Colony stimulating factor-1 receptor drives glomerular parietal epithelial cell activation in focal segmental glomerulosclerosis

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Parietal epithelial cells (PECs) are kidney progenitor cells with similarities to a bone marrow stem cell niche. In focal segmental glomerulosclerosis (FSGS) PECs become activated and contribute to extracellular matrix deposition. Colony stimulating factor-1 (CSF-1), a hematopoietic growth factor, acts via its specific receptor, CSF-1R, and has been implicated in several glomerular diseases, although its role on PEC activation is unknown. Here, we found that CSF-1R was upregulated in PECs and podocytes in biopsies from patients with FSGS. Through *in vitro* studies, PECs were found to constitutively express CSF-1R. Incubation with CSF-1 induced CSF-1R upregulation and significant transcriptional regulation of genes involved in pathways associated with PEC activation. Specifically, CSF-1/CSF-1R activated the ERK1/2 signaling pathway and upregulated CD44 in PECs, while both ERK and CSF-1R inhibitors reduced CD44 expression. Functional studies showed that CSF-1 induced PEC proliferation and migration, while reducing the differentiation of PECs into podocytes. These results were validated in the Adriamycin-induced FSGS experimental mouse model. Importantly, treatment with either the CSF-1R-specific inhibitor GW2580 or Ki20227 provided a robust therapeutic effect. Thus, we provide evidence of the role of the CSF-1/CSF-1R pathway in PEC activation in FSGS, paving the way for future clinical studies investigating the therapeutic effect of CSF-1R inhibitors on patients with FSGS.

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Translational Statement

Focal segmental glomerulosclerosis (FSGS) is a frequent cause of nephrotic syndrome with unmet therapeutic needs. Podocyte injury releases colony-stimulating factor-1 (CSF-1) and induces CSF-1 receptor (CSF-1R) upregulation in glomerular parietal epithelial cells (PECs). The CSF-1/CSF-1R signaling activates PECs and contributes to the characteristic fibrotic lesion of FSGS. Administration of CSF-1R inhibitors has a consistent therapeutic effect in an experimental model of FSGS. Thus, our study paves the way for investigating the therapeutic effect of CSF-1R inhibitors on FSGS in humans.

Focal segmental glomerulosclerosis (FSGS) is a histopathologic pattern of glomerular injury rather than a specific clinicopathologic entity and is a frequent cause of nephrotic syndrome in both adults and children.[1] In both primary and secondary FSGS, podocyte damage triggers the process. Under physiologic conditions, podocytes can be replaced by differentiated parietal epithelial cells (PECs). However, in FSGS, PECs become activated (aPECs), lose their reparative functions, acquire a profibrotic and proliferative phenotype, and contribute to the glomerular extracellular matrix deposition that is a hallmark of the disease.[2] Thus, aPECs are a key effector mechanism of injury in FSGS. Current therapeutic strategies are partly effective and mainly focus on reducing podocyte damage rather than PEC

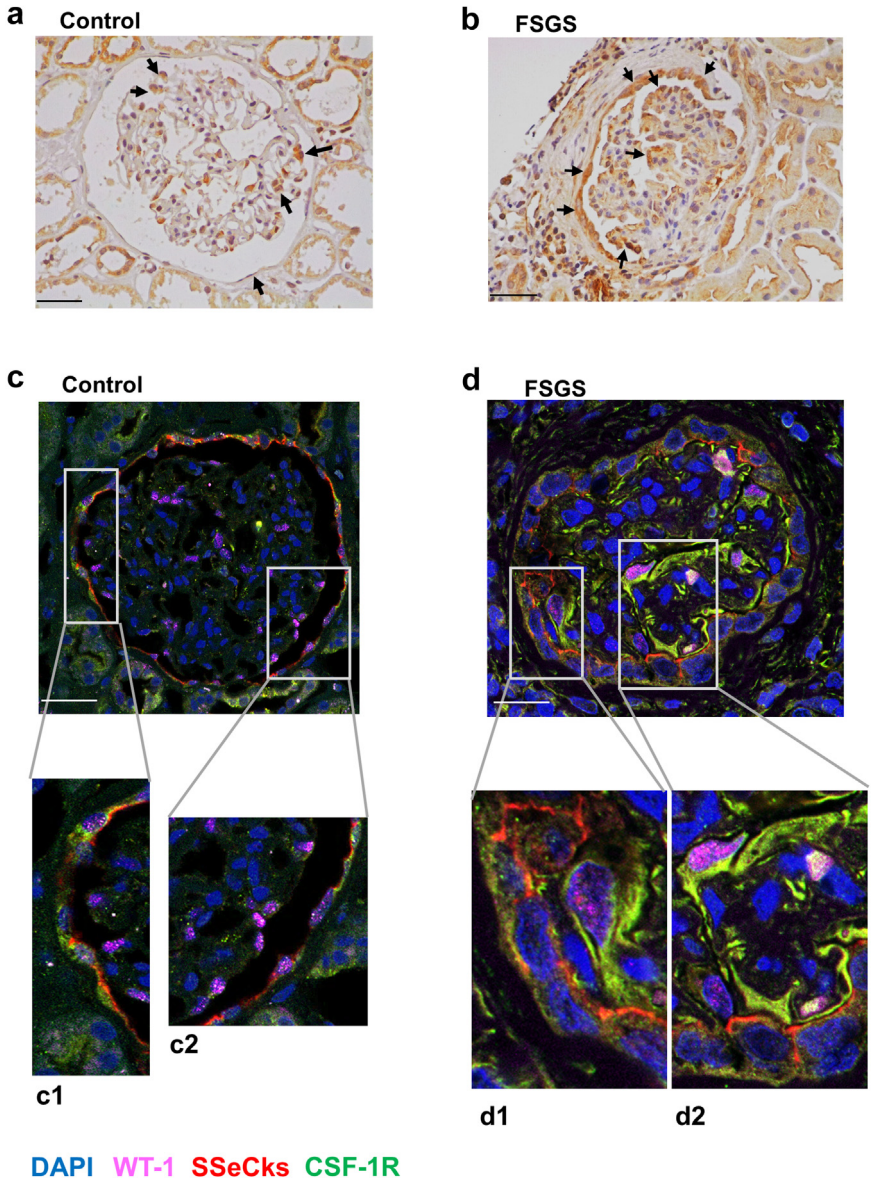

Figure 1 | Colony-stimulating factor-1 receptor (CSF-1R) expression in kidney biopsies from humans with focal segmental glomerulosclerosis (FSGS). (**a,b**) Representative images of the immunohistochemical staining of CSF-1R in human kidney biopsies from (**a**) healthy control samples and (**b**) FSGS patients. Arrows indicate the positive expression of CSF-1R. (**c,d**) Representative immunofluorescence images of the co-staining of CSF-1R (green), SSeCKS (red), and WT1 (pink) in (**c**) control and (**d**) FSGS human samples. For better visualization, higher magnifications of the selected square areas are provided in the lower panels for the (**c1,c2**) control and (**d1,d2**) FSGS human samples. Bar = 25 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

activation. There has been recent progress in identifying the molecular pathways involved in PEC activation and potential treatments.[3] However, further knowledge is needed to identify new therapeutic targets. aPECs can be identified by their *de novo* expression of CD44[4–6] through a process modulated by extracellular signal–regulated kinase (ERK).[7,8] Consequently, the ERK pathway might be a potential target in reducing PEC activation. However, it is not yet known how or which factors induce ERK1/2 activation in PECs after podocyte damage.

Colony stimulating factor-1 (CSF-1) is a cytokine originally described as a hematopoietic growth factor that acts via its unique receptor, the receptor tyrosine kinase CSF-1R. CSF-1 is constitutively produced by a variety of cells, including endothelial cells, macrophages, fibroblasts, smooth muscle cells, and osteoblasts.[9–11] This cytokine has been associated with several glomerular diseases,[12–14] although its pathogenic role has been linked to the presence of macrophages.[15–18] Some studies have reported that renal proximal tubular epithelial cell injury triggers the production of CSF-1, which acts in an autocrine and paracrine manner by its binding to CSF-1R.[19,20] CSF-1 and CSF-1R may also modulate stem and progenitor cells and their niches in some tissues,[21–23] suggesting that CSF-1/CSF-1R may have other functions in different cell types in addition to its canonical biologic functions in macrophages. Melica *et al.*[24] described a subset of PECs as renal progenitor cells that showed similarities to the

