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Rituximab targets podocytes in recurrent focal segmental glomerulosclerosis

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Abstract

Focal segmental glomerulosclerosis (FSGS) is a prevalent glomerular disease characterized by proteinuria, progression to end stage renal disease and recurrence of proteinuria after kidney transplantation in approximately one third of patients. It has been suggested that rituximab might treat recurrent FSGS through an unknown mechanism. Rituximab recognizes CD20 on B-lymphocytes but might also bind sphingomyelin-phosphodiesterase-acid-like-3b (SMPDL-3b) and regulates acid-sphingomyelinase (ASMase) activity. We hypothesized that rituximab prevents recurrent FSGS and preserves podocyte SMPDL-3b expression. We studied 41 patients at high risk for recurrent FSGS, 27 of whom were treated with rituximab at time of kidney transplant. Incidence of nephrotic-range proteinuria and change in estimated glomerular filtration rate (Δ eGFR) were analyzed. SMPDL-3b immunostaining was performed in post-reperfusion kidney biopsies. SMPDL-3b protein, ASMase activity, and cytoskeleton remodeling were studied in

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CONFLICTS OF INTEREST: A.F. and G.B. have a pending provisional patent and a pending NIH grant application on the subject matter. All other authors have declared no competing interests.

LIST OF SUPPLEMENTARY MATERIAL

Fig. S1-CD20 is not expressed in glomeruli of patients with recurrent FSGS

Fig. S2-Generation of SMPDL-3b overexpressing podocytes

Fig. S3-Rituximab does not affect podocyte expression of vinculin, podocin and nephrin

Fig. S4-Disruption of the actin cytoskeleton does not affect SMPDL-3b

Fig. S5-Generation of SMPDL-3b knockdown podocytes

cultured normal human podocytes that had been exposed to patient sera with or without rituximab. Rituximab treatment was associated with lower incidence of post-transplant proteinuria and decreased Δ eGFR. The number of SMPDL-3b⁺ podocytes in post-reperfusion biopsies was reduced in patients who developed recurrent FSGS. Rituximab partially prevented SMPDL-3b and ASMase downregulation that was observed in podocytes treated with the sera of patients with recurrent FSGS. Either SMPDL-3b overexpression or treatment with rituximab prevented disruption of the actin cytoskeleton and podocyte apoptosis induced by patient sera. This effect was diminished in cultured podocytes where the gene encoding *SMPDL-3b* was silenced. Our study suggests that treatment of high-risk patients with rituximab at time of kidney transplant might prevent recurrent FSGS by modulating podocyte function in an SMPDL-3b-dependent manner.

INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is a common glomerular disorder that clinically manifests as nephrotic syndrome and affects both pediatric and adult patients. Both primary and secondary forms of FSGS have been described, and among the primary forms several genetic mutations of proteins expressed in podocytes have been shown to cause FSGS (1). Podocytes and their foot processes comprise the outer layer of the kidney ultrafiltration barrier and form the glomerular slit diaphragm, a complex cellular structure that prevents the development of proteinuria (the leakage of protein from the blood compartment to the urinary compartment through modulation of podocyte actin cytoskeleton) (2). Although several therapeutic strategies have been shown to reduce proteinuria and preserve renal function, FSGS remains a significant cause of end-stage renal disease (ESRD) requiring dialysis or kidney transplantation (1). Recurrence of FSGS after transplantation occurs in approximately 30-40% of patients and reduces graft survival (3-5); a recurrence rate as high as 86% has been described in high-risk patients (6).

Rituximab is a monoclonal antibody directed against CD20 expressed in B-lymphocytes that has several applications in treating nephrological conditions, such as acute allograft rejection and steroid-resistant nephrotic syndrome (7). Two patients with post-transplant lymphoproliferative disorders and concomitant recurrent FSGS that had received rituximab experienced remission of nephrotic syndrome (8, 9). Since then, successful treatment of recurrent FSGS with rituximab has been reported in some (9-15), but not all instances (16). Although an infiltration of lymphocytes has been described in transplanted kidneys affected by FSGS recurrence (17), its pathogenesis has not been demonstrated to be antibody-mediated, suggesting the possibility of B-lymphocyte-independent mechanisms of rituximab action. Screening of a phage display peptide library revealed a possible cross-reactivity of rituximab with sphingomyelin-phosphodiesterase-acid-like-3b (SMPDL-3b) (18). Furthermore, *in vitro* exposure to rituximab in lymphoma cells regulates the activity of acid-sphingomyelinase (ASMase) in raft microdomains (19), which are essential for the organization of receptors and signaling molecules in highly specialized cells (20), such as the podocytes of kidney glomeruli. We hypothesized that rituximab affects the kidney filtration barrier in recurrent FSGS via the preservation of sphingolipid-related enzymes that might affect actin cytoskeleton remodeling in podocytes. Therefore, rituximab may act as a direct modulator of podocyte function, similar to what has been recently reported for cyclosporine, a calcineurin inhibitor used for immunosuppression in solid organ transplantation and in nephrotic syndrome (21).

