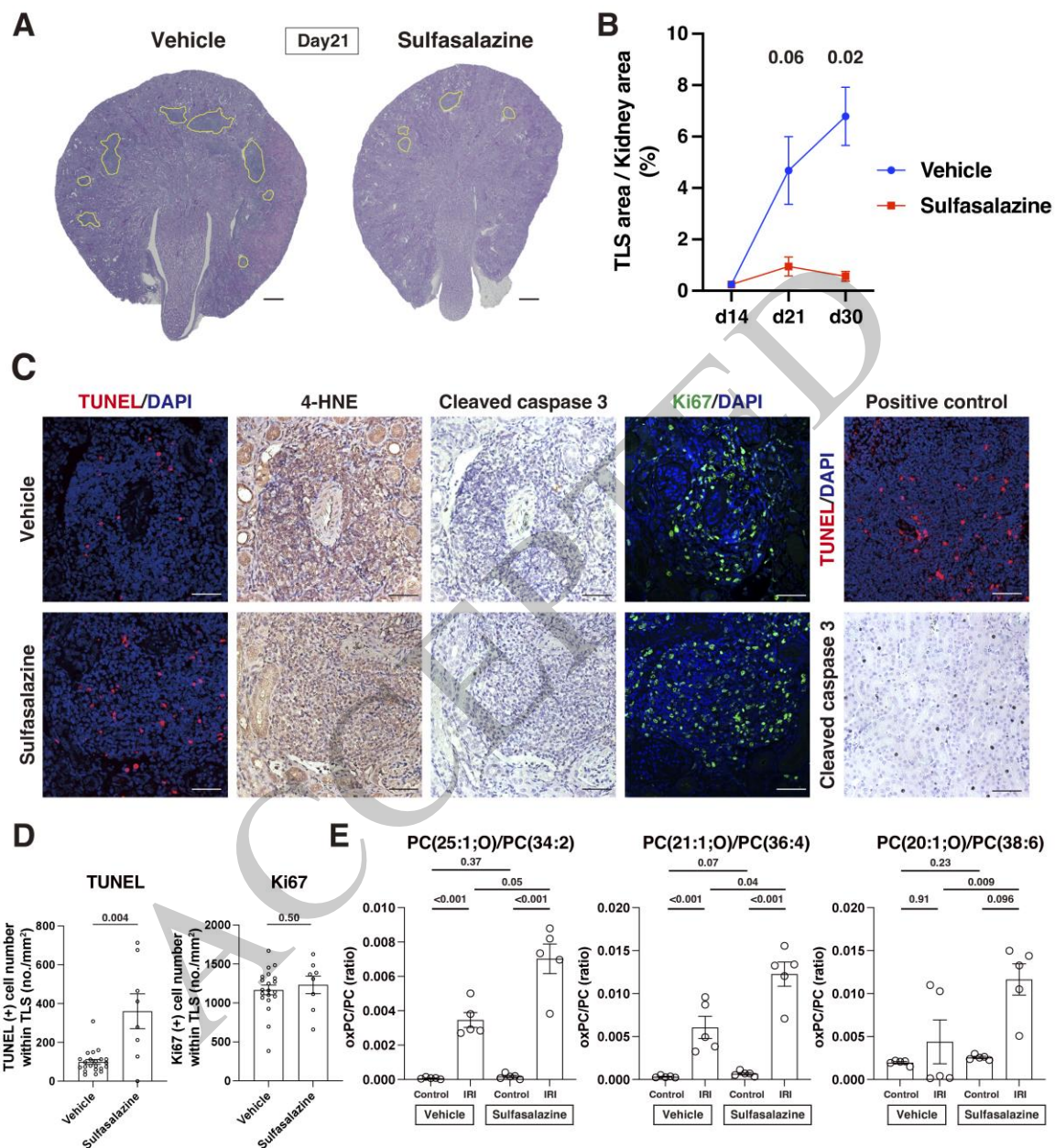


Figure 6. Urinary GSH concentrations as a potential biomarker for the presence of tertiary lymphoid structures in the kidney in mice and patients with IgA nephropathy.

(A) Urinary GSH levels at indicated time points after IRI and sham surgeries in aged mice (n = 5 in each group).

(B) Urinary GSH levels in young and aged mice at day 30 after IRI and sham surgeries (n = 5 in each group).

(C) Urinary GSH levels of mice 30 days after IRI, treated with either sulfasalazine or vehicle in the model illustrated in Figure 4 (Control: mice 30 days post-sham operation, n = 4; Vehicle: mice 30 days post-IRI, treated with vehicle from day 14, n = 5; Sulfasalazine: mice 30 days post-IRI, treated with 400 mg/kg sulfasalazine from day 14, n = 5).

(D) Representative images of PAS staining of human kidney biopsy samples with and without tertiary lymphoid structures from IgA nephropathy patients. The regions delineated by squares areas are enlarged for detailed demonstration. Tertiary lymphoid structures are encircled by dotted lines.

(E) Immunofluorescence of CD20 (red), CD3 ϵ (green), and DAPI (blue), CD21 (red), Ki67 (green), and DAPI (blue), and p75NTR (green), CXCL13 (red), and DAPI (blue) in human kidneys with tertiary lymphoid structures.

(F) Principal component plot to represent the clustering patterns of metabolite profiles in urine samples from IgA nephropathy patients with tertiary lymphoid structures [TLS (+)] and without tertiary lymphoid structures [TLS (-)] in the kidneys. Each point corresponds to a unique urine sample, and the distance between points reflects the similarity of metabolite compositions. Green and red dots represent urine samples from patients with and without tertiary lymphoid structures, respectively.

(G) Volcano plot of metabolite profiles of urine samples from IgA nephropathy patients. Each point on the plot represents a metabolite, with the x-axis indicating fold change and the y-axis indicating statistical significance. Upregulated and downregulated metabolites with $-\text{Log}_{10}(p) > 1$ and $\text{Log}_2(\text{FC}) > 1$ are highlighted in red and blue, respectively. The five metabolites exhibiting the most pronounced statistical significance are listed.

(H) GSH levels in urine samples of IgA nephropathy patients with and without tertiary lymphoid structures in the kidney (n = 18 and 28, respectively).

(I) Receive operating characteristic (ROC) curve to illustrate the performance of the logistic regression model shown in Table 2. The solid line connecting dots represents the model's discrimination, with an area under the curve (AUC) of 0.92.

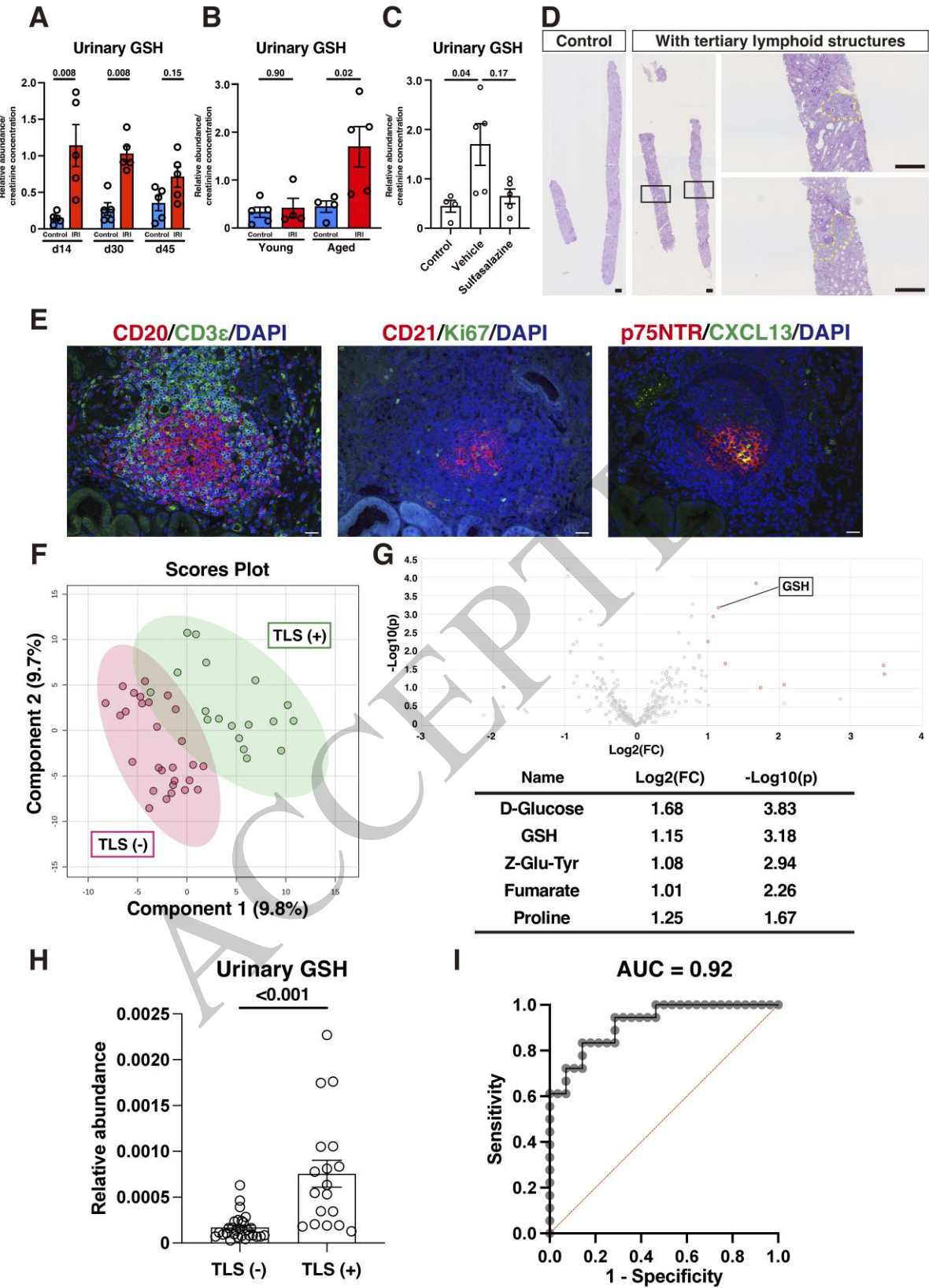
GSH levels were measured by mass spectrometry and normalized by the creatinine concentration.

Values are means $\pm$ SE. Data were analyzed by (A, B, H) Mann-Whitney U test and (C) Steel test with the vehicle-treated group as control.

GSH, glutathione reduced form. TLS, Tertiary lymphoid structures.

Scale bars: (D) 250 μm ; (E) 20 μm .

Figure 6



	Total (n=46)	Without tertiary lymphoid structures (n=28)	With tertiary lymphoid structures (n=18)
Age (year)	40 (26-49)	32 (24-42)	53 (35-70)
Gender (men/women)	17/29	7/21	10/8
Body mass index (kg/m ²)	21.3 (18.9-22.9)	20.9 (18.5-22.4)	21.9 (20.0-23.7)
Serum creatinine (mg/dL)	0.96 (0.61-1.09)	0.73 (0.58-0.92)	1.31 (0.84-1.83)
eGFR (mL/min/1.73m ²)	75 (53-94)	90 (76-102)	52 (29-64)
UPCR (g/gCr)	0.98 (0.23-0.82)	0.45 (0.18-0.63)	1.82 (0.30-2.91)
Diabetes (n (%))	3 (7)	1 (4)	2 (11)
Hypertension (n (%))	12 (26)	1 (4)	11 (61)

Table 1: Background of IgA nephropathy patients with or without tertiary lymphoid structures in the kidney.

Data are median (25th-75th percentile) or percentage.

Categorical and continuous variables were analyzed by Pearson's chi-square test and Wilcoxon rank-sum test, respectively.

eGFR: Estimate glomerular filtration rate. UPCR: Urinary protein to creatinine ratio.

Two variables	Odds ratio (95%CI)
Urinary GSH	13.72 (1.35-139.23)
eGFR	0.28 (0.07-1.07)

Table 2: Multivariable logistic regression analysis of determinants associated with tertiary lymphoid structures in the kidney.

All variables are standardized and re-defined as variables/SD. GSH: glutathione reduced form.

Glutathione synthesis via the cystine/glutamate transporter promotes the formation of tertiary lymphoid structures in the kidney

Methods



Model of tertiary lymphoid structures in kidney



Imaging mass spectrometry and metabolome analysis *in situ* hybridization to explore regulation of metabolic pathway



Analysis of urine samples from mice and humans to estimate presence of tertiary lymphoid structures in the kidney

Findings



Significant glutathione accumulation and depletion of cysteine, as well as the accumulation of 4-HNE and 8-OHdG, was observed in tertiary lymphoid structures in the kidney. In addition, kidneys with tertiary lymphoid structures exhibited higher glutathione concentrations than healthy kidneys.



Dendritic cells and fibroblasts in tertiary lymphoid structures expressed the cystine/glutamate transporter and supplied synthesized glutathione to lymphocytes which lacked its expression.



Pharmacological inhibition of the cystine/glutamate transporter prevented tertiary lymphoid structure formation in the kidney.



Enhanced glutathione synthesis in tertiary lymphoid structures was reflected in elevated urinary glutathione concentrations in mice and humans, which effectively detected the presence of tertiary lymphoid structures in the kidney in IgA nephropathy patients.

Conclusions: Glutathione significantly accumulated within tertiary lymphoid structures in the kidney. Inhibition of the cystine/glutamate transporter prevented the formation of tertiary lymphoid structures. Urinary glutathione served as a biomarker to detect tertiary lymphoid structures in the kidney.

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Supplemental Material

Glutathione synthesis via the cystine/glutamate transporter promotes the formation of tertiary lymphoid structures in the kidney

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Supplemental table 1: Changes in serum creatinine, eGFR, and urinary protein levels in IgA nephropathy patients at baseline and one year after renal biopsy.

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Supplemental Methods

Animals

We purchased 12-month-old C57BL6J male mice from Japan SLC. All mice were maintained under specific-pathogen-free conditions in the animal facility of Kyoto University. All animal experiments were approved by the Animal Research Committee, Graduate School of Kyoto University, and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA). For generating P0-Cre/tdTomato mice, transgenic mice expressing Cre under the control of the P0 promoter (P0-Cre mice)¹ were mated with R26 tdTomato mice² to obtain double-transgenic mice.

Kidney injury models

Unilateral ischemia–reperfusion injury (IRI) was induced as described previously.³ Briefly, mice were maintained at 37°C under anesthesia with 2% isoflurane inhalation. The left kidney was exteriorized through a small incision. IRI was induced by clamping the unilateral renal pedicles for 45 min. Adenine nephropathy was induced by feeding a 0.20% adenine diet (Research Diets, New Brunswick, NJ, USA) as previously described.⁴

Compound administration

Sulfasalazine (catalog S0883; Sigma-Aldrich, Saint Louis, MO, USA) (400 mg/kg) was administered to the mice by intraperitoneal injection daily from day14 to day30 after IRI (n = 6). Fresh sulfasalazine solution was prepared every day in 10% DMSO and 90% sterile phosphate-buffered saline.