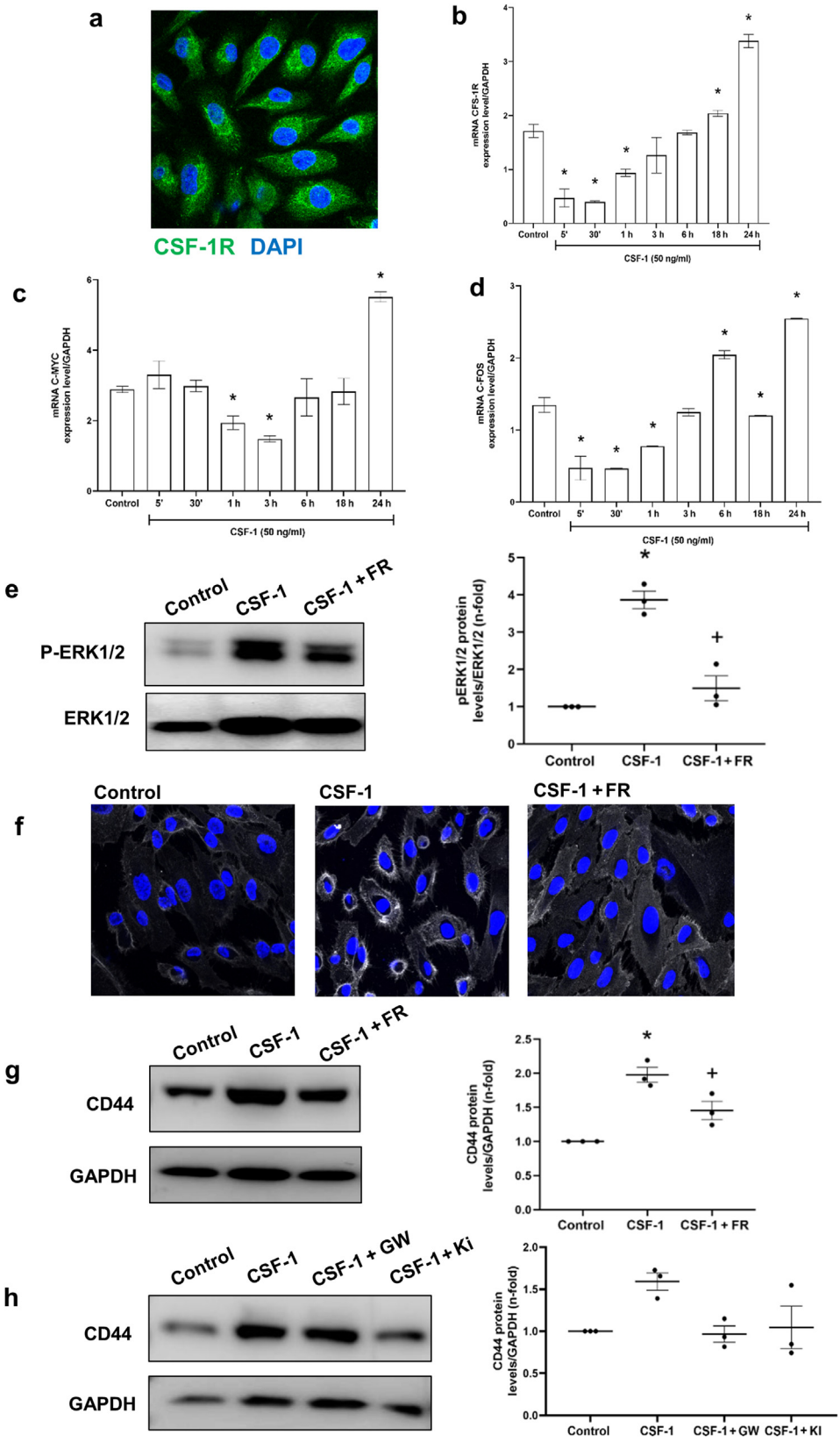

Figure 2 | Colony-stimulating factor-1 receptor (CSF-1R) downstream response after CSF-1 stimulation *in vitro*. (**a**) Representative images of CSF-1R staining (green) in isolated human parietal epithelial cells showing a dotted distribution. (**b**) mRNA expression levels of CSF-1R and its downstream targets (**c**) c-myc and (**d**) c-fos after 5 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 18 hours, or 24 hours of stimulation with 50 ng/ml of CSF-1. (**e**) Representative image of extracellular signal–regulated kinase phospho-ERK (pERK1/2) detection (continued)

bone marrow stem cell niche. Overall, these findings provide a rationale to investigate the CSF-1/CSF-1R role in the dysfunctional activation of PECs in FSGS. Therefore, the purposes of the present work were to (i) evaluate the functional and molecular effects of modulating the CSF-1/CSF-1R pathway on PECs, (ii) to evaluate the therapeutic effect of inhibiting the CSF-1/CSF-1R axis in an experimental model of FSGS, and to (iii) provide a pathogenic mechanism connecting CSF-1/CSF-1R with aPECs.

METHODS

Study design

Formalin-fixed paraffin-embedded human kidney biopsies were obtained from a local biobank and blindly processed, as approved by our institutional review board (PR043/17). Biopsy samples came from 12 patients with primary or hereditary FSGS. Normal kidney tissues obtained from nephrectomies were used as controls ($n = 3$). Relevant clinical information of FSGS patients is summarized in Supplementary Table S1.

For *in vitro* studies, human PECs were isolated from fresh kidney tissue obtained immediately after a nephrectomy for a clinical indication as previously described[25,26] in accordance with the recommendations of the Ethics Committee of our hospital (PR362/19). The human podocyte cell line was provided by Moin Saleem (Children's Renal Unit and Academic Renal Unit, University of Bristol, Southmead Hospital) and Luigi Gnudi (School of Cardiovascular and Metabolic Medicine and Science, King's College London Medical School).[27]

All procedures on animals were performed according to the recommendations of the European Research Council for the Care and Use of Laboratory Animals. The FSGS experimental protocol was approved by the local Animal Ethics Committee of the University of Barcelona (reference 431/18).

RNA sequencing and data analysis

Libraries were prepared using the TruSeq Stranded mRNA Library Prep kit (96 samples, reference 20020595, or 48 samples, reference 20020594), according to the manufacturer's protocol, to convert total RNA into a library of template molecules with a known strand origin that is suitable for subsequent cluster generation and DNA sequencing.

Statistical analysis

Results are presented as the median and quartiles for groups with >3 samples and as the mean ± SEM for groups with ≤3 samples. Statistical analysis was performed using GraphPad Prism 8. Group means were compared with Student's t-test or analysis of variance for parametric values or the Mann–Whitney U test for nonparametric values and Tukey's multiple comparison test. Differences were considered statistically significant with a crude $P \leq 0.05$ and an adjusted $P \leq 0.1$. A detailed description of methods is included in the Supplementary Methods and Supplementary Tables S1 and S2.

RESULTS

FSGS is associated with an increased expression of CSF-1R in PECs and podocytes

We evaluated CSF-1R expression in kidney biopsies from patients with FSGS and in kidney tissue from the experimental FSGS mouse model, using immunohistochemistry to define CSF-1R glomerular localization and immunofluorescence to identify the cells expressing CSF-1R. The immunohistochemistry assays of control samples showed a constitutive glomerular expression, with scarce positive cells localized mainly in the tuft (Figure 1a). CSF-1R expression was increased in human FSGS, with a prominent expression in the PECs and in the periphery of the tuft that suggested expression in the podocytes (Figure 1b). We next determined the cell types expressing CSF-1R by co-staining specific podocyte (WT1 [mammalian Willm's tumor 1]) and PEC (SSeCKS) markers. The images confirmed co-localization of CSF-1R with both cell types (Figure 1c and d). Specifically, glomeruli from the controls showed a positive staining for CSF-1R (green) in some PECs (red) and a few podocytes (pink) (Figure 1c and the enlarged images in panels c1 and c2). By contrast, CSF-1R expression in the FSGS biopsies was amplified in the PECs and especially in the podocytes (Figure 1d and the enlarged images in panels d1 and d2).

PECs constitutively express CSF-1R, whereas the CSF-1 protein activates the downstream response that upregulates CD44 expression

Similar to the results from the kidney tissues, we observed constitutive CSF-1R expression *in vitro* in the isolated human PECs (Figure 2a). Incubation with CSF-1 induced increasing CSF-1R mRNA upregulation (Figure 2b).

To verify that the expression of the receptor correlated with its activity after ligand binding, we measured the expression of 2 downstream targets of the receptor tyrosine kinase: *c-myc* and *c-fos* (Figure 2c and d). We observed rapid and significant changes in the mRNA expression profiles, with some oscillations. To further explore the downstream signaling pathway of CSF-1R, we focused on the ERK1/2 pathway. The activation of CSF-1R by its ligand triggers the dimerization and autophosphorylation of the receptor as well as the activation of downstream signaling cascades such as the ERK1/2 and PI3K-AKT pathways.[28] Our *in vitro* data showed that the addition of CSF-1 to human PECs activated the ERK1/2 pathway, as demonstrated by the significant increase in phospho-ERK1/2 levels (Figure 2e) when compared with the

Figure 2 | (continued) by Western blot and the corresponding quantification. (**f**) Representative images of CD44 staining. (**g**) Total protein levels of CD44 in cells treated with 50 ng/ml of CSF-1 and with or without FR180204 (10 μM). (**h**) Total protein levels of CD44 in cells treated with 50 ng/ml of CSF-1 for 24 hours and with or without a CSF-1R inhibitor (GW2580 or Ki20227). Data are expressed as the mean ± SEM of 3 per group and as the median and quartiles of > 3 per group. *$P < 0.05$ versus control; +$P < 0.05$ versus CSF-1. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

