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JAK-STAT signaling is activated in the kidney and peripheral blood cells of patients with focal segmental glomerulosclerosis.

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Abstract

Focal segmental glomerular sclerosis (FSGS) is a devastating disease with limited treatment options and poor prognosis. Activated JAK-STAT signaling has been implicated in other kidney diseases. Since new technologies allow us to better evaluate changes in systemic and renal JAK-STAT activity as it relates to kidney function, we examined this in 106 patients with biopsy proven FSGS compared to 47 healthy control individuals. Peripheral immune function was assessed in peripheral blood mononuclear cells by phosphoflow studies before and after cytokine stimulation. Kidney JAK-STAT activity was measured by immunofluorescence and by transcriptomics. A STAT1 activity score was calculated by evaluating message status of downstream targets of pSTAT 1. Peripheral blood mononuclear cells were found to be upregulated in terms of pSTAT production at baseline in FSGS and to have limited reserve to respond to various cytokines. Increased staining for components of the JAK-STAT system in FSGS by microscopy was found. Furthermore, we found transcriptomic evidence for activation of JAK-STAT that increased pSTAT 1 and pSTAT 3 in glomerular and tubulointerstitial sections of the kidney. Some of these changes associated with the likelihood of remission of proteinuria and progression of disease. JAK-STAT signaling is altered in patients with FSGS as compared to healthy controls with activated peripheral immune cells, increased message in the kidney and increased activated proteins in the kidney. Thus, our findings support immune activation in this disease and point to the JAK-STAT pathway as a potential target for treatment of FSGS.

Keywords

focal segmental glomerulosclerosis; lymphocytes; gene expression; cell signaling

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Introduction:

Focal segmental glomerulosclerosis (FSGS) is a group of diseases sharing a common glomerular lesion of segmental glomerular sclerosis and hyalinosis.¹ It accounts for approximately 20% of cases of nephrotic syndrome in children and nearly 40% in adults.² Over the last 30 years, the prevalence of FSGS in United States has increased from 14% to 20%³ among primary glomerulonephritis, and it is the leading glomerular disorder causing end-stage renal disease (ESRD)⁴ in the US.

Traditionally, idiopathic FSGS, a podocytopathy,⁵ is thought to be caused, at least in part, by a disorder of circulating lymphocytes or T cell dysfunction, potentially through the release of an unidentified “permeability factor”,⁶ which is toxic to the glomerular ultrafiltration barrier.⁷ Many attempts have been made to elucidate the nature of this factor, although it remains elusive.⁸ Knowledge about disorders of circulating lymphocytes in FSGS is even more limited due to limitations in research technology. Human peripheral blood mononuclear cells exist in a heterogeneous pool comprised of 70% T cells, 10% B cells, and another 10% monocytes. In 2000, utilizing three channels as the most advanced technology for flow cytometry at that time, Stachowski et al.⁹ found the activity of T-helper-1 (Th1) and T-helper-2 (Th2) cells and specific lymphocyte subsets, namely CD45RA+CD4+ (“naive” helper T cells, suppressor-inducer), CD45RA+CD8+ (“naive” cytotoxic T cells, cytotoxic-effector) CD45RO+CD4+ (“memory” helper T cells) were predictive of steroid sensitivity in FSGS. The work nicely suggested a strategy to further study the function of PBMCs as a disease pathway and biomarker of FSGS.

Phosphoflow is a more recently developed research strategy based on flow cytometry. It can simultaneously distinguish cell subsets in PBMCs and quantify the phosphorylation levels of intracellular signaling proteins such as STAT.¹⁰ Present phosphoflow platforms allow us to precisely separate PBMCs into subsets as CD4+, CD4+CD45RA+, CD4+CD45RA-, CD8+, CD8+CD45RA+, CD8+CD45RA-, NK cells, B lymphocytes, and monocytes. Janus kinase signal transducer and activator of transcription (JAK/STAT) is a major pathway that responds to and transduces inflammatory signals from cells through extracellular ligands such as cytokines and chemokines.¹¹

Recently, compelling preliminary data in kidney cell specific over-expression and knock out transgenic animal models, as well as in genetic profile analysis in human samples have revealed that JAK/STAT is extensively activated in diabetic nephropathy, ADPKD, HIVAN, AKI, and obstructive uropathy,¹² in which the JAK2/STAT3 pathway is the most involved. This has already resulted in active trials of medications that inhibit the JAK-STAT pathway in diabetic nephropathy and kidney transplantation^{13,14}. Thus far, in primary glomerular disease, only one study analyzing the genomic profile of PBMCs from three IgAN patients highlighted the role of STAT1 in the exacerbation of gross hematuria.¹⁵ It thus seemed prudent to explore JAK/STAT in mediating the immune response systemically or locally in the kidney as a major role in the pathogenesis of idiopathic FSGS.¹⁶

In this study, we, for the first time, examined the phosphorylation of STAT1, STAT3 and STAT5 in each of these subsets with or without in vitro cytokine stimulation. We also

explored JAK/STAT expression and content in the kidney and associated it with disease activity and outcomes (Figure 1).