We found that the number of SMPDL-3b⁺ podocytes in post-reperfusion biopsies is reduced in patients that later experience recurrent FSGS. Serum collected in the pre-transplant setting from these patients that would ultimately develop recurrent FSGS was used to culture

normal human podocytes and caused a downregulation of SMLPD-3b protein and ASMase activity *in vitro*, a phenomenon that was prevented by rituximab. Although podocytes exposed *in vitro* to the sera of affected patients experienced a marked disruption of the actin cytoskeleton, pretreatment with rituximab or overexpression of SMPDL-3b partially prevented this phenotype. Rituximab directly affects podocyte function in an SMPDL-3b-dependent manner, and could therefore represent a valid treatment strategy to prevent recurrent FSGS after kidney transplantation.

RESULTS

Rituximab treatment is associated with reduced incidence of recurrent FSGS after kidney transplantation

We evaluated the effect of rituximab induction in pediatric and young-adult patients who had received kidney transplantation. These patients were at high-risk because of their young age (<25 years old) and because they progressed rapidly to end-stage renal disease from the time of diagnosis of FSGS (less than 7 years). The research protocol was approved by the institutional review board and ethics committee. From 2004 through to 2009, 27 consecutive FSGS patients who received one dose of rituximab (375 mg/m²) within 24 hours of kidney transplantation per protocol (rituximab group) were compared with 14 consecutive transplantation recipients that did not receive rituximab (historical control group, 1999–2003). Progression to end-stage renal disease was comparable (Table 1). All patients received combined thymoglobulin (1 mg/kg, 3–5 doses) and daclizumab (1 mg/kg, 2 doses) as standard induction protocol for immunosuppression (22); alemtuzumab (0.4 mg/kg) induction for one patient in each group; patients with delayed graft function received 1–5 additional doses of thymoglobulin (1 mg/kg). Tacrolimus (target trough level of 5 to 7 ng/ml), mycophenolate (500 mg twice a day), and corticosteroids (methylprednisolone, 4 mg/day) were used for maintenance of immunosuppression. Biopsies obtained 1–2 hours post-reperfusion, but prior to rituximab infusion, were available from 20 out of 27 patients in the rituximab group. Additional biopsies were performed after transplantation when clinically indicated. Nine out of 14 patients in the historical group underwent a kidney biopsy after transplantation, and 5 out of these 9 (56%) showed histological evidence of recurrent FSGS. Eleven out of the 27 patients in the rituximab treatment group were biopsied for either rising creatinine or proteinuria, and 5 out of these 11 (45%) showed histological evidence of recurrent FSGS.

To assess the effect of rituximab on peripheral B-lymphocytes, determination of CD19 (expressed during all stages of B cell differentiation except plasma cells) was performed by flow cytometry (FACS, BD, Franklin Lakes, New Jersey) in cells obtained from peripheral blood. FSGS recurrence was defined as spot urine protein-to-creatinine ratio >3.5 g/g during the first 30 days after transplantation in any morning collection. The need for plasmapheresis, as determined by the physician, was also used as a surrogate marker for recurrent disease. Patients with FSGS and with a spot urine protein-to-creatinine ratio of less than 3.5 g/g during the first 30 days after transplantation and no need for plasmapheresis were classified in the non-recurrent group. Changes in renal function from baseline were evaluated at 3 and 6 months using the differences of estimated glomerular filtration rate from the baseline post transplantation value at 1 month (Δ eGFR) (23, 24). For those patients with native kidney proteinuria at time of transplantation (21.4% in the historical group and 24.1% in the rituximab group), a stent was placed in the transplanted kidney for measurement of transplant proteinuria and removed in the outpatient follow-up clinic 2–3 weeks later. Two patients from the historical group (2 out of 14, 14%) and three patients from the rituximab group (3 out of 27, 11%) received a preemptive transplant.