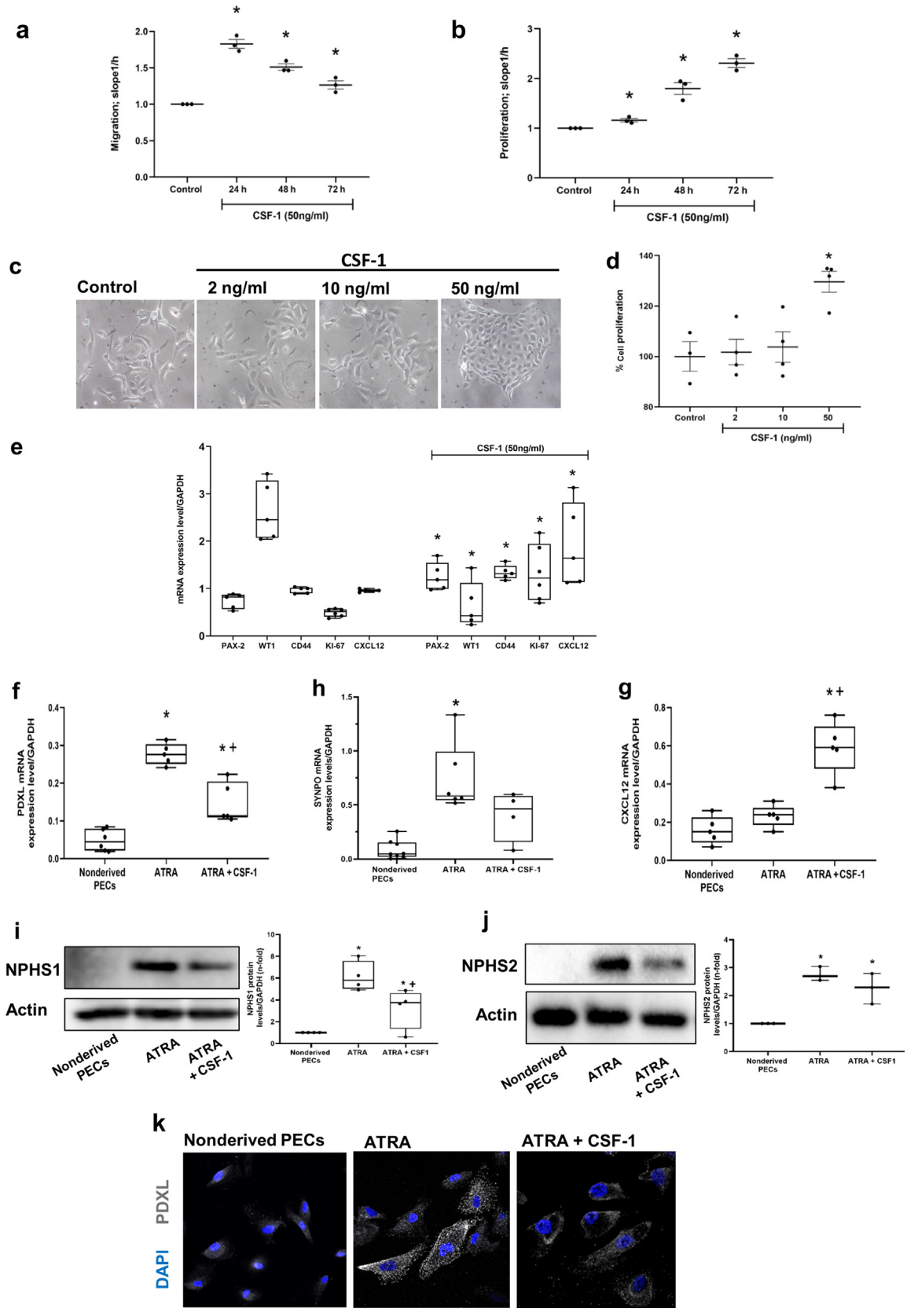

Figure 3 | Colony-stimulating factor-1 (CSF-1) effects on cell proliferation and migration and on the levels of podocyte markers. (**a**) Migration measurement and (**b**) proliferation rate of parietal epithelial cells (PECs) after 24, 48, or 72 hours of stimulation with 50 ng/ml of CSF-1. (**c**) Representative images of PEC growth and colony appearance and (**d**) proliferation (by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay quantification) after 5 days of CSF-1 protein stimulation at concentrations of 0, 2, 10, and 50 (continued)

untreated cells. This activation was accompanied by increased levels of the CD44 protein, as detected by immunohistochemistry and Western blotting (Figure 2f and g). The blocking of ERK activation with the ERK-selective inhibitor FR180204 reduced the CD44 upregulation induced by CSF-1 and ERK phosphorylation, thus demonstrating that the effect of CSF-1 on CD44 is mediated, at least in part, by the induction of ERK1/2. Furthermore, we investigated whether CSF-1R inhibitors modified CD44 expression in cultured PECs. Our data showed that CSF-1 induced CD44 expression, which was partially abrogated by the specific inhibitors *in vitro* (Figure 2h).

CSF-1 protein induces PEC migration and proliferation *in vitro* and reduces the levels of podocyte markers

To verify the activation of PECs, we tested the ability of the CSF-1 protein to induce changes in the migration and proliferation of cultured PECs. After 24 hours of incubation, CFS-1 induced significant PEC migration and proliferation (Figure 3a and b). Figure 3c shows representative images of the growth progression at 5 days in the presence of increasing CSF-1 concentrations. The percentage of proliferation was measured with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Figure 3d) and by cell counting (Supplementary Figure S1A). Both showed a positive correlation between cell proliferation and CSF-1 concentration. The addition of CSF-1R inhibitors abrogated the proliferation induced by CSF-1 (Supplementary Figure S1B).

$CD133^+/CD24^+$ PECs are podocyte precursors.[29,30] Based on this premise, we tested whether CSF-1 modified the expression of podocyte markers in these cells. To this end, we used 2 approaches *in vitro*. We first analyzed whether CSF-1 affected the expression levels of the markers associated with podocyte transition in naive PECs (Figure 3e). We observed that CSF-1 significantly reduced the expression of the podocyte marker *WT1* and increased those of the activation markers *CD44*, *KI-67*, and *PAX-2*. Interestingly, other studies have described the loss of *WT1* and the re-expression of PAX-2 in FSGS-associated lesions.[31] CSF-1 has also been reported to induce *CXCL12* expression, which limits podocyte regeneration after glomerular injury.[32] In the second approach, we used a podocyte differentiation protocol based on exposing primary PECs to all-trans retinoic acid in a VRAD medium.[25] As previously shown by our group and other authors, all-trans retinoic acid administration significantly induced the expression of the podocyte marker podocalyxin. We observed that exposure to CSF-1 in this context reduced the expression of the *podocalyxin* and *synaptopodin* gene (Figure 3f and g) as well as the protein expression of NPHS1, NPHS2 (as seen by Western blot, Figure 3i and j), and PDXL (Figure 3k) but increased *CXCL12* gene expression (Figure 3h), suggesting that CSF-1 may play a negative role in the progenitor-to-podocyte transition.

CSF-1 induces gene transcriptional regulation in PECs

To investigate the functional changes induced by CSF-1 through its specific receptor in PECs, we performed an RNA sequencing (RNA-seq) experiment (Figure 4, Supplementary Figure S2, and Supplementary Table S3). Normalized gene expression profiles were compared between cells treated with CSF-1 for 24 hours (T24H sample) and untreated cells (CTL sample). Two hundred twenty-seven differentially expressed genes were identified when considering a probability of differential expression of >0.9 and a value of $|M|$ of >1. Among these genes, 187 were upregulated and 40 were downregulated (Figure 4a). To explore potential functional mechanisms, the 227 differentially expressed genes were subjected to a Gene Ontology (GO) enrichment analysis. Fifty-six GO terms in the Biological Process ontology and 6 GO terms in the Molecular Function ontology showed a false discovery rate < 0.05. These top significant GO terms are shown in Figure 4b. GO annotation and the over-representation analysis (ORA) results revealed that the differentially expressed genes were specially involved in pathways associated with interferon (IFN; e.g., negative regulation of the type I IFN-mediated signaling pathway, responses to type I IFN, cellular responses to type I IFN, the type I IFN signaling pathway, and positive regulation of IFN-β production), among others. The key upregulated genes involved in these pathways are *ISG15*, *MX1*, *IFI44*, *IFI44L*, *IFITM1*, *IFIT1*, *IFIH1*, and *IFI27* (Figure 4a), which overlap with the biologic processes of IFN. The effects of CSF-1 on the modulation of these genes were confirmed by reverse transcriptase quantitative polymerase chain reaction (Figure 4c).

CSF-1R inhibition reduces glomerulosclerosis in the FSGS mouse model *in vivo*

Next, we decided to test the effect of CSF-1R inhibition *in vivo* in an experimental mouse model of FSGS induced by Adriamycin (ADR). To evaluate the functional role of CSF-1R in the pathogenesis of FSGS, mice were treated with a specific CSF-1R inhibitor (either GW2580 or Ki20227) at an early phase of FSGS induction by ADR. As seen in the human samples, the control animals displayed a constitutive glomerular expression of CSF-1R that was increased in the ADR-treated mice (Figure 5). This expression was significantly reduced in the mice treated with specific CSF-1R inhibitors. These data were confirmed by the analysis of *CSF-1R* gene expression (Figure 5a–c). Immunofluorescence co-

Figure 3 | (continued) ng/ml, respectively. (**e**) mRNA expression levels of the markers *WT1*, *CD44*, *PAX2*, *KI67*, and *CXCL12* in control cells and cells treated with 50 ng/ml of CSF-1 for 24 hours. (**f**) mRNA expression levels of *PDXL*, (**g**) *Synaptopodin*, and (**h**) *CXCL12* in nondifferentiated cells or cells treated with all-trans retinoic acid (ATRA) for 5 days to promote podocyte differentiation in the presence or absence of 50 ng/ml of CSF-1. Total protein levels of (**i**) NPHS1 and (**j**) NPHS2 in cells treated with 50 ng/ml of CSF-1. Data are expressed as the median and quartiles of >3 per group and as the mean ± SEM of 3 per group. $^*P < 0.05$ versus control; $+P < 0.05$ versus ATRA. (**k**) Representative images of PDXL immunohistochemistry. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

