

**Supplemental file for**

***In vivo* expansion of regulatory T cells via an engineered IL-2 mutein to suppress autoimmune glomerulonephritis**

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## **Supplemental methods for animal experiments**

### ***Assessment of disease severity.***

24-h urine and blood specimens were sampled prior to and following immunization, with weekly sampling intervals. Proteinuria and serum creatinine, urea, albumin, and total cholesterol were quantified using an automated biochemical analyzer (UniCel DxC 600 Synchron; Beckman Coulter, Inc.). Kidney tissues were evaluated using light microscopy, immunofluorescence, and electron microscopy. For light microscopic analysis, kidney tissues were fixed in 10% neutral buffered formalin, paraffin-embedded, and sectioned (3  $\mu\text{m}$  thick). Tissue sections were subjected to staining with periodic acid–Schiff (PAS) or Periodic-acid silver methenamine (PASM), and a minimum of 100 glomeruli per section were examined to assess the crescents lesion. For immunofluorescence analysis, frozen kidney sections (5  $\mu\text{m}$  thick) were acetone-fixed and incubated with anti-C5b-9 antibodies (sc-66190; Santa Cruz) overnight at 4°C, following incubation of goat anti-mouse IgG H&L (AF555) highly cross-adsorbed secondary antibody (A32727; Invitrogen). Anti-Rat IgG (FITC) (F6258; Sigma-aldrich) and anti-Sheep IgG H&L (AF555) (A-21436; Invitrogen) antibodies were used for detecting the deposition of rat and sheep IgG. The mean immunofluorescence intensity of C5b-9 deposits was quantified by Image-Pro Plus (v6.0; Media Cybernetics). For electron microscopic analysis, samples were sequentially fixed in 3% glutaraldehyde and 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon 812 resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate before examination under a transmission electron microscope (JEM-1230; JEOL).

To assess podocyte foot process effacement, two glomeruli were randomly chosen from specimens. For each glomerulus, 15–20 non-overlapping digital images were acquired at 13,000 $\times$  magnification. The number of foot processes per micrometer of glomerular basement membrane (GBM) and GBM thickness were calculated using ImageJ (v1.48, NIH, Bethesda, MD). The FPW was calculated using the arithmetic mean formula:

$$PFW = \frac{4}{\pi} \times \left( \frac{\sum GBM \text{ length}}{\sum \text{foot process count}} \right)$$

A correction factor of  $\pi/4$  was applied to account for random foot process orientation in ultrathin sections.

### ***Immunohistochemistry***

Kidney sections were dehydrated and subjected to antigen retrieval using one of the following methods: (1) heat-induced epitope retrieval in citric acid buffer (pH 6.0) for CD4 and CD8 detection, or EDTA buffer (pH 9.0) for nephrin staining, both performed under high-temperature and high-pressure conditions for 4 minutes; or (2) enzymatic antigen retrieval using 0.04% pepsin at 37°C for 30 minutes for CD68 detection. After blocking endogenous peroxidase with 3% H<sub>2</sub>O<sub>2</sub> and nonspecific binding with 3% BSA, the sections were incubated overnight at 4°C with the following antibodies: anti-CD4 (25229S; Cell Signaling Technology), anti-CD8 (sc-53063; Santa Cruz), anti-CD68 (MCA341GA; Bio-Rad), or anti-nephrin (ab216341; Abcam) antibodies. Staining was further visualized using 3,3'-diaminobenzidine (DAB). For quantitative analysis, a minimum of 10 random glomeruli per sample were evaluated. CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD68<sup>+</sup> macrophages were quantified as the number of positive cells per glomerular or peri-glomerular cross-section. Nephrin expression was measured as mean integrated optical density (IOD) per glomerular cross-section by Image-Pro Plus.