Patients from the two groups were matched for age, race, gender, donor age, nephrectomy of the native kidneys, and time to ESRD (Table 1). There was a higher percentage of deceased donors in the rituximab group ($p<0.01$), reflecting the change in United Network for Organ Sharing (UNOS) policy allowing pediatric patients a higher priority. Complete depletion of CD19+ cells was observed in rituximab-treated patients after 2 weeks of treatment ($p<0.001$). A single dose of rituximab administered within 24 hours of transplantation was associated with a reduction in the incidence of nephrotic range proteinuria between days 3 and 30 after transplantation ($p<0.05$). Similarly, rituximab administration was associated with a decreased prescription for plasmapheresis ($p<0.05$). While control patients experienced a loss of eGFR, the eGFR remained unchanged at 6 months in rituximab-treated patients ($p<0.01$). Although a trend to higher graft survival was observed in the rituximab-treated group at 6 and 12 months, this did not reach statistical significance. The impact of rituximab was conserved after multivariable analysis that adjusted for the difference in donor type (living versus deceased). No opportunistic infections or post-transplant lymphoproliferative disorders complicating single dose rituximab infusion were observed.

Rituximab binds to SMPDL-3b in podocyte lipid rafts

We first investigated if CD20, the traditional ligand of rituximab, is expressed in podocytes. Podocytes from normal human kidney tissue sections were identified as glomerular cells positive for the podocyte marker synaptopodin and did not express CD20 (Fig. 1A). Human lymph node tissue was used as a positive control. Given that podocytes express B7-1/CD80 in proteinuric but not normal states (25), we tested CD20 expression in post-reperfusion biopsies from three patients with recurrent FSGS. CD20 was absent in biopsies of patients with recurrent FSGS, suggesting that podocytes do not express CD20 in both healthy and diseased conditions (Fig. S1). Conversely, SMPDL-3b was expressed in the glomeruli and was partially colocalized with synaptopodin (Fig. 1A). An irrelevant IgG isotype was used as negative control (Figure 1A). Fluorescently labeled rituximab bound to podocytes in kidney tissue sections (Fig. 1B); this could be prevented by pre-incubation of the antibody with a linear SMPDL-3b blocking peptide (18). IgG1, a nonspecific antibody, and an anti-CD20 monoclonal antibody unable to recognize SMPDL-3b did not bind to podocytes in tissue sections (Fig. 1B).

Rituximab was also able to bind to differentiated human podocytes in culture, with Raji lymphoma cells as positive controls, and this binding was prevented by the SMPDL-3b blocking peptide (Fig. 1C), suggesting that rituximab also binds to SMPDL-3b in cultured podocytes. Cultured human podocytes did not express either of the two CD20 mRNA transcript variants (40 or 50 kDa) (Fig. 1D) or the CD20 protein (Fig. 1E), compared with Raji lymphoma cells as positive controls. However, SMPDL-3b was expressed in human podocytes as determined by RT-PCR and Western blot (Fig. 1F).

Separation of cell membranes (ME) from cytosolic (CE) fractions of lysed differentiated human podocytes as well as in SMPDL-3b-transfected human embryonic kidney (HEK) 293 cells revealed a predominant plasma membrane localization of the SMPDL-3b 50 kDa isoform (Fig. 1G). Lipid raft separation from podocyte and HEK293 cell lysates revealed a unique localization of SMPDL-3b to flotillin-1-positive lipid raft fractions (Fig. 1H), suggesting a potential role of SMPDL-3b in raft domain organization. HEK293 cells transfected with green-fluorescent protein (GFP)-SMPDL-3b were used for immunoprecipitation experiments with either rituximab or a negative interleukin (IL)-8 monoclonal antibody control. Two bands at approximately 70 and 80 kDa were observed in cell lysates corresponding to the expression of GFP-tagged SMPDL-3b isoforms; whereas a single band was detected in the eluate from the rituximab immunoprecipitate at 80 kDa, indicating that rituximab binds the 50 kDa isoform (Fig. 1I).

SMPDL-3b is downregulated in podocytes in post-reperfusion biopsies of patients who develop recurrent FSGS

Post-reperfusion kidney biopsies obtained prior to rituximab infusion were available from 12 patients with non-recurrent FSGS and 8 patients with recurrent FSGS. A mean of 13 glomeruli per patient biopsy were analyzed. The number of SMPDL-3b⁺ synaptopodin⁺ cells per glomerulus was lower (* $p < 0.05$) in biopsies from patients with recurrent disease when compared with nonrecurrent biopsies (Fig. 2A, B).

Rituximab prevents SMPDL-3b and ASMase downregulation in serum-treated podocytes

Human podocytes were cultured overnight in the presence of serum from normal subjects ($n=5$) or from patients with either recurrent ($n=12$) or non-recurrent ($n=10$) FSGS. A significant downregulation of SMPDL-3b mRNA ($p < 0.01$, Fig. 2C) and protein (50 kDa isoform) (** $p < 0.01$, Fig. 2D) was observed when cultured with recurrent FSGS serum and compared to normal human serum. Conversely, SMPDL-3b mRNA ($p = 0.08$, Fig. 2C) and protein ($p = 0.23$, Fig. 2D) was not significantly downregulated when podocytes were cultured in the presence of serum from patients with non-current FSGS when compared to normal human sera (Fig. 2C, D).