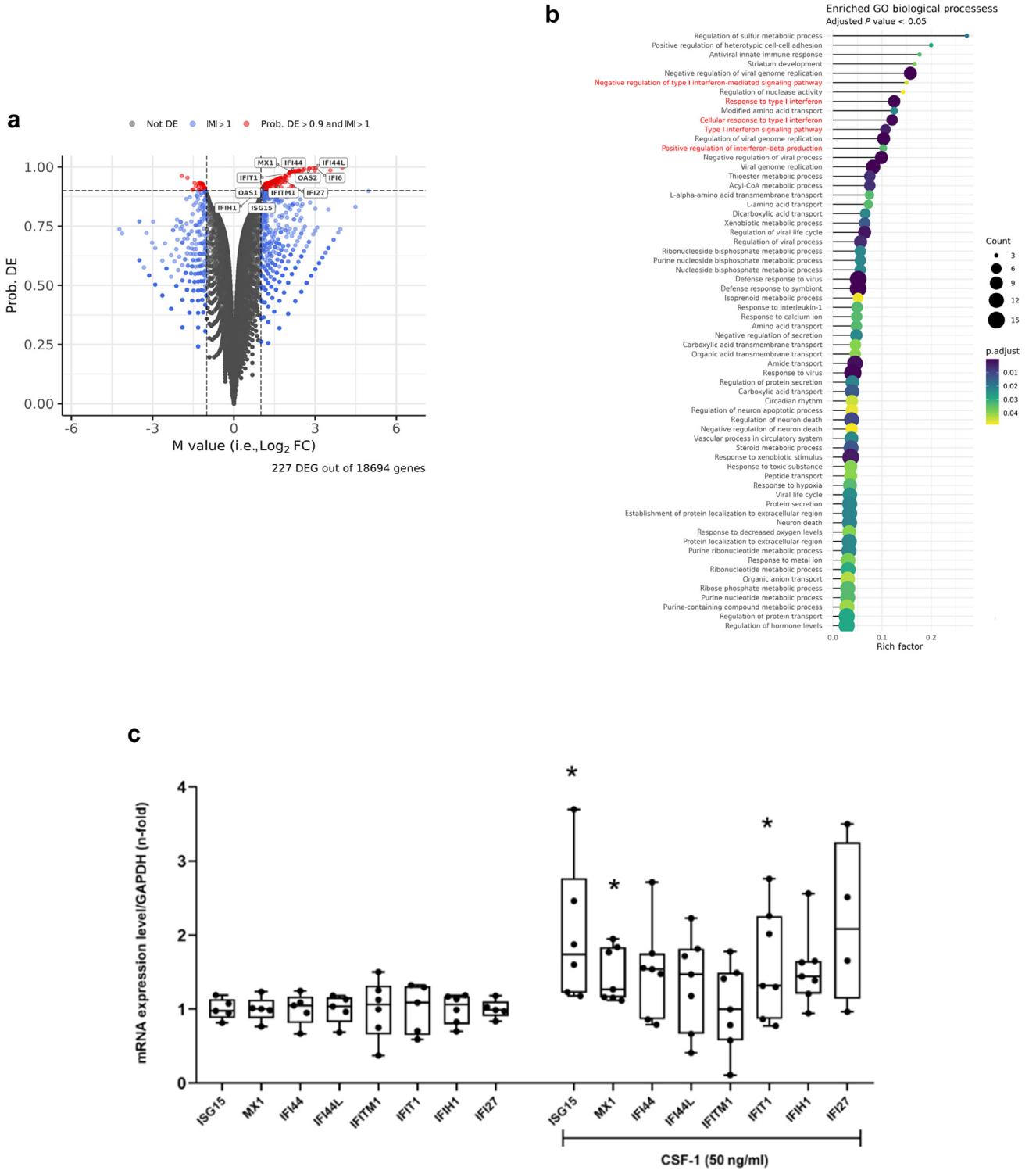

Figure 4 | Colony-stimulating factor-1 (CSF-1) induces modifications in gene expression. (**a**) Volcano plot of differentially expressed genes (DEGs) in cells treated with CSF-1 for 24 hours (T24H). The probability of differential expression (Prob DE) between T24 and the basal time point (Control) was estimated using the NOISeq approach. The M value indicates the log2 fold change. Genes were differentially expressed if Prob DE $>$ 0.09 and the absolute M value $>$ 1. Some DEGs are shown in red, whereas the genes with high levels of the log2 fold change are shown in blue. (**b**) Enriched Biologic Process (BP) concepts from Gene Ontology (GO) enrichment analysis for the 227 DEGs. Significant GO terms were determined by performing an over-representation analysis with clusterProfiler. The GO terms showing a false discovery rate (FDR) $<$ 0.05 were considered to be enriched. The number of genes related to each GO term and its FDR are indicated by the size of the circle and its color, respectively. Interferon (IFN) biologic processes are highlighted in red. (**c**) Kidney expression of IFN-inducible genes in human parietal epithelial cells with or without CSF-1 treatment (50 ng/ml) for 24 hours. Data are expressed as the median and quartiles of 5–6 per group. * $P < 0.05$ versus control.

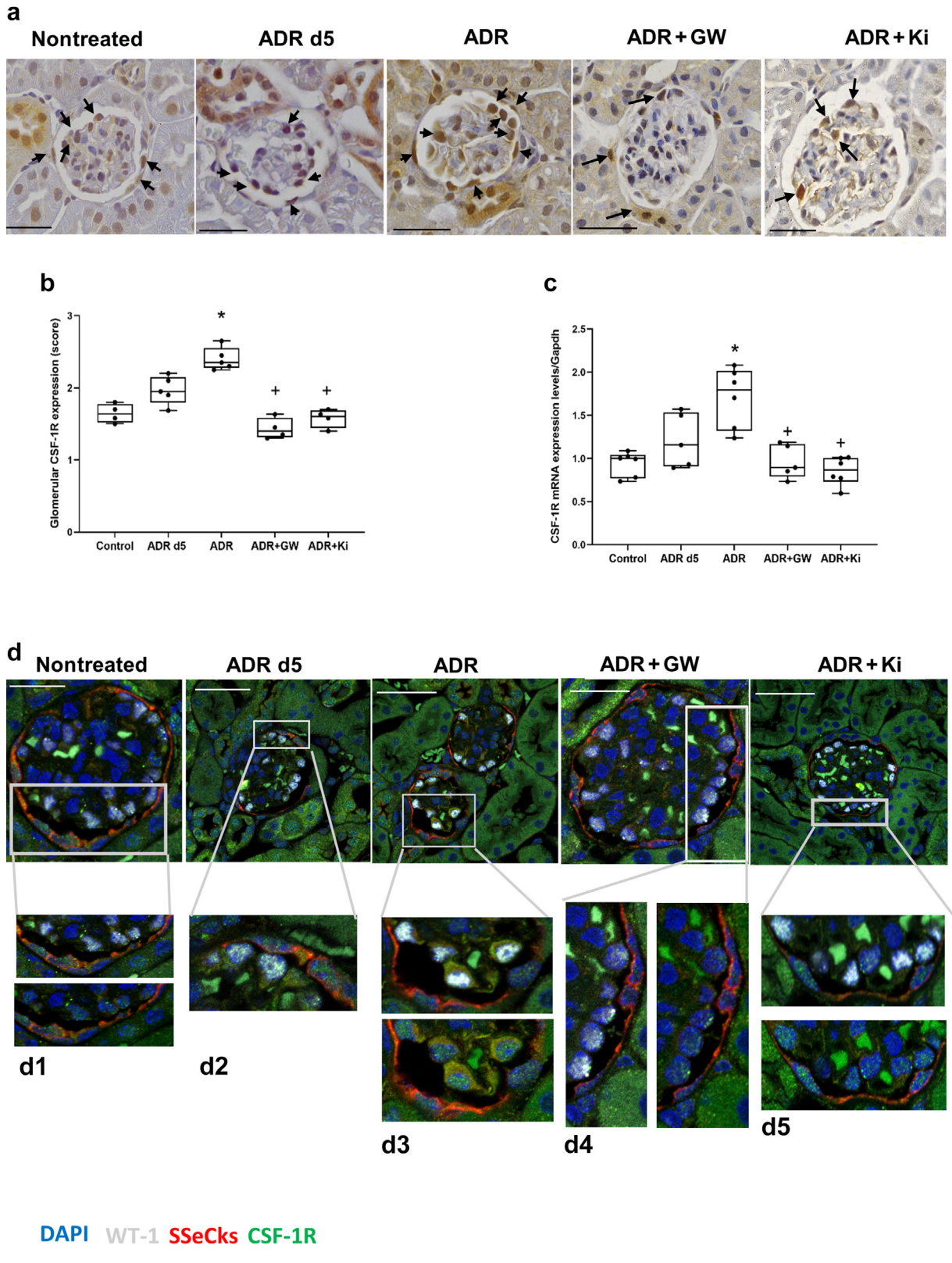

Figure 5 | Colony-stimulating factor-1 receptor (CSF-1R) kidney expression in the focal segmental glomerulosclerosis (FSGS) mouse model. (**a**) Representative images of the immunohistochemical staining of CSF-1R in the glomeruli of untreated animals or Adriamycin (ADR)-treated mice with or without the administration of CSF-1R inhibitors (GW2580 or Ki20227). Arrow indicates in brown the positive expression of CSF-1R in both the Bowman's capsule and the inner tuft. (**b**) Immunohistochemical quantification by semiquantitative scores. (**c**) CSF-1R kidney mRNA expression levels in each mouse group. Data are expressed as the median and quartiles of 4–5 animals (continued)

staining and confocal microscopy analysis showed that both PECs (Figure 5d, red) and podocytes (Figure 5d, white) expressed CSF-1R (Figure 5d, green). Altogether, our data indicate that FSGS is associated with CSF-1R overexpression in PECs and podocytes. Given the heterogeneity of PECs conforming the Bowman's capsule, we verified the expression of the CSF-1R through co-staining with accepted markers for different PEC subtypes (Supplementary Figure S3). The images demonstrated that both flat PECs (SSecCks-positive cells; Figure S3A) and transitional PECs (podocalyxin-positive cells; Figure S3B) but not cuboidal PECs (LTA-positive cells; Figure S3C) expressed CSF-1R and exhibited a pathologic response after ADR treatment. Additionally, we subjected isolated PECs to ADR treatment, which promoted the overexpression of the *CSF-1* gene, corroborating the increase in the cytokine expression in response to ADR (Supplementary Figure S4A).

Interestingly, the ADR-injected mice treated with either CSF-1R inhibitor showed a significant decrease in glomerulosclerosis compared with the ADR-injected mice used as controls, as observed by the periodic acid–Schiff staining (Figure 6a and b) and the analysis of the mRNA expression of different profibrotic tissue markers such as *transforming growth factor-β1*, *CTGF*, *Col4A1*, and *Fn1* (Figure 6c). The therapeutic effect of the CSF-1R inhibitors was also confirmed by the preservation of the glomerular filtration rate and albuminuria (Figure 6d and e). In addition, treatment with the CSF-1R inhibitors reduced the weight loss induced by ADR (Supplementary Figure S4B).

CSF-1R inhibition reduces phospho-ERK levels and PEC activation and is associated with the preservation of podocytes in the FSGS mouse model

The treatment with ADR to induce FSGS in mice was also associated with a prominent increase of CD44 staining at the glomeruli, mainly in the PECs (Figure 7a). The positive staining was detected in approximately 70% of the glomeruli on day 15 after FSGS induction and decreased to 40% in the animals treated with a CSF-1R–specific inhibitor (Figure 7b). We also estimated the degree of CD44 overexpression by assessing the extent of CD44 staining in the Bowman's capsule. For this purpose, we separated the positive glomeruli depending on whether the staining extent was >50% or <50% (Figure 7c). The administration of CSF-1R inhibitors (GW2580 or Ki20227) significantly reduced both the percentage of $CD44^{+}$ glomeruli and the CD44 staining of the Bowman's capsule boundary. Overall, these results suggested that CSF-1R inhibition reduced the amount of aPECs in our experimental FSGS model. We then determined the association between *CD44* upregulation and ERK activation and whether CSF-1R inhibitors could modify phospho-ERK (pERK) levels as we found in our *in vitro* experiments. We observed a significant increase in ERK1/2 activation in a similar pattern as that of CD44 in PECs, which was significantly reduced in the animals treated with the CSF-1R inhibitors (Figure 7d and e). This suggested the involvement of CSF-1R in ERK activation and, consequently, the fate of aPECs. We examined podocyte preservation by WT1 glomerular expression, finding a significant increase in podocyte numbers with CSF-1R inhibition compared with ADR-treated mice and similar to controls (Figure 7f and g). Simultaneously, PEC migration from Bowman's capsule decreased (Supplementary Figure S5). Immunohistochemistry showed PECs in the Bowman's capsule in control mice, whereas ADR-treated mice exhibited SSeCKS and claudin-1–positive cells in the glomerular tuft. CSF-1R inhibition resulted in the near absence of PEC markers outside Bowman's capsule.