Methods

Patients

As the initial part of this study, 15 adult patients with biopsy-proven idiopathic FSGS were evaluated and provided their blood for analysis of PBMCs (Peripheral blood cells, Figure 1). All subjects gave informed consent. Age (42.9 ± 14.56 years old) and gender (10 males and 10 females) matched healthy controls (total $n=20$) were also studied. Patients were not on any active immunotherapy for at least the prior six months. Their general characteristics are listed in Table 1A. Kidney tissue from nine FSGS patients were studied by immunohistochemistry for JAK1, JAK2, STAT3, pSTAT3 and pSTAT1. Their baseline characteristics are also summarized in Table 1B. Five patients had both PBMCs assayed and biopsy slides stained. A separate group of 72 patients (Table 1C) with biopsy proven FSGS from NEPTUNE were evaluated. NEPTUNE, part of the National Institutes of Health (NIH) Rare Disease Clinical Research Network (RDCRN), is a multicenter prospective cohort study enrolling patients with proteinuric glomerular disease and performing comprehensive clinical and molecular phenotyping. Patients with secondary glomerular disease (such as diabetic kidney disease, lupus nephritis, and amyloidosis) were excluded. Comparable healthy tissue was obtained from living transplant donors for comparison with NEPTUNE ($n=6$) FSGS biopsy samples (Transcriptional group, with added patients from ECRB, Figure 1). Biopsy material from both cohorts was micro-dissected into glomerular and tubulointerstitial compartments prior to transcriptional profiling as previously described.^{17,18} Twelve of the NEPTUNE subjects had biopsy slides available from that same biopsy further assessed for JAK-STAT component and CD3 staining (immunohistochemistry group, Figure 1). The Institutional Review Boards of Stanford University Medical Center and participating centers in NEPTUNE approved all of these studies.

Phosphoflow assay

PBMCs were isolated using density gradient separation Ficoll-Paque Plus (Amersham Biosciences) and then cryopreserved. For evaluation, cells were thawed, stored at room temperature for 1 hour and counted. 0.5×10^6 viable cells/mL were plated in a 96-well plate. These cells were then stimulated with 10^4 U/ml of IFN α , 50 ng/ml of IFN γ , 50 ng/ml of IL-6, 50 ng/ml of IL-7, 50 ng/ml of IL-10, 50 ng/ml of IL-2, and 50 ng/ml of IL-21 prior to paraformaldehyde fixation and methanol permeabilization. Each well was then bar-coded using a combination of Pacific Orange and Alexa-750 dyes (Invitrogen, Carlsbad, CA), pooled in tubes, washed with FACS buffer (PBS supplemented with 2% FBS and 0.1% sodium azide), and stained with the following antibodies: BV421 to CD3, PerCP-Cy5.5 to CD4, PerCP-Cy5.5 to CD20, PE-Cy7 to CD33, BV510 to CD45RA, AlexaFluor488 to pSTAT-1, AlexaFluor 647 to pSTAT-3, PE to pSTAT-5 (all from BD Biosciences, San Jose, CA). Following resuspension in FACS buffer, 100,000 cells per stimulation condition were collected using DIVA 6.0 software on an LSRII flow cytometer (BD Biosciences). Data acquisition was performed using FlowJo v 9.3, gating on live cells based on forward versus side scatter profiles, then on singlets using forward scatter area versus height, followed by

cell subset-specific gating. Unstimulated cell populations were compared after log normalization. Following cytokine stimulation, the fold change was calculated as any stimulant status/non stimulant status. Fold change in any subset under any stimulant between control and FSGS was compared. The subset percentage of PBMCs is shown in Supplement Table 1. Phosphorylation of individual STAT1, 3, and 5 proteins in PBMCs from FSGS (n=15) stimulated in vitro by IFN α , IFN γ , IL-6, IL-7, IL-10, IL-2, and IL-21 were compared with healthy controls (n=20) (see Figure 2).

Immunohistochemistry staining for CD3, JAK1, JAK2, STAT3, pSTAT3 and pSTAT1

Starting with unstained paraffin fixed slides, antigen retrieval was done with citrate solution at pH 6.0. Normal serum was used to block the antigens for 30 minutes at room temperature. Primary antibodies-JAK1(Cell signaling #3344), JAK2 (Cell signaling #3230), STAT3 (Cell signaling #9139), pSTAT3 (Cell signaling #9145), CD3 (DAKO CD3 Clone F7.28) and pSTAT1 (Cell signaling #8826) were applied with a dilution of 1:200, 1:100, 1:2000, 1:200, and 1:200 respectively for 60 minutes at room temperature. Second antibodies to rabbit Ig or mouse Ig (Vector labs) were incubated for 30 minutes at room temperature. DAB was incubated for 3 minutes at room temperature to develop the staining. Hematoxylin was counter-stained for 1 minute at room temperature.

