COMPLETE METHODS

Suppressor of cytokine signaling-1 (SOCS1) peptidomimetic limits progression of diabetic nephropathy

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Peptide synthesis

Palmitoylated peptides derived from mouse SOCS1 KIR sequence[1,2] (residues 53-68; mutant inactive F→A) were synthesized and rhodamin-labeled (ProteoGenix), then dissolved in 1% DMSO in saline solution and filter-sterilized.

Diabetes model and treatments

All animal studies were performed according to the Directive 2010/63/EU of the European Parliament and were approved by the Institutional Animal Care and Use Committee (IIS-Fundacion Jimenez Diaz). Experimental diabetes model of insulin deficiency[2,3] was induced in male Apolipoprotein E (ApoE)-deficient mice (Jackson Laboratory) by two daily intraperitoneal injections of streptozotocin (STZ, 125 mg/kg/day; Sigma-Aldrich) in 10 mmol/L citrate buffer. Animals maintained on standard diet were monitored every 2-3 days for body weight and non-fasting blood glucose (glucometer strips). Diabetes was defined as blood glucose >19.4 mmol/L. Severely hyperglycemic mice (glucose>29 mmol/L) were given subcutaneous intermittent low dosages of long acting insulin (1–1.5 IU) to maintain blood glucose levels within a more tolerable range[3,4].

In a first experiment (early model), 8-week-old mice (n=18) were made diabetic and after 2 weeks of STZ injection were randomized to receive either vehicle (0.1% DMSO in saline solution, 200µL; n=10) or SOCS1-derived peptide (65 µg/day in 200µL; n=8) administered intraperitoneally every second day for 6 weeks. Age-matched non-diabetic mice (n=5) receiving citrate buffer alone were used as control.

In a parallel experiment (late model), 22-week-old ApoE mice (n=19) were made diabetic and after 2 weeks of STZ injection were treated with vehicle (0.1% DMSO in saline solution, 200µL; n=8), SOCS1 peptide (65 µg/day in 200µL; n=6), and mutant inactive peptide (65 µg/day in 200µL; n=5) administered intraperitoneally every second day for additional 10 weeks.

Clinical signs of toxicity related to treatment such as weight gain/loss, abnormal behavior, discoloration of urine, stool and fur were monitored throughout the studies.

At 16 and 34 weeks of age, 16 hour-fasted mice were anesthetized (100 mg/kg ketamine and 15 mg/kg xylazine) and saline perfused. After collection of blood and urine samples, the mice were killed and kidneys were harvested, then snap-frozen for RNA/protein expression studies or stored in 4% paraformaldehyde for histology.

Biodistribution and pharmacokinetics

For localization experiments, mice received a single intraperitoneal injection of rhodamine-labeled SOCS1 peptide, then sacrificed at 0, 3, 6, 16 and 24 h after injection (n=3 animals each time point). The liver, kidneys and spleen were removed and immediately frozen until analysis. Tissue biodistribution was evaluated *ex vivo* by confocal microscopy. Additionally, fluorescence intensity in homogenized samples was measured (λemission=540nm; λexcitation=570nm) and data normalized to the weight of organ/tissue.

For pharmacokinetics, fluorescence in plasma and urine samples taken at different time points (0.5, 1, 2, 3, 4, 6, 8, 16, 20 and 24 h; n=2 each) was measured. Plasma fluorescence data versus time were plotted, and the data were fitted into a straight line to calculate the plasma half-life of peptide.

Blood and urine determinations

Serum concentrations of total cholesterol, LDL, HDL and triglyceride were determined by autoanalyzer. ELISA kits were used to measure glycated hemoglobin A1c (Gentaur) and urine albumin (Cell Trend). Serum and urine creatinine values were determined by creatininase enzymatic method (Abcam). Single-cell suspensions from EDTA-buffered blood were treated with erythrocyte lysis buffer, then stained with antibodies to CD115 and Ly6C (eBioscience) and analyzed by flow cytometry[5,6].

Histology and immunohistochemistry

Paraffin-embedded kidney sections (3 μm) were stained with periodic acid-Schiff (PAS) to evaluate renal pathology. Renal lesions were and blindly graded (0-3 scale) according to the extent of glomerular changes (hypertrophy, hypercellularity, mesangial expansion and capillary dilation; 30 glomeruli), tubular lesions (atrophy and degeneration; 20 fields at 40X magnification), and interstitial damage (fibrosis and infiltration; 20 fields at 40X magnification)[3,4]. Glomerular area and PAS^+ mesangial area were quantified by a computerized image analysis system (MetaMorph; Molecular Devices). Collagen content was examined by picrosirius red staining. Immunodetection of phosphorylated STAT1 (P-STAT1 (Tyr701); Invitrogen), phosphorylated STAT3 (P-STAT3 (Ser727); Cell Signaling), macrophages (F4/80; Serotec) and T lymphocytes (CD3; DAKO) was performed by indirect immunoperoxidase technique. Positive staining (>20 fields at 20X magnification; 2-3 tissue slices/mice) were quantified using Image Pro-Plus analysis software (Media Cybernetics), and positive area was expressed as percentage of total area. $P\text{-}STAT^+$, $F4/80^+$ and $CD3^+$ cells were expressed as number per glomerular cross-section and number of interstitial cells per mm^2.