CSF-1R inhibition reduces inflammation in the FSGS mouse model

Based on RNA-seq data, we explored CSF-1 involvement in FSGS transcriptional regulation and its response to CSF-1R inhibition. A significant increase in IFN-associated genes occurred 15 days after ADR administration. Notably, CSF-1R inhibitors prevented upregulation in injured mice (Figure 8), indicating a direct link between CSF-1R pathway activation and IFN family gene transcription in FSGS. We assessed inflammation in our FSGS model by measuring macrophage infiltration and inflammatory cytokine levels (Supplementary Figure S6). Treatment with the specific CSF-1R inhibitor GW2580 reduced infiltrating macrophages and proinflammatory cytokine levels in FSGS.

Crosstalking between injured podocytes and PECs

To delve into CSF-1/CSF-1R axis communication between damaged podocytes and PECs, we performed an *in vitro* approach using conditioned medium from ADR-stimulated podocytes on PECs cultured with or without CSF-1R inhibitors (Figures 9 and 10). First, the results demonstrated that ADR promoted the expression of the CSF-1/CSF-1R system in podocytes and the release of CSF-1 into the medium (Figure 9a–d). Second, the results demonstrated that conditioned medium incubation induced CSF-1R expression in PECs (Figure 9e) and promoted a genetic activation toward an activated phenotype as evidenced by the significant increase in the expression of *CD44*, *collagen 4A1*, and *transforming growth factor-β* (Figure 9f–h). The upregulation of these genes was significantly reversed by the administration of CSF-1R inhibitors, particularly by GW2580, as observed in *in vivo* experiments. Further, both inhibitors reversed the increase of WT1 (Figure 9i). The inhibitor GW significantly

Figure 5 | (continued) per group. *$P < 0.05$ versus control; +$P < 0.05$ versus ADR. (**d**) Representative immunofluorescence images obtained by confocal microscopy of podocytes (WT1, white), parietal epithelial cells (SSeCKS, red), and CSF-1R (white) in each mouse group. The lower panels in triple or double staining show a higher magnification of the selected areas for better visualization (**d1–d5**). Mouse groups: untreated; ADR d5, treated with Adriamycin and sacrificed on day 5 post-treatment; ADR, treated with Adriamycin; ADR+GW, treated with Adriamycin and GW2580; and ADR+KI, treated with Adriamycin and Ki20227. Bar = 25 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

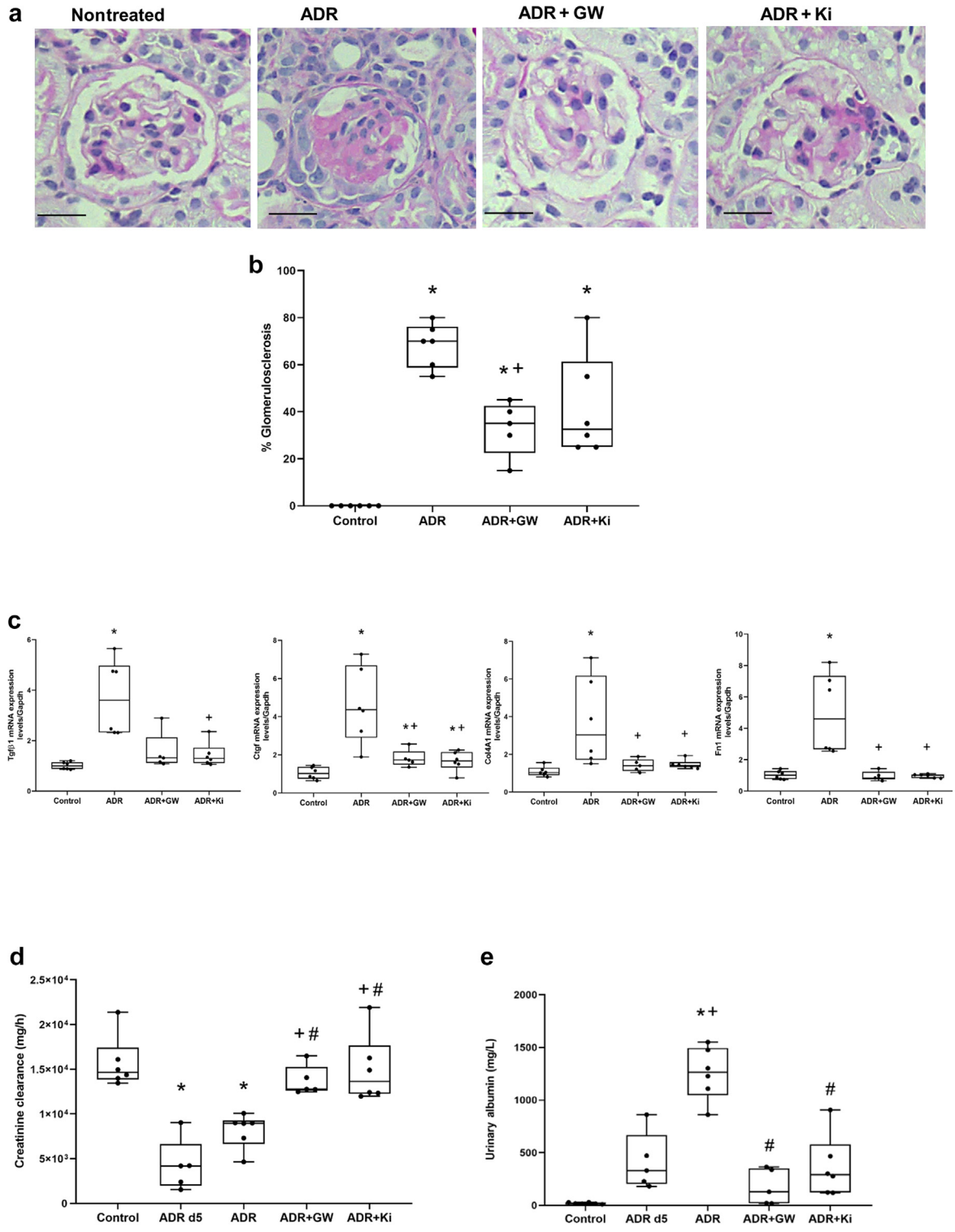

Figure 6| Colony-stimulating factor-1 receptor (CSF-1R) inhibition affects glomerulosclerosis in the focal segmental glomerulosclerosis mouse model. (**a**) Representative images of the glomerulus subjected to periodic acid–Schiff staining and (**b**) the percentage of affected glomeruli. (**c**) mRNA expression levels of profibrotic genes such as *transforming growth factor-β1*, *CTGF*, *Col4a1*, and *Fn1* in each mouse group. Measurements of (**d**) creatinine clearance and (**e**) albuminuria in mice. Mouse groups: untreated; ADR d5, treated with Adriamycin and sacrificed on day 5 post-treatment; ADR, treated with Adriamycin; ADR+GW, treated with Adriamycin and the CSF-1R inhibitor GW2580; and ADR+KI, treated with Adriamycin and the CSF-1R inhibitor Ki20227. Data are expressed as the median and quartiles of 5–6 animals per group. *$P < 0.05$ versus control; +$P < 0.05$ versus ADR d5; #$P < 0.05$ versus ADR. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