RNA Preparation and Microarray Expression

Kidney tissue was obtained by biopsy, stored in RNAlater® (ThermoFisher), and manually micro-dissected into tubulointerstitial (TI) and glomerular compartments. In terms of glomerular injury, manual glomerular microdissection captures glomeruli with an open Bowman's space. Therefore, across samples, our glomerular expression studies are enriched for the transcriptomes of functioning glomeruli, rather than for globally sclerosed glomeruli. Microdissected renal biopsy specimens were processed and analyzed using Affymetrix Human Gene ST 2.1 Array platforms (ThermoFisher). Probe sets were annotated to Entrez Gene IDs using custom CDF version 19 generated from the University of Michigan Brain Array group.¹⁹ Kidney tissue was processed as previously described.¹⁵ Expression data was quantile normalized and batch corrected using COMBAT.²⁰ Differential gene expression across the transcriptome was compared between patients with FSGS and living donors using the SAM method;²¹ genes were defined as differentially expressed with q-value 0.05. CEL files are accessible in GEO²²²⁰ for the ERCB dataset (GSE10498, GSE104948) and for the NEPTUNE dataset under reference series GSE104066. Differential expression analysis was performed using the SAM function in Multiple Experiment Viewer (MeV) v4.9.

Functional enrichment analysis and STAT1 dependent signature generation

Functional enrichment and upstream regulator analyses were performed in Ingenuity Pathways Analysis (Qiagen, Inc.). A STAT1 dependent signature was derived using STAT1 target genes identified from 1441 potential target genes from ChIP-Seq and limiting the search space to the 20 genes with the highest fold change after treatment of HeLa cells with interferon gamma.²³ Of the 20 genes, only 17 were annotated on a test dataset and carried forward. The 17 gene set was used to generate STAT1 signature scores across samples from the transcriptional data. The STAT1 scores were generated by creating Z-scores for each of

the 17 genes in the network and then using the average Z-score of 17 genes as a transcriptional measure of STAT1 activation.

Statistical Analysis

Descriptive statistics, including mean and SD for normally distributed variables, median and interquartile range (IQR) for skewed variables and proportions for categorical variables were used to characterize participant characteristics. Differences between subjects and controls in baseline and stimulated phosphoflow activity were compared with Wilcoxon rank-sum analysis. Spearman's rank correlation coefficient (ρ) was used to evaluate the relationship between JAK-STAT1 activation score and baseline eGFR and UPCR. Kaplan-Meier survival curves were generated by tertile of JAK-STAT1 activation score for time to complete remission (defined as first UPCR <0.3 mg/mg) and the composite of 40% decline in eGFR from baseline or ESRD. Differences between the curves were tested using the log-rank test. Additionally, separate Cox proportional hazards models were fit to estimate the hazard ratio for complete remission and the composite of 40% decline in eGFR from baseline or ESRD using JAK-STAT1 activation score as a continuous variable. Unadjusted models and models adjusted for baseline eGFR and UPCR were fit. Analyses were performed using both STATA, v12 and R.

Results

Phosphoflow Studies

Fifteen subjects with FSGS were compared with 20 healthy, adult controls. Baseline subsets of peripheral blood mononuclear cells were similar, with a trend towards fewer CD4⁺ lymphocytes and more CD8⁺ lymphocytes in FSGS (Supplement Table 1). Under non-stimulated conditions, pSTAT1 was significantly greater in FSGS subjects in CD4⁺Tcells, CD8⁺Tcells and monocytes. A similar trend was seen for pSTAT3 but did not reach significance. pSTAT5 was significantly increased in B cells and in CD4⁺ lymphocytes, restricted to those that were CD45RA marker positive (Figure 2, Supplement Table 2). However, with cytokine stimulation, there was a generalized, highly significant pattern of reduced ability to increase pSTATs 1, 3 and 5 in FSGS patients as compared to controls, suggesting a generalized defect or fatigue in the system. This was most notable among the CD4⁺ lymphocytes, and again mainly within the CD45RA⁺ subset (Figure 3). It was variable as to whether the decreased fold change left the cellular pSTAT higher or lower than control subjects post stimulation, but frequently the levels were lower or the same, (see Supplement Table 3) There was correlation between higher creatinine in these subjects and reduced ability to increase STAT phosphorylation in the CD4 and CD45⁺ lymphocytes. Greater proteinuria was significantly and inversely correlated with the ability of cytokines to stimulate phosphorylation of STAT1 or STAT3 in a variety of PBMC subtypes, shown by heat maps (Supplement figure 1). The generalized pattern of increased basal stat activation and decreased fold change could be seen in other glomerular disease patients, such as patients with lupus nephritis and membranous nephropathy, but cell lines involved and specific cytokine pathways behaved differently. (Supplemental Figure 2)

Immunohistochemistry of FSGS subjects

Unstained slides of nine subjects with FSGS were assessed for STAT3, JAK2, pSTAT1 and pSTAT3 and compared to staining in control subjects. As demonstrated in Figure 4, staining was somewhat decreased for JAK 2, but increased for STAT 3 as well as for pSTAT1 and pSTAT3. Semi-quantitative analysis (Figure 5) demonstrated significantly greater staining for pSTAT1 and pSTAT3 in FSGS subjects in the glomerulus, tubules and vessels of the kidney. While pSTAT3 appears restricted to glomerular endothelial cells, vasculature and tubule cells, pSTAT1 staining was more generalized, including podocytes and mesangial cells.