Renal histochemistry

Harvested kidney samples were fixed in 10% formalin solution, embedded in paraffin, sectioned

(2.0 μm), and stained with periodic acid–Schiff (PAS). All PAS-stained samples were analyzed using a Keyence BZ-X710 all-in-one microscope and Zeiss Axio Imager A2 microscope.

Renal immunofluorescence

Immunofluorescence studies of mouse and human kidneys were performed as previously described.⁵ The harvested kidney samples were fixed in 4% paraformaldehyde, incubated in 20% sucrose in PBS overnight, and incubated in 30% sucrose overnight at 4°C. OCT-embedded kidneys were cryosectioned into 6.0- μm sections. Formaldehyde-fixed and paraffin-embedded kidney samples were sectioned at a 4.0- μm thickness and used for immunofluorescence analysis. The sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval at 15 minutes at 110°C with the citrate buffer or 10 minutes at 95°C with Dako Target Retrieval Solution, pH 9 (catalog S2367; Dako, Glostrup, Denmark) was performed for deparaffinized and rehydrated sections. These sections were blocked with 5% serum appropriate for secondary antibodies for 1 hour at room temperature and then incubated with primary antibodies for overnight at 4°C. The following primary antibodies were used for immunostaining: anti-CD3 ϵ (catalog ab5690; Abcam, Cambridge, UK), anti-CD20 (catalog 14-0202; eBioscience, San Diego, CA), anti-B220 (catalog 557390; BD PharMingen, San Diego, CA, USA), anti-CD11c (catalog 550283; BD PharMingen), anti-rBAT (catalog 16343-1-AP; Proteintech, Rosemont, IL, USA), anti-MRP1 (catalog ALX-801-007; Enzo Life Sciences, Farmingdale, NY, USA), anti-p75NTR (catalog AF1157; R&D Systems, Minneapolis, MN, US. catalog ab52987; Abcam), anti-TIM-1/KIM1/HAVCR (catalog AF1817; R&D Systems), Cy3-conjugated anti- α -smooth muscle actin (αSMA) (catalog C6198; Sigma-Aldrich), anti-CD11b (catalog 14-0112-82; eBioscience), anti-PDGFR β (catalog 12-1402; eBioscience), and anti-CXCL13 (catalog MAB8012; R&D Systems). Staining was visualized using appropriate secondary antibodies. Lotus tetragonolobus lectin, FITC conjugate (catalog FL1321; Vector Laboratories, Newark, CA, USA) was also used for staining. Cell nuclei were counterstained with DAPI and mounted in mounting medium

(Fluoromount, catalog K024; Diagnostic BioSystems, Pleasanton, CA, USA). Immunofluorescence samples were analyzed using an OLYMPUS FV1000-D confocal microscope and Zeiss LSM900 confocal microscope.

Immunohistochemistry

For immunohistochemistry of mouse and human kidneys, formaldehyde-fixed and paraffin-embedded sections were deparaffinized and rehydrated, as described above, for immunofluorescence. After rehydration, endogenous peroxidase was blocked with 3% H₂O₂, and antigen retrieval was performed as described above for immunofluorescence. The sections were stained with the following primary antibodies: anti-8-Hydroxy-2'-deoxyguanosine [N45.1] (catalog ab48508; Abcam), anti-4-Hydroxynonenal (catalog ab46545; Abcam), anti-GPX4 (catalog ab125066; Abcam), anti-GCLM (catalog ZRB2011; Sigma-Aldrich), and anti-Cleaved caspase 3 (catalog 9664; CST). Antibody labeling was detected using a Histofine Simple Stain MAX-PO(R) (catalog 414341; NICHIREI BIOSCIENCES, Tokyo, Japan) and a Diaminobenzidine (DAB) Substrate Kit (catalog SK-4100; Vector Laboratories) for rabbit primary antibodies and a Histofine Simple Stain AP(M) (catalog 414241; NICHIREI BIOSCIENCES) and an ImmPACT Vector Red Substrate Kit (catalog SK-5105; Vector Laboratories) for mouse primary antibodies. Sections were counterstained using Mayer's Hematoxylin Solution (catalog 131-09665, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and mounted in malinol (catalog 20091; MUTO PURE CHEMICALS, Tokyo, Japan). For TUNEL staining, antigen retrieval was performed using 20 µg/mL Proteinase K (catalog 3115836001; Roche, Basel, Switzerland) at 37°C for 30 minutes. TUNEL staining was performed with the In Situ Cell Death Detection Kit, TMR Red (catalog 12156792910; Roche) according to the manufacturer's instructions.

RNAscope *in situ* hybridization

In situ hybridization was performed using RNAscope Multiplex Fluorescent Assay V2 (catalog 323100; Advanced Cell Diagnostics [ACD], Newark, CA, USA) on mouse kidney samples fixed with 4% paraformaldehyde for 24 h, OCT-embedded, cryosectioned at 10- μ m thickness, or fixed with 10% neutralized formaldehyde for 24 h, paraffin-embedded, and sectioned at 4.0- μ m thickness, according to the manufacturer's instructions. The following RNAscope Target Probes were used: Mm-Slc7a11 (catalog 42251; ACD) and Mm-Itgax-C2 (catalog 311501-C2; ACD).

Measurement of the size of tertiary lymphoid structures

The sizes of tertiary lymphoid structures in the kidney were examined in PAS-stained sections of injured kidneys as previously described⁶. Briefly, the size of tertiary lymphoid structures was defined as the total cumulative size of the sizes of tertiary lymphoid structures in the renal cortex of the sample. Images that included tertiary lymphoid structures were taken at the same size and resolution, and its size was measured by an experienced renal pathologist using the Adobe Photoshop software (Adobe Inc., San José, CA, USA) .

Real-time RT-PCR analysis

RNA extraction and real-time RT-PCR were performed as described previously⁶. The primer sequences are listed in the Supplementary Table S1. The expression levels were normalized to those of mouse *Gapdh*.

Imaging mass spectrometry

Matrix-assisted laser desorption/ionization-time of flight imaging Mass spectrometry (MALDI-IMS) of murine kidneys was performed as previously reported.^{7,8} Briefly, freshly frozen tissues were sectioned at a thickness of 8.0 μ m using a cryostat (CM 3050, Leica) . Frozen sections were thawed and mounted onto indium-tin-oxide (ITO) -coated glass slides (Bruker Daltonics, Billerica, MA, USA) , followed by spray-coating with 9-aminoacridine as the matrix (10 mg/mL, dissolved

in 80% ethanol). MALDI imaging was performed using a MALDI-TOF mass spectrometer (UltraFlextreme, Bruker Daltonics) equipped with an Nd:YAG laser and an Orbitrap mass spectrometer (Q-Exactive Focus, Thermo Fisher Scientific, Waltham, MA, USA) combined with atmospheric pressure matrix-assisted desorption/ionization (AP-SMALDI10, TransMIT). Data were acquired in the negative mode with raster scanning at a pitch distance of 50 μm . Image reconstruction was performed using the FlexImaging 4.1 software (Bruker Daltonics) and Image Quest 1.1 (Thermo Fisher Scientific). Amino acids were imaged by MALDI-IMS using an on-tissue chemical derivatization method as previously described.⁹

Desorption Electrospray Ionization-Multiple-Reaction-Monitoring Mass Spectrometry (DESI-MRM) data acquisition was performed by integrating a Waters DESI-XS ionization source with a Waters Xevo TQ Absolute (TqA) mass spectrometer. Methanol/water (98:2 v/v) was delivered from the Waters nanoAcquity Binary Solvent Manager through an M-Class Symmetry C18 column to the DESI sprayer at a flow rate of 2 $\mu\text{L}/\text{min}$. High voltage applied to the sprayer generated an electrospray that was pneumatically directed at a 75° angle toward the tissue section using a nitrogen gas stream. For glutathione detection, MRM parameters (m/z 307.76>178.81) were set in positive ion detection mode. The DESI spray voltage, Heater Transfer Line (HTL) temperature, and nitrogen gas flow were set to 0.8 kV, 150 °C, and 0.15 MPa, respectively, to ensure optimal performance in negative ionization mode. These optimized settings were saved in a Waters .ipr file. Prior to biological sample analysis, the motion and precise alignment of the x,y stage in the optimized DESI-MRM setup was verified using black ink (m/z 666.06) as a test analyte. The DESI-MRM experiment configuration was completed using HDImaging v1.7 and the DESI Method Editor (Waters).

Metabolome analysis

Comprehensive metabolome analysis of murine kidneys was performed as previously reported.¹⁰ Briefly, frozen kidney tissues were dissolved in 500 μL of methanol containing internal standard

(IS) using a homogenizer (Finger Masher, AM79330, Sarstedt, Tokyo, Japan). Equal volumes of ultrapure water and 0.4× volume of chloroform were then added and mixed. The mixture was then centrifuged at $15,000 \times g$ for 15 min at 4°C. The resulting supernatant was filtered through an ultrafilter tube (Ultrafree-M C, UFC3 LCC NB; Human Metabolome Technologies, Tsuruoka, Japan). The filtrate was then concentrated by nitrogen flow-assisted evaporation on a heating block (DTU-28N, TAITEC, Koshigaya City, Japan). The concentrated filtrate was resuspended in 50 µL of ultrapure water for subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) and ion chromatography-high resolution (IC-HR)-MS analytical procedures.

The metabolome analysis of anionic metabolites was performed using an Orbitrap mass spectrometer (Q-Exactive Focus, Thermo Fisher Scientific) connected to a high-performance IC system (ICS-5000+, Thermo Fisher Scientific). The ion chromatography system was equipped with an anion electrolytic suppressor (Dionex AERS 500; Thermo Fisher Scientific) to convert the potassium hydroxide gradient to pure water before entering the mass spectrometer. Separation was performed using a Dionex IonPac AS11 -HC-4 µm IC column (Thermo Scientific) with an IC flow rate of 0.25 mL/min, and the post column was supplemented with a methanol make-up flow at 0.18 mL/min for IC separation. The concentration gradient conditions for potassium hydroxide were as follows: 1 mM to 100 mM (0-40 min), 100 mM (40-50 min), and 1 mM (50.1-60 min); column temperature was 30°C. The Orbitrap mass spectrometer was operated in ESI negative mode for all detections. A full mass scan (m/z 70-900) was performed at a resolution of 70,000. The automatic gain control target was set to 3×10^6 ions with a maximum ion implantation time of 100 ms. Ionization parameters of the ion source were as follows: spray voltage 3 kV, transfer temperature 320°C, S-Lens level 50, heater temperature 300°C, sheath gas 36, and aux gas 10.

LC-MS/MS was used for metabolomic analysis of cationic metabolites. A triple quadrupole mass spectrometer (LCMS-8060, SHIMADZU) equipped with a direct-going electrospray (ESI) ion source was used in the positive and negative ESI and multiple reaction monitoring modes.

Samples were prepared on a Discovery HS F5-3 column (2.1 ID × 150 mm L, 3-μm particle, Sigma-Aldrich) using a step gradient of mobile phase A (0.1% formic acid) and mobile phase B (0.1% acetonitrile). A step gradient of mobile phase A:mobile phase B was performed at a ratio of 100:0 (0-5 min), 75:25 (5-11 min), 65:35 (11-15 min), 5:95 (15-20 min), 100:0 (20-25 min), flow rate of 0.25 mL/min and column temperature of 40°C.

Oxidized lipid analysis

Lipids and oxidized lipids were extracted by adding 100 μL of a 1:1 (v/v) solution of 1-butanol and methanol, containing 100 μM butylated hydroxytoluene (BHT) and 100 μM ethylenediaminetetraacetic acid (EDTA), to the cell pellets. The mixture was vortexed for 10 s, sonicated for 15 min in an ice-cooled sonic bath, and then centrifuged at 16,000 × g for 10 minutes at 20 °C. The resultant supernatant was transferred into a 0.2 mL glass insert equipped with a Teflon-lined cap for subsequent LC ESI-MS analysis.

The analysis of (oxidized) lipids utilized a Q-Exactive Focus mass spectrometer (Thermo Fisher Scientific) linked to an Ultimate 3000 HPLC system (Thermo Fisher Scientific). Chromatographic separation was achieved on a Thermo Scientific Accucore C18 column (2.1 × 150 mm, 2.6 μm). Mobile phase A comprised 10 mM ammonium formate in 50% acetonitrile with 0.1% formic acid, while mobile phase B consisted of 2 mM ammonium formate in a blend of acetonitrile, isopropyl alcohol, and water (10:88:2, v/v/v) with 0.02% formic acid. The applied step gradient proceeded as follows: from 65:35 at onset to 40:60 over 0 to 4 min, shifting to 15:85 from 4 to 12 minutes, then to 0:100 from 12 to 21 minutes, maintained at 0:100 from 21 to 24 min, reverted to 65:35 over 24 to 24.1 min, and finally to 100:0 from 24.1 to 28 min, with a flow rate of 0.4 mL/min and a column temperature maintained at 35°C.

The Q-Exactive Focus mass spectrometer was operated in both ESI positive and negative ion modes. A full mass scan (m/z 250–1100) was followed by three rapid data-dependent MS/MS scans with resolutions of 70,000 and 17,500. The instrument settings included an automatic gain

control target of 1×10^6 ions and a maximum ion injection time of 100 ms. Ion source adjustments were set with a spray voltage of 3 kV, a transfer tube temperature of 285°C, an S-Lens level of 45, a heater temperature of 370°C, a sheath gas flow rate of 60, and an auxiliary gas flow rate of 20. Data analysis was conducted using Qual Browser (Thermo Fisher Scientific) specifically for the assessment of oxidized lipids.

Urine metabolome analysis

Frozen urine (50 μ L) was mixed with 500 μ L of methanol containing IS and a standard (2-morpholinoethanesulfonic acid), followed by the addition of equal volumes of ultrapure water and 0.4 volume of chloroform (LC/MS grade, FUJIFILM Wako Pure Chemical Corporation). The resulting suspension was centrifuged at $2,800 \times g$ for 15 min at 4°C. After centrifugation, the aqueous phase was ultrafiltered in ultrafiltration tubes (Ultrafree MC-PLHCC; Human Metabolome Technologies). The filtrate was then concentrated by nitrogen flow-assisted evaporation on a heating block (DTU-28N, TAITEC). The concentrated filtrate was dissolved in 50 μ L of ultrapure water and analyzed using IC-HR-MS and LC-MS/MS. Urinary metabolite levels were calculated as relative values and were corrected for urinary creatinine concentrations. Data were analyzed using MetaboAnalyst 5.0 software (<https://www.metaboanalyst.ca>).