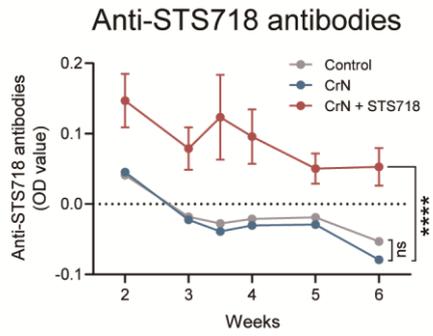
### ***Circulating antibody and cytokines detection by enzyme-linked immunosorbent assay***

Antibodies towards full-length  $\alpha 3(\text{IV})\text{NC1}$  and STS718 in rat experimental crescentic glomerulonephritis model were analyzed by ELISA.  $\alpha 3(\text{IV})\text{NC1}$  (2  $\mu\text{g}/\text{ml}$ , home-made) and STS718 (2  $\mu\text{g}/\text{ml}$ ) were coated overnight on the 96-well plates. Plasma samples were diluted 1:100 in PBS containing 0.1% Tween-20, and incubated in plates for 1 hour at 37°C. After washing, alkaline phosphatase (AP)-conjugated rabbit anti-rat IgG (A6066, Sigma-Aldrich), diluted 1:5000 in PBS with 0.1% Tween-20, was added and incubated for 45 minutes at 37°C. The reaction was developed using p-nitrophenyl phosphate in substrate buffer, and absorbance was measured spectrophotometrically at 405 nm. The levels of circulating cytokines were detected by commercial ELISA kits

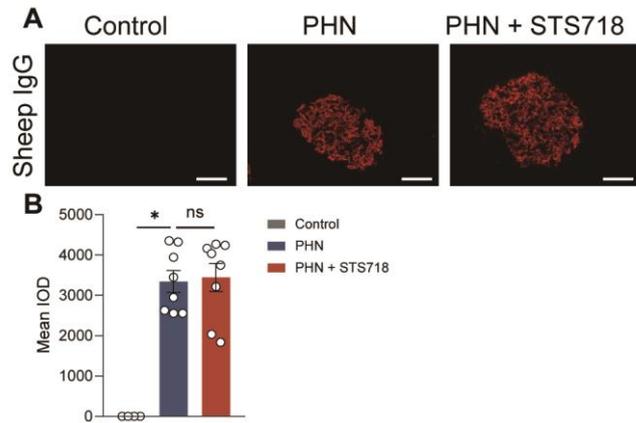
following the manufacturer's protocols: Rat TNF- $\alpha$  ELISA kit (E-HSEL-R0001, Elabscience), Rat IL-10 ELISA kit (E-HSEL-R0005, Elabscience), Rat TGF- $\beta$ 1 ELISA kit (CSB-E04727r, CUSABIO)