Transcriptomic analysis

Glomerular (n=48) and tubulointerstitial (n=67) profiles from 72 subjects with FSGS were compared to profiles from healthy kidney donor biopsies (n=6 for glomeruli and n=5 for tubulointerstitium). Analysis of the differentially expressed genes in FSGS demonstrated a significant enrichment of JAK-STAT signaling pathway by IPA pathway analysis in the tubulointerstitium ($p=1.08E-04$). In addition to JAKSTAT pathway enrichment, upstream analysis in IPA identified predicted activation of JAK-STAT signaling pathway nodes, IFNG and STAT1 in the tubulointerstitium (Activation Z-score: 4.7 and 3.106, enrichment p-value: $2.56E-07$) (Figure 6) A similar finding was found in the glomerular tissue (Figure 7). To represent a predicted activation at the patient level, a transcriptional assessment of JAK-STAT activation was performed. We used this composite score comprised of 17 genes as representative of STAT1 activation (Supplement Table 4). In the NEPTUNE cohort, the STAT1 activation score was elevated in FSGS patients relative to healthy living donor biopsies in both glomerular and TI sections (Supplemental Figure 3). Evaluation of an independent cohort of FSGS patients, from ECRB (baseline characteristics seen in Supplement Table 5), confirmed this elevation in STAT1 activation scores in tissue transcriptomic data from FSGS patients compared to living donors, seen in both glomerular and TI sections. Within ECRB, an analysis of diabetic subjects with similar CKD and proteinuria (Supplement Table 6) demonstrated increased STAT1 activations scores as well, (Supplemental Figure 4)

Immunohistochemistry of FSGS subjects with genomic analysis

In 12 subjects who were characterized by their glomerular gene expression of JAK1 or STAT 1 to be either high (top quartile) or low (bottom quartile), unstained slides were stained in dual fashion for both CD3 and for pSTAT1. As depicted in Figure 8, in all FSGS patients, staining for JAK1 is positive and frequently co-locates with CD3 + staining lymphocytes. In these selected subjects, greater staining for both CD3+ cells and pSTAT1 appears to be present in subjects who demonstrated higher transcriptional levels of JAK1 compared to lower transcriptional levels of JAK1, and greater degree of this dual staining was also seen for subjects with greater transcriptional levels of STAT1 compared to low STAT1 subjects. Semi-quantification results are shown in Table 2.

Correlation of clinical outcomes with transcriptomic analyses

Subjects with greater STAT1 activation (S17 z scores) were seen to have higher levels of baseline serum creatinine (Figure 9A and 9C), and higher levels of baseline proteinuria (Figure 9B and 9D). Given the longitudinal follow up data available in NEPTUNE, we assessed whether the STAT1 signature score associated with further disease progression or remission of proteinuria. The highest tertile of scores from the tubular compartment was associated with higher rate of achieving the 40% loss of eGFR/ESRD, (HR-2.10 (1.16, 3.82), p-value 0.01), and this held up with adjustment for baseline creatinine and proteinuria. While not statistically significant, there was a trend towards lower rates of complete remission in the highest tertiles from both the glomerular and tubular compartments (HR 0.68 (0.41, 1.14), p-value 0.14 and HR 0.73 (0.45, 1.18), p-value 0.08), but this was lost when adjusted for baseline characteristics.(Figure 10).

Discussion

This study evaluated systemic and intrarenal components of the JAK-STAT signaling pathway in patients with biopsy proven FSGS and active disease, characterized by mild renal dysfunction and proteinuria. Dramatic differences were found in comparison to healthy subjects without kidney disease. In the peripheral blood, there were numerous differences in baseline phosphorylation of STAT 1, 3, 5 showing increased activity among several types of PBMCs, particularly CD4+ T lymphocytes. After exposure to cytokines, PBMCs of patients with FSGS were significantly less able to increase their phosphorylation of STATs. This pattern is similar to that seen in other systemic autoimmune diseases, such as rheumatoid arthritis and systemic lupus, where increased chronic inflammation appears be associated with a decreased ability to respond to acute stimulation with various cytokines.²⁴⁻²⁷ We also found other glomerular diseases may also have altered responses to pSTAT stimulation, similar in some cells with some cytokines, but others responses were distinct (Supplemental figure 2). The finding of diminished cytokine responsive phosphorylation of STATs in PBMCs among our FSGS patients was associated with increased levels of proteinuria and decreased levels of kidney function.