Cell cultures

The murine mesangial cell line SV40 MES 13 (CRL-1927; American Type Culture Collection) was maintained in 3:1 mixture of DMEM and Ham's F12 medium containing 14 mmol/L HEPES and 5% FBS (Life Technologies). The murine proximal tubuloepithelial MCT line[4,7] was cultured in RPMI 1640 containing 10% FBS. Bone marrow-derived macrophages were cultured for 7 days in DMEM with 10%

FBS and supplemented with 10% L929-cell conditioned medium as a source of macrophage colony stimulating factor[5]. All culture media were supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L L-glutamine (Life Technologies).

Quiescent cells (24 h in medium without FBS) were treated for 90 min with different peptide concentrations (25-200 µg/mL) before stimulation with murine recombinant cytokines (10^3 U/mL IFNγ plus 10^2 U/mL IL-6; PeproTech) and high-glucose concentration (30 mmol/L D-glucose).

Transfection of small-interfering RNA

MCT cells grown to 60-70% confluence were transfected with 30 nmol/L of small interfering RNA (siRNA) targeting STAT1 or negative control scramble siRNA (Ambion) using Lipofectamine RNAiMAX reagent (Life Technologies)[4]. Transfected cells (silencing efficiency 70%) were pretreated with SOCS1 peptide (100 µg/mL) for 90 min before 16 h of stimulation with high-glucose concentration.

mRNA expression analysis

Total RNA from mouse kidney and cultured cells was extracted with Tryzol (Life Technologies)[3,7]. Target gene expression (*Stat1, Stat3, Socs1, Kim1, ColI, Fn, Tgfβ, Tnfα, Ccl2, Ccl5, Cxcl10, ArgI* and *ArgII*) was analyzed by real-time quantitative PCR (Applied Biosystem) and normalized to 18S housekeeping gene.

Protein expression analysis

Total proteins from kidney tissue and cultured cells were homogenized in ice-cold buffer containing 1% Triton X-100, 0.5% NP-40 and protease inhibitors. Cell conditioned media were collected for chemokine and fibronectin expression.

Proteins were resolved on SDS-PAGE gels, transferred onto polyvinylidene fluoride membranes and immunoblotted for P-STAT1 (Invitrogen), P-STAT3, STAT3 (Cell Signaling), fibronectin (Millipore Corporation), STAT1, TGFβ, arginase I and II (Santa Cruz Biotechnology), using α-tubulin (Sigma-Aldrich) or β-actin (Santa Cruz Biotechnology) as loading controls and peroxidase/biotin conjugated secondary antibodies (Amersham). Immunoblots were quantified using Quantity One software (Bio-Rad Laboratories).

CCL2 levels and CCL5 levels in renal samples and cell supernatants were measured using mouse ELISA kits (BD Biosciences and eBiosciences, respectively).

Immunofluorescence analysis was performed in fixed, permeabilized cells by incubation with P-STAT1 and P-STAT3 antibodies, followed by conjugated secondary antibodies (Alexa Fluor 488; Invitrogen) and nuclear counterstaining (4',6-diamidino-2-phenylindole; Sigma-Aldrich). Images were captured using a confocal fluorescent microscope (Leica).

Cell migration and proliferation assays

Mesangial cell migration was measured by the wound-healing assay[2,8]. Briefly, cells in a confluent monolayer were serum-depleted, followed by a wound injury using a plastic pipette tip, and then preincubated with peptides before cytokine stimulation. Images were collected during the healing period (0-24 h) using a phase contrast microscope (Nikon), and the remaining wound areas were quantified and normalized to time 0 values.

For cell viability/proliferation studies, cells were maintained for 48 h in culture medium alone (viability) and supplemented with either cytokines or high-glucose (proliferation) in the presence of different peptide concentrations, and then assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

Statistical analysis

Results are presented as individual data points and mean±SEM. Each experimental condition was analyzed in duplicate/triplicate. Statistical analysis was performed using Prism 5 (GraphPad Software Inc). Data passed the D'Agostino and Pearson omnibus normality test and were tested for homogeneity of variance with the Bartlett test. Pearson's correlation analysis were performed for normally distributed parameters. Differences across groups were considered significant at $P<0.05$ using either unpaired Student's t test or one-tail ANOVA followed by post-hoc Bonferroni pairwise comparison test.