Cell Culture

DC2.4 cells, mouse dendritic cells, were purchased from Sigma-Aldrich (catalog SCC142) and cultured with RPMI-1640 (catalog R0883; Sigma-Aldrich) supplemented with 10% FBS (catalog CCP-F BS-BR-500; Cosmo Bio, Tokyo, Japan), 1X L-Glutamine (catalog TMS-002-C; Sigma-Aldrich), 1X non-essential amino acids (catalog 06344-56; Nacalai Tesque, Kyoto, Japan), 1X HEPES Buffer Solution (catalog 17557-94; Nacalai Tesque), 100 μ g/ml penicillin-streptomycin (catalog 26253-84; Nacalai Tesque), and 0.0054X β -Mercaptoethanol (catalog 133-14571; FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer's instructions.

C3H10T1/2 cells, mouse embryonic fibroblasts, were purchased from the Health Science Research Resources Bank (HSRRB, Osaka, Japan) and cultured with DMEM low glucose (catalog 08490; Nacalai Tesque) supplemented with 10% FBS and 100 µg/ml penicillin-streptomycin. Cells were incubated at 37°C in a 5% CO₂ incubator.

Knockdown of Slc7a11 in DCs and fibroblasts by siRNA

Slc7a11 knockdown in DC2.4 cells and C3H10T1/2 cells was performed as previously described.^{11 12} Briefly, DC2.4 cells and C3H10T1/2 cells were seeded at a density of 4×10^4 cells/well in a 12-well plate using the complete media described earlier. After 48 hours, cells were transfected with ON-TARGETplus Mouse Slc7a11 SMARTpool siRNA (catalog L-047420-01-0005; Horizon Discovery, Cambridge, UK) or a non-targeting control siRNA (catalog D-001810-10-05; Horizon Discovery) using the Lipofectamine 3000 Transfection Kit (catalog L3000-015; Thermo Fisher Scientific), according to the manufacturer's instructions. After siRNA treatment, the culture medium of DC2.4 cells and C3H10T1/2 cells was replaced with fresh medium containing 8 ng/mL recombinant murine TNF- α (catalog 315-01A; PeproTech, Cranbury, NJ, USA) and 10 ng/mL IFN- γ (catalog 315-05; PeproTech) to induce xCT expression. Cells were harvested and analyzed 48 hours post-transfection.

T Cell Isolation from murine spleens by magnetic-activated cell sorting

T cells were isolated from murine spleen using magnetic-activated cell sorting (MACS) by negative selection according to the manufacturer's instructions, with the Pan T Cell Isolation Kit II, mouse (catalog 130-095-130; Miltenyi Biotec, Bergisch Gladbach, Germany). Spleens were harvested from mice and single-cell suspensions were prepared by gently mashing the tissue through a 40 µm cell strainer using the plunger of a syringe. Erythrocytes were lysed using RBC lysis buffer (catalog 130-094-183; Miltenyi Biotec) for 5 minutes at room temperature, followed by washing with MACS buffer (PBS containing 0.5% bovine serum albumin and 2 mM EDTA).

The resulting splenocytes were counted and resuspended in MACS buffer at a concentration of 10^8 cells per 400 μ L. Cells were then incubated with a biotin-antibody cocktail (50 μ L per 10^8 cells) for 5 minutes at 4°C to label non-T cells. Subsequently, anti-biotin microbeads (100 μ L per 10^8 cells) were added and incubated for an additional 10 minutes at 4°C. The cell suspension was then passed through an LS column placed in a MACS separator. The flow-through containing the unlabeled T cells was collected. The purity of isolated T cells was confirmed by flow cytometry analysis using anti-CD3 antibody, consistently yielding >98% purity. The entire isolation procedure was completed within 4 hours to minimize cellular stress and maintain T cell functionality.

Co-culture Experiments

Co-culture experiment of T cells with DC2.4 cells and C3H10T1/2 cells were performed as previously described.¹³ 48 hours post-siRNA treatment, the culture media of DC2.4 cells and C3H10T1/2 cells were removed and replaced with co-culture medium consisting of RPMI-1640 supplemented with 10% FBS, 1X L-Glutamine, 1X non-essential amino acids, 1X HEPES buffer solution, and 100 μ g/mL penicillin-streptomycin, without β -Mercaptoethanol. T cells were then added to each well at a 1:4 ratio of DCs or fibroblasts to T cells and co-cultured for 24 hours at 37°C in a 5% CO₂ incubator. To maintain the viability of T cells, 1 μ g/mL anti-CD3 antibody (catalog 16-0032-82; eBioscience) and 2 μ g/mL anti-CD28 antibody (catalog 16-0281-85; eBioscience) were added to the co-culture medium. 24 hours after starting the co-culture, intracellular glutathione levels of T cells were measured by fluorescence-activated cell sorting (FACS) using the glutathione detection reagent ThiolTracker Violet (catalog T10095; Invitrogen, Waltham, MA, USA). For inducing oxidative stress, we treated cells by 200 μ M tert-Butyl Hydroperoxide (TBHP, catalog 180342500; Thermo Fisher Scientific) at 37°C for 1 hour and analyzed intracellular glutathione levels. For sulfasalazine treatment, dendritic cells or fibroblasts were pre-treated with 500 μ M sulfasalazine or vehicle (DMSO) for 24 hours, based on a previous

study,¹³ before initiating co-culture with T cells. Intracellular glutathione levels were then analyzed after 24 hours using ThiolTracker Violet as described above.

Mononuclear cell isolation from the kidney, spleen, and peripheral blood

Single-cell suspensions of the kidneys, spleen, and peripheral blood were obtained in the previously described method.⁴ Briefly, after the collection of 100 μ L of blood, mice were perfused with Hank's Balanced Salt Solution (HBSS, catalog 17461-05; Nacalai Tesque) and then the kidneys and spleens were isolated. The kidneys were minced finely and homogenated in 5 ml of FACS buffer (HBSS with 2% FCS and 1% EDTA). The homogenate was centrifuged at 500g for 7 minutes under 4°C and resuspended in 5ml of collagenase I solution (1 mg/mL collagenase in HBSS) for 15 minutes at 37°C, with gentle stirring. Digested kidneys were then centrifuged at 400g for 5 minutes. The resulting pellets were resuspended in 10 mL of FACS buffer and incubated on ice for 15 minutes. Suspended solution was filtered through a 70- μ m cell strainer. The suspension is centrifuged at 400g for 5 minutes under 4°C and resuspended in 1mL of RBC lysis buffer for 1 minutes. 4 mL of FACS buffer was added and centrifuged at 400g for 5 minutes. A single-cell suspension of the kidney digestion was obtained by resuspending the pellet in 1 mL of FACS buffer and filtering through a 70- μ m cell strainer.

The spleens were mechanically minced firmly using the plunger of a syringe in 4 mL of FACS buffer and filtered through a 40- μ m cell strainer. The suspension was centrifuged at 400g for 5 minutes under 4°C and resuspended in 1 mL of FACS buffer. 4mL of RBC lysis buffer was added and incubated for 2 minutes under room temperature. The suspension was centrifuged at 400g for 5 minutes under 4°C and washed twice. A single-cell suspension of splenocytes was generated by resuspending the pellet in 1 mL of FACS buffer.

For peripheral blood, 1 mL of FACS buffer was added to 100 μ L of peripheral blood with 10 U of heparin sodium (catalog 224122458; Mochida, Japan) and centrifuged at 400g for 5 minutes under 4°C. 1 mL of RBC lysis buffer was added and incubated for 10 minutes under room

temperature. The solution was centrifuged at 400g for 5 minutes under 4°C. A single-cell suspension of blood cells was obtained by resuspending the pellet in 1 mL of FACS buffer.

Flow cytometric analysis

Single-cell suspensions were blocked with FcR Blocking Reagent (Miltenyi Biotec) at 4°C for 10 minutes, followed by staining with a cocktail of conjugated antibodies against the following proteins: mouse CD45 (clone 30-F11, Pacific Blue conjugate, catalog 103112, and APC-Cy7 conjugate, catalog 103115; BioLegend, San Diego, CA, USA) , CD3 ϵ (clone 145-2C11, FITC conjugate, catalog 100306; BioLegend) , CD19 (clone 1D3, PE conjugate, catalog 152408; BioLegend) , CD11b (clone M1/70, APC conjugate, catalog 101211; BioLegend) , and CD11c (clone N418, APC-Cy7 conjugate, catalog 117323; BioLegend) . Staining was performed at 4°C for 30 minutes. For oxidative stress measurement, cell suspensions were first incubated with 5 μ M CellRox Green Reagent (catalog C10444; Invitrogen) at 37°C for 30 minutes, followed by antibody staining. For intracellular glutathione level measurement in co-cultured T cells using ThiolTracker Violet, cells were first stained with the antibody cocktail, followed by incubation with 10 μ M ThiolTracker Violet at 37°C for 30 minutes. T cells were isolated by excluding CD11b (+) CD11c (+) cells in co-culture with DCs, or by including CD45 (+) cells in co-culture with fibroblasts. Flow cytometric analyses were conducted using a FACS Aria Fusion (BD Biosciences) and analyzed with FlowJo software (Becton Dickinson Co.) .

Reanalysis on single-nucleus RNA-sequencing dataset of aged injured kidneys with tertiary lymphoid structures

We reused the single-nucleus RNA-sequencing Seurat object of aged injured kidneys with tertiary lymphoid structures 30 days after a 45-minute IRI that we previously reported.¹⁴ We showed Uniform Manifold Approximation and Projection (UMAP) plots and gene expression patterns in each cluster.

Statistics

Results are presented as the mean $\pm$ SE. Statistical analysis was performed with non-parametric tests, using Mann-Whitney U test (Figures 1J, 4C, 5D, 6A, 6B, 6G, Supplemental Figure 3B, 8C, 9C, 10B, 10E, 11, 14), multiple Mann-Whitney U test (Figure 5B), Steel test (Figures 4E, 4F, 4H, 6C, Supplemental Figure 4B, 8E, 9E, 9F, 13), non-parametric trend test (Figure 3A, 3E, 3I, 3M, Supplemental Figure 6B), 2-way ANOVA after aligned rank transform¹⁵ (Figure 5E), nonparametric multiple comparison with Dunnett-type contrasts using the nparcomp package in R¹⁶ (Figure 1G), Pearson's chi-square test (Table 1), Wilcoxon rank-sum test (Table 1, Supplemental Figure 16, Supplemental Table 1), and multivariate logistic regression analysis (Figure 6H and Table 2) as indicated in the figure legends, with significance set at $P < 0.05$. Statistical analyses were performed using JMP version 11 (SAS Institute Inc., Cary, NC, USA), GraphPad Prism for Mac (version 10; GraphPad Software, La Jolla, CA, USA), and R v4.4.0.

All patients followed by steroid treatment (n=9)			
	Baseline	One year	p value
Serum creatinine (mg/dL)	1.31 (0.86-1.06)	1.27 (0.82-1.12)	0.840
eGFR (mL/min/1.73m ²)	57.6 (52.6-69.2)	56.8 (49.7-70.3)	0.820
UPCR (g/gCr)	1.71 (0.35-2.00)	0.62 (0.12-0.39)	0.004

Responders to steroid treatment (n=7)			
	Baseline	One year	p value
Serum creatinine (mg/dL)	1.44 (0.91-1.56)	1.38 (0.89-1.58)	0.625
eGFR (mL/min/1.73m ²)	49.7 (36.4-59.0)	49.6 (35.3-66.3)	>0.999
UPCR (g/gCr)	1.451(0.51-1.94)	0.27 (0.14-0.34)	0.016

Supplemental table 1: Changes in serum creatinine, eGFR, and urinary protein levels in IgA nephropathy patients at baseline and one year after renal biopsy.

Of the 18 patients with tertiary lymphoid structures in the kidney recruited in Figure 6, nine patients were treated by steroid therapy and had urine samples available at both the time of diagnosis and one year later. Seven patients showed a favorable response to steroid treatment, with more than a 50% reduction in urinary protein levels.

Data are median (25th-75th percentile) or percentage.

Data were analyzed by Wilcoxon rank-sum test.

eGFR: Estimate glomerular filtration rate. UPCR: Urinary protein to creatinine ratio.

Supplemental Table 2: Primer sequences used for real-time PCR

Gene	Sequence (5'-3')	
	Forward	Reverse
<i>Gapdh</i>	ACGGCAAATTCAACGGCACAGTCA	TGGGGGCATCGGCAGAAGG
<i>Slc7a11</i>	CTTTTGTTTCGAGTCTGGGTGGAAC	GTTCCAGGATGTAGCGTCCAAATG
<i>Abcc1</i>	ACAACCTGCGCTTCAAGATCAC	AAAGCCCTTTAGGTGAGCAAGC
<i>Cxcl13</i>	CGTGCCAAATGGTTACAAAGATT	GTGGCTTCAGGCAGCTCTTC
<i>Ccl19</i>	CCTGGGAACATCGTGAAAGC	TGGAGGTGCACAGAGCTGATA
<i>Ifng</i>	CTCATGGCTGTTTCTGGCTGTTAC	TTTCTTCCACATCTATGCCACTTG
<i>Ngfr</i>	AGAGAAACTGCACAGCGACAG	TAGAGGTTGCCATCACCTTGAG
<i>Cd4</i>	AAGAACTGGTTCGGCATGACAC	TGCAAAGTTGAGTGGGAAGGAG
<i>Cd19</i>	CCTGGGCATCTTGCTAGTGATTG	AAGCATTCCACCGGAACATCTC
<i>Itgax</i>	TCTTGGTCTGAACAAGCACTGTG	AAGTTGAGGCGAAGAGTGATCG
<i>Havcr1</i>	TCTATGTTGGCATCTGCATCG	GAAGGCAACCACGCTTAGAGA
<i>Col1a1</i>	AGCCGCAAAGAGTCTACATGTC	TAGGCCATTGTGTATGCAGCTG
<i>Gpx4</i>	ATGCACGAATTCTCAGCCAAGG	TTGGTGACGATGCACACGAAAC
<i>Acsf4</i>	CGACAACATCTGTGCTTTTGCC	AGCTTCCATGGCGGGATTATTG