Following the finding of dysregulation of JAK-STAT in the peripheral blood cells, we next evaluated the kidney. Utilizing immunohistochemistry, consistently increased staining for selected components of JAK-STAT signaling was seen. Thus, staining for JAK1, JAK 2, STAT3, pSTAT1, and pSTAT3 demonstrated that JAK and STAT proteins are indeed found in both the normal and FSGS involved kidney (in both the glomerulus and interstitial area), but increased phosphorylation and activation was demonstrated in FSGS patients, quite diffusely for pSTAT 1, and more limited to vascular and tubular sections for pSTAT3.

To evaluate changes in JAK-STAT activity in FSGS further, transcriptomic profiles in glomerular and tubulointerstitial sections of tissue from patients were compared to healthy controls. These studies strongly support that several components of this signaling pathway are upregulated and result in increasing mRNA levels for many of the known downstream targets of pSTAT1 and pSTAT3. Furthermore, a score derived from tallying expected downstream targets of STAT activation also was significantly increased in FSGS patients as compared to healthy kidney donors. Separately, we found that this score was similar to that

seen in diabetic nephropathy (supplemental Figure 5), where manipulation of JAK STAT may favorably influence clinical parameters¹². Furthermore, the association of these scores with increasing serum creatinine and increasing levels of proteinuria as well as adverse longitudinal outcomes suggests that activation of STATs may be an important marker and mediator of kidney injury and scar.

Finally, to try to integrate the findings of dysregulated PBMCs and increased renal activity of JAK-STAT signaling, dual staining for pSTAT1 and lymphocytes was utilized in subjects with either high or low message levels for JAK1 or STAT1. The results of this staining support the hypothesis that an influx of lymphocytes may be a local stimulus to increasing renal activity of pSTAT1. Further, higher message in the kidney associated with greater infiltration of lymphocytes and more intense pSTAT staining.

JAK-STAT is a major pathway that responds to and transduces inflammatory signals from extracellular ligands such as cytokines and chemokines.²⁸ This pathway is active in multiple inflammatory pathways, and in cancers. In diabetic nephropathy, basic studies in preclinical models demonstrate increased activation in both the glomerulus and tubulointerstitium and agents that limit STAT activation (JAK inhibitors) have been associated with more mild progression in animals.¹³ A recent pilot human study of JAK inhibition in diabetic nephropathy demonstrated significant reductions in proteinuria.²⁹ Furthermore, by western blot, Savin et al showed that cytokine receptor-like factor-1 (CRLFF-1), a cytokine known for B-cell stimulation, identified as a potential plasma permeability factor in FSGS,³⁰ could increase phosphorylation of STAT3 in mouse peripheral blood cells and renal cortex.³¹ This finding accompanied activated podocytes, and increased glomerular albumin permeability, which was alleviated by a JAK2 inhibitor. Thus, there is significant support for the idea that abnormalities in STAT phosphorylation can be important in the development and progression of FSGS. Indeed, our findings suggest that this pathway is altered in many kidney diseases, but the specific pathways and factors altering function are likely to be somewhat distinct in each disease.

Our study is limited by the depth in which we could further evaluate this hypothesis. Only certain molecules are available to be analyzed using all three available technologies (phosphoflow, immunohistochemistry and transcriptomic analyses). Thus, following and assessing all specific regulators of the JAK-STAT system could not be accomplished here, but will be an important goal of subsequent studies.

