



Research Article

# Sphingosine-1-phosphate receptor modulator FTY720 attenuates experimental myeloperoxidase-ANCA vasculitis in a T cell-dependent manner

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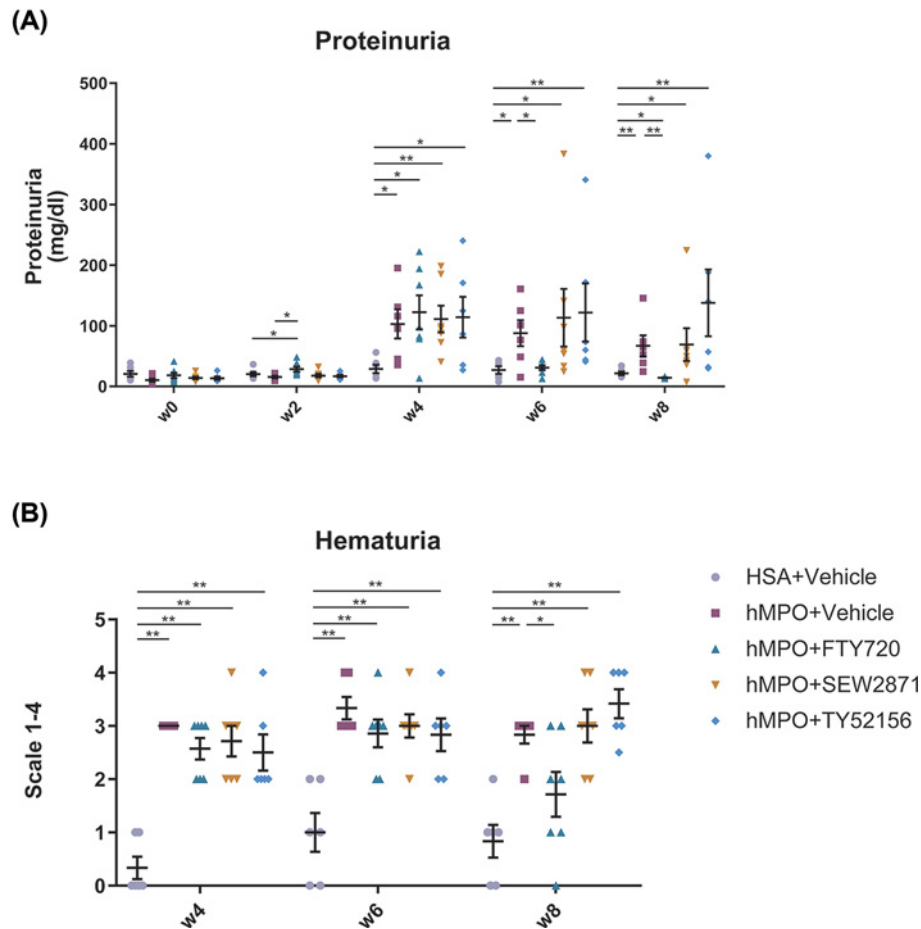
Sphingosine-1-phosphate (S1P) is a pleiotropic lysosphingolipid derived from the metabolism of plasma membrane lipids. The interaction between S1P and its ubiquitously expressed G-protein-coupled receptors (S1PR1-5) is crucial in many pathophysiological processes. Emerging evidence suggested a potential role for S1P receptors in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). In the present study, we investigated the effects of three different S1P receptors modulators (FTY720, SEW2871 and TY52156) in a recognized rat model of experimental autoimmune vasculitis (EAV). The effects of treatments were evaluated with clinico-pathological parameters including hematuria, proteinuria, crescent formation, pulmonary hemorrhage, etc. *In vitro* functional studies were performed in a Jurkat T-cell line following stimulations of serum from myeloperoxidase-AAV patients. We found that only the FTY720 treatment significantly alleviated hematuria and proteinuria, and diminished glomerular crescent formation, renal tubulointerstitial lesions and pulmonary hemorrhage in EAV. The attenuation was accompanied by less renal T-cell infiltration, up-regulated mRNA of S1PR1 and down-regulated IL-1 $\beta$  in kidneys, but not altered circulating ANCA levels, suggesting that the therapeutic effects of FTY720 were B-cell independent. Further *in vitro* studies demonstrated that FTY720 incubation could significantly inhibit the proliferation, adhesion, and migration, and increase apoptosis of T cells. In conclusion, the S1P modulator FTY720 could attenuate EAV through the reduction and inhibition of T cells, which might become a novel treatment of ANCA-associated vasculitis.

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## Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of autoimmune diseases comprising granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), characterized by necrotizing inflammation of small vessels [1]. Kidneys and lungs are the most frequently affected organs with necrotizing crescentic



**Figure 1. FTY720 attenuated proteinuria and hematuria in EAV**

The proteinuria was tested in 24 h rat urine collected at week 0, 2, 4, 6, 8 and the dipsticks were used to detect hematuria after EAV established at week 4, 6, 8. Treatments were started at week 4. FTY720 attenuated proteinuria as early as week 6 (A) and alleviated hematuria at week 8 (B). Data represent means  $\pm$  standard error from six to seven rats per group. \* $P < 0.05$ , \*\* $P < 0.01$  compared with HSA or hMPO treated rats.

glomerulonephritis and pulmonary hemorrhage. ANCA specific for myeloperoxidase (MPO) or proteinase 3 (PR3) are the main pathogenic autoantibodies in AAV. Conventional treatments, including glucocorticoids and cyclophosphamide, could induce remission in most patients but adverse effects are common [2]. Thus, access to improved therapies remains an unmet need.

Sphingosine-1-phosphate (S1P) is a biologically active lipid produced by phosphorylation of sphingosine by sphingosine kinases (SphKs). S1P binds to five related G-protein-coupled receptors (GPCRs) termed S1PR1-5 and regulates diverse cellular processes in neovascularization, lymphocyte trafficking, inflammation and tumor growth [3–5]. The intervention of S1P and related components have been implicated in several immune-mediated disorders including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases and ischemic stroke based on the lymphocyte homing from circulation and hence immunosuppression [6]. Notably, the immunomodulatory drug FTY720 (Fingolimod, an S1P analogue) has been approved for oral treatment of relapsing-remitting multiple sclerosis due to its impressive efficacy and good tolerability [7].

Although the pathogenesis of AAV is not fully understood, increasing clinical and experimental evidence indicates the vital role of T-cell autoimmunity [8]. Helper and cytotoxic T cells recognize MPO as a vessel-planted antigen, inducing the activation of autoreactive B cells, production of ANCA and recruitment of leukocytes to destroy vasculature. Depletion of CD4+, CD8+ T cells or their cytokines could attenuate the injury in murine anti-MPO glomerulonephritis [9–11]. It is reasonable to speculate that S1P–S1PR1 dependent T cells depletion could improve

the condition of AAV. Furthermore, as a downstream product of C5a-primed neutrophils, S1P was found in our previous studies to enhance the activation of neutrophils and interact with its receptors (S1PR1 and S1PR3) to maintain the stability of human glomerular endothelial cells (GENC) stimulated with ANCA [12,13]. In the present study, we hypothesized that the intervention of S1P-related targets would ameliorate AAV. We tested this hypothesis in an experimental autoimmune vasculitis (EAV) rat model using three agents: FTY720 (modulator of S1PR1, 3, 4 and 5), SEW2871 (S1PR1 agonist) and TY52156 (S1PR3 antagonist), due to their potential immunosuppressive and vascular protective effects.

## Materials and methods

### Animals

Six-week-old female WKY rats were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). They were fed with standard solid laboratory chow and tap water ad libitum with controlled temperature, 50% relative humidity and a 12-h light/dark cycle. During housing, rats were monitored daily for health status. All sections of animal experiments adhered to the ARRIVE guidelines and were performed in Laboratory Animal Facility of Peking University First Hospital. The study was approved by Laboratory Animal Ethics Committee of Peking University First Hospital and all experiments were in accordance with Laboratory Animal Care and Use Committee of Peking University First Hospital (number: J201760).

### Experimental autoimmune vasculitis induction

The rat model of MPO-ANCA associated vasculitis has been described previously by Little et al. [14,15]. Briefly, WKY rats were intramuscularly and subcutaneously immunized with 1600 µg/kg human MPO (hMPO; 475911, Merck Nottingham, U.K.) in Freund's complete adjuvant, along with 1 µg of Pertussis toxin (P7208, Sigma-Aldrich, St. Louis, Missouri, U.S.A.) i.p. twice post immunization. Equivalent human serum albumin (HSA) immunized rats were included as controls. All rats were killed by inhaling carbon dioxide at week 8.