The downregulation of SMPDL-3b induced by recurrent FSGS sera was prevented by rituximab (** $p < 0.01$); however, rituximab did not significantly affect SMPDL-3b mRNA expression ($p = 0.60$, Fig 2C) or protein level ($p = 0.32$, Fig 2D) in podocytes treated with serum from patients with non-recurrent FSGS, similar to normal human sera. These data suggest that rituximab binding to SMPDL-3b might prevent its degradation that would otherwise occur in recurrent FSGS. In order to appreciate the variability in the expression of SMPDL-3b in normal podocytes exposed to the sera of different patients at baseline or in response to rituximab, a Western blot with both non-recurrent ($n=4$) and recurrent ($n=4$) consecutive patients is shown (Fig. 2E).

ASMase protein level and activity were also analyzed in human podocyte cultures. Exposure of normal podocytes to non-recurrent human sera did not affect ASMase protein level when compared to normal human serum ($p = 0.55$, Fig. 2F). However, there was a significant reduction in the amount of ASMase protein (52 and 54 kDa isoforms) in podocytes treated with sera from patients with recurrent FSGS, as compared to normal and non-recurrent sera treatments ($p < 0.05$, Fig. 2F). Rituximab was able to prevent this down-regulation primarily by preserving the 52 kDa ASMase isoform ($p < 0.05$). Rituximab also preserved ASMase enzymatic activity to normal levels in podocytes treated with serum from recurrent FSGS patients ($p < 0.01$, Fig. 2G). Overall, a modulation of sphingolipid-related proteins occurs in podocytes exposed to the sera of patients with recurrent FSGS, suggesting their role in the pathogenesis of recurrent FSGS and as targets of rituximab treatment.

Both rituximab and SMPDL-3b prevent the serum-induced loss of podocyte stress fibers

When human podocytes were cultured overnight in the presence of recurrent FSGS serum, a marked disruption of stress fibers was observed by the quantitative confocal image analysis of phalloidin-stained podocytes ($n=12$) ($p < 0.001$, Fig. 3A, B). Non-recurrent FSGS sera ($n=10$) did not significantly affect podocyte cytoskeleton remodeling when compared to NHS ($p = 0.16$ Fig. 3B). We noted a significant correlation ($p < 0.001$) between the loss of stress fibers and the urinary protein/creatinin ratio after transplantation (Fig. 3C), suggesting that disruption of stress fibers *in vitro* could be utilized as a prediction bioassay for recurrence of proteinuria after transplantation in FSGS patients. Rituximab partially prevented the loss of stress fibers in podocytes cultured in recurrent FSGS serum ($p < 0.01$, Fig. 3D). SMPDL-3b overexpression also protected against the disruption of stress fibers by recurrent FSGS sera ($p < 0.001$, Fig. 3E). Proof of SMPDL-3b overexpression was obtained

by Western blot (Fig. S2) The protective effect of rituximab on the disruption of stress fibers observed after exposure to recurrent FSGS sera was not dependent on the regulation of the expression of vinculin, podocin, or nephrin (Fig. S3), which were found to be modulated after exposure to nephrotic human sera (26, 27). These data suggest that recurrent FSGS sera may affect podocyte actin cytoskeleton through a sphingolipid-dependent, slit-diaphragm-independent mechanism. The effect of recurrent FSGS sera on SMPDL-3b shown in Fig. 2 is upstream of actin cytoskeleton remodeling, as treatment with cytochalasin D (200 μ M for 6 hours) disrupted the actin cytoskeleton without affecting SMPDL-3b (Fig. S4).

Rituximab protects podocyte actin cytoskeleton and viability through SMPDL-3b

We tested whether the protective effect of rituximab on the podocyte actin cytoskeleton was preserved in cells treated with recurrent FSGS sera where *SMPDL-3b* expression was silenced (Fig. S5). *SMPDL-3b* knock-down cell lines (siSMP) showed a conserved actin cytoskeleton when exposed to non-recurrent FSGS human sera (n=10) (Fig. 4A, B); whereas exposure to recurrent FSGS sera (n=12) resulted in a more profound disruption of actin fibers when compared with non-targeting (NT) control cells ($p<0.05$, Fig. 4B). Rituximab could not prevent the recurrent sera-induced disruption of stress fibers in siSMP cells ($p=0.8$, Fig. 4A, B), suggesting that SMPDL-3b mediates the protective effect of rituximab on the podocyte actin cytoskeleton.