In sum, systemic abnormalities in PBMCs, particularly T cell regulation and activity, are paralleled by changes in glomerular and interstitial activity of the JAK-STAT system. These changes are associated with important clinical features among patients with FSGS, suggesting a significant role in disease pathogenesis. This provides justification for further exploration of this pathway and encouragement for clinical studies that aim to alter activity of the JAK-STAT pathway, particularly phosphorylation of STAT 1 and STAT 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Malaga-Dieguez L, Bouhassira D, Gipson D, Trachtman H. Novel Therapies for FSGS: Preclinical and Clinical Studies. *Advances in chronic kidney disease*. 2015;22(2):e1–e6. [PubMed: 25704355]
2. Kitiyakara C, Kopp JB, Eggers P. Trends in the epidemiology of focal segmental glomerulosclerosis. *Seminars in nephrology*. 2003;23(2):172–182. [PubMed: 12704577]
3. McGrogan A, Franssen CF, de Vries CS. The incidence of primary glomerulonephritis worldwide: a systematic review of the literature. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2011;26(2):414–430.
4. Maisonneuve P, Agodoa L, Gellert R, et al. Distribution of primary renal diseases leading to end-stage renal failure in the United States, Europe, and Australia/New Zealand: results from an international comparative study. *Am J Kidney Dis*. 2000;35(1):157–165. [PubMed: 10620560]
5. Fogo AB. Causes and pathogenesis of focal segmental glomerulosclerosis. *Nature reviews. Nephrology* 2015;11(2):76–87. [PubMed: 25447132]
6. Bao L, Haas M, Pippin J, et al. Focal and segmental glomerulosclerosis induced in mice lacking decay-accelerating factor in T cells. *J Clin Invest*. 2009;119(5):1264–1274. [PubMed: 19349693]
7. Shalhoub RJ. Pathogenesis of lipoid nephrosis: a disorder of T-cell function. *Lancet*. 1974;2(7880):556–560. [PubMed: 4140273]
8. Wada T, Nangaku M. A circulating permeability factor in focal segmental glomerulosclerosis: the hunt continues. *Clin Kidney J*. 2015;8(6):708–715. [PubMed: 26613029]
9. Stachowski J, Barth C, Michalkiewicz J, et al. Th1/Th2 balance and CD45-positive T cell subsets in primary nephrotic syndrome. *Pediatr Nephrol*. 2000;14(8–9):779–785. [PubMed: 10955927]
10. Krutzik PO, Crane JM, Clutter MR, Nolan GP. High-content single-cell drug screening with phosphospecific flow cytometry. *Nat Chem Biol*. 2008;4(2):132–142. [PubMed: 18157122]
11. O'Shea JJ, Plenge R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. *Immunity*. 2012;36(4):542–550. [PubMed: 22520847]
12. Brosius FC 3rd, He JC. JAK inhibition and progressive kidney disease. *Curr Opin Nephrol Hypertens*. 2015;24(1):88–95. [PubMed: 25415616]
13. Brosius FC, Tuttle KR, Kretzler M. JAK inhibition in the treatment of diabetic kidney disease. *Diabetologia*. 2016;59(8):1624–1627. [PubMed: 27333885]
14. Vincenti F, Silva HT, Busque S, et al. Evaluation of the effect of tofacitinib exposure on outcomes in kidney transplant patients. *Am J Transplant*. 2015;15(6):1644–1653. [PubMed: 25649117]
15. Cox SN, Sallustio F, Serino G, et al. Activated innate immunity and the involvement of CX3CR1-fractalkine in promoting hematuria in patients with IgA nephropathy. *Kidney Int*. 2012;82(5):548–560. [PubMed: 22572859]
16. Reggiani F, Ponticelli C. Focal segmental glomerular sclerosis: do not overlook the role of immune response. *J Nephrol*. 2016.
17. Gadegbeku CA, Gipson DS, Holzman LB, et al. Design of the Nephrotic Syndrome Study Network (NEPTUNE) to evaluate primary glomerular nephropathy by a multidisciplinary approach. *Kidney Int*. 2013;83(4):749–756. [PubMed: 23325076]
18. Sampson MG, Robertson CC, Martini S, et al. Integrative Genomics Identifies Novel Associations with APOL1 Risk Genotypes in Black NEPTUNE Subjects. *J Am Soc Nephrol*. 2016;27(3):814–823. [PubMed: 26150607]
19. Sandberg R, Larsson O. Improved precision and accuracy for microarrays using updated probe set definitions. *BMC Bioinformatics*. 2007;8:48. [PubMed: 17288599]

20. Chen C, Grennan K, Badner J, et al. Removing batch effects in analysis of expression microarray data: an evaluation of six batch adjustment methods. *PLoS One*. 2011;6(2):e17238. [PubMed: 21386892]
21. Rapaport F, Khanin R, Liang Y, et al. Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. *Genome Biol*. 2013;14(9):R95. [PubMed: 24020486]
22. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 2002;30(1):207–210. [PubMed: 11752295]
23. Shoguchi E, Tanaka M, Shinzato C, Kawashima T, Satoh N. A genome-wide survey of photoreceptor and circadian genes in the coral, *Acropora digitifera*. *Gene*. 2013;515(2):426–431. [PubMed: 23266624]
24. Popa C, Netea MG, Barrera P, et al. Cytokine production of stimulated whole blood cultures in rheumatoid arthritis patients receiving short-term infliximab therapy. *Cytokine*. 2005;30(2):72–77. [PubMed: 15804598]
25. Pavon EJ, Garcia-Rodriguez S, Zumaquero E, et al. Increased expression and phosphorylation of the two S100A9 isoforms in mononuclear cells from patients with systemic lupus erythematosus: a proteomic signature for circulating low-density granulocytes. *J Proteomics*. 2012;75(6):1778–1791. [PubMed: 22230807]
26. Ramirez-Velez G, Medina F, Ramirez-Montano L, et al. Constitutive phosphorylation of interferon receptor A-associated signaling proteins in systemic lupus erythematosus. *PLoS One*. 2012;7(7):e41414. [PubMed: 22859983]
27. Hale MB, Krutzik PO, Samra SS, Crane JM, Nolan GP. Stage dependent aberrant regulation of cytokine-STAT signaling in murine systemic lupus erythematosus. *PLoS One*. 2009;4(8):e6756. [PubMed: 19707593]
28. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annu Rev Med*. 2015;66:311–328. [PubMed: 25587654]
29. Tuttle KBF, Adler S et al. Baricitinib in diabetic kidney disease: results from a phase 2, multi-center, randomized, double-blind, placebo-controlled study. *Diabetes*. 2015;64(Suppl 1):114–LB.
30. Savin VJ, Sharma M, Zhou J, et al. Renal and Hematological Effects of CLCF-1, a B-Cell-Stimulating Cytokine of the IL-6 Family. *J Immunol Res*. 2015;2015:714964. [PubMed: 26146641]
31. McCarthy ET, Sharma M, Savin VJ. Circulating permeability factors in idiopathic nephrotic syndrome and focal segmental glomerulosclerosis. *Clin J Am Soc Nephrol*. 2010;5(11):2115–2121. [PubMed: 20966123]