References

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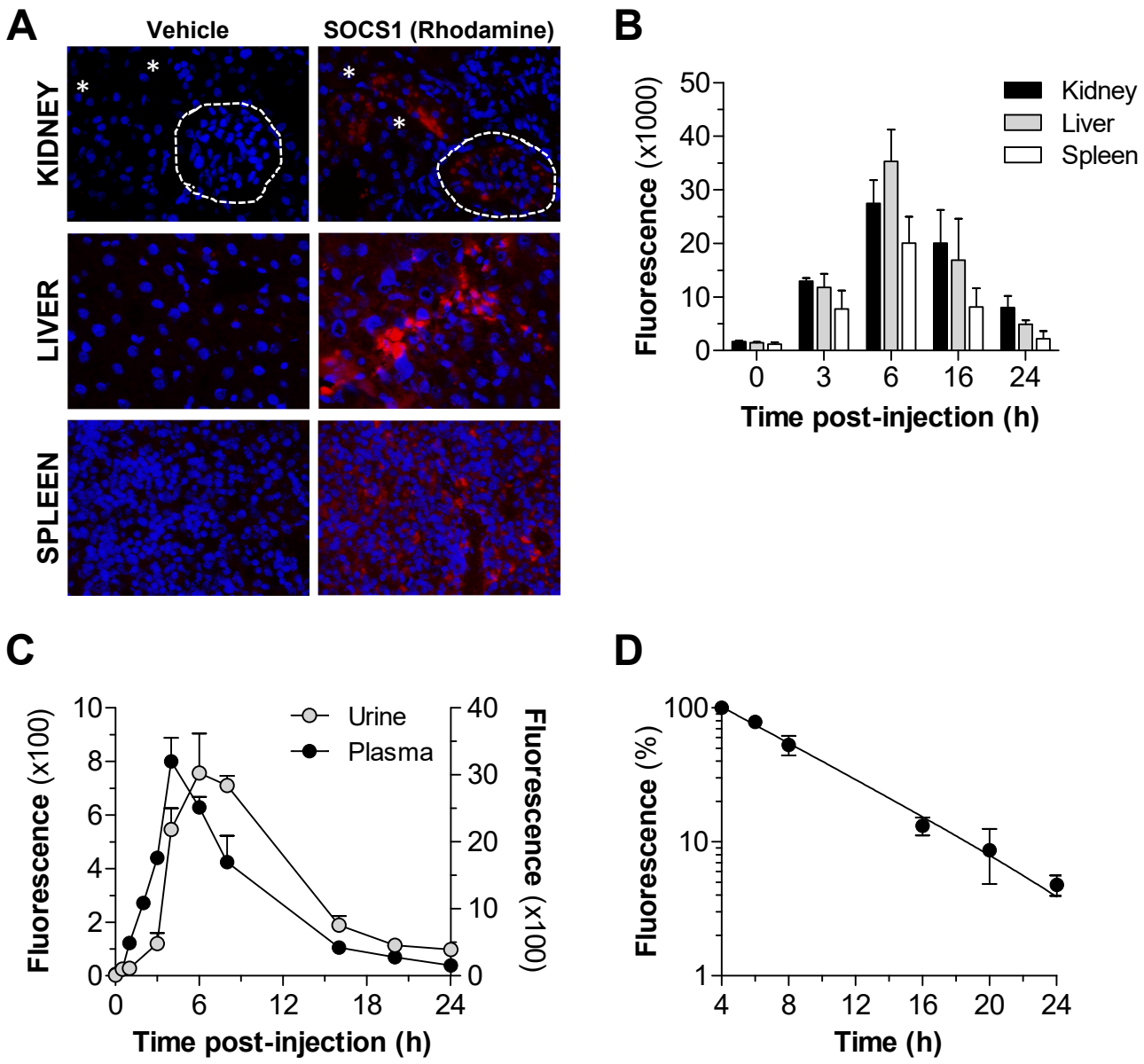
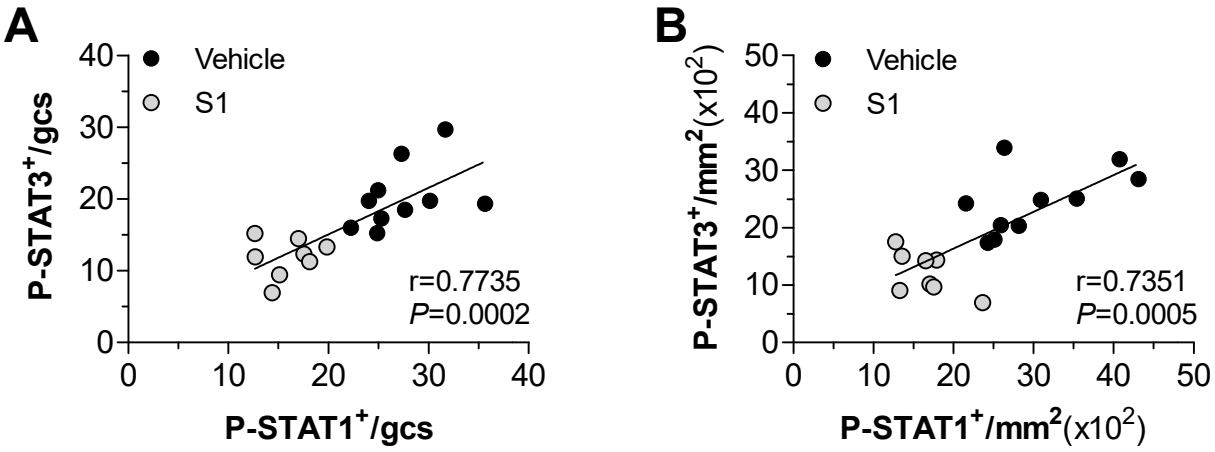
Supplemental Figure S1
(Recio et al.)