### Reagents and treatment protocol

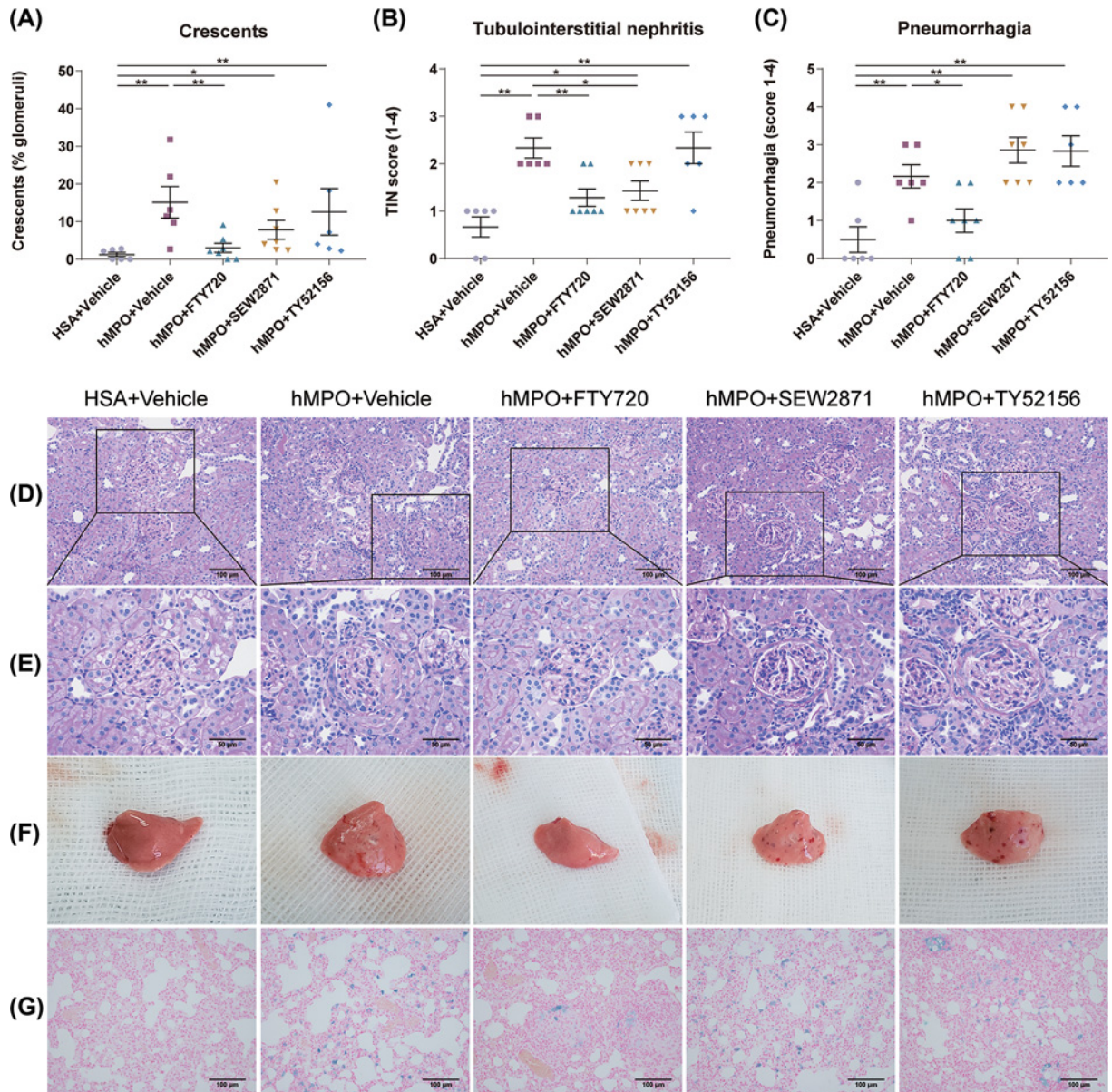
S1P modulators obtained from APExBIO (Houston, Texas, U.S.A.) were prepared in a mixture of 10% dimethyl sulfoxide, 40% PEG300 and 50% phosphate-buffered saline solution according to the product reports. The EAV rats were randomly divided into four groups and treated with vehicle, FTY720 1 mg/kg, SEW2871 0.5 mg/kg or TY52156 1 mg/kg, respectively ( $n = 6-7$  per group, the dosages of treatments were derived from previously reported rodent experiments [16–18]), starting when the disease was well established at week 4. All rats including normal controls got vehicle or drugs i.p. five times per week until killed at week 8. Pharmacological treatments were performed by staff in the laboratory animal center and the experimenters were blinded while processing data.

### Urine and blood analysis of EAV rats

Hematuria was assessed semi-quantitatively (0–4) with dipstick (URIT, Shenzhen, China), and proteinuria was assessed by ELISA (9040, Chondrex, Washington, U.S.A.) following the manufacturer's introductions. Blood samples were taken from the orbital vein under the anesthesia with pentobarbital (40 mg/kg i.p., P3636, Sigma-Aldrich, St. Louis, Missouri, U.S.A.). Circulating anti-hMPO antibody titers were measured with ELISA as previously described [14]. Ninety-six-well plates were coated with 2 µg/ml hMPO, incubated with gradient dilutions of rat serum, developed with alkaline phosphatase-conjugated goat antibodies specific for rat IgG (1:10000, A6066, Sigma-Aldrich, St. Louis, Missouri, U.S.A.) and pNPP-Na substrate, finally analyzed spectrophotometrically at OD 405 nm. Peripheral lymphocyte subsets were determined using flow cytometry at sacrifice. After red blood cells lysis, leukocytes were stained with anti-rat CD3 FITC, anti-rat CD4 APC-Cy7, anti-rat CD8a PerCP, anti-rat CD45RA PE-Cy7 or isotype antibodies (all BioLegend, San Diego, California, U.S.A., detailed information was in Supplementary Table S1). Data were analyzed by FlowJo software 7.6 (Tree Star, Ashland, Oregon, U.S.A.).

### Histology and immunohistochemistry

Kidneys and lungs were harvested at the time of killing and subsequently assessed for the histopathological lesions as described by Little et al. [14,15]. After fixing in 4% paraformaldehyde and embedding in paraffin, tissues were cut as 3 µm sections for light microscopic studies. We used periodic acid–Schiff (PAS) stain to assess the renal involvement in EAV rats. Developments of crescentic glomerulonephritis and tubulointerstitial nephritis (TIN) were evaluated as the percentage of crescents and previously described global visual analogue score (0–3): 0 = no TIN; 1 = a single focus of TIN; 2 = <25% tubulointerstitium involved; 3 = ≥25% tubulointerstitium involved. In addition to renal pathology,