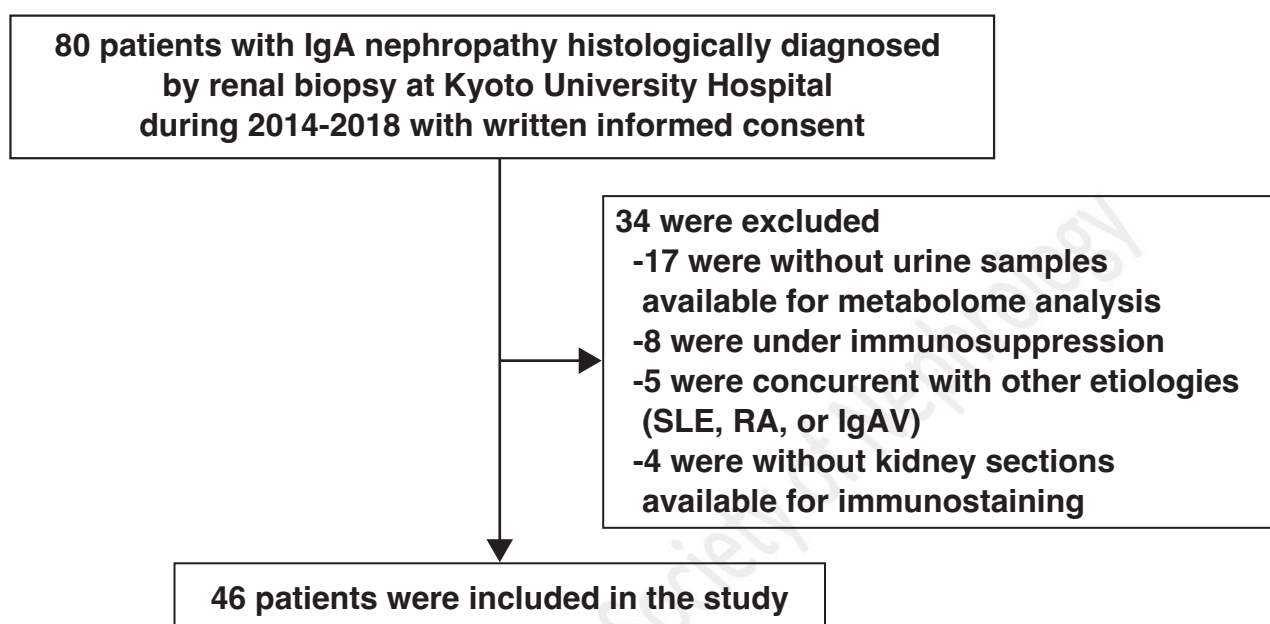
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Supplemental Figure 1

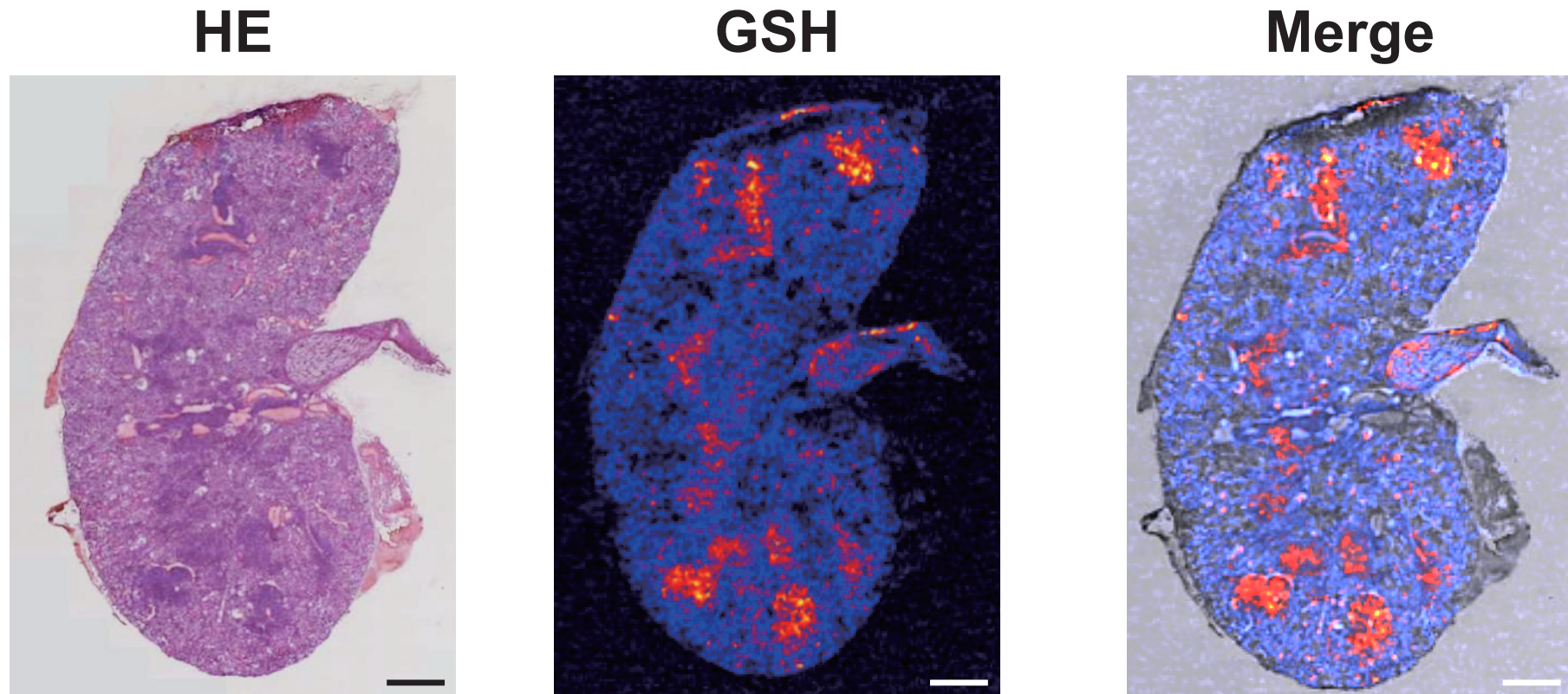


Supplemental Figure 1. Enrollment for histological evaluation of tertiary lymphoid structures in the kidney and metabolome analysis of urine samples.

80 patients were diagnosed as IgA nephropathy by renal biopsy at Kyoto University Hospital between 2014 and 2018 with written informed consent for the use of biosamples in the clinical study. 46 patients were finally included in the study to evaluate tertiary lymphoid structures in the kidney and analyze urine metabolite profiles.

LN: Lupus nephritis, RA: Rheumatoid arthritis, IgAV: IgA vasculitis.

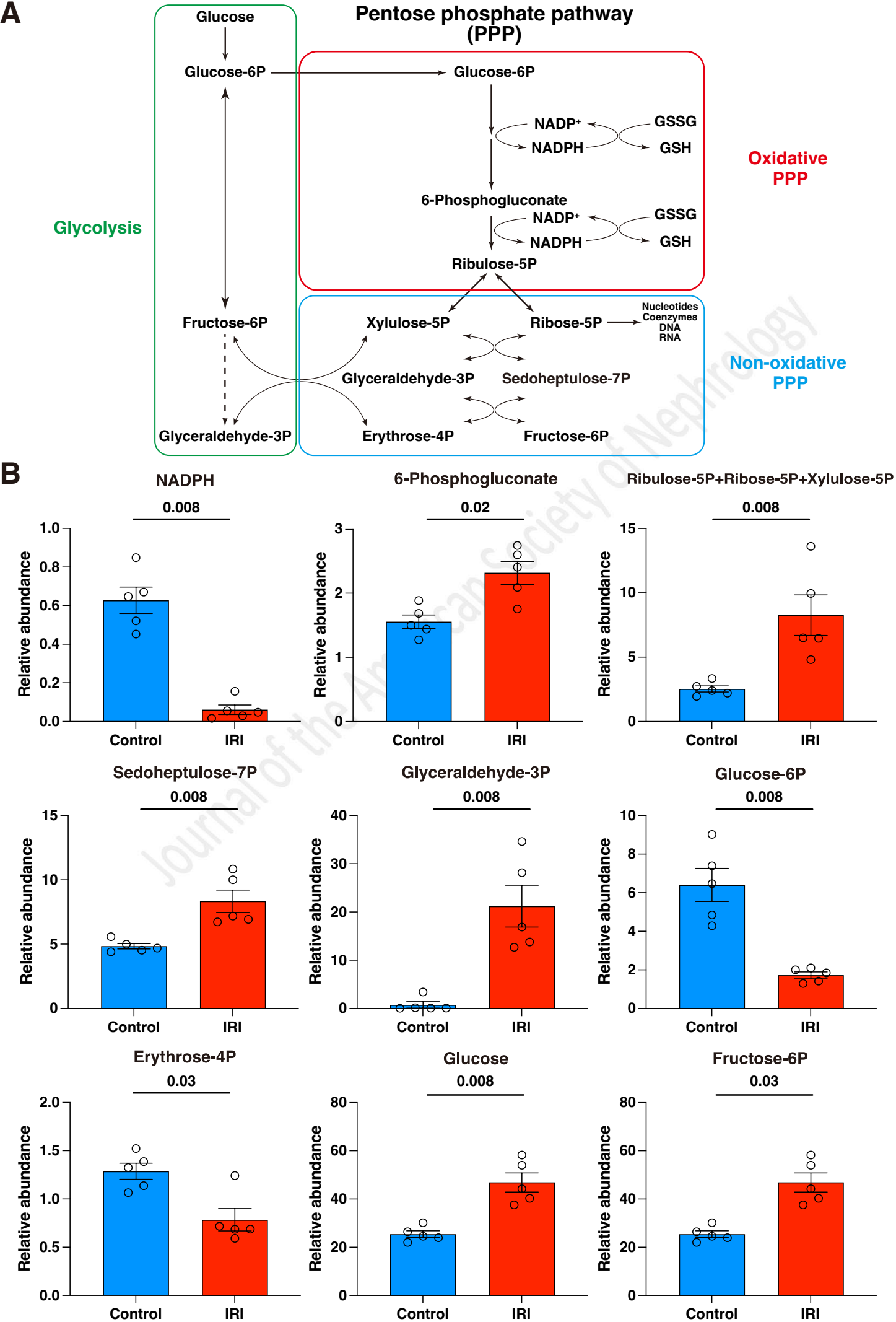
Supplemental Figure 2



Supplemental Figure 2: DESI-imaging mass spectrometry of GSH in the kidney with tertiary lymphoid structures.

HE staining and Desorption Electrospray Ionization (DESI) – Imaging Mass Spectrometry of GSH in the kidneys 45 days after IRI. GSH accumulation was observed within tertiary lymphoid structures. Scale bars: 1000 μm .

Supplemental Figure 3



Supplemental Figure 3. Pentose phosphate pathway is upregulated in tertiary lymphoid structures in the kidney.

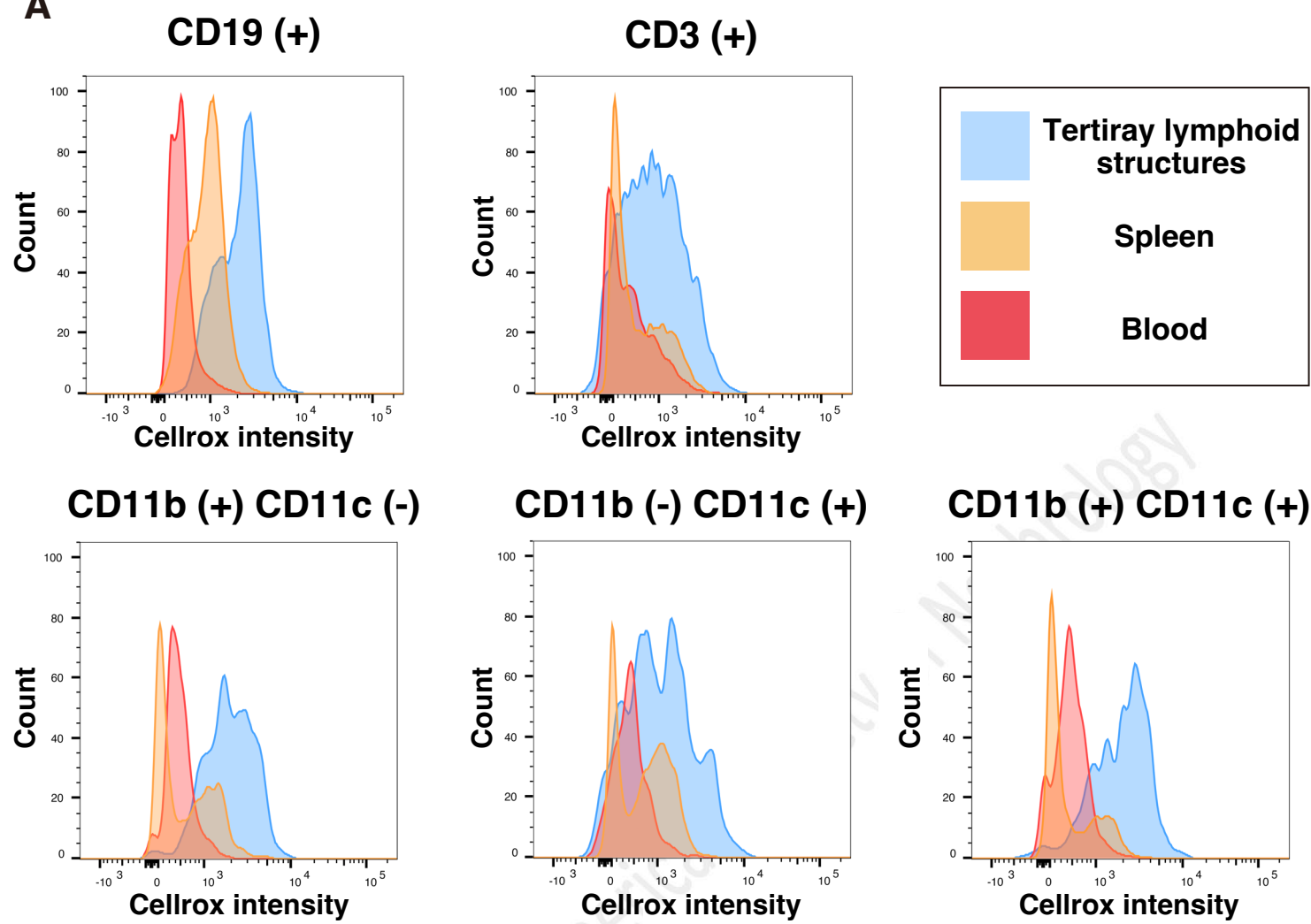
(A) Overview of the pentose phosphate pathway (PPP) with key metabolites. NADPH is generated via oxidative PPP and used for the conversion of GSSG to GSH. Ribose-5P, which is essential for the synthesis of nucleotides and cell proliferation, is produced via non-oxidative PPP.

(B) Metabolome analysis of key metabolites in PPP in the kidneys 45 days after sham surgery and IRI (n = 5 in each group).