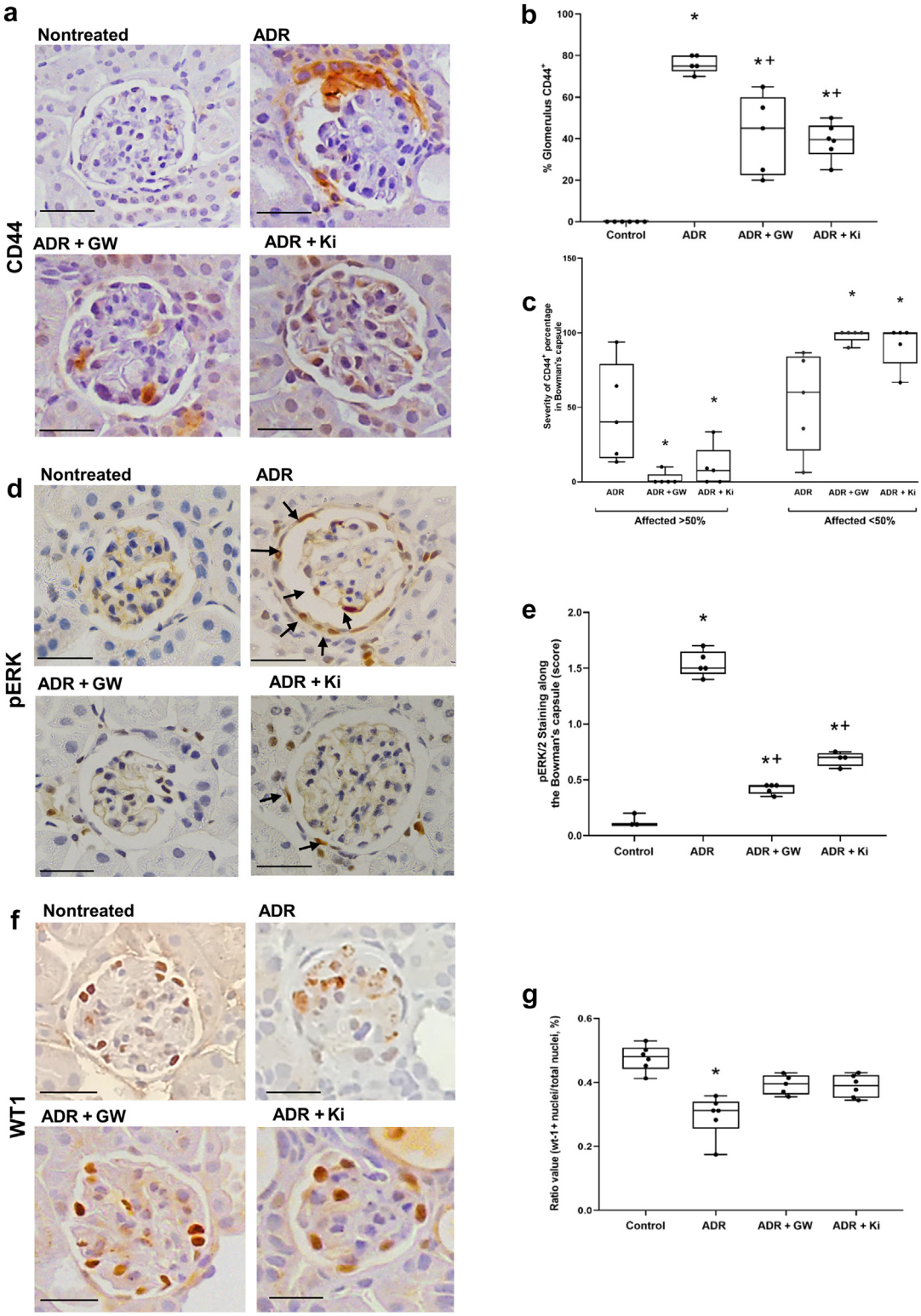

Figure 7 | CD44 and phospho-ERK1/2 kidney expression in the focal segmental glomerulosclerosis mouse model. (**a**) Representative images of the immunohistochemical staining of CD44 in the glomerulus. (**b**) Total percentage of $CD44^+$ glomeruli. (**c**) Percentage of $CD44^+$ staining in the affected glomerulus of untreated animals or Adriamycin (ADR)-treated mice with or without treatment with a colony-stimulating factor-1 receptor inhibitor (GW2580 or Ki20227). (**d**) Representative images of the immunohistochemical detection of pERK1/2. Arrow indicates in brown the positive expression of pERK1/2 in both the Bowman's capsule and the inner tuft. (**e**) Positive staining quantification by semiquantitative scores in the glomeruli in each mouse group. (**f**) Representative images of WT1 expression in the inner tuft of the glomerulus. (**g**) Podocyte quantification by the ratio of $WT1^+$ nuclei-to-total nuclei. Mouse groups: untreated; ADR, treated with Adriamycin; ADR+GW, treated with Adriamycin and GW2580; and ADR+KI, treated with Adriamycin and Ki20227. Data are expressed as the median and quartiles of 3–5 animals per group. *$P < 0.05$ versus untreated; +$P < 0.05$ versus ADR. Bar = 25 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

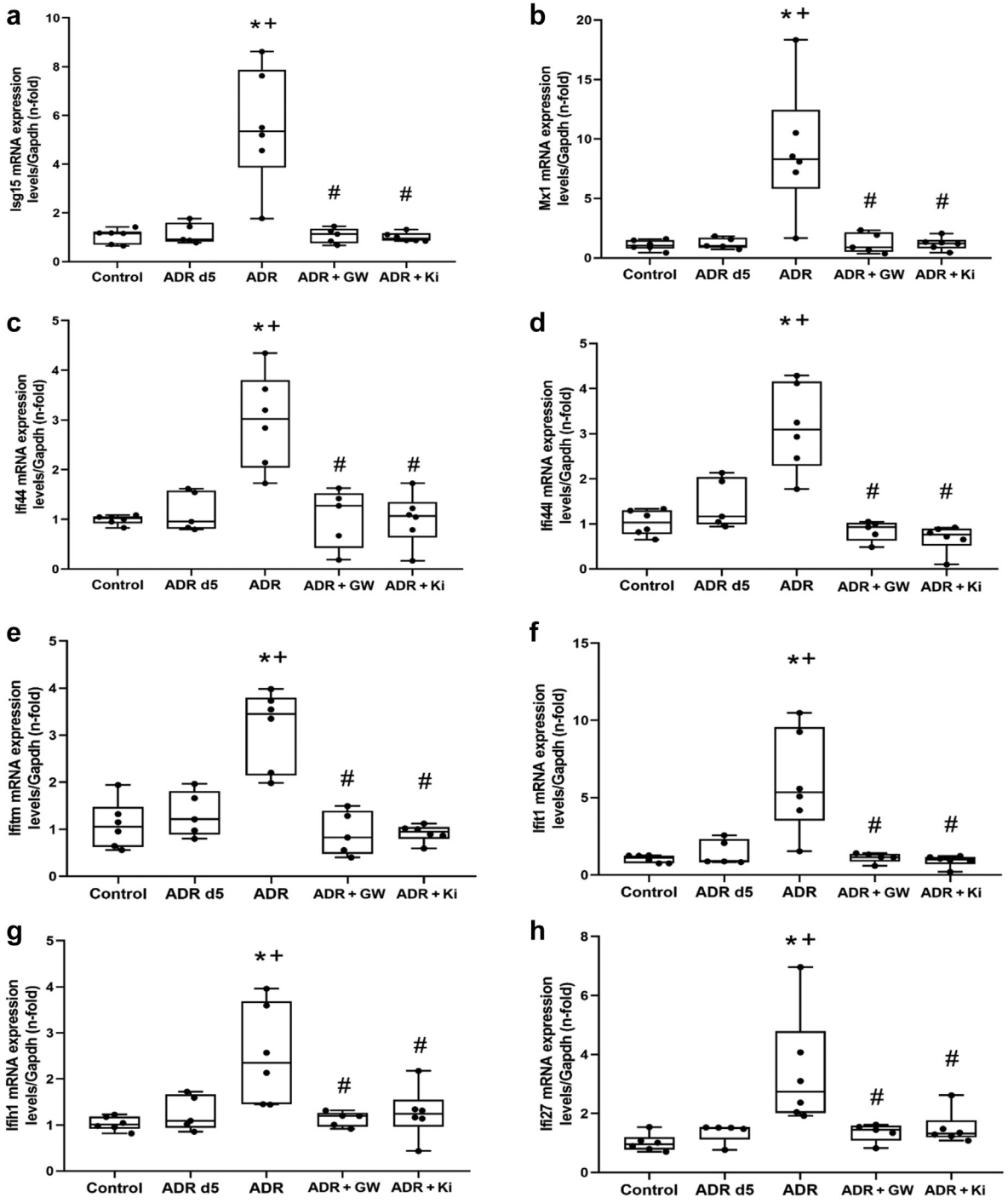

Figure 8 | Kidney expression of interferon-inducible genes in the focal segmental glomerulosclerosis mouse model. (**a**) *ISG15*, (**b**) *MX1*, (**c**) *IFI44*, (**d**) *IFI44L*, (**e**) *IFITM*, (**f**) *IFIT1*, (**g**) *IFIH1*, and (**h**) *IFI27* mRNA expression levels in the kidneys from the animal experimental groups. Mouse groups: untreated; ADR d5, treated with Adriamycin and sacrificed on day 5 post-treatment; ADR, treated with Adriamycin; ADR+GW, treated with Adriamycin and GW2580; and ADR+KI, treated with Adriamycin and Ki20227. Data are expressed as the median and quartiles of 5–6 animals per group. *$P < 0.05$ versus control; +$P < 0.05$ versus ADR d5; #$P < 0.05$ versus ADR.

downregulated the caspase-3 gene (Figure 9j). Finally, we observed IFN-inducible gene upregulation in PECs treated with the conditioned medium from damaged podocytes, especially at 24 hours (Figure 10), and the decrease of this upregulation by the CSF-1R inhibitors. These findings also underscore the consistency between the *in vivo* and *in vitro* results.

DISCUSSION

Here, we described the role of the CSF-1/CSF-1R pathway in the pathogenesis of FSGS and the relevance of the CSF-1/CSF-1R axis in the crosstalk between damaged podocytes and PECs. First, human FSGS was associated with an upregulation of CSF-1R expression in PECs and podocytes. Second, the