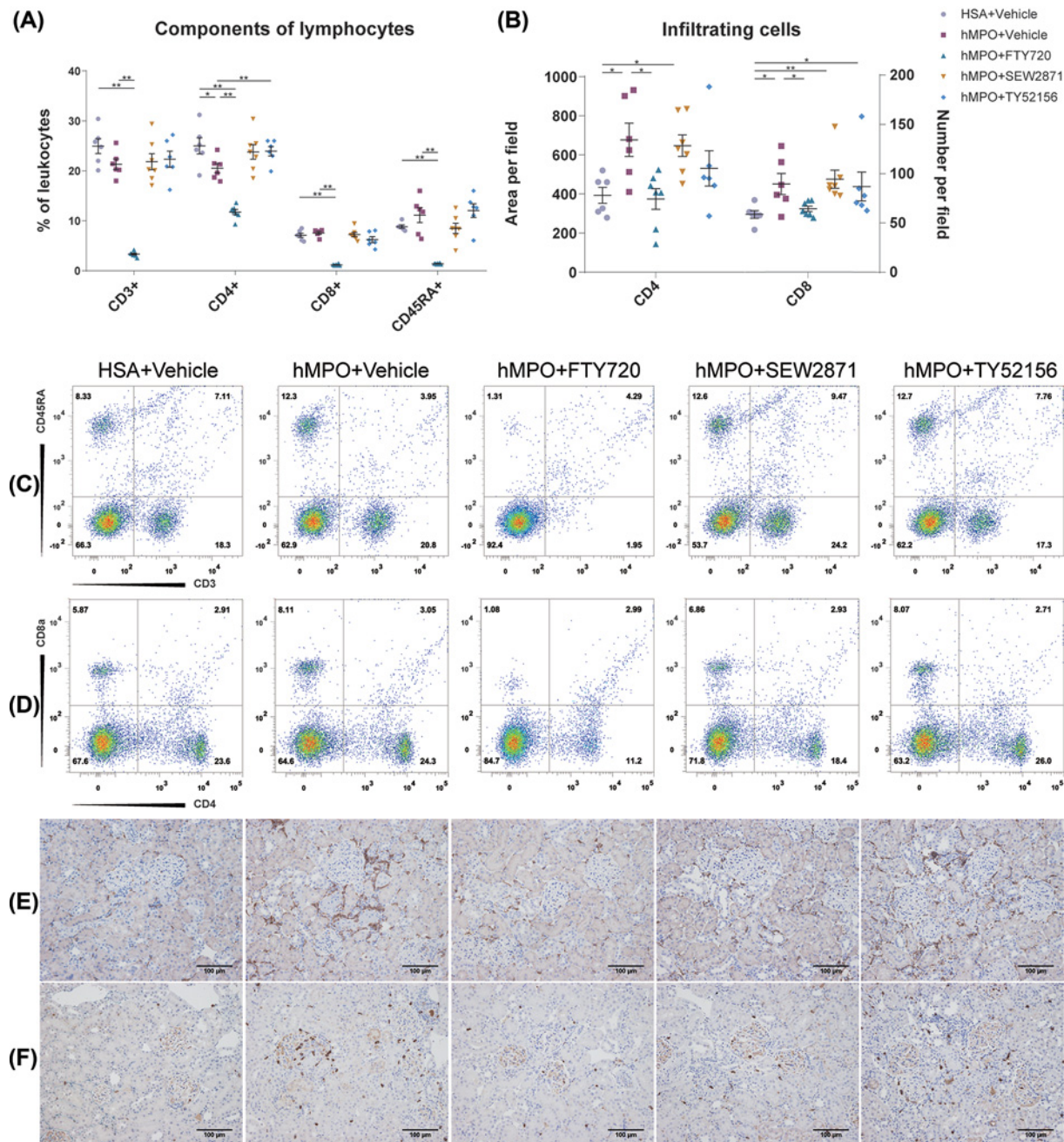


**Figure 2. S1P modulators attenuated renal and pulmonary histopathological lesions in EAV**

(A) FTY720 treatment reduced fractions of crescents in glomeruli. (B) FTY720 and SEW2871 treatments both alleviated tubulointerstitial nephritis score. (C) FTY720 attenuated pulmonary hemorrhage. (D and E) PAS staining of kidney sections after sacrifice. (F) Lung macroscopic photos at sacrifice. (G) Perl's Prussian blue staining of lung haemorrhage after killing. Blue color represents hemosiderin depositions. Red color represents nucleus. (D and G) Bar = 100  $\mu$ m; (E) Bar = 50  $\mu$ m. Data represent means  $\pm$  standard error from six to seven rats per group; \* $P$ <0.05, \*\* $P$ <0.01 compared with HSA- or hMPO-treated rats.

the development of pulmonary vasculitis in EAV was graded according to the amount of lung surface bleeding: 0 = no hemorrhage; 1 = a single hemorrhage; 2 = 2–5 hemorrhage; 3 = 6–12 hemorrhage and 4 = >12 hemorrhage. Lung hemorrhage was also verified with Perl's Prussian blue stain (G1426, Solarbio, Beijing, China) following the manufacturer's instruction.

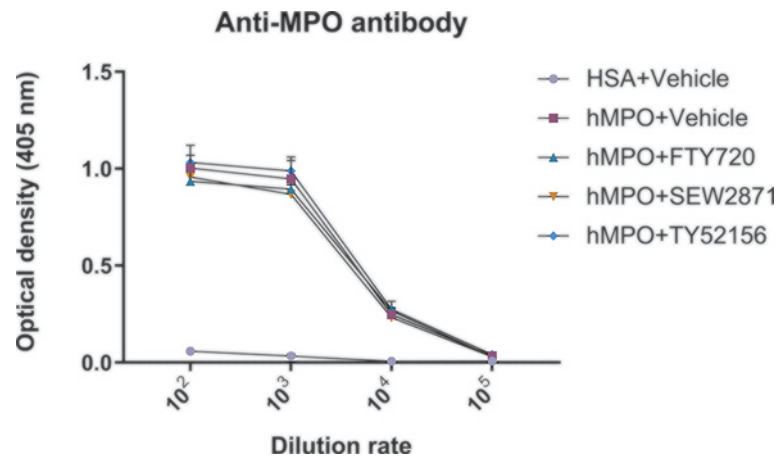
Infiltration of lymphocytes into the kidney was quantified with immunohistochemistry as previously described [19]. After antigen retrieved in citrate buffer (0.01 M, pH 6.0), endogenous peroxidase activity quenched and non-specific staining blocked, renal slides were incubated with primary antibodies for CD4 (1:100, Cell Signaling Technology, Danvers, Massachusetts, U.S.A.), CD8 (1:100, Invitrogen, Waltham, Massachusetts, U.S.A.) or CD68 (1:100, Bio-Rad, Hercules, California, U.S.A., detailed information was in Supplementary Table S1) overnight at



**Figure 3. FTY720 reduced circulating lymphocyte fraction and renal T-cell infiltration**

(A) FTY720 treatment reduced all measured T-cell fractions in blood. (B) Renal infiltration of CD4+ and CD8+ T cells in EAV was reduced by FTY720 treatment. (C and D) Representative scatter diagrams of circulating lymphocyte fractions. (E and F) Immunohistochemistry staining for CD4+ T cells (upper) and CD8+ T cells (lower) in renal specimens; bar = 100 μm. Data represent means ± standard error from six to seven rats per group; \* $P < 0.05$ , \*\* $P < 0.01$  compared with HSA- or hMPO-treated rats.

4°C and secondary antibodies (PV9001 for rabbit antibodies and PV9002 for mouse antibodies, ZSGB-Bio, Beijing, China) at 37°C for 20 min. The chromogenic reaction was developed with fresh hydrogen peroxide plus 3-3'-diaminobenzidine tetra hydrochloride solution for 30 s and hematoxylin for 8 min. Specimens were rinsed with phosphate-buffered saline (PBS) between each step. The staining results were evaluated by Image Pro Plus (version 6.0; Media Cybernetics, Dallas, Texas, U.S.A.) as areas or quantity of infiltrating lymphocytes in view of the microscopic appearance.



**Figure 4. Little effect of S1P modulators was found on hMPO-specific antibodies productions**

The gradient diluted serum and relative optical density value were adopted to detect the production of hMPO-specific antibodies but no difference was found among drug treated groups. Data represent means  $\pm$  standard error from six to seven rats per group; \* $P < 0.05$ , \*\* $P < 0.01$  compared with HSA- or hMPO-treated rats.

## Cell culture

We chose commonly used Jurkat cell lines (E6-1) as a prototypical T-cell line to investigate multiple events in T-cell biology [20,21]. The cell line was obtained from the Cell Resource Center, Peking Union Medical College (which is the headquarter of National Infrastructure of Cell Line Resource, NSTI) on September 15th, 2014. The cell line was checked free of mycoplasma contamination by PCR and culture. Its species origin was confirmed with PCR. The identity of the cell line was authenticated with STR profiling (FBI, CODIS). E6-1 T cells were cultured in advanced RPMI-1640 medium (12633020, Gibco, Thermo Fisher Scientific, Waltham, MA, U.S.A.) containing 10% fetal bovine serum (10099141, Gibco), 1% penicillin/streptomycin solution (15140122, Gibco) at 37°C with 5% CO<sub>2</sub>. Human glomerular endothelial cells (GEnC; ScienCell, San Diego, California, U.S.A.) were grown at 37°C and 5% CO<sub>2</sub> in endothelial cell medium (ECM; ScienCell San Diego, California, U.S.A.) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 1% endothelial cell growth factor according to the manufacturer's instructions.