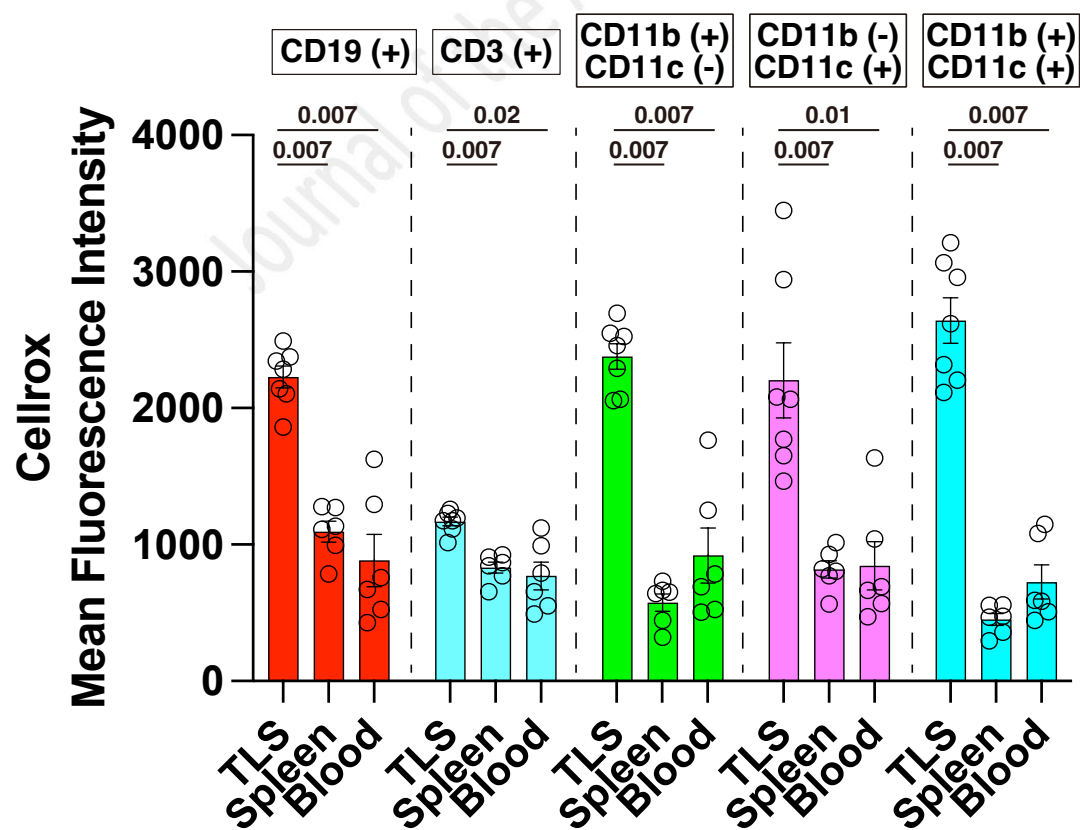
Values are means $\pm$ SE. Data were analyzed by Mann-Whitney U test.

Supplemental Figure 4

A



B



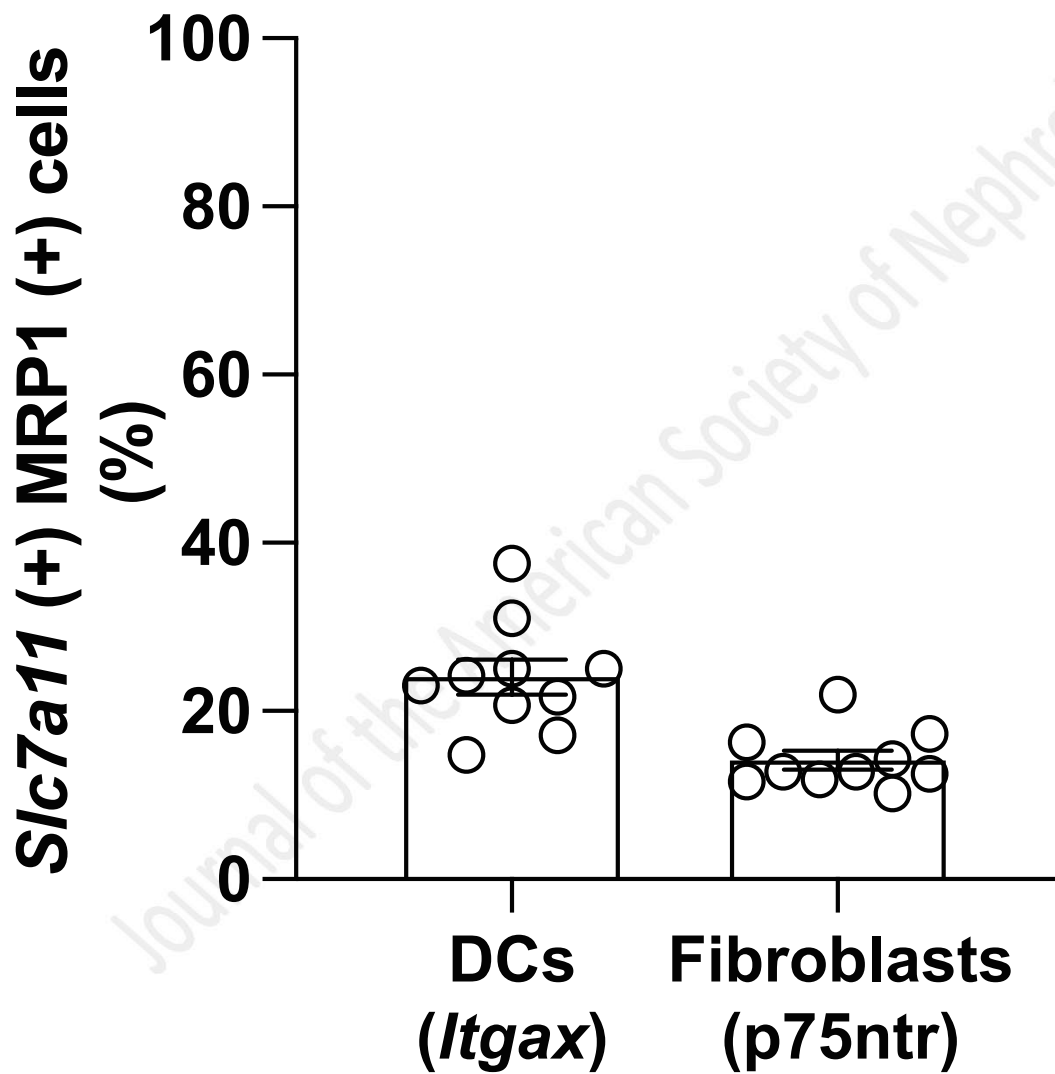
Supplemental Figure 4. Higher oxidative stress accumulates in immune cells within tertiary lymphoid structures compared with those within the spleen and peripheral blood.

(A) Representative histogram of Cellrox intensity in CD19 (+), CD3 (+), CD11b (+) CD11c (-), CD11b (-) CD11c (+), and CD11b (+) CD11c (+) cells derived from tertiary lymphoid structures (n = 7), spleen (n = 6), and peripheral blood (n = 6) of aged mice after 45 minute unilateral IRI.

(B) The quantitative mean fluorescence intensity (MFI) of Cellrox in each immune cell subpopulation derived from tertiary lymphoid structures, the spleen, and peripheral blood.

Cells are gated on CD45 (+) and analyzed accordingly.

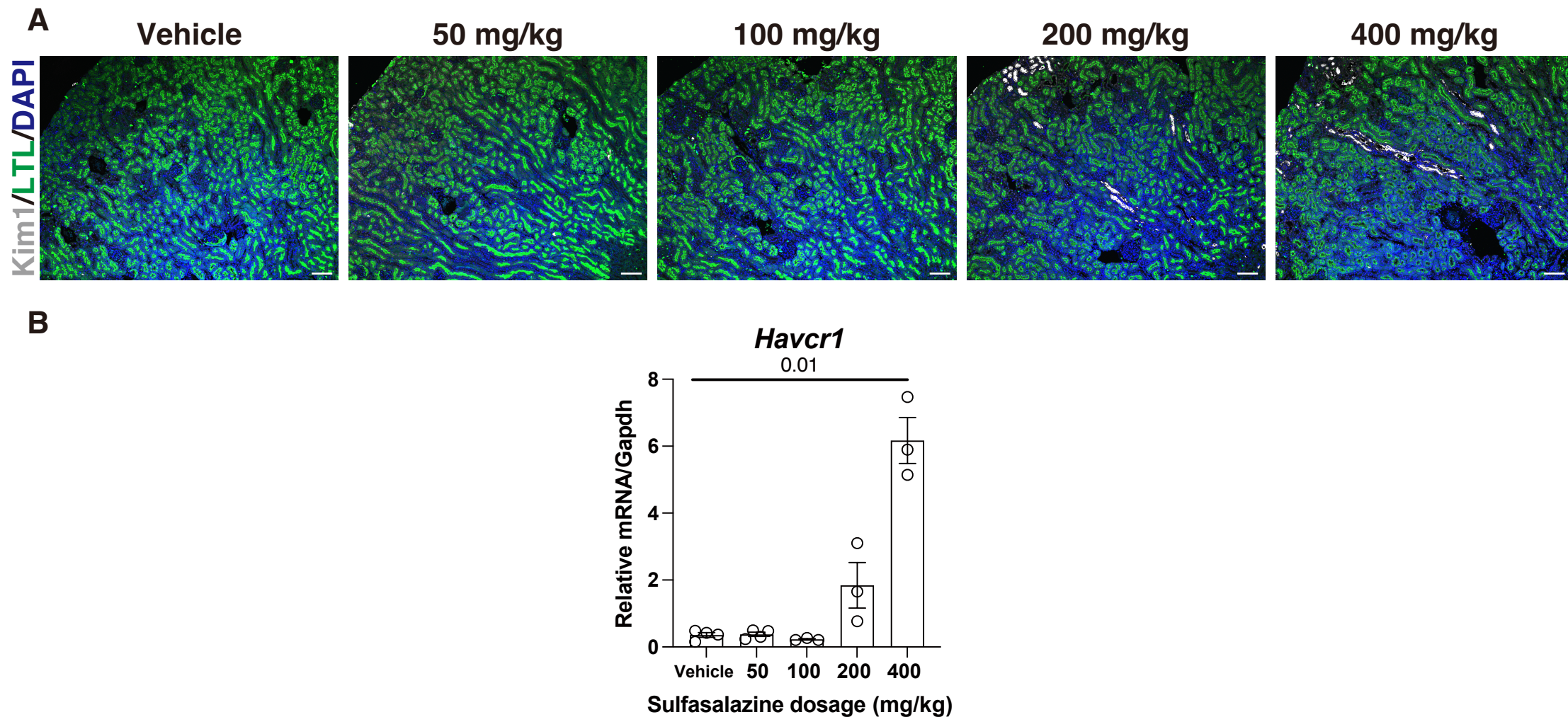
Values are means $\pm$ SE. Data were analyzed by (B) Steel test with the group of tertiary lymphoid structures as control. TLS, tertiary lymphoid structures.



Supplemental Figure 5. The percentages of *Slc7a11* (+) MRP1 (+) dendritic cells and fibroblasts within TLSs.

The number of *Slc7a11* (+) MRP1 (+) *Itgax* (+) cells and *Slc7a11* (+) MRP1 (+) *p75ntr* (+) cells within TLSs were counted and the percentage of these cells per *Itgax* (+) or *p75ntr* (+) cells were calculated. Ten TLS lesions from five mice were randomly selected and used for analysis. Counting and calculation was performed by using ImageJ software.

Supplemental Figure 6



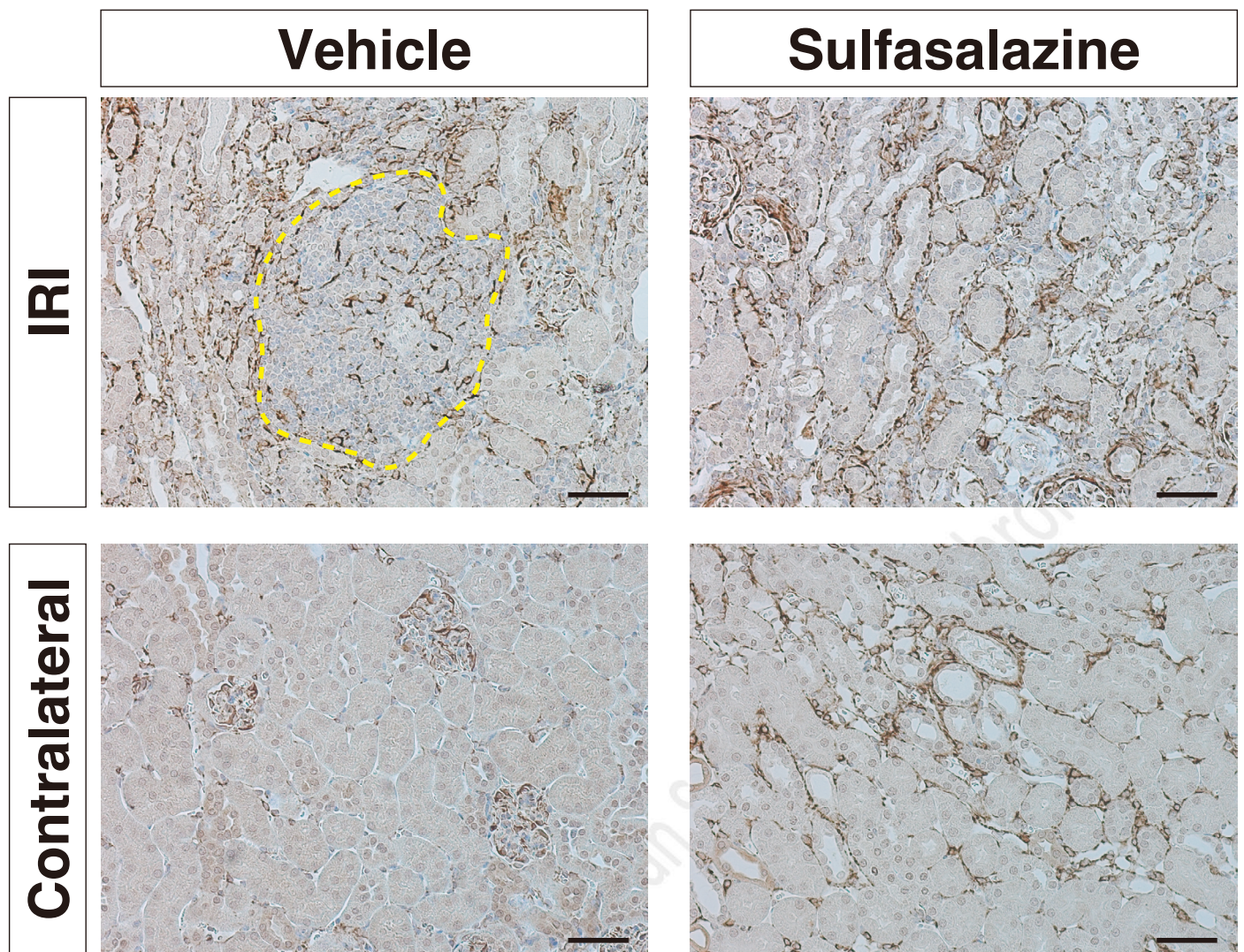
Supplemental Figure 6. Tubular injury in the kidney following different dosages of sulfasalazine-treatment.

(A) Immunofluorescence of Kim1 (white), LTL (green), and DAPI (blue) in the kidneys of mice treated daily with varying dosages of sulfasalazine for 16 days. The 400g/kg dosage corresponds to that used in the experiment described in Figure 4.