#### **Detection of Treg cells by flow cytometry**

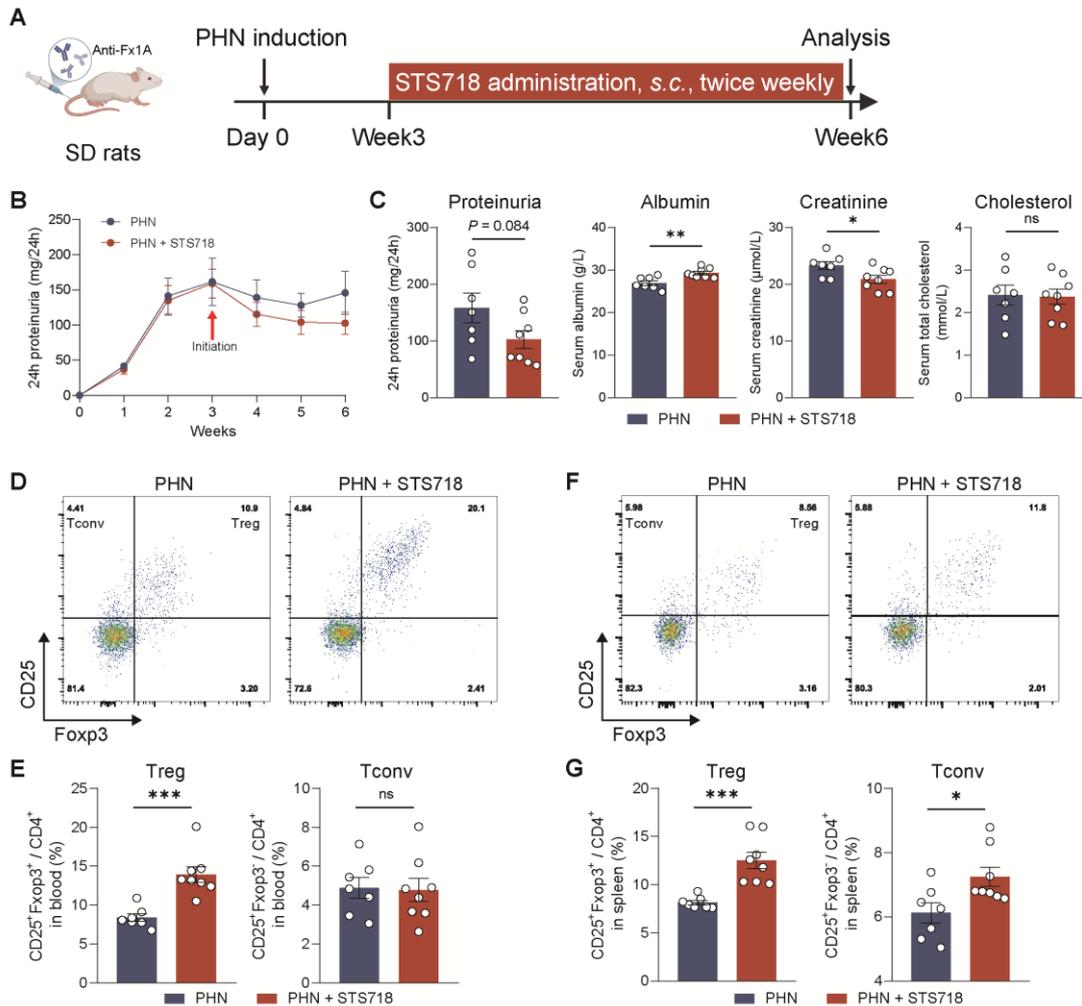
The proportion of circulating Treg cells in rat autoimmune glomerulonephritis models was detected by flow cytometry. 40  $\mu$ L whole blood was acquired from rats and stained with antibodies against CD4 and CD25 for 20 minutes. Following red blood cell lysing (00-4300-54; Invitrogen) and washing, cells were then fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (00-5523-00; Invitrogen) for 45 minutes, and stained with antibodies against Foxp3 for 30 minutes. Cells were analyzed using FACS Verse (BD Biosciences) and FlowJo software v10.8. The proportions of Treg cells and Tconv cells were presented as CD25<sup>+</sup>Foxp3<sup>+</sup> expression and CD25<sup>+</sup>Foxp3<sup>-</sup> in gated CD4<sup>+</sup> T cells, respectively.



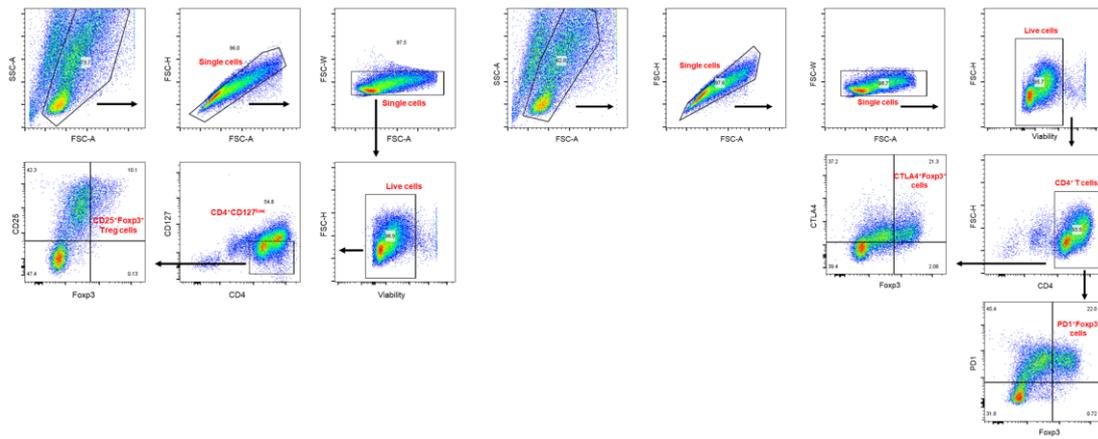
**Figure S1. Circulating antibodies against ST5718 were measured.** Data are expressed as mean  $\pm$  SEM; statistical significance in was determined by Two-way ANOVA test with Dunnett's multiple comparisons test.