## Qualification of FTY720 effects on T cells

The effects of gradient concentration of FTY720 (100 nM, 1  $\mu$ M and 10  $\mu$ M according to the pharmacokinetic modeling of FTY720 in rats [22]) on T cells were detected by assessing proliferation, apoptosis, adhesion and migration two times independently. Serum was collected from six healthy volunteers and six MPO-AAV patients before immunosuppressive therapies. After drawn into red topped tubes, the whole blood was left at room temperature for 20 min to clot. The serum was centrifuged at 2000 g for 10 min at 4°C and stored in aliquot at -80°C until use. When testing, after rapid thawing at 37°C, the frozen specimens were transferred immediately onto ice before use within 1 h. Repeated freeze/thaw cycles were avoided. Informed consent was signed by each participant. The characteristics of AAV patients were listed in Table 1.

## Viability, proliferation and apoptosis

A total of  $3 \times 10^5$  T cells were pretreated with FTY720 or vehicle for 1 h at 37°C and then stimulated with 10% MPO-AAV or healthy serum at 37°C with 5% CO<sub>2</sub> for 24 h. We detected cells viability with Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) at different time-points (4, 6, 12 and 24 h) and counted live cells by Cellometer Auto 2000 (Nexcelom, Lawrence, Massachusetts, U.S.A.) with acridine orange/propidium iodide staining (AO/PI, CS2-0106, Nexcelom) to assess the proliferation of T cells [23]. In addition to the number of live cells, we also measured adenosine triphosphate (ATP) with Promega Celltiter (PR-G7570, Fisher Scientific, Pittsburgh, U.S.A.) by Mithras LB 940 (Berthold. Technologies GmbH & Co. KG, Germany) and early apoptosis using the PE Annexin V Apoptosis Detection Kit (559763, BD biosciences, San Jose, California, U.S.A.) by BD FACSVerser (BD biosciences) following the manufacturer's instructions, which reflected energy metabolism and cells death.

**Table 1** General data of the MPO-AAV patients

Parameters	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Statistics
Gender (female/male)	Female	Male	Female	Male	Female	Female	4/2
Age at diagnosis (years)	79	58	75	62	14	39	54.5 ± 9.94
MPO-ANCA titer (RU/ml)	200	52	98	200	200	56	149 (55.0–200)
Hematuria (cells/HPF)	118.7	10	36.2	287.5	6.7	22.5	29.4 (9.18–161)
Proteinuria (g/24 h)	1.52	2.84	0.44	3.39	0.51	2.36	1.84 ± 0.50
Scr (μmol/l)	747.6	907.63	761.9	232.3	63.9	61.1	462.4 ± 157.3
BUN (mmol/l)	36.56	39.57	40.48	24.3	6.57	6.94	30.4 (6.85–39.8)
eGFR (ml/min/1.73 m <sup>2</sup> )	4.094	4.577	4.115	25.326	95	109.95	15.0 (4.11–98.7)
ESR (mm/h)	94	21	23	42	8	33	36.8 ± 12.4
CRP (mg/l)	5.46	2.01	3.28	1.4	0.14	1.81	2.35 ± 0.75
BVAS	23	19	22	27	22	14	21.2 ± 1.78
Renal biopsy (crescents/glomeruli)	n/a	n/a	n/a	14/23	19/50	14/29	n/a

Statistics are shown as mean ± standard error or median (interquartile range) for different distributions.

Abbreviations: BUN, blood urea nitrogen; BVAS, Birmingham Vasculitis Activity Score; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; HPF, high power field; n/a, not applicable; RU/ml, relative units per millilitre; Scr, serum creatinine.

## Adhesion

We prepared a co-culture system of human glomerular endothelium monolayer and T cells as previously described [24]. Briefly, endothelial monolayers were grown on wells of Costar 96-well black transparent-bottom plates (Corning Life Sciences, Corning, New York, U.S.A.) and starved with FBS-free ECM for 12 h. T cells were pretreated with FTY720 or vehicle for 16 h at 37°C. To avoid the effects of proliferation effects on subsequent experiments, remaining live cells were washed and resuspended to  $3 \times 10^5$ /ml. Resuspended T cells were stained with 5 μM Cell tracker green (C2925, Invitrogen, Waltham, Massachusetts, U.S.A.) for 45 min at 37°C and added to the wells with MPO-AAV or healthy serum stimulations to adhere for 1 h at 37°C. Non-adherent cells were removed by extensive washing. Adherent T cells were reflected by fluorescence intensity (FI) measured with a microplate fluorescence reader (TriStar Multimode Microplate Reader LB941, Berthold Technologies, Bad Wildbad, Germany) with filters of 490 nm (excitation) and 520 nm (emission).

## Migration

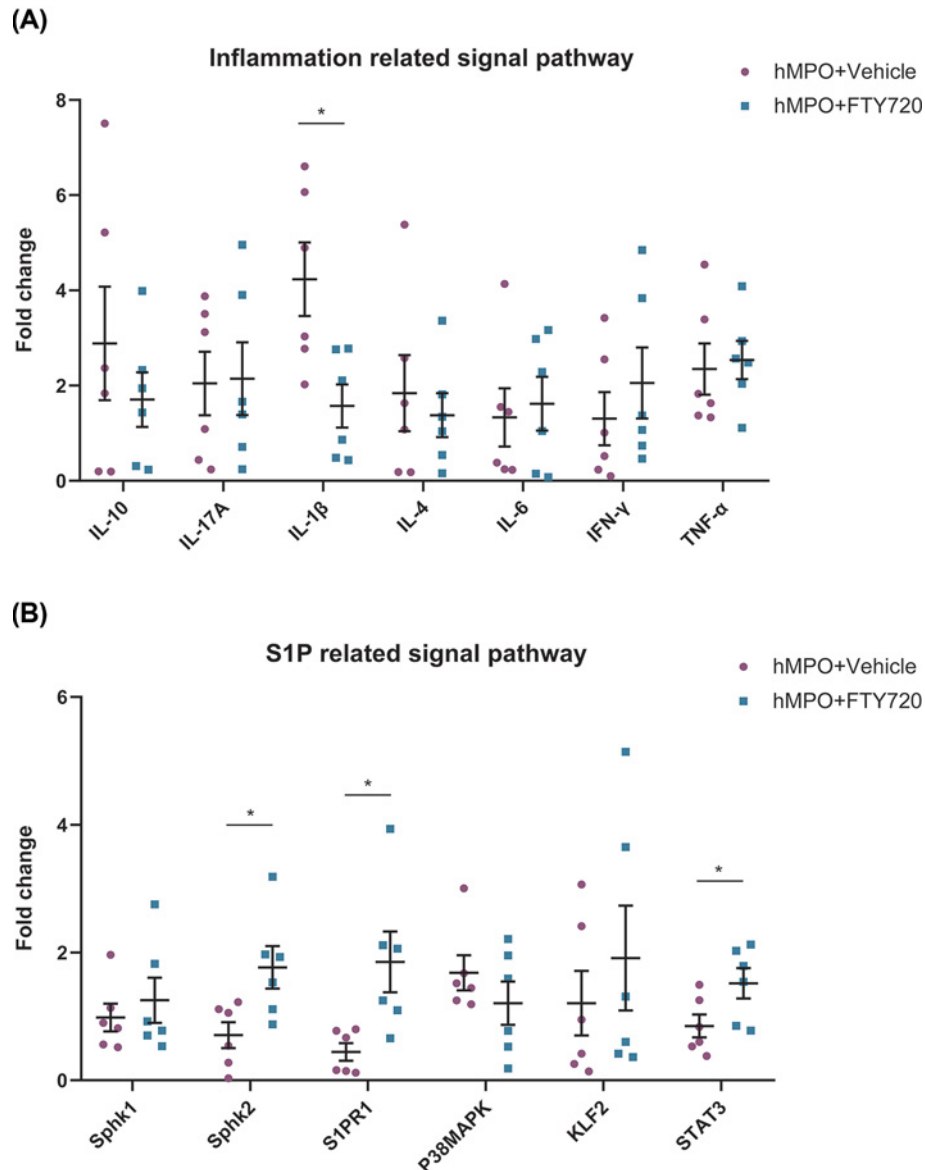
The migration assay was performed using 24-well Costar transwell plates with 8 μm-pore polycarbonate membranes inserts (3422, Corning Life Sciences, Corning, New York, U.S.A.), as previously described [24,25]. T cells were pretreated with FTY720 or vehicle for 16 h at 37°C. After washing and uniformly resuspending,  $3 \times 10^5$  live T cells were added to the upper chamber with 10% MPO-AAV or healthy serum added to the lower chamber for 4 h at 37°C. Migrated T cells in the lower chamber were counted with AO/PI staining as above-mentioned.