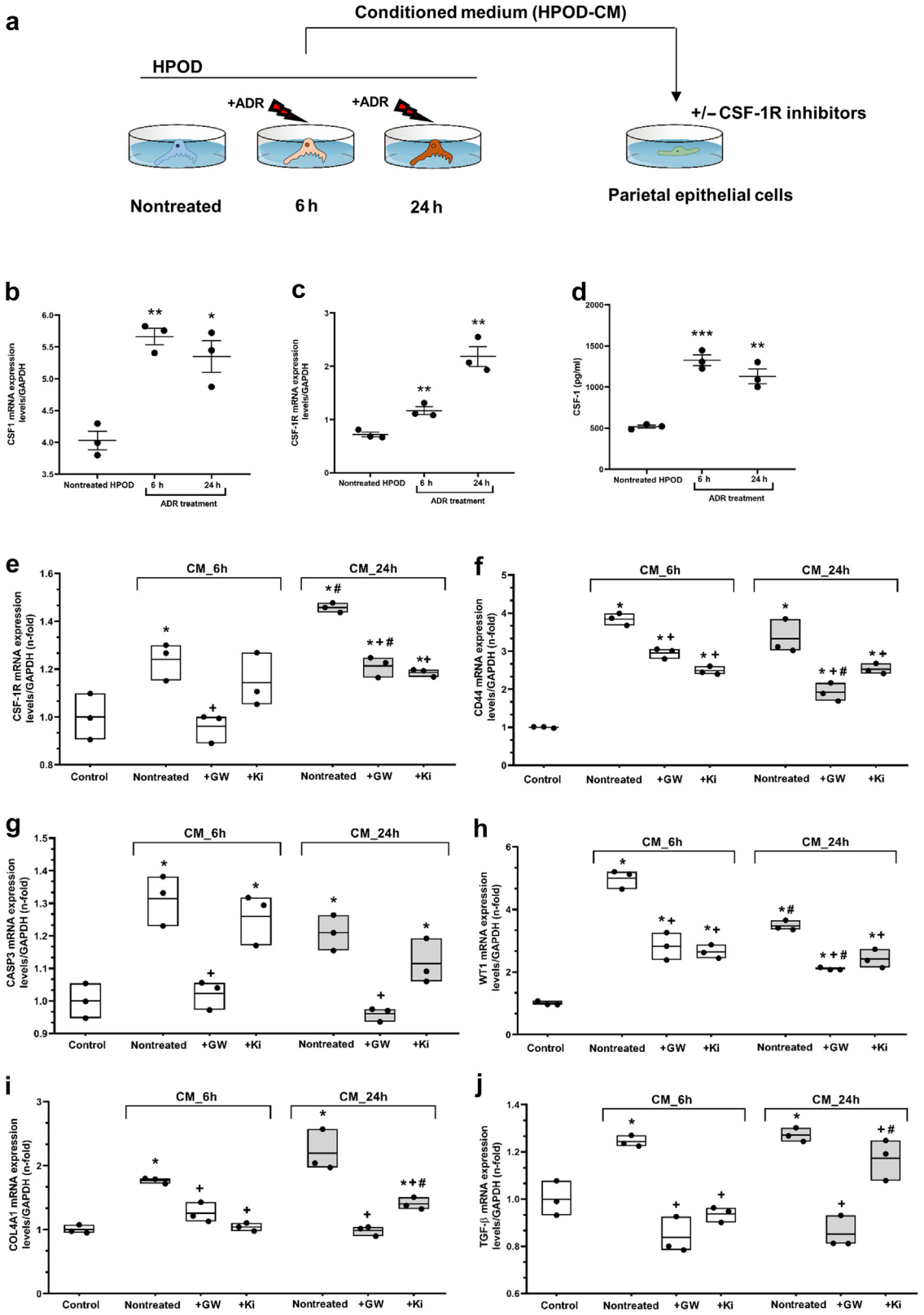

Figure 9 | Crosstalking between injured podocytes and parietal epithelial cells. (**a**) Experimental design outline. (**b**) mRNA expression levels of colony-stimulating factor-1 receptor (CSF-1R), (**c**) colony-stimulating factor-1 (CSF-1) and (**d**) secreted CSF-1 protein levels in nontreated podocytes and podocytes treated with Adriamycin (ADR) during 6 hours or 24 hours. Data are expressed as the mean ± SEM of 3 per group. *$P < 0.05$ versus nontreated human podocytes (HPOD). (**e–j**) mRNA expression levels of *CSF-1R*, *CD44*, *Col4A1*, *transforming growth factor–β* (*TGF-β*), *WT1*, and *Caspase-3* in control parietal cells, nontreated cells and cells pretreated with CSF-1R inhibitor (GW2580 or Ki20227) and incubated with conditioned medium (CM) from podocytes damaged with ADR during 6 hours or 24 hours. Data are expressed as the median and quartiles of 3 per group. *$P < 0.05$ versus control; +$P < 0.05$ versus nontreated cells; #$P < 0.05$ versus the homologous group.

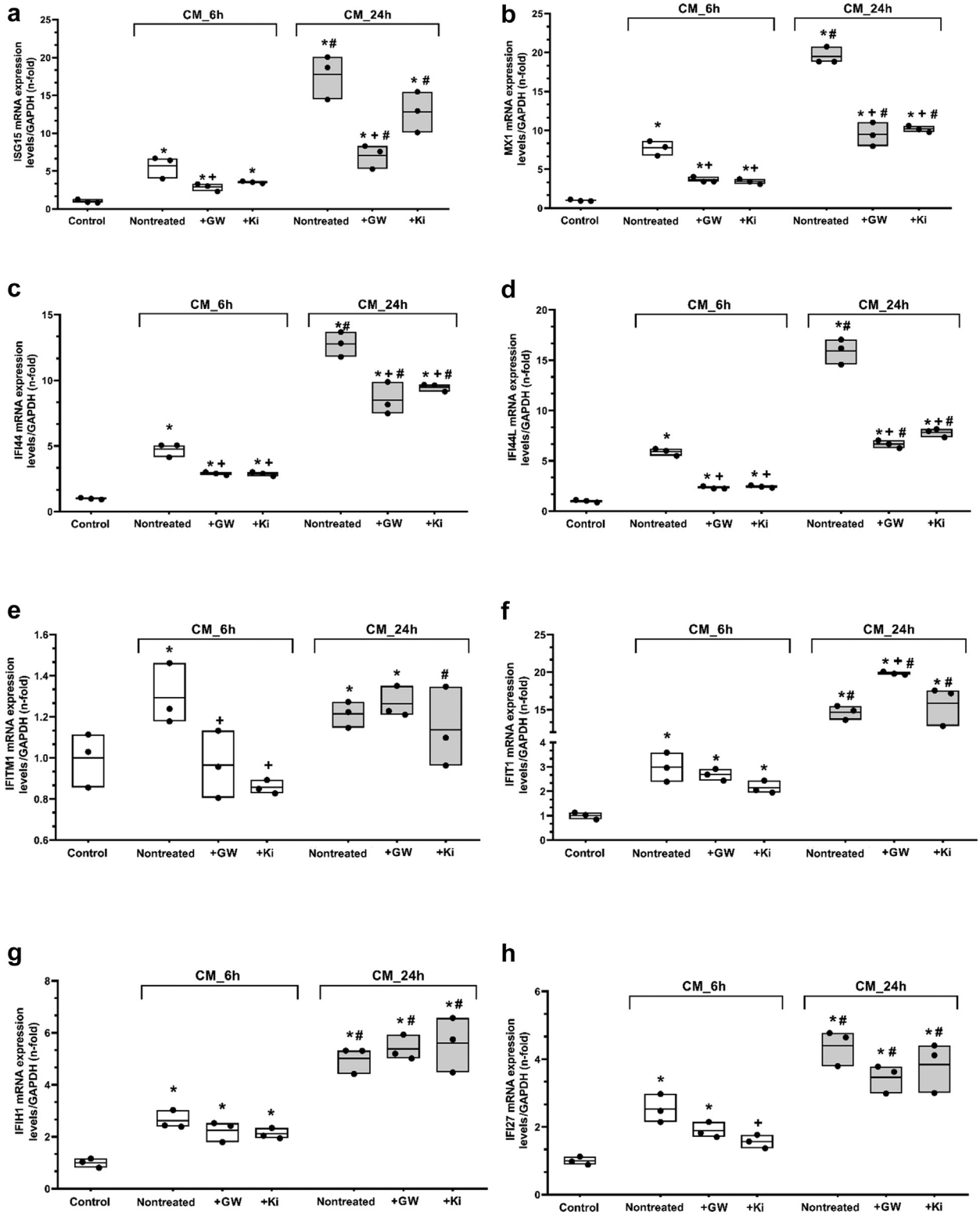

Figure 10 | Interferon-inducible expression genes in conditioned medium experiments. (**a**) *ISG15*, (**b**) *MX1*, (**c**) *IFI44*, (**d**) *IFI44L*, (**e**) *IFITM*, (**f**) *IFIT1*, (**g**) *IFIH1*, and (**h**) *IFI27* mRNA expression levels in control parietal cells, nontreated cells, and cells pretreated with colony-stimulating factor-1 receptor inhibitor (GW2580 or Ki20227) and incubated with conditioned medium (CM) from podocytes damaged with Adriamycin during 6 hours or 24 hours. Data are expressed as the median and quartiles of 3 per group. *$P < 0.05$ versus control; +$P < 0.05$ versus nontreated cells; #$P < 0.05$ versus the homologous group.

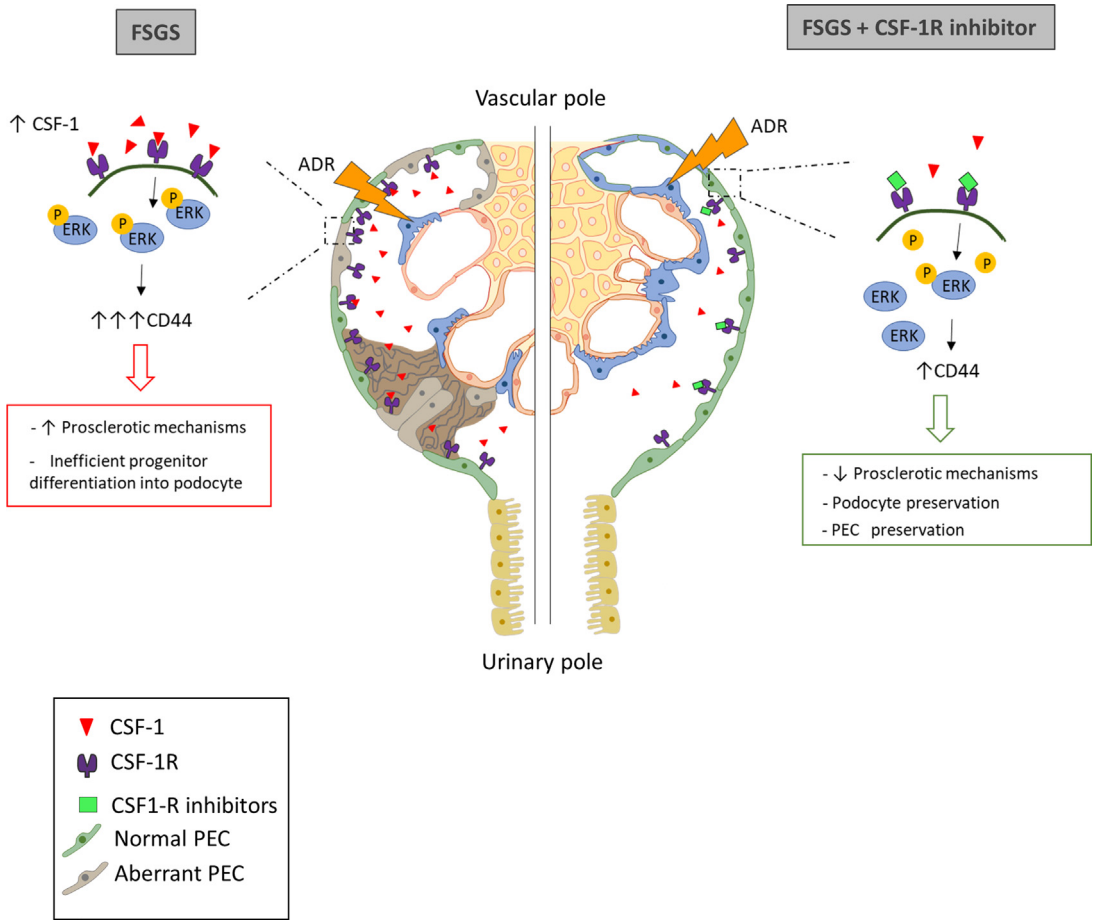

Figure 11 | Schematic illustration of the observations described in this study. Podocyte injury induces colony-stimulating factor-1 (CSF-1) and CSF-1 receptor (CSF-1R) upregulation in parietal epithelial cells (PECs) and podocytes. The activation of CSF-1/CSF-1R signaling activated the ERK pathway, leading to CD44 upregulation and providing a profibrotic PEC phenotype. Administration of CSF-1R inhibitors produces a therapeutic effect in FSGS by blocking PEC activation and by maintaining PEC capacity to restore podocyte loss. ADR, Adriamycin; ERK, extracellular signal–regulated kinase; FSGS, focal segmental glomerulosclerosis.