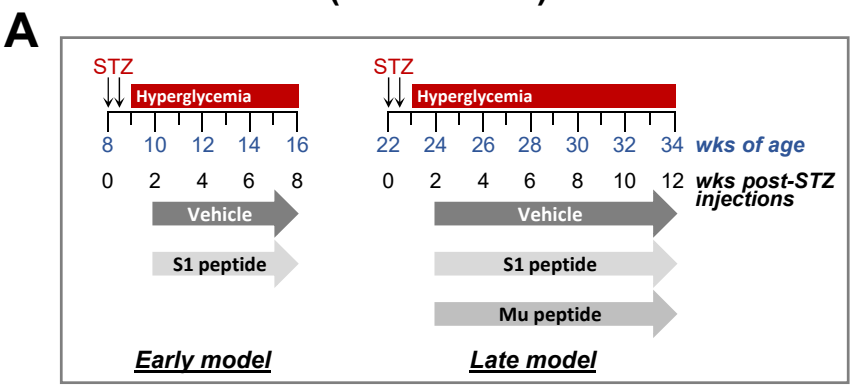
Biodistribution and pharmacokinetics of SOCS1 mimetic peptide in ApoE-deficient mice. (A) *Ex vivo* fluorescence microscopy images of mouse tissue sections at 6 h of intraperitoneal injection of either vehicle or rhodamine-labeled SOCS1 peptide (magnification x200; red, S1; blue, DAPI labeled nuclei). Dotted lines indicate glomerular area; asterisks indicate tubular cells. **(B)** Quantification of red fluorescence in tissue samples over time (n=4) was expressed as relative fluorescence per g of tissue. (**C**) Fluorescence in plasma and urine at different time points (n=2-3 per time point). (**D**) Plasma fluorescence data were normalized, plotted in a logarithmic scale versus time post-injection, and fitted by nonlinear regression to a one-phase exponential decay function (R^2=0.976).

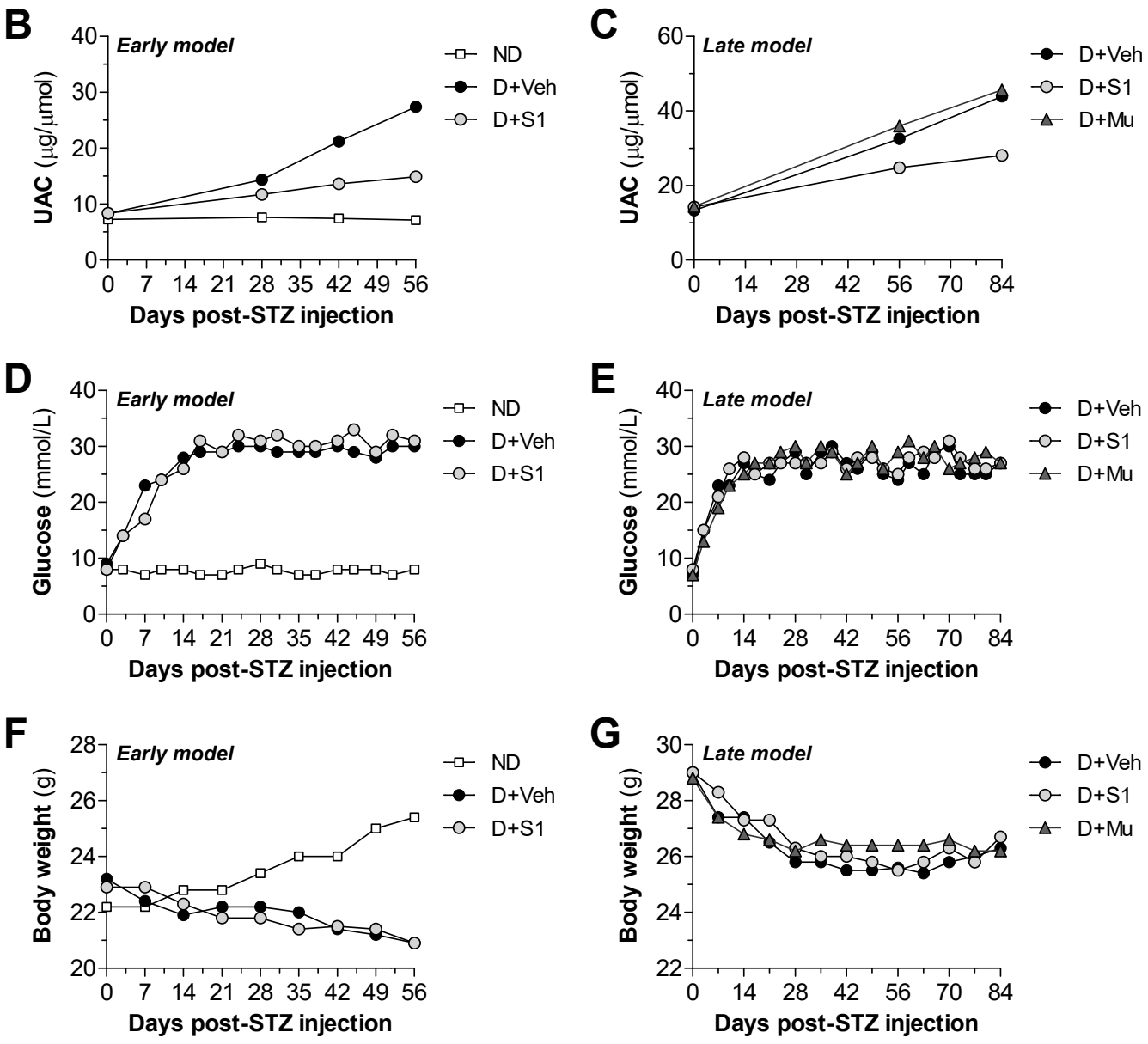
Supplemental Figure S2
(Recio et al.)