(B) *Havcr1* mRNA levels in the kidneys of mice treated with varying dosages of sulfasalazine (Vehicle: n = 4; 50mg/kg: n = 3; 100mg/kg: n = 3; 200mg/kg: n=3; and 400mg/kg: n = 3).

Values are means ± SE. Data were analyzed by non-parametric trend test.

Scale bars: 100 μ m.

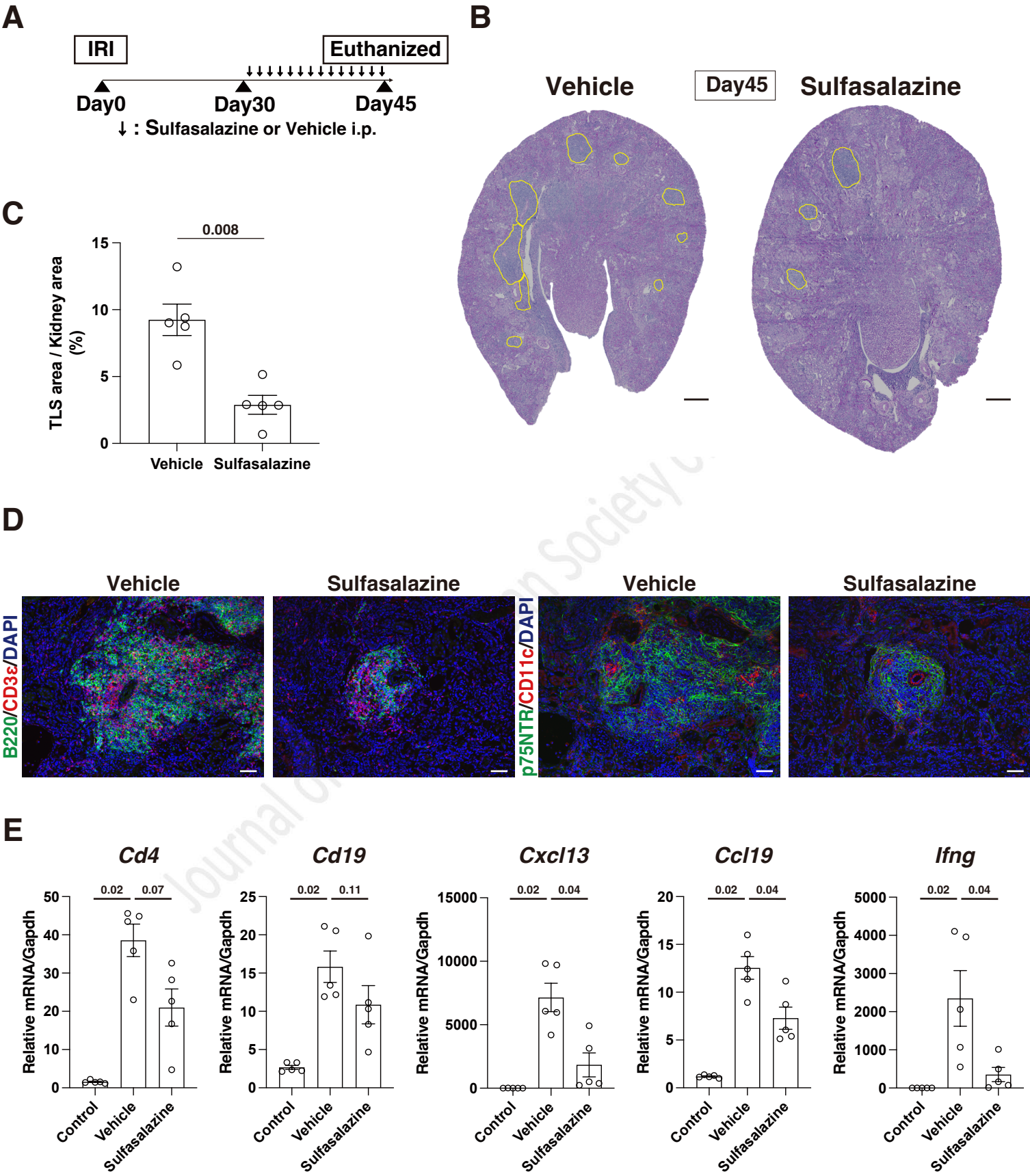


Supplemental Figure 7. Desmin expression in the kidney of mice treated with sulfasalazine or vehicle after IRI.

Desmin expression was analyzed in the kidneys at Day30 after IRI treated with sulfasalazine or vehicle. Both the IRI-affected kidneys and contralateral kidneys were examined. Desmin was detected by DAB staining (brown). Sections were counterstained with eosin. Tertiary lymphoid structures are encircled by dotted lines.

Scale bars: 50 μ m.

Supplemental Figure 8



Supplemental Figure 8. Sulfasalazine treatment contributes to partial reversion of tertiary lymphoid structures in the kidney.

(A) Experimental protocol for (B-E).

(B) Representative images of the kidneys 45 days after IRI treated with sulfasalazine or vehicle from day 30 in Periodic acid–Schiff (PAS) staining.

Tertiary lymphoid structures are encircled by yellow lines.

(C) Cumulative sizes of tertiary lymphoid structures per kidney cortex area (n = 5 in each group).

(D) Immunofluorescence of B220 (green), CD3 α (red), and DAPI (blue) and p75NTR (green), CD11c (red), and DAPI (blue) in the kidneys 45 days after IRI treated with sulfasalazine or vehicle from day 30.

(E) *Cd4*, *Cd19*, *Cxcl13*, *Ccl19*, and *Ifng* mRNA levels in the IRI kidneys (Control: kidneys 45 days after sham operation, n = 5; Vehicle: kidneys 45 days after IRI treated with vehicle from day30, n = 5; Sulfasalazine: the kidneys 45 days after IRI treated with sulfasalazine 400 mg/kg from day 30, n = 5).

Values are means $\pm$ SE. Data were analyzed by (C) Mann-Whitney U test and

(E) Steel test with the vehicle-treated group as control.

Scale bars: (B) 300 μ m; (D) 50 μ m.

A UUO Euthanized
Day0 Day14
↓ : Sulfasalazine or Vehicle i.p.

B Vehicle Day14 Sulfasalazine

C

0.002

TLA area / Kidney area (%)

Vehicle Sulfasalazine

D Vehicle Sulfasalazine Vehicle Sulfasalazine

B220/CD3ε/DAPI p75NTR/CD11c/DAPI

E

Cxcl13 0.02 0.01
Relative mRNA/Gapdh
Control Vehicle Sulfasalazine

Ccl19 0.02 0.22
Relative mRNA/Gapdh
Control Vehicle Sulfasalazine

Ifng 0.02 0.02
Relative mRNA/Gapdh
Control Vehicle Sulfasalazine

F

GSH 0.01 0.02
Relative abundance
Control Vehicle Sulfasalazine

GSSG 0.01 1.00
Relative abundance
Control Vehicle Sulfasalazine

Cystine 0.01 0.02
Relative abundance
Control Vehicle Sulfasalazine

Cysteine 1.00 0.22
Relative abundance
Control Vehicle Sulfasalazine

Supplemental Figure 9. Sulfasalazine treatment prevents the formation of tertiary lymphoid structures in unilateral ureteral obstruction model.

(A) Experimental protocol for (B-F).

(B) Representative images of the kidneys 14 days after unilateral ureteral obstruction (UUO) treated with sulfasalazine or vehicle in Periodic acid–Schiff (PAS) staining. Tertiary lymphoid structures are encircled by yellow lines.

(C) Cumulative sizes of tertiary lymphoid structures per kidney cortex area (n = 6 in each group).

(D) Immunofluorescence of B220 (green), CD3 ϵ (red), and DAPI (blue) and p75NTR (green), CD11c (red), and DAPI (blue) in the kidneys 14 days after UUO treated with sulfasalazine or vehicle.

(E) *Cxcl13*, *Ccl19*, and *Ifng* mRNA levels in the kidneys after UUO (Control: normal kidneys, n = 5; Vehicle: kidneys 14 days after UUO treated with vehicle, n = 6; Sulfasalazine: the kidneys 14 days after UUO treated with sulfasalazine 400 mg/kg, n = 5).

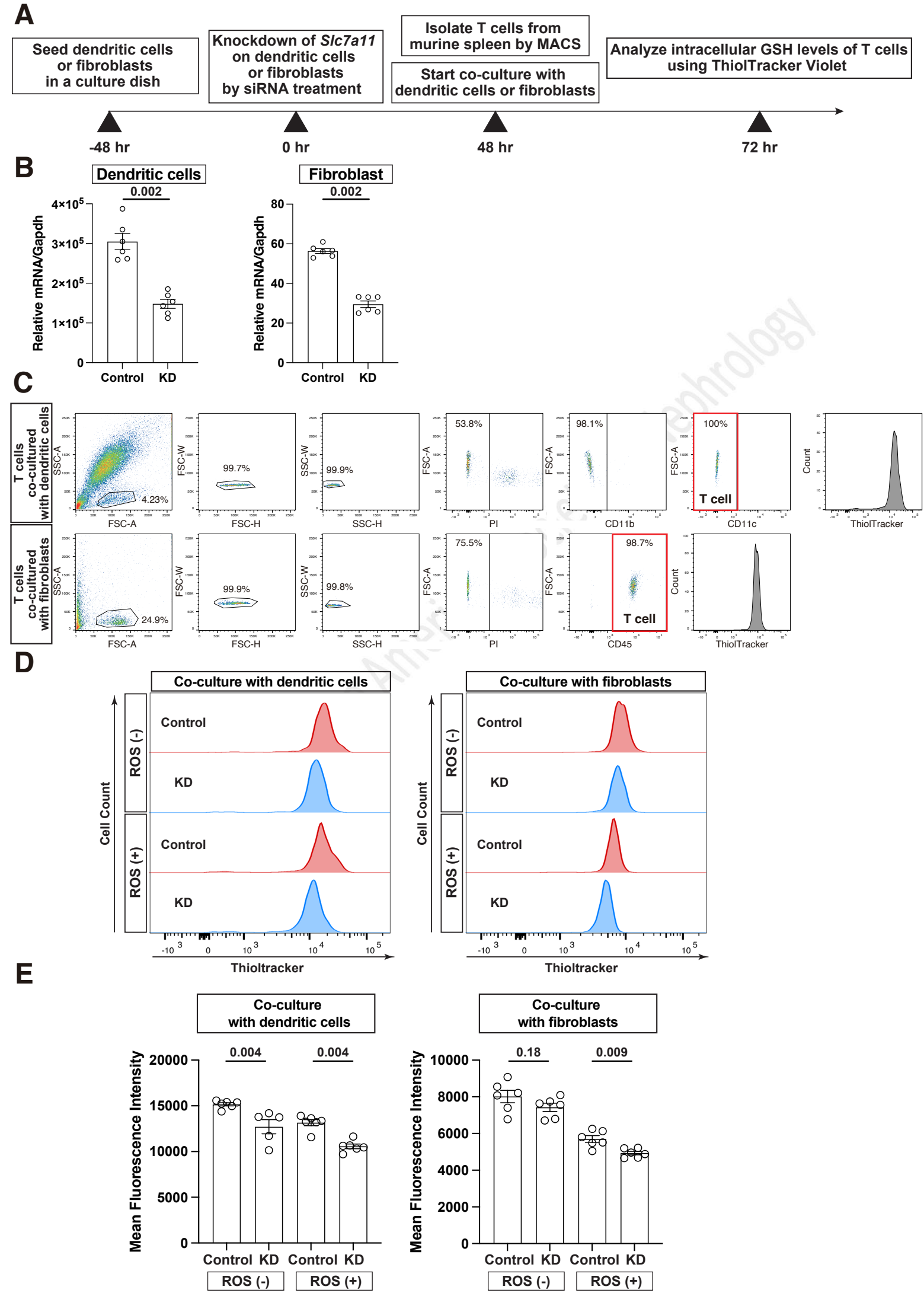
(F) GSH, GSSG, cystine, and cysteine levels in the kidneys after UUO (n = 6 in each group).

Values are means $\pm$ SE. Data were analyzed by (C) Mann-Whitney U test and (E, F) Steel test with the vehicle-treated group as control.

UUO, Unilateral ureteral obstruction. GSH, Glutathione reduced form. GSSG, Glutathione oxidized form.

Scale bars: (B) 300 μ m; (D) 50 μ m.

Supplemental Figure 10



Supplemental Figure 10. Intracellular glutathione levels significantly decrease in T cells co-cultured with *Slc7a11*-knockdown dendritic cells or fibroblasts.

(A) Experimental protocol for (B-E).

(B) *Slc7a11* mRNA levels in dendritic cells and fibroblasts treated with *Slc7a11* siRNA or non-targeting probe (n = 6 in each group).

(C) Representative FACS plot for the identification of T cells co-cultured with dendritic cells or fibroblasts and the evaluation of ThiolTracker Violet (glutathione detection reagent) in T cells. T cells co-cultured with dendritic cells or fibroblast were identified by excluding CD11b (+) and CD11c (+) cells or including CD45 (+) cells, respectively.

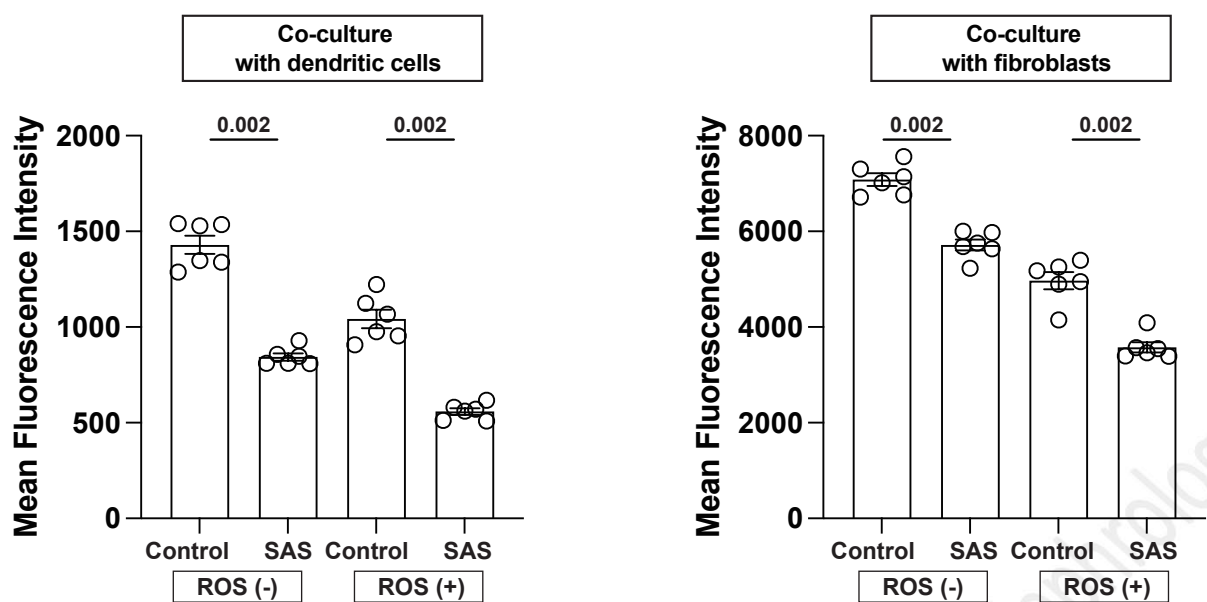
(D) Representative histogram of mean fluorescence intensity (MFI) of ThiolTracker Violet in T cells co-cultured with dendritic cells or fibroblasts under normal condition or oxidative stress (after TBHP treatment).