## Real-time quantitative polymerase chain reaction

Total RNA was extracted from rat kidneys using RNAprep pure Tissue Kit (DP431, TIANGEN, Beijing, China). After reverse transcription of RNA (4368814, Applied Biosystems, Foster City, California, U.S.A.), the mRNA level of each target gene was quantified on ViiATM7 Dx Real-Time PCR (Applied Biosystems) using SYBR Green Universal PCR Master Mix (A25918, Applied Biosystems) according to the manufacturer's instructions. Every reaction contained 10 μl 2 × PowerUp SYBR Green Master Mix, 1 μl each of 10 μM forward and reverse primers and 5 ng cDNA in 8 μl ddH<sub>2</sub>O. The cycling conditions were as follows: an activation of uracil-DNA glycosylase at 50°C for 2 min, an initial denaturation at 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 60 s. The fold change of expression levels was calculated using the comparative ΔCt method. Samples were calculated with normalization to the commonly used reference genes GAPDH and PPIA (rat endogenous reference genes primers; Sangon Biotech, Shanghai, China) [26]. Every targeted gene was independently tested three times. Gene-specific primers were listed in Table 2.

## Statistics

Experimental statistics are shown as mean ± standard error for variables with Gaussian distribution and median (interquartile range, IQR) for the others. Multiple functional studies of T cells were analyzed with one-way analysis of



**Figure 5. Involved signal pathways in the therapeutic effect of FTY720**

To further explore potentially involved signal pathways participating in the renal improvement of FTY720 treatment, related signaling pathways of inflammation (A) and S1P (B) were analyzed on mRNA level. FTY720 treated kidneys exhibited up-regulated S1PR1, SphK2, STAT3 and down-regulated IL-1 $\beta$ . Data represent means  $\pm$  standard error from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  compared with hMPO- and vehicle-treated rats.

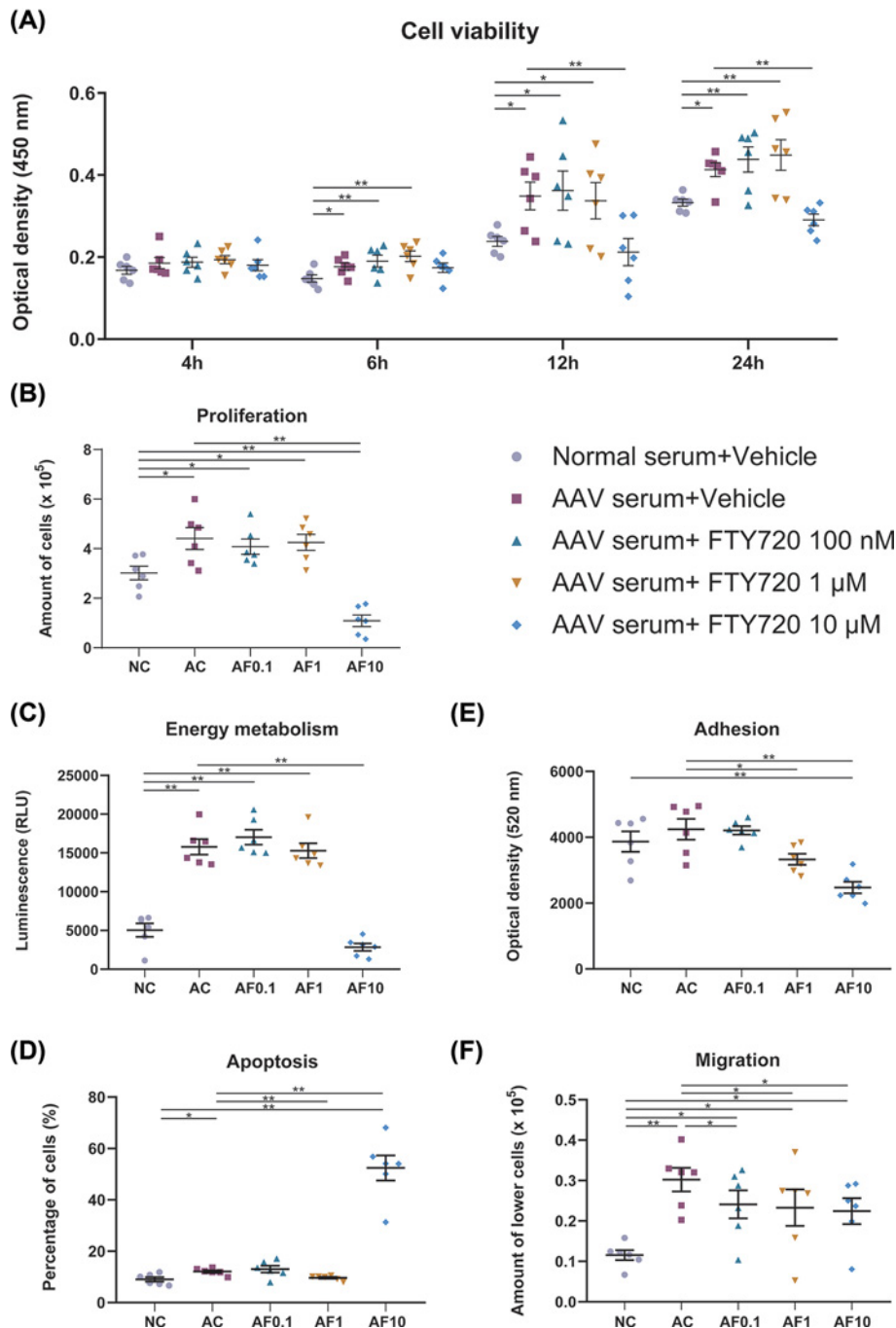
variance followed by original false discovery rate (FDR) correction of Benjamini and Hochberg. All other comparisons between two groups were evaluated using the non-parametric Mann–Whitney  $U$  test. Results were considered significant at  $P < 0.05$ . Data analysis was performed with SPSS version 20.0 (SPSS, Chicago, Illinois, U.S.A.).

## Results

### FTY720, but not other S1P modulators, alleviated the urinary manifestations in EAV

By week 4, all hMPO-immunized rats developed proteinuria and hematuria (Figure 1). FTY720 completely abolished proteinuria to the level in HSA-immunized rats as early as 2 weeks after commencing treatments, which persisted until killing ( $14.4 \pm 0.66$  versus  $66.9 \pm 17.3$ , compared with those treated with vehicle at week 8,  $P < 0.01$ , Figure 1A).





**Figure 6. Effects of FTY720 treatment on a typical T cell line with AAV serum stimulation**

A total of  $3 \times 10^5$  Jurkat E6-1 T cells were adopted to investigate the direct effect of FTY720 treatment on T cells stimulated with MPO-AAV serum. High dose FTY720 induced extensive inhibition of T-cell viability over time (A) determined by CCK-8 assays at different time points), proliferation (B and C) with suppressed energy metabolism, determined by AO/PI and Promega luciferase assays after 24 h FTY720 treatments), apoptosis (D) determined by Annexin V staining after 24 h FTY720 treatments), adhesion (E) determined by cell track green staining after 16 h FTY720 treatments) and migration (F) determined by transwell experiments after 16 h FTY720 treatments). NC, normal serum control. AC, AAV serum control. AF0.1, AAV serum and 100 nM FTY720 treatment. AF1, AAV serum and 1  $\mu$ M FTY720 treatment. AF10, AAV serum and 10  $\mu$ M FTY720 treatment. RLU, relative light unit. Data are means  $\pm$  standard error from one representative experiment of two independent experiments; \* $P$ <0.05, \*\* $P$ <0.01 compared with normal or AAV serum treatments.