Correlation of STAT activation levels in diabetic mice. Pearson's correlation analysis of STAT1 versus STAT3 activation in glomerular **(A)** and tubulointerstitial **(B)** compartments.

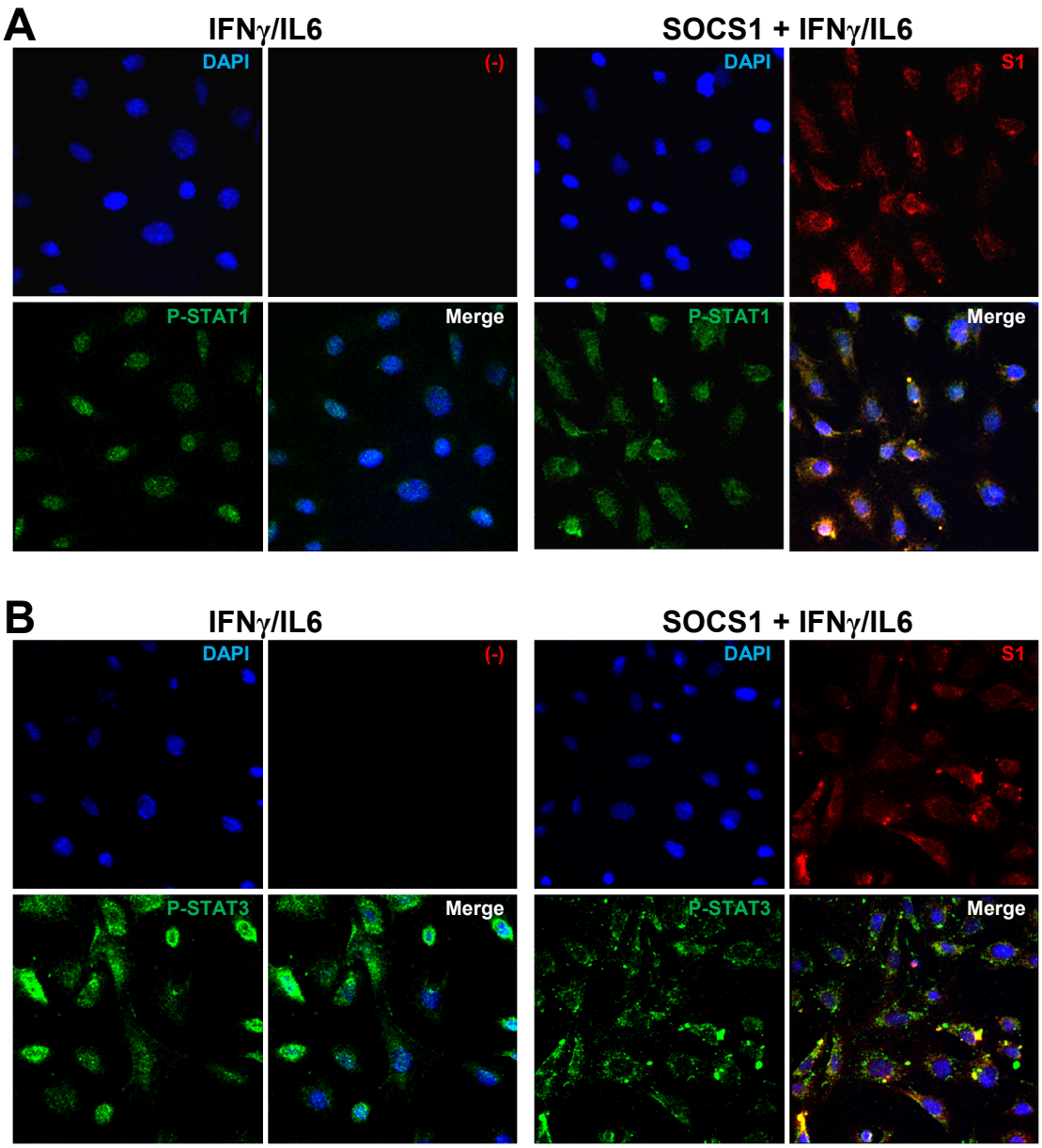
Supplemental Figure S3
(Recio et al.)