(E) The quantitative MFI of ThiolTracker Violet in T cells co-cultured with dendritic cells or fibroblasts (n = 5 or 6 in each group).

Values are means $\pm$ SE. Data were analyzed by (B, E) Mann-Whitney U test.

KD, Knockdown; MACS, Magnetic activated cell sorting; FACS, Fluorescence activated cell sorting; ROS, redox oxidative stress; PI, Propidium iodide.

Supplemental Figure 11

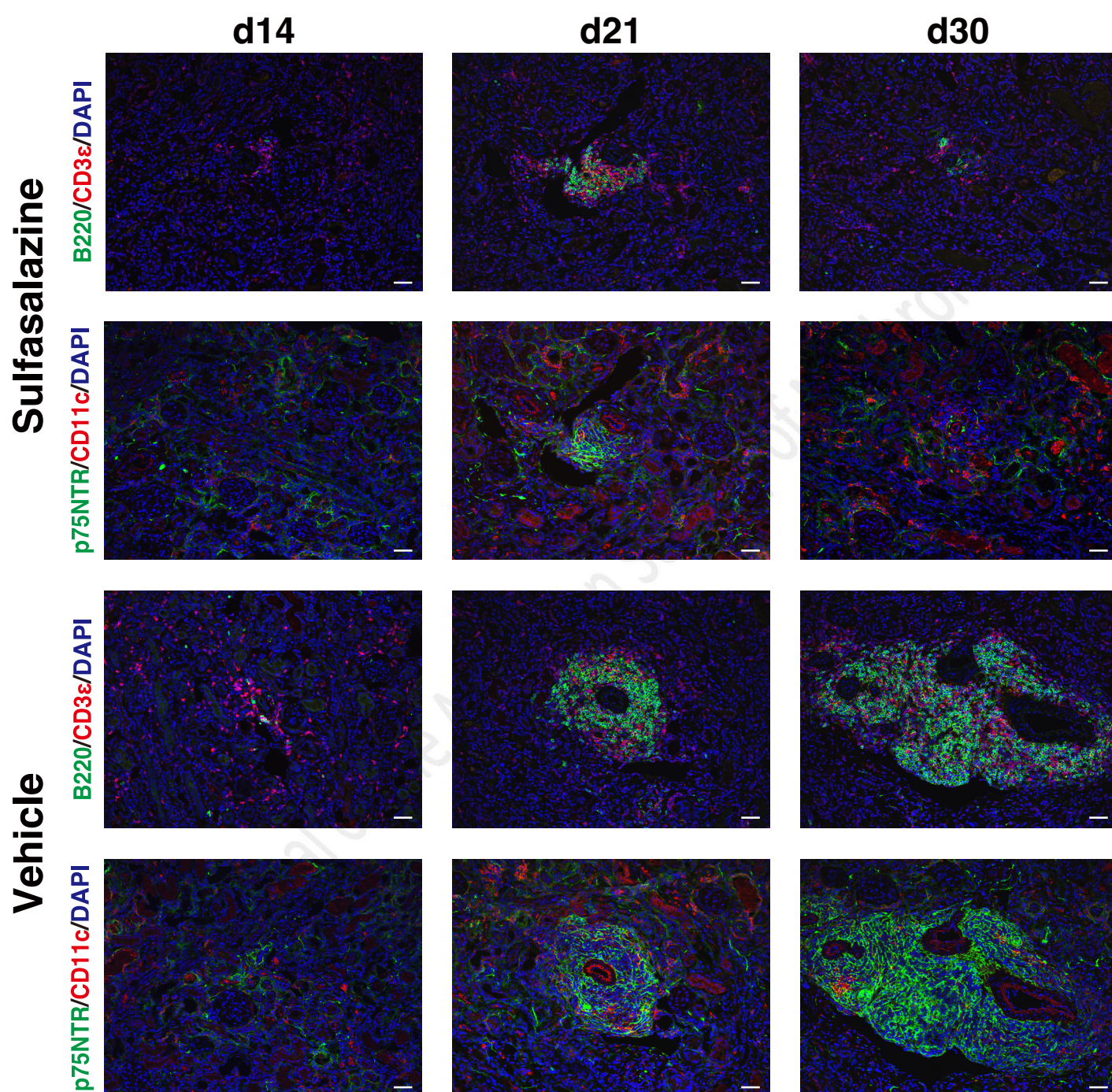


Supplemental Figure 11. Intracellular glutathione levels of T cells co-cultured with dendritic cells or fibroblasts pre-treated with sulfasalazine.

The quantitative MFI of ThiolTracker Violet in T cells co-cultured with dendritic cells or fibroblasts treated with sulfasalazine or vehicle (n = 6 in each group).

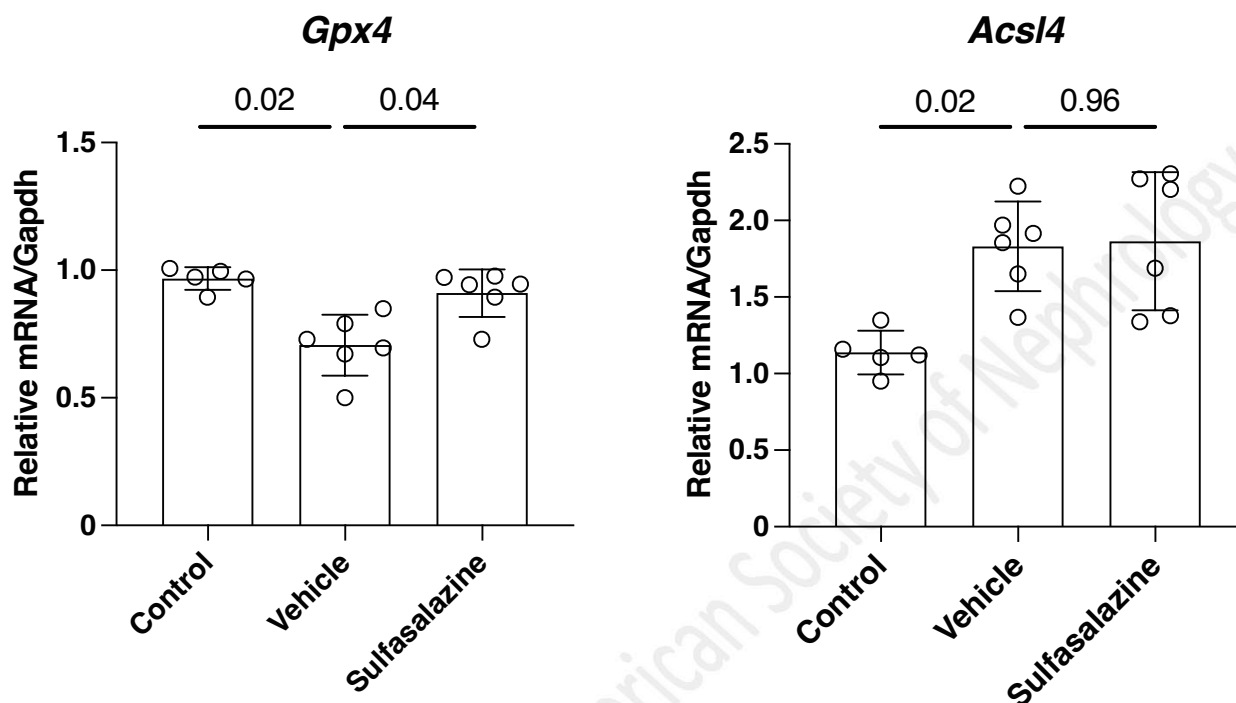
Values are means ± SE. Data were analyzed by Mann-Whitney U test.

SAS, sulfasalazine.



Supplemental Figure 12. Time course immunostaining of the kidneys treated with sulfasalazine or vehicle after IRI.

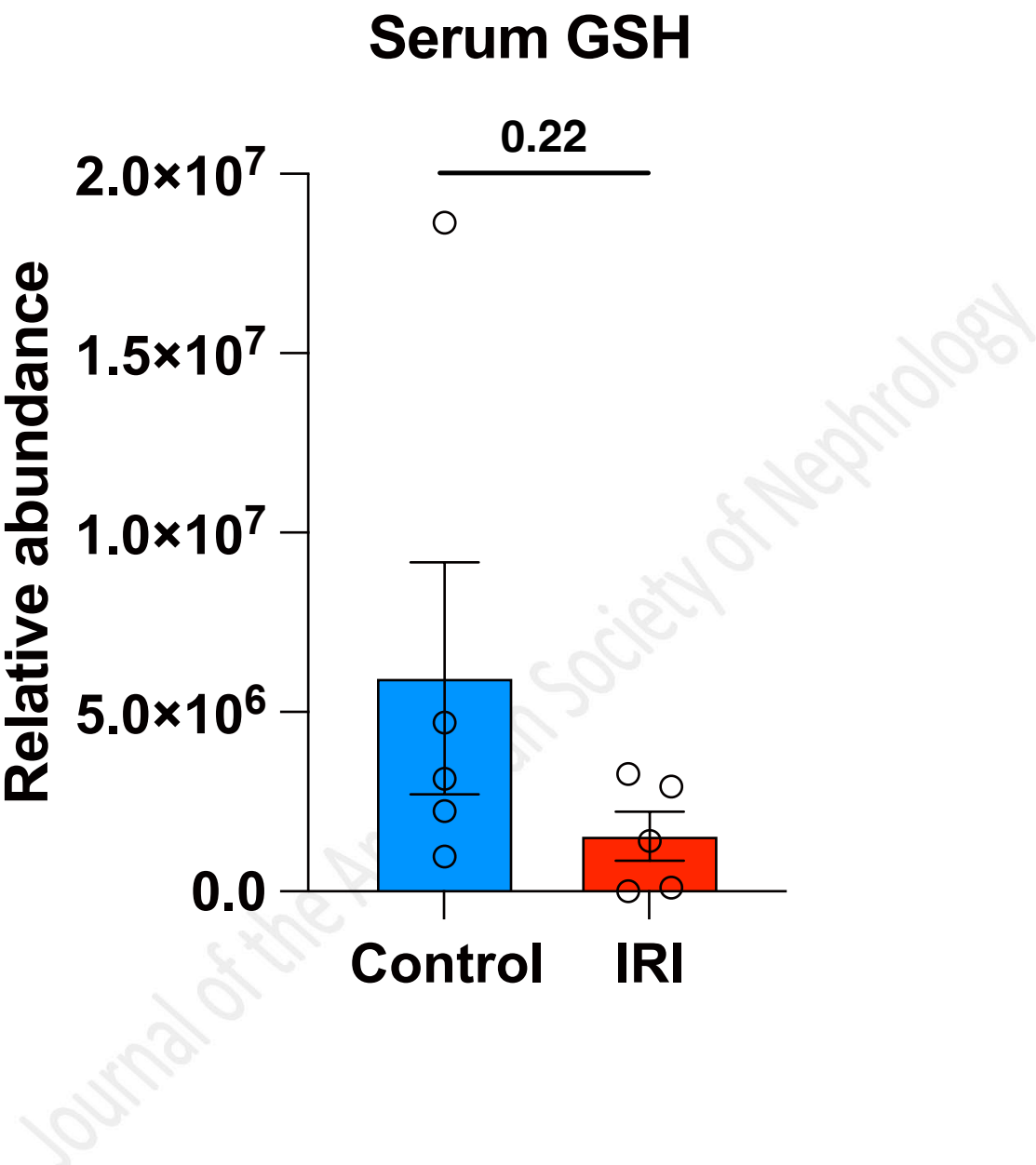
Immunofluorescence of B220 (green), CD3 ϵ (red), and DAPI (blue) and p75NTR (green), CD11c (red), and DAPI (blue) in the kidneys at Day14, Day21, and Day30 after IRI treated with sulfasalazine or vehicle. Scale bars: 50 μ m.



Supplemental Figure 13. Alterations of *Gpx4* and *Acsf4* expression following sulfasalazine treatment.

Gpx4 and *Acsf4* mRNA levels in the IRI kidneys (Control: kidneys 30 days after sham operation, n = 5; Vehicle: kidneys 30 days after IRI treated with vehicle from day14, n = 6; Sulfasalazine: the kidneys 30 days after IRI treated with sulfasalazine 400 mg/kg from day 14, n = 6).

Values are means ± SE. Data were analyzed by Steel test with the vehicle-treated group as control.

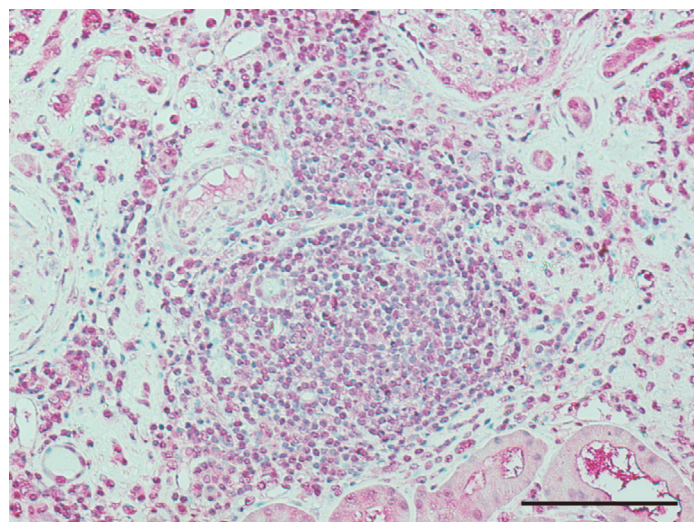
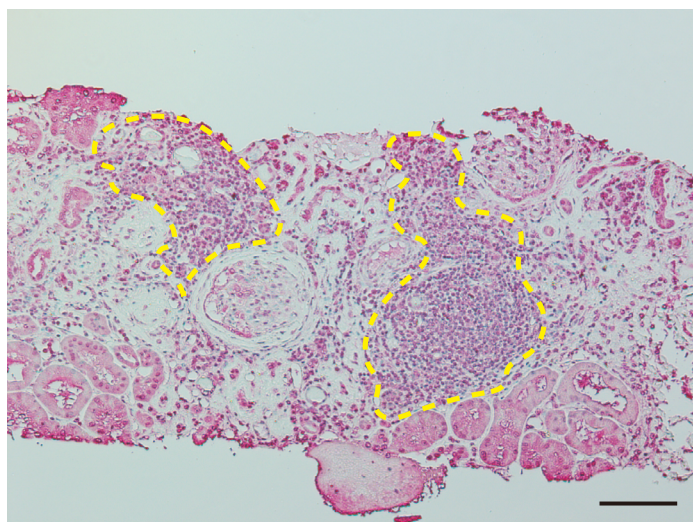


Supplemental Figure 14. Serum GSH concentrations do not increase in mice with tertiary lymphoid structures in the kidney.