CSF-1 protein induced molecular, phenotypic, and functional changes in PECs, leading to their activation and a consequent profibrotic phenotype as well as reductions in podocyte markers. Third, the activation of CSF-1/CSF-1R signaling induced the ERK pathway, leading to CD44 upregulation in PECs. Finally, CSF-1R inhibitors provided a therapeutic effect and podocyte preservation in an experimental model of ADR-induced FSGS (Figure 11).

CSF-1/CSF-1R expression in kidney tissue has classically been attributed to the presence of macrophages and dendritic cells.[9–11] However, CSF-1 expression has also been found in tubular epithelial cells after acute injury, whereas CSF-1 intraglomerular expression has been reported in cases of IgA nephropathy and lupus nephritis.[15,19,33,34] Interestingly, podocytes produce low levels of CSF-1 and increase its production in response to inflammatory stimuli[34]; however, currently no data are available on CSF-1R in this setting. In FSGS, podocytes can be damaged by several mechanisms, but PECs are the key effector cells in the pathogenesis of the disease. However, it has not been stablished whether CSF-1/CSF-1R contributes to the activation of PECs.

Specifically, PECs undergo a phenotypic shift from an epithelial to a mesenchymal state, expressing α-smooth muscle actin and platelet-derived growth factor receptor-β (PDGFR-β) in FSGS.[3] A positive correlation exists between CSF-1 and α-smooth muscle actin upregulation in pathologic glomeruli,[34] suggesting the potential involvement of CSF-1 in PEC activation and FSGS formation.

We observed consistent CSF-1R overexpression in PECs and in some podocytes in both human and experimental FSGS. CSF-1 has a direct effect on PECs; *in vitro*, human PECs exhibited functional CSF-1R, responding rapidly to CSF-1 by activating downstream oscillatory signaling molecules like c-fos and c-myc, similar to reported in other cells such as monocytes.[35]

The PECs activated in response to podocyte injury express CD44 *de novo*,[4–6] coinciding with ERK pathway activation and the development of a prosclerotic, migratory phenotype fostering the pathology.[36] Although pERK plays a central role in generating profibrotic PECs, information is lacking on the mediators inducing ERK1/2 activation. We hypothesized CSF-1/CSF-1R as a candidate for promoting

ERK pathway activation. CSF-1R, a PDGFR family of class III receptor tyrosine kinase, activates downstream pathways like PI3K and ERK1/2, as observed in macrophages and bone marrow progenitors.[28,37] Our *in vivo* results demonstrated that using specific CSF-1R inhibitors in FSGS, CD44 and pERK1/2 levels are reduced, along with sclerotic events. *In vitro* studies in human PECs supported these findings, indicating a cellular and molecular effect of CSF-1R inhibition on CD44 expression.

The CSF-1R axis is important in maintaining and modulating some stem and progenitor cells and regulating their niches.[22,23,38] PECs, as progenitor cells, naturally migrate to the glomerular tuft, differentiating into podocytes.[39] We investigated the impact of CSF-1 on PEC differentiation and migration, finding that CSF-1/CSF-1R pathway activation inhibited *in vitro* progenitor-to-podocyte transition by downregulating the genes related to podocyte transition (such as podocalyxin or WT1) and inducing the expression of *CXCL12*, a limiting factor in the podocyte–progenitor turnover.[32] CSF-1 is known for regulating migration and mediating proliferation in various cell types, including macrophages,[40] osteoclasts, and different human cancer cells.[23,41,42] The CSF-1/CSF-1R pathway also prompted human PEC migration *in vitro*, confirmed by our data on CSF-1 promoting both PEC migration and proliferation, favoring a migratory phenotype.

CSF-1 induced differential gene expression in human PECs, as revealed by RNA-seq analysis. The genes and the associated biologic processes encompassed those driving epithelial-to-mesenchymal transition and encoding IFN-stimulated cytokines, among others. IFN and CSF-1R are known to synergistically act in certain pathologic situations.[43–45] with compelling data linking IFN and FSGS.[46–49] We confirmed that *in vitro*, CSF-1 stimulates the transcription of genes associated with IFN in human PECs.

We validated our hypothesis regarding the involvement of CSF-1/CSF-1R in FSGS pathogenesis using an animal model. In mice, a single ADR dose induces podocyte injury, progressive podocyte loss, proteinuria, and characteristic FSGS histology.[50] We designed an interventional study with 2 CSF-1R inhibitors (GW2580 and Ki20227), with relevant specificity differences. GW2580 is highly specific for CSF-1R, whereas Ki20227 also inhibits vascular endothelial growth factor receptor-2, c-KIT, and platelet-derived growth factor-β (PDGFR-β).[51,52] Given the recent identification of PDGF/PDGFR-β crosstalk as a significant pathogenic mechanism in FSGS,[3] the availability of a specific CSF-1R inhibitor is crucial. Our results demonstrated that both inhibitors reduced proteinuria and FSGS, preserved podocyte numbers, and minimized PEC activation. *In vivo*, we confirmed the differential gene expression of IFN-stimulated cytokine-encoding genes, consistent with RNA-seq analysis.

We further explored the CSF-1R expression in PEC subtypes in FSGS *in vivo* model. Positive expression was noted in flat and transitional PECs, whereas CSF-1R was negative in cuboidal ones, making the flat and transitional subtypes potential candidates for migration into the glomerulus. These findings agree with studies reporting flat and transitional PECs subtypes as pathogenic effector cells in FSGS.[6] As in cuboidal PECs, LTA-labeled proximal tubular cells did not express CSF-1R. However, the proximal tubular cells immediately adjacent to the glomerulus express CSF-1,[19,20] and therefore we cannot rule out these cells as an additional paracrine source of CSF-1 in FSGS.

Finally, we investigated the CSF-1/CSF-1R axis in the crosstalk between damaged podocytes and PECs. ADR promoted CSF-1 release from podocytes to the medium that was able to activate PECs, thus suggesting that in addition to the autocrine effect, there is also a paracrine activation of the CSF-1/CSF-1R pathway.

Our study has limitations. Because CSF-1/CSF-1R signaling promotes myeloid progenitor differentiation into monocytes, macrophages, and dendritic cells and considering the involvement of the CSF-1/CSF-1R axis in macrophage recruitment to the kidneys in glomerular diseases, an additional beneficial effect of CSF-1R inhibition based on these mechanisms cannot be ruled out. We assessed macrophage infiltration in our FSGS model, confirming that the highly specific CSF-1R inhibitor GW2580 reduced macrophages and renal inflammation. However, our *in vitro* data on the direct effect of CSF-1 on PECs remained independent of macrophage presence.

In conclusion, our study provides new insights into the pathologic mechanisms involved in FSGS. This is the first evidence of the role of the CSF-1/CSF-1R pathway in PEC activation, paving the way for future clinical studies investigating the therapeutic effect of CSF-1R inhibitors on FSGS in humans.

DISCLOSURE

All authors declared no competing interests.

DATA STATEMENT

Clinical samples and experimental protocols underwent review and approval by the following public committees and procedures: IDIBELL Institutional Review Board (PR043/17), Ethics Committee of University Hospital of Bellvitge (PR362/19), and Animal Ethics Committee of the University of Barcelona (Reference 431/18). The RNA-seq data repository is presently under submission to the editorial board as Supplementary Table S3. The data sets analyzed in this study are available from the Gene Expression Omnibus repository under accession number GSE255489.

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AUTHOR CONTRIBUTIONS

JMC and AS conceived the original idea and supervised the research. AS designed the research studies. JMC coordinated the human samples and data. JMC and AS wrote the manuscript. AMan, JD, and AMad provided human samples and helped with the human studies. SC and FV contributed with human ethical requirements and nephrectomies samples. RG was responsible for the animal experiments. LM-V contributed to the animal experiments. SR-M, ND, AA-G, and MJ performed the *in vitro* data and analyzed data. JLM performed the bioinformatics analysis. CV performed the histological assays, and RG, ND, AA-G, and AS performed the histological quantification. MCF and MR-O provided critical feedback and helped shape the research, analysis, and manuscript. CC contributed to the *in vitro* design and experiments of crosstalking data. All authors corrected the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Figure S1. Colony-stimulating factor-1 protein increases parietal epithelial cell number *in vitro*.

Supplementary Figure S2. Enrichment analysis using the Gene Ontology terms to improve the interpretation of the biologic processes.

Supplementary Figure S3. Colony-stimulating factor-1 receptor kidney expression in parietal epithelial cell types.

Supplementary Figure S4. Adriamycin treatment directly promotes colony-stimulating factor-1 receptor (CSF-1R) synthesis in parietal epithelial cells and CSF-1R inhibition preserves mice body weight in the focal segmental glomerulosclerosis model.

Supplementary Figure S5. Colony-stimulating factor-1 receptor inhibition prevented the migration of parietal epithelial cells from the Bowman's capsule.

Supplementary Figure S6. CSF-1R inhibition modulates inflammation in FSGS mouse model.

Supplementary Methods.

Supplementary References.

Supplementary File (Excel)

Supplementary Table S1. Relevant clinical information of focal segmental glomerulosclerosis patients at histologic diagnosis.

Supplementary Table S2. Primer sequences used in the study.

Supplementary Table S3. Differential expression and biologic significance analysis.

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