**Table 2 Gene-specific primers**

Name	Gene ID	Forward primer 5'-3'	Reverse primer 5'-3'
IFN- $\gamma$	25712	AAGACAACCAGGCCATCAGCAAC	GAACCTGGCGATGCTCATGAATGC
IL-10	25325	ACTGGCTGGAGTGAAGACCA	CATGTGGGTCTGGCTGACTG
IL-17A	301289	GACTCTGAGCCGCAATGAG	CATGTGGTGGTCCAACCTCC
IL-1 $\beta$	24494	CTCACAGCAGCATCTCGACAAGAG	CACACTAGCAGGTCGTCATCATCC
IL-4	287287	ACAAGGAACACCACGGAGAA	GGAGGTACATCACGTGGGAA
IL-6	24498	AGGAGTGGCTAAGGACCAAGACC	TGCCGAGTAGACCTCATAGTGACC
KLF2	306330	CGGCAAGACCTACACCAAGAGTTC	CGCACAAAGTGGCACTGGAAGG
P38MAPK	81649	TGCGGCTGCTGAAGCACATG	AACTGAACGTGGTCATCGGTAAGC
S1PR1	29733	TTCCGCAAGAATCTCCAAGGC	CAGCACAGCCAGAACCAGGAAG
SphK1	170897	GCCAACACATACCTCGTTCCTCTG	AAGCAGCACCAGCACCAGAAC
SphK2	308589	AGGTCTCAGGCTGTGGCACTC	TCGGAGGAGACACATGAGAGCAG
STAT3	25125	CCAGTCGTGGTGATCTCCAACATC	CAGGTTCCAATCGGAGGCTTAGTG
TNF- $\alpha$	24835	CGTCGTAGCAAACCAACCAAG	CCCTTGAAGAGAACCTGGGA

Abbreviations: IFN- $\gamma$ , interferon  $\gamma$ ; IL, interleukin; KLF2, Krüppel-like factor 2; P38MAPK, p38 mitogen-activated protein kinases; SphK, sphingosine kinase; STAT3, signal transducer and activator of transcription 3; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

Hematuria was also attenuated by FTY720 at week 8 (2.0 (1.0–3.0) versus 3.0 (2.75–3.0), compared with those treated with vehicle,  $P < 0.05$ , Figure 1B). SEW2871 or TY52156 treatment did not show obvious improvements in urinary manifestations of EAV.

## Effects of S1P receptor modulators on renal and pulmonary histology in EAV

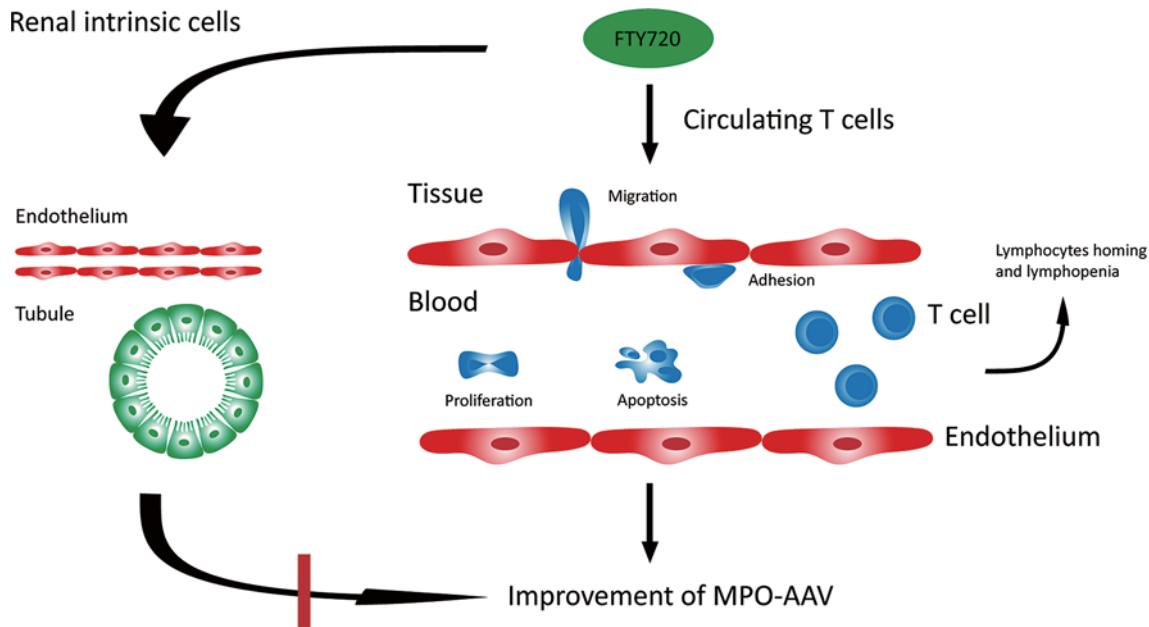
Kidneys and lungs were the most commonly affected organs in AAV. To evaluate these histopathological lesions in EAV rat models, we assessed glomerular crescent formation, TIN score and pulmonary hemorrhage after killing. Crescents were found in all hMPO-immunized rats treated with vehicle, SEW2871 or TY52156 (Figure 2A,D,E). FTY720 significantly reduced the proportion of crescents ( $3.0\% \pm 1.2\%$  versus  $15.1\% \pm 4.2\%$ ,  $P < 0.01$ , compared with those treated with vehicle). Both FTY720 and SEW2871 attenuated the tubulointerstitial lesions in EAV (1.0 (1.0–2.0) versus 2.0 (2.0–3.0),  $P < 0.01$ ; 1.0 (1.0–2.0) versus 2.0 (2.0–3.0),  $P < 0.05$ , compared with those treated with vehicle, Figure 2B). Consistent with the attenuation of renal lesions, pulmonary hemorrhage was also improved in EAV rats treated with FTY720 (1.0 (0.0–2.0) versus 2.0 (1.75–3.0),  $P < 0.05$ , compared with those treated with vehicle, Figure 2C,E,G).

## Treatment of FTY720 decreased circulating lymphocyte fraction and renal T-cell infiltration

FTY720 is known to elicit a reversible redistribution of lymphocytes from circulation to secondary lymphoid tissues [27]. In consideration of the pathogenic roles of T cells in AAV, we used immunohistochemistry to assess whether lymphopenia induced by FTY720 (Figure 3A,C,D) was associated with a commensurate reduction in renal T-cell infiltration and further attenuated vasculitic lesions in AAV. We found that FTY720 treatment reduced CD4+ and CD8+ T-cell infiltration in kidneys (Figure 3B,E,F), whereas SEW2871 or TY52156 did not. Renal infiltrating macrophages were also assessed using immunohistochemistry but no significant difference was found between vehicle and FTY720 treated groups (Supplementary Figure S1).

## Treatment of FTY720 did not affect the production of ANCA in EAV

ANCA produced by B cells is important in the development of AAV. All hMPO-immunized rats developed high titer anti-hMPO antibodies by week 8. Although B cells fraction was reduced in the circulation (Figure 3A,C), no significant difference of hMPO–ANCA production was observed among the four treated groups (Figure 4).



**Figure 7. Proposed working model for the role of FTY720 in MPO-ANCA vasculitis**

For circulating lymphocytes, FTY720 or its phosphorylated products could induce lymphocytes homing and inhibit the T cells functions. For renal intrinsic cells, phosphorylated FTY720 could attenuate local damage partially resulting from the activation of S1PR1. But single activation of S1PR1 or inhibition of S1PR3 was not sufficient to improve the established phenotype of MPO-AAV.

## The therapeutic effect of FTY720 was accompanied by altered S1P and inflammation signals

Direct renal protection of S1P intervention has been reported in some diseases including renal ischemia–reperfusion injury (IRI) and lupus nephritis [28]. We speculated that there were *in situ* signal changes contributing to the amelioration of EAV in FTY720-treated rats. To test the effect of FTY720 on renal gene activation in AAV, we analyzed S1P and inflammation related mRNA changes in kidneys. Compared with vehicle-treated EAV rats, rats treated with FTY720 exhibited up-regulated expression of S1PR1, SphK2 and STAT3, and down-regulated IL-1 $\beta$  (Figure 5).

## FTY720 showed extensive inhibition of T cells *in vitro*

Given the therapeutic effect of FTY720 in EAV and previous studies of FTY720 on lymphocytes [29], we hypothesized that FTY720, apart from its established effects on lymphocyte homing, ameliorated EAV by inhibiting T cells directly. We tested this hypothesis using four readouts: proliferation, apoptosis, adhesion and migration.

### Proliferation

To simulate the *in vivo* condition of AAV, we co-incubated commonly used Jurkat E6-1 T cells with a gradient of FTY720 and 10% serum from six patients with MPO-AAV. The proliferation was recorded as live cell counts. At a low concentration of FTY720, no obvious alteration of proliferation among treatment groups was observed. But at a high concentration of FTY720, the proliferation of E6-1 cells was significantly inhibited, which was consistent with a reduction in cell viability (Figure 6A,B). We also found a similar effect on ATP measurements, indicating that FTY720 also inhibits cellular energetics and affects cell viability *in vitro* (Figure 6C).