**Figure S2. Glomerular sheep IgG deposits in passive Heymann nephritis (PHN) was detected. (A)** Representative immunofluorescence staining of sheep IgG deposits within glomerulus. **(B)** Shown is quantification of glomerular sheep IgG deposits. Data are expressed as mean  $\pm$  SEM; statistical significance was determined by Kruskal-Wallis's test with Dunn's multiple comparisons test.



**Figure S3. Therapeutic intervention with STS718 in rats with established membranous glomerulonephritis.** (A) Schematic strategy to induce passive Heymann nephritis (PHN). Three weeks after immunization, Sprague-Dawley (SD) rats ( $n = 7\sim 8$  per group) were treated with subcutaneous dose of 0.3 mg/kg STS718 twice weekly. (B-C) 24-hour proteinuria, serum albumin, serum total cholesterol, and serum creatinine were measured. (D, F) Representative flow cytometry analyses of  $CD4^+CD25^+Foxp3^+$  Treg cells and  $CD4^+CD25^+Foxp3^-$  Tconv cells from blood and spleen, and quantified results were shown in (E, G). Data are expressed as mean  $\pm$  SEM; statistical significance was determined by unpaired t test.



**Figure S4. Gating strategy for cell subsets in human naïve T cells upon STS718 stimulation.**

**Supplemental Table 1. Information for antibodies or relevant agents in flow cytometry.**

| Antibodies or relevant agents     | Source                    | STS718 experiments  |
|-----------------------------------|---------------------------|---|
| Streptavidin-PE                   | 554061, BD Biosciences    | Screening   |
| THE™ V5 Tag Antibody [iFluor 647] | A01805-100, GenScript     |   |
| CD3-BV421                         | E-AB-F1230Q2, Elabscience | Bioactivity in human PBMCs (panel 1)  |
| CD8-PE                            | AN00427D, Elabscience     |   |
| CD56-AF488                        | E-AB-F1270L, Elabscience  |   |
| pSTAT5-AF647                      | 562076, BD Biosciences    |   |
| CD4-BV421                         | 562424, BD Biosciences    | Bioactivity in human PBMCs (panel 2)  |
| CD25-AF647                        | 356128, Biolegend         |   |
| pSTAT5-PE                         | 612567, BD Biosciences    |   |
| Foxp3-AF488                       | 320112, Biolegend         |   |
| CD4-FITC                          | 100406, Biolegend         | Treg selectivity in mice  |
| CD25-APC                          | 102012, Biolegend         |   |
| Foxp3-PE                          | 320008, Biolegend         |   |
| IL4-APC                           | 504106, Biolegend         |   |
| IFN $\gamma$ -BV421               | 505829, Biolegend         |   |
| IL17A-PE                          | 506904, Biolegend         |   |
| CD4-Pacific Blue™                 | 317429, Biolegend         | Treg selectivity in cynomolgus monkeys                                      |
| CD25-AF647                        | 356128, Biolegend         |   |
| Foxp3-AF488                       | 320112, Biolegend         |   |
| CD8-BV605™                        | 344742, Biolegend         |   |
| CD4-FITC                          | E-AB-F1352C, Elabscience  | Treg expansion in human naïve T cells and PBMCs of healthy donors (panel 1) |
| CD127-Percp/Cy5.5                 | E-AB-F1152J, Elabscience  |   |
| CD25-PE-Cy7                       | E-AB-F1194H, Elabscience  |   |
| Foxp3-PE                          | 320008, Biolegend         |   |
| CD4-FITC                          | E-AB-F1352C, Elabscience  | Treg expansion in human naïve T cells and PBMCs of healthy donors (panel 2) |
| PD1-PE-Cy7                        | 561272, BD Biosciences    |   |
| CTLA4-APC                         | 369612, Biolegend         |   |
| Foxp3-PE                          | 320008, Biolegend         |   |
| CD4-APC                           | E-AB-F1109E, Elabscience  | Treg expansion in human PBMCs of patients                                   |
| CD25-FITC                         | E-AB-F1194C, Elabscience  |   |