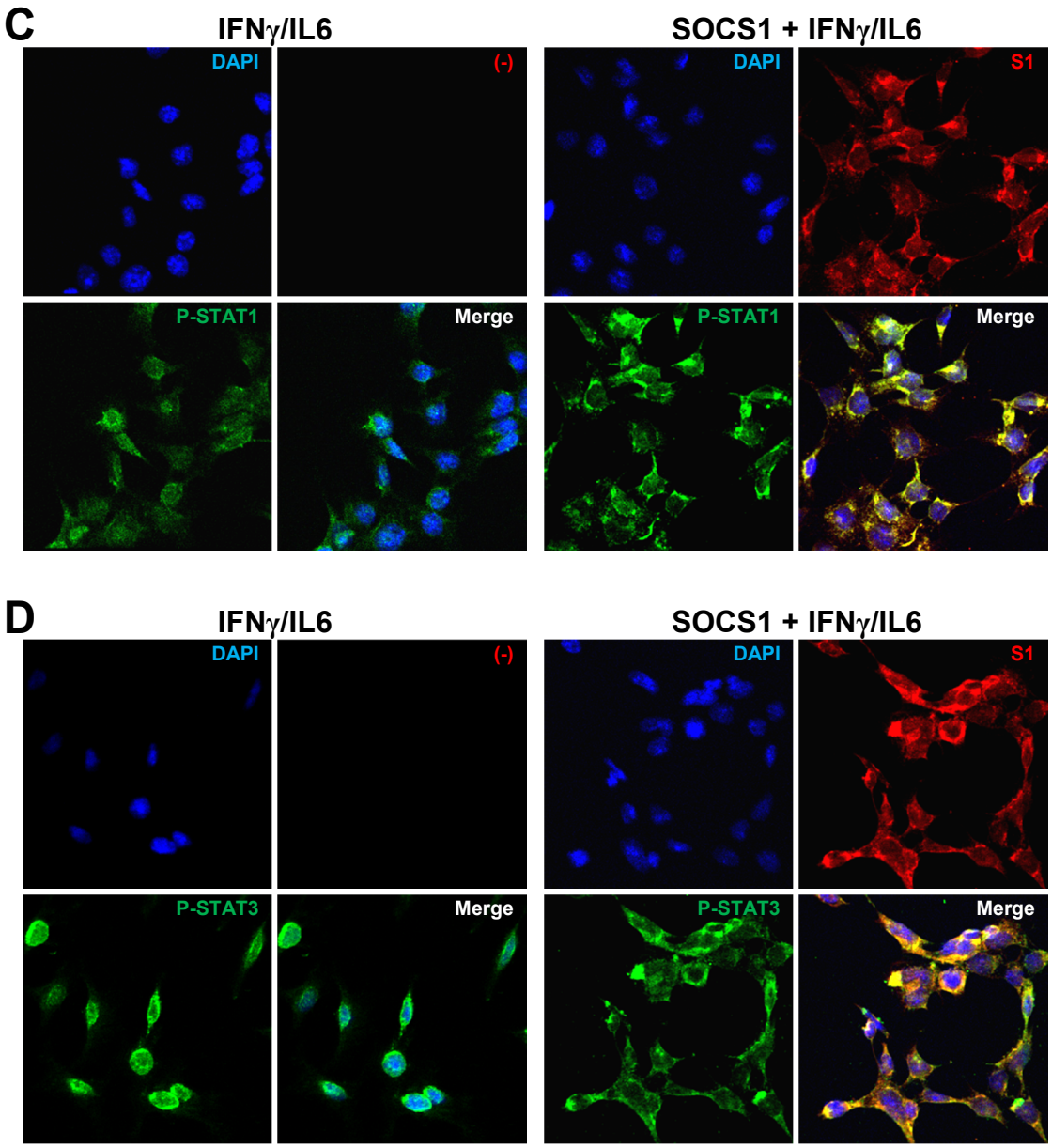


Experimental model of diabetes in ApoE-deficient mice and treatments. (A) Scheme of experimental procedure. **(B-G)** Evolution of urine albumin-to-creatinine ratios **(B**, **C),** blood glucose levels **(D**, **E)** and body weights **(F**, **G)** in the experimental groups of mice at early and late treatments. Average values of 3-10 animals per group are represented. ND, non-diabetic; D, diabetic; Veh, vehicle; S1, SOCS1 peptide; Mu, mutant peptide.