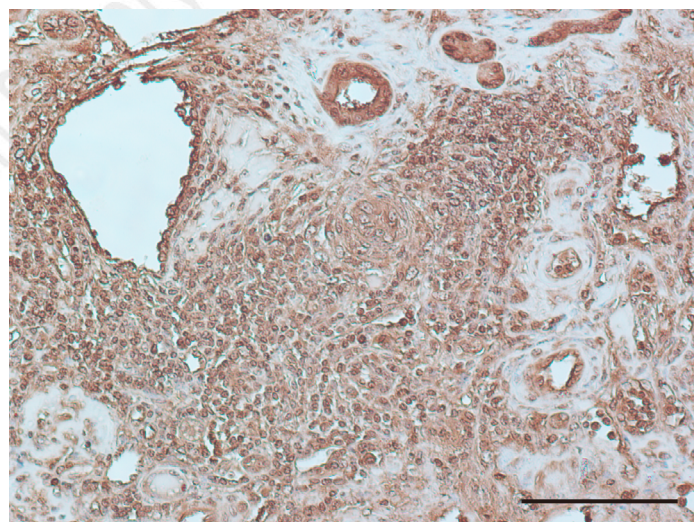
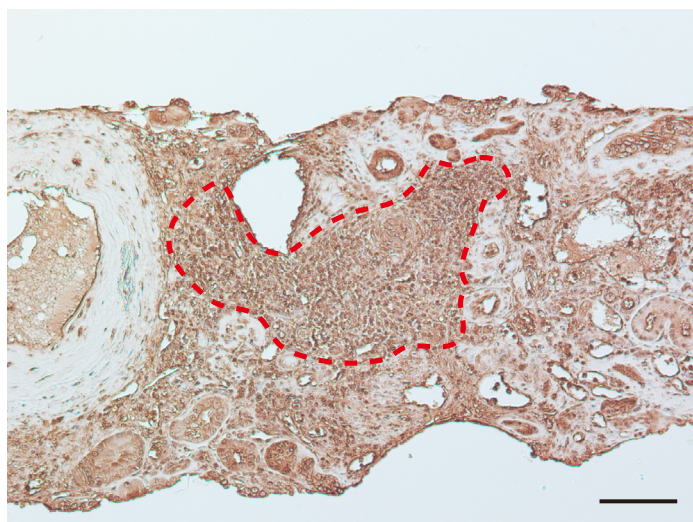
Serum GSH levels of aged mice at day 45 after IRI and sham surgeries (n = 5 in each group). GSH levels were measured by mass spectrometry. Values are means ± SE. Data were analyzed by Mann-Whitney U test. GSH, Glutathione reduced form.

IgA Nephropathy

8-OHdG



4-HNE



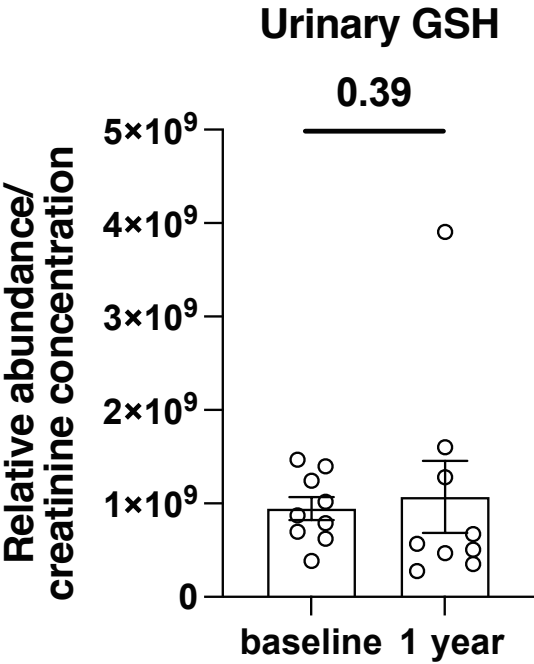
Supplemental Figure 15. 8-OHdG and 4-HNE accumulate within tertiary lymphoid structures in the kidney of IgA nephropathy patients.

Immunohistochemistry of 8-OHdG and 4-HNE in kidney biopsy samples of IgA nephropathy patients with TLSs. 8-OHdG and 4-HNE were detected by alkaline phosphatase (red) and DAB (brown) staining, respectively. Sections were counterstained with eosin. Tertiary lymphoid structures are encircled by dotted lines.

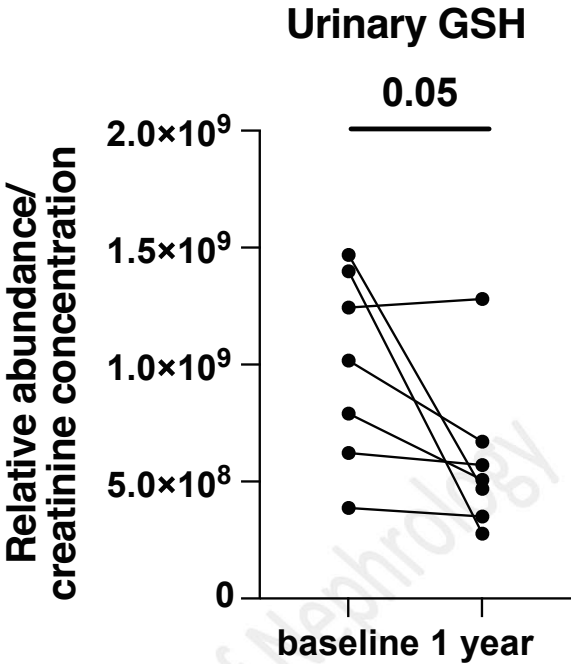
Scale bars: 50 μ m.

Supplemental Figure 16

A



B



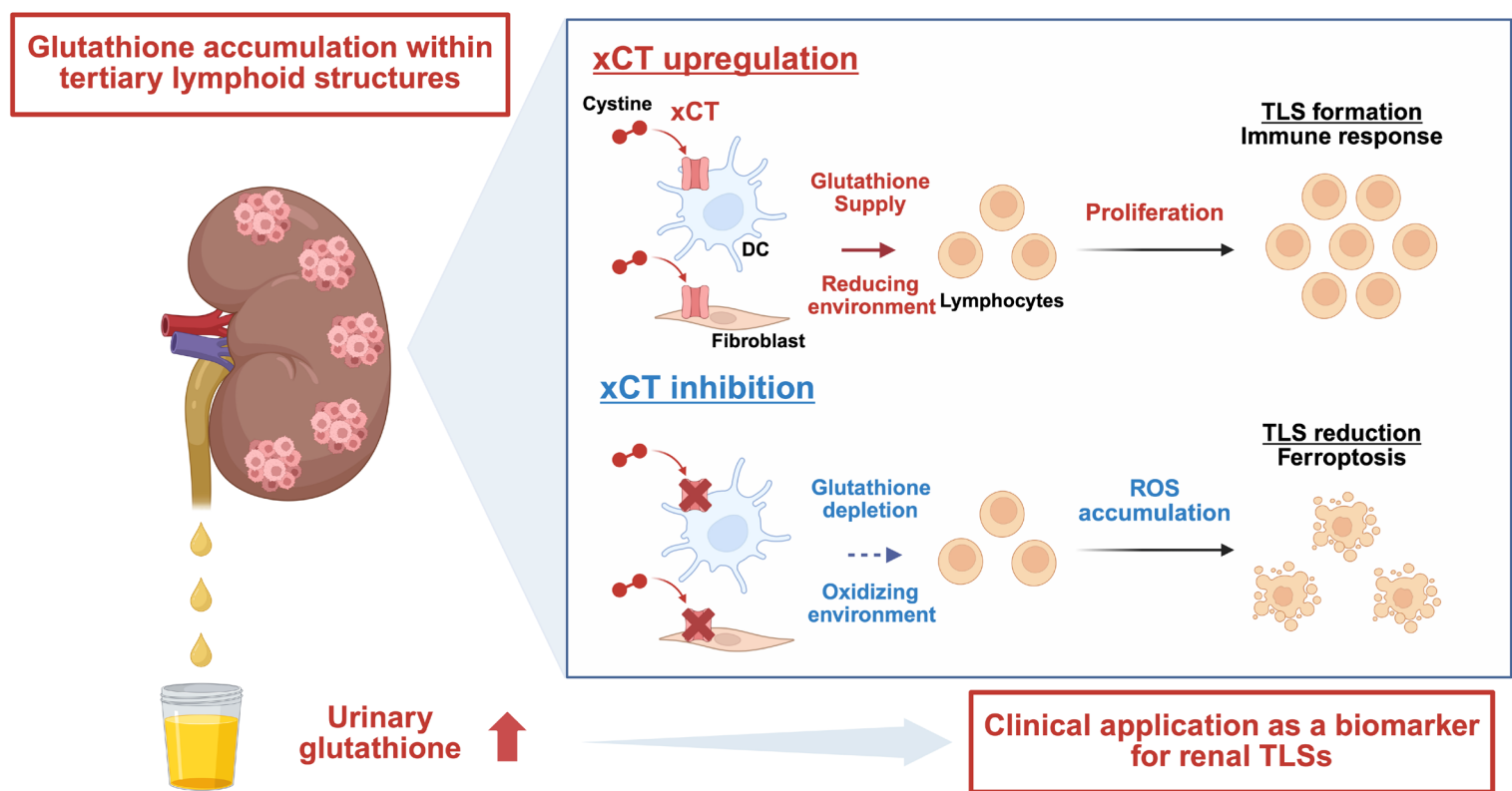
Supplemental Figure 16. Urinary GSH concentrations at baseline and one year after diagnosis in IgA nephropathy patients with tertiary lymphoid structures.

(A) GSH levels in urine samples from IgA nephropathy patients with tertiary lymphoid structures in the kidney. Nine patients were recruited from those with tertiary lymphoid structures shown in Figure 9, who underwent steroid treatment, with urine samples available for analysis at both diagnosis and one year later.

(B) Comparison of urinary glutathione levels at baseline and one year later among seven patients who exhibited more than 50% decrease in urinary protein levels following steroid treatment.

Values are presented as means $\pm$ SE. Data were analyzed by (A, B) Wilcoxon signed-rank sum test.

GSH, Glutathione reduced form.



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Supplemental Figure 17. A scheme showing metabolic microenvironment of tertiary lymphoid structures and clinical potential of urinary glutathione as a biomarker to detect tertiary lymphoid structures in the kidney.

Our study unveiled a unique metabolic microenvironment within tertiary lymphoid structures in the kidney. The distinctive metabolic signature of tertiary lymphoid structures is the accumulation of glutathione. The glutathione synthesis pathway plays a pivotal role in the formation of tertiary lymphoid structures, acting as a resilience mechanism to oxidative stress and ferroptosis. Urinary glutathione holds important clinical promise as a biomarker for identifying tertiary lymphoid structures in the kidney.

Glutathione synthesis via the cystine/glutamate transporter promotes the formation of tertiary lymphoid structures in the kidney

JASN
Journal of the American Society of Nephrology

Basic Research

Methods



Model of tertiary lymphoid structures in kidney



Imaging mass spectrometry and metabolome analysis
In situ hybridization to explore regulation of metabolic pathway



Analysis of urine samples from mice and humans to estimate presence of tertiary lymphoid structures in the kidney

Findings



Significant glutathione accumulation and depletion of cysteine, as well as the accumulation of 4-HNE and 8-OHdG, was observed in tertiary lymphoid structures in the kidney. In addition, kidneys with tertiary lymphoid structures exhibited higher glutathione concentrations than healthy kidneys.



Dendritic cells and fibroblasts in tertiary lymphoid structures expressed the cystine/glutamate transporter and supplied synthesized glutathione to lymphocytes which lacked its expression.



Pharmacological inhibition of the cystine/glutamate transporter prevented tertiary lymphoid structure formation in the kidney.



Enhanced glutathione synthesis in tertiary lymphoid structures was reflected in elevated urinary glutathione concentrations in mice and humans, which effectively detected the presence of tertiary lymphoid structures in the kidney in IgA nephropathy patients.

Conclusions: Glutathione significantly accumulated within tertiary lymphoid structures in the kidney. Inhibition of the cystine/glutamate transporter prevented the formation of tertiary lymphoid structures. Urinary glutathione served as a biomarker to detect tertiary lymphoid structures in the kidney.

Hiroyuki Arai, Yuki Sugiura, Shinya Yamamoto, et al. **Glutathione Synthesis via the Cystine/Glutamate Transporter Promotes the Formation of Tertiary Lymphoid Structures in the Kidney.** JASN DOI: 10.1681/ASN.0000000825. Visual Abstract by Anna Gaddy, MD



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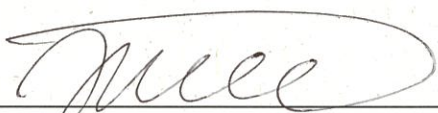
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Name: Shingo Fukuma

Manuscript ID: JASN-2025-000331R1

Manuscript Title: Glutathione synthesis via the cystine/glutamate transporter promotes the formation of tertiary lymphoid structures in the kidney

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Name: Shima Goto

Manuscript ID: JASN-2025-000331R1

Manuscript Title: Glutathione synthesis via the cystine/glutamate transporter promotes the formation of tertiary lymphoid structures in the kidney

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Name: Ryo Kamimatsuse

Manuscript ID: JASN-2025-000331R1

Manuscript Title: Glutathione synthesis via the cystine/glutamate transporter promotes the formation of tertiary lymphoid structures in the kidney

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Name: Makiko Kondo

Manuscript ID: JASN-2025-000331R1

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Name: Rae Maeda

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Name: Yuta Matsuoka

Manuscript ID: JASN-2025-000331R1

Manuscript Title: Glutathione synthesis via the cystine/glutamate transporter promotes the formation of tertiary lymphoid structures in the kidney

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Name: Hiroyuki Neyama

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Name: Yuki Sato

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Name: Naoya Toriu

Manuscript ID: JASN-2025-000331R1

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Name: Shinya Yamamoto

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Name: Motoko Yanagita

Manuscript ID: JASN-2025-000331R2

Manuscript Title: "Glutathione Synthesis via the Cystine/Glutamate Transporter Promotes the Formation of Tertiary Lymphoid Structures in the Kidney

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Name: Takahisa Yoshikawa

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Manuscript Title: Glutathione Synthesis via the Cystine/Glutamate Transporter Promotes the Formation of Tertiary Lymphoid Structures in the Kidney

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