### Apoptosis

Since there is inhibition of proliferation, we went on to assess whether FTY720 treatment induces apoptosis using Annexin V staining. Similar to proliferation, only the high concentration of FTY720 increased early apoptosis ( $52.4\% \pm 4.9\%$  versus  $12.1\% \pm 0.5\%$ , compared with those treated with vehicle,  $P < 0.01$ , Figure 6D, Supplementary Figure S2).

## Adhesion

Glomerular infiltration by CD4+ and CD8+ T cells is crucial in the pathogenesis of AAV and is related to the adhesion of T cells to glomerular endothelium for further local injury [10,30]. Hence co-incubation of FTY720 pretreated T cells and human glomerular endothelial cells was performed. Pretreated T cells were uniformly resuspended to avoid the effect of FTY720 on proliferation and apoptosis. We found an increasing amount of T cells adhered to endothelium following MPO-AAV serum stimulation. But following FTY720 pretreatment, the adhesion of live T cells was reduced in a dose-dependent manner ( $4242.8 \pm 314.4$  without FTY720;  $4209.2 \pm 126.5$  at 100 nM;  $3328.9 \pm 167.8$  at 1  $\mu$ M;  $2473.8 \pm 174.5$  at 10  $\mu$ M; Spearman's  $r = -0.781$ ,  $P < 0.01$ , Figure 6E).

## Migration

The reduction of circulating cells could partially explain the decreased infiltration of T cells in renal tissues. But the effect of FTY720 on the migration of T cells in AAV, which is a crucial component of interstitial infiltration, is unknown. We resuspended FTY720 pretreated live T cells and quantified migration using a transwell system. We found that MPO-AAV serum increased the migration of T cells across polycarbonate membranes, which was partially reduced by all doses of FTY720 (Figure 6F).

## Discussion

As a bioactive lipid mediator that regulates diverse biological processes in health and diseases [3,6], S1P interacts with its receptors S1PR1–5 and generates many physiological functions including vascular homeostasis and immunomodulation [31]. However, the role of the S1P–S1PR axis in ANCA-associated vasculitis is not fully clear yet.

In the actively immunized rat model of MPO-AAV, three modulators of S1P receptors were administered to rats with established vasculitis [32]. We found that FTY720 (a modulator of S1PR1, 3, 4 and 5) improved established glomerulonephritis and pulmonary hemorrhage. FTY720 treatment also decreased renal infiltration by T cells with little effect on ANCA production, indicating that T cells rather than B cells might play an important role in the therapeutic effect of FTY720 in AAV.

Cell-mediated immune responses are an essential aspect of the pathogenesis of AAV [8]. Transfer of MPO or MPO<sub>409–428</sub> (an immunodominant MPO CD4+ T-cell epitope) specific CD4+ T cell could induce crescentic glomerulonephritis in  $\mu$ MT<sup>-/-</sup> (B cell deficient) or *Rag2*<sup>-/-</sup> (T/B cell deficient) mice [9,30,33]. MPO<sub>431–439</sub>-specific CD8+ T cells also mediated glomerular injury when MPO was planted in glomeruli [10]. Additionally, several studies suggested that Th1, Th17, Treg and their cytokines were involved in the development of AAV to various degrees [11,34–36]. S1P and its receptors are the major regulators of lymphocyte egress from tissues (especially the thymus and secondary lymphoid organs) into the circulation [29,37]. Inhibition of S1PR1 induces reversible lymphocytes homing [27]. FTY720 was phosphorylated by SphK2 and tested as an unselective agonist of S1P receptors (S1PR1, 3, 4, 5) and as a selective functional antagonist of the S1PR1 subtype by inducing receptor down-regulation [7]. Therefore, functional blockage of S1PR1 by FTY720 and consequent T-cell lymphopenia partially explained the alleviation of AAV in our study, underlining the importance of T cells in this model. We further explored the effect of FTY720 on T cells following stimulation by AAV serum and found that high dose FTY720 (10  $\mu$ M) markedly inhibited T-cell biology. This concentration of FTY720 is achieved in rats' kidney and spleen after administration and has been adopted in several other studies [22,38,39]. FTY720 might decrease circulating T cells via inducing lymphocytes emigration, inhibiting proliferation and upregulating apoptosis. Through its effect on adhesion and migration, FTY720 may limit the contact between T cells and glomerular endothelium and reduce renal T-cell infiltration. However, although the FTY720 could reduce the pathogenic effect of T cells in AAV, our findings cannot demonstrate the relative contribution of T-cell homing versus direct inhibition on T cells to the therapeutic effect, which is a limitation of the current study.

Accumulating evidence supports the benefit of modulating S1P and its receptors in kidney diseases [28]. S1PR1 protects ischemia–reperfusion injury via maintaining endothelial barrier integrity and stress-induced cell survival in *Rag1*<sup>-/-</sup> mice, indicating an *in situ* protection independent of T and B lymphocytes [40–42]. Similar renal protection was reported in cisplatin-induced tubule injury, chronic kidney disease with 5/6 nephrectomy, anti-thy 1 glomerulosclerosis, lupus nephritis and diabetic nephropathy [16,17,43–47]. Most studies of S1P in kidney diseases focused on two aspects: renal protection (for endothelium, podocytes and tubular cells) and lymphocyte depletion, which often involved FTY720 for the seemingly contradictory effects on S1PR1. In some studies, the FTY720 was regarded as an S1PR1 antagonist inducing circulating lymphocytes depletion [46,48,49], whereas in other studies, the therapeutic effect of FTY720 was attributed to activating S1PR1 in resident cells and could be restrained by S1PR1 knockout or

antagonism [42,43,50]. In our studies, we found that S1PR1 and related signal transducer and activator of transcription 3 (STAT3) were up-regulated, indicating activation of S1PR1 in FTY720 treated kidneys [51]. But little alleviation in EAV was found following activation of S1PR1 by SEW2871 (similar dosages were reported to activate S1PR1 and exert protective effects in rat models [17,52]), which suggests that activation of S1PR1 alone was not sufficient to improve the established phenotype of AAV compared with FTY720-induced lymphocyte depletion and inhibition (Figure 7).

S1P axis modulators such as FTY720 have been applied in numerous clinical trials and approved for the treatment of relapsing-remitting multiple sclerosis in 2010 [6,7]. The reversal of renal manifestations of EAV, qualified oral applicability and good tolerability supports FTY720 as a novel therapy in AAV, while precise and comprehensive understandings of S1P and related components need further studies.

In conclusion, S1P receptor modulator FTY720 is an effective treatment of established EAV, a rat model of MPO–ANCA vasculitis. This effect is mediated through depletion of circulating lymphocytes, reduction of renal T-cell infiltration and direct effects on T-cell proliferation, apoptosis, adhesion and migration. It has the potential as an additional therapeutic agent in AAV.

## Clinical perspectives

- Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of rapidly progressive autoimmune diseases with adverse prognosis. Given frequent relapses and adverse side effects of conventional treatments, access to improved therapies remains an unmet need.
- We show that the S1P receptor modulator FTY720 is an effective treatment of established EAV, a rat model of MPO–ANCA vasculitis. This effect is mediated through depletion of circulating lymphocytes, reduction of renal T-cell infiltration and direct effects on T-cell proliferation, apoptosis, adhesion and migration, with little effect on the production of ANCA.
- Already approved for use in humans, FTY720 shows potential as an additional therapeutic agent in AAV.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## Author Contribution

M.C., M.A.L. and M.H.Z. designed the study; L.Y.W. carried out experiments, analyzed the data and made the figures; X.J.S. proposed ideas of functional experiments *in vitro*; C.W., S.F.C. and Z.Y.L. provided methods of EAV model; L.Y.W., M.C., M.A.L. and M.H.Z. drafted and revised the paper. All authors approved the final version of the manuscript.

## Ethics Approval

This research was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Peking University First Hospital.