|  |                        |                                 |
|--|------------------------|---------------------------------|
| Foxp3-PE                                       | 320008, Biolegend      |                                 |
| CD4-PerCP/Cyanine5.5                           | 201520, Biolegend      | Treg detection in rat models    |
| CD25-PE  | 12-0390-82, Invitrogen |                                 |
| Foxp3-AF647                                    | 320014, Biolegend      |                                 |
| Zombie NIR™ Fixable Viability Kit              | 423106, Biolegend      | Cell viability staining         |
| Fixation Buffer                                | 554655, BD Biosciences | <i>In vitro</i> pSTAT5 assays   |
| Phosflow™ Perm Buffer III                      | 558050, BD Biosciences |                                 |
| Foxp3/Transcription Factor Staining Buffer Set | 00-5523-00, Invitrogen | Detection for Treg cells        |
| Cell Activation Cocktail                       | 423304, Biolegend      | Detection of Th1/Th2/Th17 cells |
| Cyto-Fast™ Fix/Perm Buffer Set                 | 426803, Biolegend      |                                 |

**Supplemental Table 2. Demographic information of enrolled patients with membranous nephropathy.**

|                                     | MN1    | MN2    | MN3   | MN4   | MN5  |
|-------------------------------------|--------|--------|-------|-------|------|
| Gender                              | Male   | Female | Male  | Male  | Male |
| Age, year                           | 60     | 32     | 47    | 42    | 67   |
| Serum creatinine, $\mu\text{mol/L}$ | 372.8  | 236.6  | 237.7 | 85    | 91   |
| Serum albumin, g/L                  | 16.5   | 37.9   | 17.5  | 25.4  | 29.5 |
| Proteinuria, g/day                  | 13.78  | 2.81   | 20.47 | 5.89  | 2.4  |
| Anti-PLA2R1 antibody, RU/mL         | 101.57 | 191    | 1078  | 229.9 | 1500 |

MN, membranous nephropathy; PLA2R1, phospholipase A2 receptor 1.

**Supplemental Table 3. Demographic information of enrolled patients with anti-GBM disease.**

|                                      | GBM1   | GBM2   | GBM3   | GBM4   | GBM5   | GBM6   |
|--------------------------------------|--------|--------|--------|--------|--------|--------|
| Gender                               | Female | Female | Female | Female | Female | Female |
| Age, year                            | 70     | 35     | 37     | 46     | 51     | 28     |
| Serum creatinine, $\mu\text{mol/L}$  | 450.9  | 84.92  | 333.7  | 275    | 357.9  | 318.7  |
| eGFR, mL/min per 1.73 m <sup>2</sup> | 7.98   | 76.806 | 14.479 | 17.174 | 12.143 | 16.306 |
| Serum urea, mmol/L                   | 25.66  | 8.26   | 15.55  | 10.06  | 23.8   | 19.65  |
| Anti-GBM antibody, RU/mL             | 162    | 127    | 112    | 26     | 87     | 180    |
| Crescents on kidney biopsy, %        | 76.5   | 40     | 62.2   | 52.2   | 71.4   | 71.4   |

eGFR, estimated glomerular filtration rate; GBM, glomerular basement membrane.