Supplemental Figure S4
(Recio et al.)

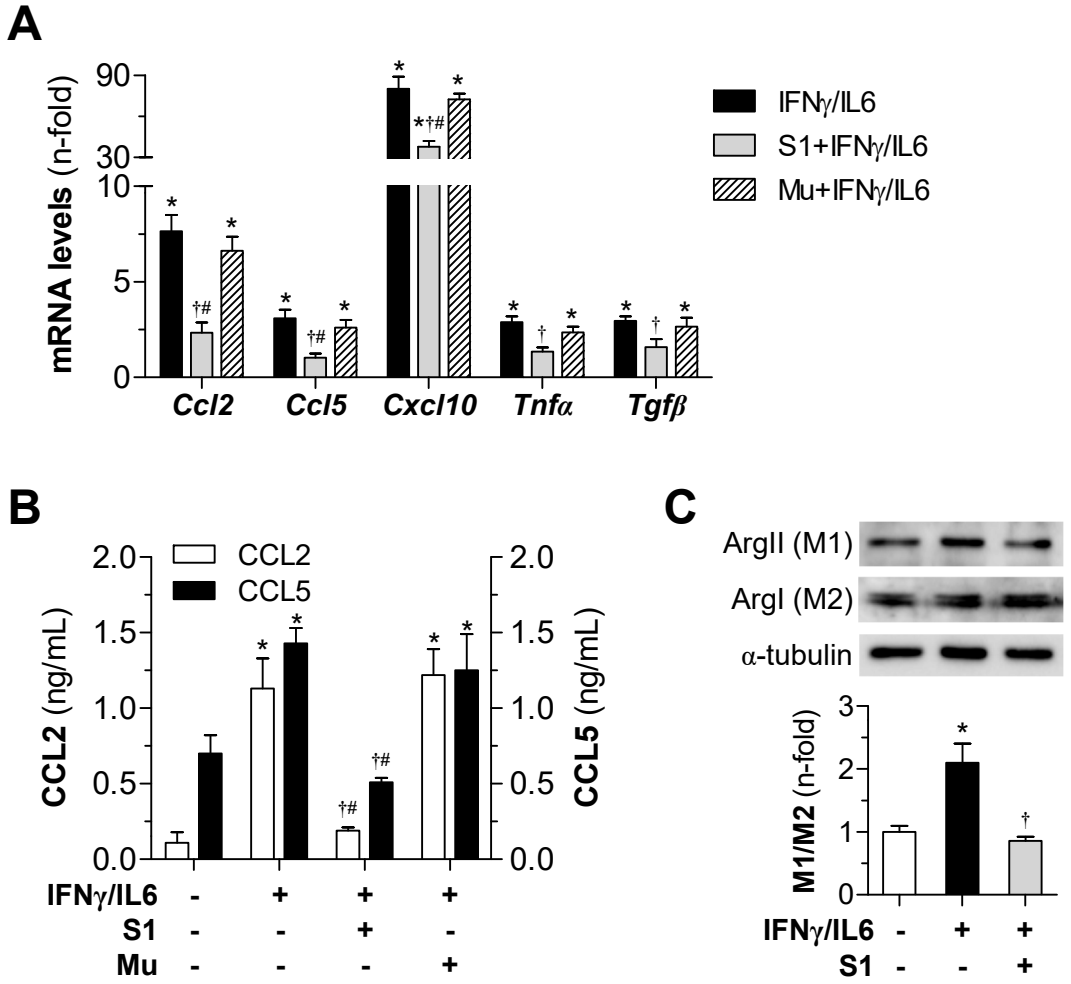


Supplemental Figure S4
(Recio et al.)