## Abbreviations

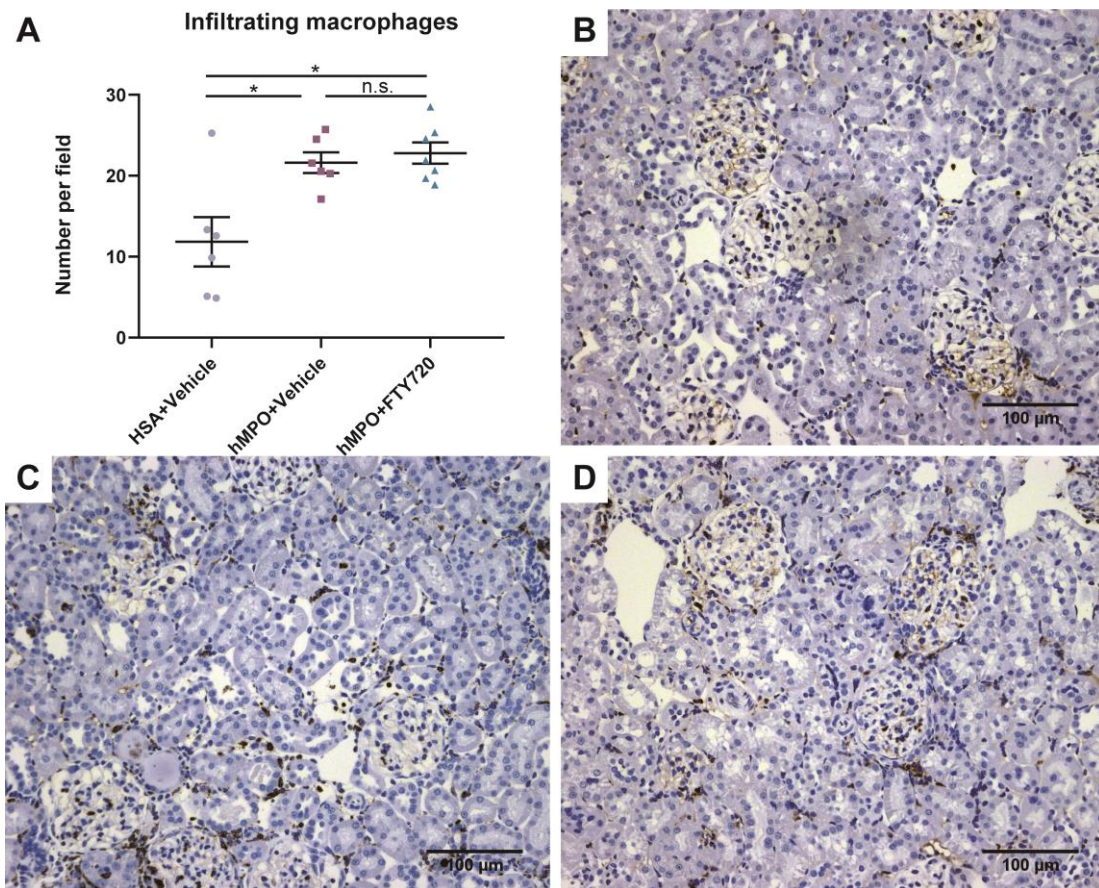
AAV, ANCA-associated vasculitis; ANCA, Anti-neutrophil cytoplasmic antibody; EAV, experimental autoimmune vasculitis; EGPA, eosinophilic granulomatosis with polyangiitis; GEnC, glomerular endothelial cells; GPA, granulomatosis with polyangiitis; GPCR, G-protein-coupled receptor; KLF2, Krüppel-like factor 2; MPA, microscopic polyangiitis; MPO, myeloperoxidase; P38MAPK,

p38 mitogen-activated protein kinases; PR3, proteinase 3; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; SphK, sphingosine kinase; STAT3, signal transducer and activator of transcription 3; TIN, tubulointerstitial nephritis.

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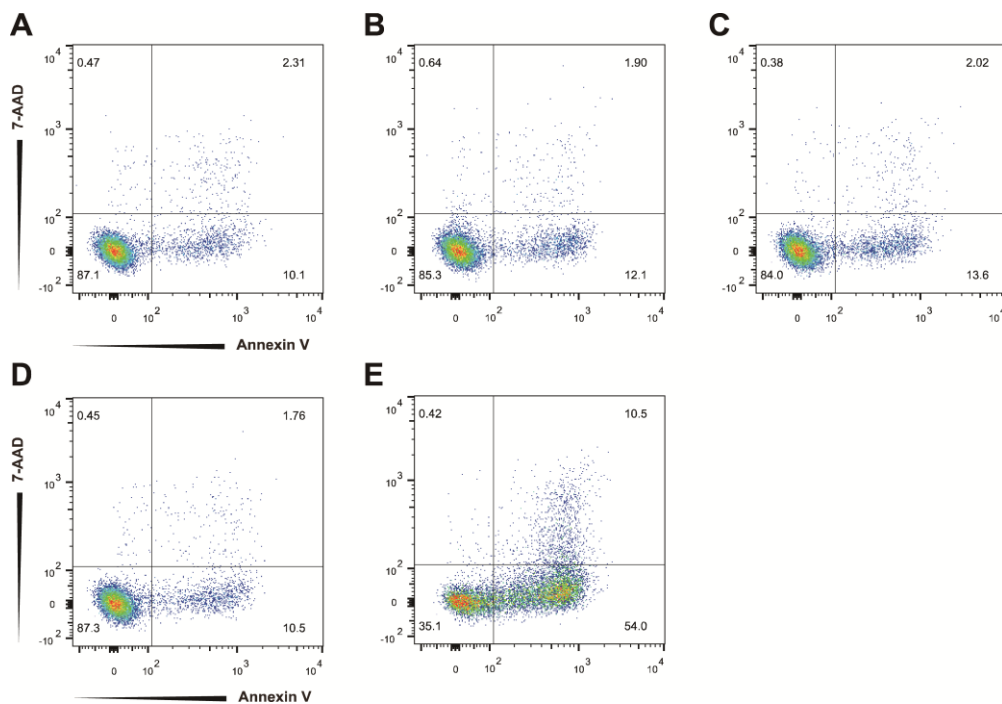
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**Figure S1. FTY720 did not influence the renal infiltration of CD68+ macrophages.**

(A) An exacerbated renal infiltration of macrophages was found in EAV, which was not alleviated with FTY720 treatment. Representative immunohistochemistry staining for CD68+ macrophages in renal specimens of normal control (B), EAV (C) or EAV treated with FTY720 (D), Bar = 100 μm. Data represent means ± standard error from six to seven rats per group. \* $P < 0.05$ , \*\* $P < 0.01$  compared with HSA or hMPO treated rats. n.s. no significance.





**Figure S2. Representative flow cytometry scatter plots of E6-1 T cells apoptosis upon FTY720 treatment.**

(A) T cells treated with normal serum and vehicle. (B) T cells treated with AAV serum and vehicle. (C) T cells treated with AAV serum and 100 nM FTY720. (D) T cells treated with AAV serum and 1  $\mu$ M FTY720. (E) T cells treated with AAV serum and 10  $\mu$ M FTY720. Viable cells were PE Annexin V and 7-AAD negative. Early apoptotic cells were PE Annexin V positive and 7-AAD negative. Late apoptotic or already dead cells were PE Annexin V and 7-AAD positive. Shown are representative scatter plots out of six samples per group.

**Table S1. Primary antibodies used for flow cytometry or immunohistochemistry**

Antibody	Host	Catalog number	Dilution	Application
FITC anti-rat CD3	Mouse	201403	1:200	Flow cytometry
FITC Mouse IgM, $\kappa$ Isotype Ctrl	Mouse	401606	1:200	Flow cytometry
APC/Cy7 anti-rat CD4	Mouse	201518	1:50	Flow cytometry
APC/Cy7 Mouse IgG1, $\kappa$ Isotype Ctrl	Mouse	400128	1:50	Flow cytometry
PerCP anti-rat CD8a	Mouse	201712	1:50	Flow cytometry
PerCP Mouse IgG1, $\kappa$ Isotype Ctrl	Mouse	400148	1:50	Flow cytometry
PE/Cy7 anti-rat CD45RA	Mouse	202316	1:100	Flow cytometry
PE/Cy7 Mouse IgG1, $\kappa$ Isotype Ctrl	Mouse	400126	1:100	Flow cytometry
CD4 (D7D2Z) monoclonal antibody	Rabbit	25229	1:100	Immunohistochemistry
CD8 (SP16) monoclonal antibody	Rabbit	MA5-14548	1:100	Immunohistochemistry
CD68 (ED1) monoclonal antibody	Mouse	MCA341R	1:100	Immunohistochemistry