Figure 1.
Work flow of FSGS JAK-STAT evaluation

Figure 2. Unstimulated phosphoflow. Baseline of pSTAT1 (2A), pSTAT3 (2B), and pSTAT5 (2C) in different cell subsets of B lymphocytes, CD4+, CD4+CD45RA+, CD4+CD45RA-, CD8+, CD8+CD45RA+, CD8+CD45RA-, NK cells, and monocytes in FSGS were compared with controls. * compared with control, $p < 0.05$.

Figure 3.

Changes of pSTAT1, and pSTAT3 of B lymphocytes, CD4+, CD4+CD45RA+, CD4+CD45RA-, CD8+, CD8+CD45RA+, CD8+CD45RA-, NK cells, and monocytes stimulated by IFN α , IFN γ , IL-6, IL-7, IL-10, IL-2, and IL-21 were compared between FSGS and control. P values were determined by Wilcoxon rank sum test. P values lower than 0.05 were filtered and shown in a heatmap format (left panel). Direction of increased or decreased STAT phosphorylation in comparison to controls is represented by blue or red. Correlation analysis between 24 hour urine protein excretion, or serum creatinine with any fold change described above were conducted and the four significant correlations that overlapped with the significant change of pSTAT1 are shown in Figure 3A–D. Detailed raw

data in change of pSTAT1, and pSTAT3 in any cell subset under any stimulant between control and FSGS were expressed as Mean $\pm$ SEM in supplement file on request. P values from those comparisons are also provided in supplement file on request.

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Figure 4.

Representative images of IHC stain of JAK1, JAK2, pSTAT1, STAT3 and pSTAT3 in control kidney and FSGS patient. A: Control JAK1, B: Control JAK2, C: Control pSTAT1, D: Control STAT3, E: Control pSTAT3, F: FSGS JAK1, G: FSGS JAK2, H: FSGS pSTAT1, I: FSGS STAT3, J: FSGS pSTAT3. (200×) K: Control JAK1, L: Control JAK2, M: Control pSTAT1, N: Control STAT3, O: Control pSTAT3, P: FSGS JAK1, Q: FSGS JAK2, R: FSGS pSTAT1, S: FSGS STAT3, T: FSGS pSTAT3. (400×)

Figure 5.

Semi-quantification of the JAK2, pSTAT1, STAT3, and pSTAT3 immunohistochemistry staining in FSGS patients (n=9). Healthy non-neoplastic kidney dissected from tumor nephrectomy was used as control. For JAK2 (A), the scoring was used as 0: none; +1 <25% cells positive with weak intensity; +2 26–100% cells positive with weak intensity; +3 26–100% cells positive with strong intensity. For pSTAT1 (B), the scoring was used as 0: none; +1 <50% cells positive with weak intensity; +2 51–100% cells positive with a stronger intensity; +3 51–100% cells positive with further stronger intensity. For STAT3 (C), the scoring was used as 0: none; +1 100% cells positive with weak intensity; +2 100% cells positive with a stronger intensity and the similar intensity between nuclear staining and cytoplasmic staining; +3 51–100% cells positive with a strong intensity and stronger intensity in nuclear staining than cytoplasmic staining. For pSTAT3 (D), the scoring was used as 0: none; +1 <10% cells positive with weak intensity; +2 11%–74% cells positive with weak intensity; +3 >75% cells positive with a strong intensity.

Figure 6. Differentially expressed transcripts in FSGS were enriched in the JAK-STAT pathway in the tubulointerstitium. (A) Signaling network nodes highlighted in purple indicate those genes that were differentially expressed in FSGS relative to living donor controls, while shading of the nodes indicates up-regulation (red) or down-regulation (green) in FSGS relative to living donor controls. (B) Upstream regulator analysis identified a predicated activation of IFNG and STAT1 in the tubulointerstitium with evidence supporting activation of STAT1 indicated (C).

Figure 7. Differentially expressed transcripts in FSGS were enriched in the JAK-STAT pathway in the glomeruli. (A) Signaling network nodes highlighted in purple indicate those genes that were differentially expressed in FSGS relative to living donor controls, while shading of the nodes indicates up-regulation (red) or down-regulation (green) in FSGS relative to living donor controls. (B) Predicated activation of STAT related signaling nodes in the glomeruli with evidence supporting activation of STAT1 indicated (C). Network supporting STAT1 activation in the glomeruli. Orange and blue connections indicate that STAT1 can induce or inhibit expression of downstream genes. These findings are consistent with STAT1 activation. Yellow connections indicate findings that are inconsistent with STAT1 activation, while grey lines are findings that neither support nor reject the predicated activation of STAT1.