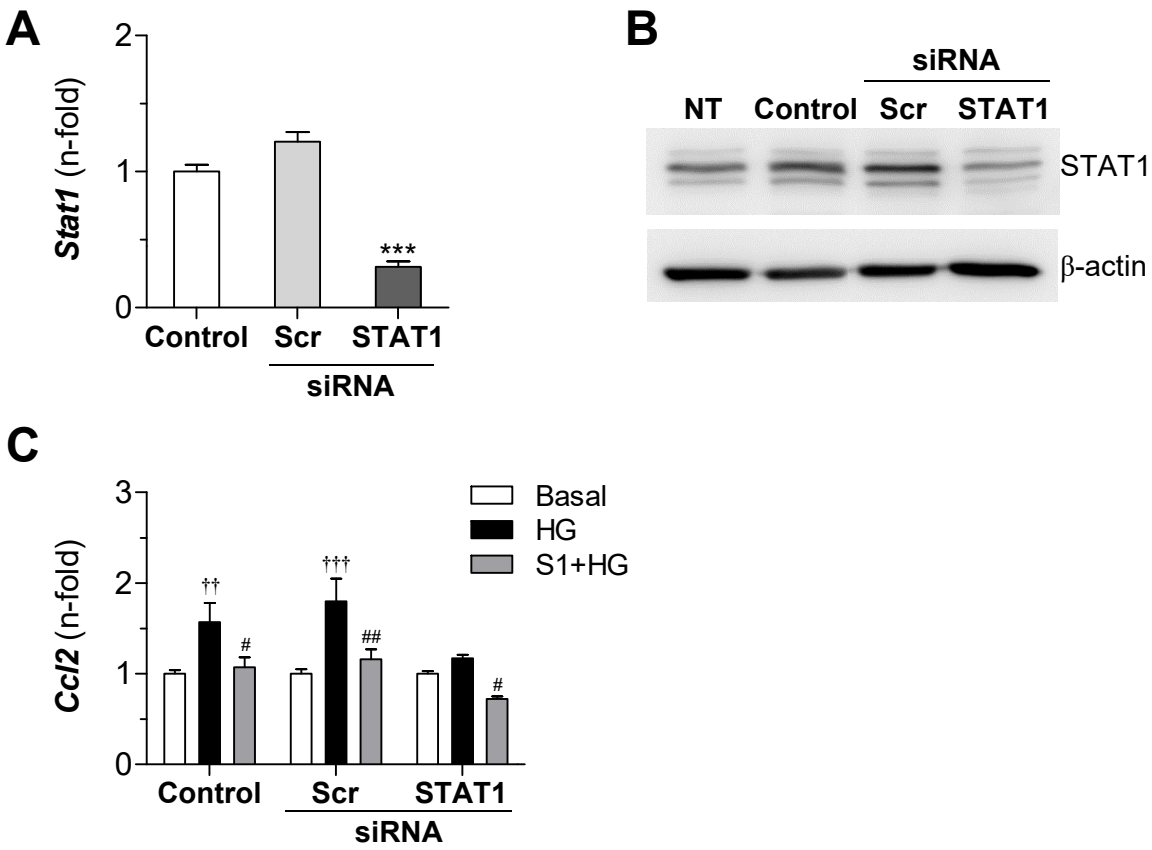
SOCS1-derived peptide inhibits STAT activation in cultured renal cells. Cultured mesangial cells (MC) **(A, B)** and tubuloepithelial cells (MCT) **(C, D)** were stimulated with cytokines (10^3 U/mL IFNγ plus 10^2 U/mL IL-6; 60 min) in the absence or presence of rhodamine-labeled peptide (S1, 100μg/mL). Cells were then fixed and stained for P-STAT1 **(A, C)** and P-STAT3 **(B, D)**. Representative confocal fluorescence images of four separate experiments (red, S1; green, P-STAT1/P-STAT3; blue, DAPI stained nuclei).

Supplemental Figure S5
(Recio et al.)



SOCS1 peptide inhibits cytokine mediated responses *in vitro*. Quiescent cells were preincubated with SOCS1 peptide sequences (S1 and Mu; 100 μg/mL, 90 min) before cytokine stimulation (10^3 U/mL IFNγ plus 10^2 U/mL IL-6). **(A)** Real-time PCR analysis of indicated genes in MC at 24 h of stimulation. Values normalized by 18S are expressed as fold increases over basal conditions (arbitrarily set to 1). **(B)** CCL2 and CCL5 concentrations in supernatants from cytokine-stimulated MCT measured by ELISA. **(C)** Protein expression of arginase isoforms (ArgII and ArgI) in mouse macrophages. Representative immunoblots and summary of the relative levels of M1 (ArgII) and M2 (ArgI) markers are shown. Bars represent the mean±SEM of duplicate determinations from 4-6 independent experiments. *P<0.05 versus Basal, †P<0.05 versus Cytokines, #P<0.05 versus Mu.

Supplemental Figure S6
(Recio et al.)



STAT1 silencing and SOCS1 peptide similarly inhibit CCL2 expression *in vitro*. MCT were transfected with vehicle (Control), scramble siRNA (Scr) and specific siRNA for STAT1 (30 nmol/L, 24 h). **(A)** Gene expression levels of STAT1 in unstimulated MCT. RT-PCR data normalized by 18S are expressed as fold increases versus Control. **(B)** Representative immunoblot of STAT1 protein expression (n=3) in non-transfected (NT) and transfected cells. **(C)** Real-time PCR analysis of CCL2 in MCT at 16 h of stimulation with high-glucose (HG, 30 mmol/L D-glucose) in the presence or absence of SOCS1 peptide (100 μg/mL, 90 min pretreatment). Bars represent the mean±SEM of duplicate determinations from 3-5 independent experiments. ***P<0.001 versus Control transfection; †P<0.05, †† P<0.01 versus Basal; #P<0.05, ## P<0.01versus HG.