Figure 8.

Dual IHC stain of pSTAT1 (red) and CD3 (grey) in 12 FSGS patient from NEPTUNE cohort according to isolated glomerular genetic analysis results for JAK1, JAK2 or STAT1 expression. A and G: two FSGS patients with low glomerular expression of JAK1; B and H: two FSGS patients with high glomerular expression of JAK1; C and I: two FSGS patients with low glomerular expression of JAK2; D and J: two FSGS patients with high glomerular expression of JAK2; E and K: two FSGS patients with low glomerular expression of STAT1; F and L: two FSGS patients with high glomerular expression of STAT1 (200×). A1 to L1 are corresponding images for the cases labeled as A to L (200×) under higher magnification (400×).

Figure 9.

Correlation of JAK-STAT1 activation score from glomerular compartment with baseline eGFR (panel A) and baseline UPCR (panel B) and JAK-STAT activation score from tubular compartment with baseline eGFR (panel C) and baseline UPCR (panel D) in NEPTUNE patients.

Outcome	Predictor	Unadjusted		Adjusted*	
		HR (95% CI)	P value	HR (95% CI)	P value
Complete Remission	S17 score, glom	0.68 (0.41, 1.14)	0.14	0.73 (0.45, 1.18)	0.20
	S17 score, tub	0.86 (0.64, 1.2)	0.31	1.11 (0.77, 1.60)	0.57
Composite of 40% decline and ESRD	S17 score, glom	1.82 (0.75, 4.38)	0.18	1.26 (0.51, 3.09)	0.61
	S17 score, tub	2.08 (1.41, 3.08)	<0.01	2.10 (1.16, 3.82)	0.02

Figure 10.

Adjusted Kaplan Meier survival curve for complete remission by tertile of JAK-STAT1 Activation z-score (Glomerular Compartment, panel A, p-value 0.14 and Tubular Compartment, panel B, p-value 0.08). Adjusted Kaplan Meier survival curve for Composite of 40% loss in eGFR and ESRD by tertile of JAK-STAT1 Activation z-score (Glomerular Compartment, panel C, p-value 0.41 and Tubular Compartment, panel D, p-value 0.02).

Table1A.

Baseline characteristics of 15 patients with FSGS with PBMCs assayed in phosphoflow

Age at biopsy (ys)	37 (24–61)
Gender (female)	9 (60%)
African American	2 (13%)
Asian	4 (26%)
Caucasian	4 (26%)
Hispanic	5 (33%)
Sampling time after biopsy(ds)	351 (63–2884)
eGFR at PBMC sampling (ml/min/1.73m ²)	50 (26–133)
Urine protein excretion at PBMC sampling (g/g)	2.8 (0.5–6.1)

Table1B.

Baseline characteristics of 9 patients with FSGS with kidneys stained for JAK1, JAK2, STAT3, pSTAT3, and pSTAT1

Age at biopsy (ys)	56 (28–69)
Gender (female)	4 (44%)
White hispanic	4 (44%)
White non Hispanic	5 (56%)
eGFR (ml/min/1.73m ²)	76 (24–124)
Urine protein excretion (g/g)	3.5 (1.7–7.2)

Table1C.

Baseline characteristics of 72 patients with FSGS with kidney gene analysis

Age at biopsy (ys)	29 (16–55)
Gender (female)	23 (32%)
African American	27 (38%)
Hispanic	N/A
eGFR (ml/min/1.73m ²)	77 (51–103)
Urine protein excretion (g/g)	1.7 (0.9–4)

Table2.

Semi-quantification of dual staining of pSTAT1 and CD3 in NEPTUNE cohort

Gene/expression level	CD3 density	pSTAT1	dual+lymphocytes
JAK1 LOW	3	0	0
JAK1 LOW	0	2	0
JAK1 HIGH	1	1	2
JAK1 HIGH	0	1	1
JAK2 LOW	2	2	1
JAK2 LOW	3	2	2
JAK2 HIGH	0	1	0
JAK2 HIGH	3	3	3
STAT1 LOW	0	0	0
STAT1 LOW	0	1	0
STAT1 HIGH	1	3	1
STAT1 HIGH	1	1	0

For CD3 density: 0:no lymphoid aggregate (> 50 cells/focus); 1: 1 aggregate/bx; 2: 2 aggregates/bx; 3: 3 or more aggregates/core; for pSTAT1, the scoring was used as 0: none; +1 <50% cells positive with weak intensity; +2 51–100% cells, slightly stronger or 20–50% very strong; +3 51–100% cells strong; % cells dual +: 0:none; 1:< 20%; 2: 20–50%; 3: >50%