Disruption of stress fibers in podocytes exposed to serum from patients with recurrent FSGS occurred as early as 6 hours after exposure ($p<0.05$, Fig. 4C) and was amplified in siSMP cells ($p<0.05$). Disruption of stress fibers preceded the development of significant apoptosis: 24 hours after exposure to either non-recurrent or recurrent sera we observed a significant increase in apoptosis ($p<0.05$, Fig. 4D), which was amplified in siSMP cells ($p<0.05$). Although rituximab was able to partially prevent podocyte apoptosis induced by exposure to recurrent FSGS sera in control cells (non-targeting siRNA) ($p<0.01$, Fig. 4E), this was not observed in siSMP podocytes ($p=0.6$ in recurrent sera treated cells, Fig. 4F).

Therefore, our data suggest that SMPDL-3b deficiency is not sufficient to cause a phenotype in podocytes. However, downregulation of SMPDL-3b observed after exposure to patient sera renders podocytes more susceptible to actin remodeling likely caused by additional circulating permeability factors and leads to proteinuria. Rituximab prevents podocyte actin remodeling through stabilization of SMPDL-3b (Fig. 4G).

DISCUSSION

The present study unveils a mechanism by which the monoclonal antibody rituximab might prevent recurrent FSGS after kidney transplantation through a direct regulation of podocyte function. The B-lymphocyte-independent, podocyte-associated mechanism of rituximab action offers the rationale to introduce this treatment in medical conditions where B-lymphocytes do not play an apparent pathogenic role. Immunosuppressive agents are generally not effective antiproteinuric agents in recurrent FSGS. Although cyclosporine has antiproteinuric effects, these effects are clearly independent of the drug's immunosuppressive properties (21). The beneficial effect observed in case series and small uncontrolled studies when rituximab was utilized early in the course of recurrent FSGS and/or in high-risk patients, has prompted us to investigate in our FSGS patient population whether rituximab could prevent recurrent proteinuria after transplantation. Rituximab treatment has been associated with a decreased incidence of nephrotic-range proteinuria in a review of case series in patients with recurrent FSGS (28). This potential effect of rituximab in treating proteinuria, together with the lack of evidence for B-lymphocyte involvement in recurrent FSGS, led to our hypothesis that rituximab might directly affect podocyte function.

Microarray analysis of B lymphoma cell lines exposed to rituximab has revealed a broad effect on genes involved in cell proliferation, apoptosis, adhesion, kinase activation, and cell-cell contact (29). The recent evidence that rituximab can directly bind to molecules other than CD20, such as SMPDL-3b (18), and can directly affect the activity of ASMase and concomitant ceramide generation in raft microdomains (19), has prompted us to investigate the possibility of a direct action of rituximab in podocytes through modulation of SMPDL-3b and/or ASMase. Our findings suggest that rituximab is able to directly preserve SMPDL-3b and ASMase activity in podocytes that have been exposed to serum from patients with recurrent FSGS. Moreover, this demonstrates a role for SMPDL-3b in the modulation of actin remodeling in podocytes. In fact, a genetic disease characterized by excessive accumulation of sphingomyelin owing to a lack of ASMase activity (Niemann-Pick disease) causes glomerular pathology (30), which supports an important role of sphingomyelin metabolism in the pathogenesis of glomerulopathies. The fact that the downregulation of SMPDL-3b occurs within 1 to 2 hours after transplantation (Fig. 2A) suggests that degradation of SMPDL-3b occurs after exposure to circulating factors and is prevented by rituximab (Fig. 4G).

One of the major limitations for prevention of recurrent proteinuria is that the pathogenic mechanisms involved are yet to be identified. Although a circulating permeability factor has been described (31), the mechanisms responsible for podocyte damage remain unclear. The finding that the sera of patients with recurrent FSGS affect both integrin-linked-kinase activity (27) as well as SMPDL-3b and ASMase, suggests that circulating factors may affect the activity of enzymes relevant to podocyte function. The observation that rituximab affects ASMase enzymatic activity, suggests that rituximab might prevent proteinuria through enzymes in addition to those we have recently demonstrated to play a role in the pathogenesis of proteinuric kidney diseases (32, 33). Interestingly, patients treated with statins might be more resistant to the effect of rituximab (34), which further supports the possibility that rituximab function is highly dependent on cellular lipid content. Furthermore, rituximab modifies the functional organization of lipid raft microdomains in B lymphoma cells, where it allows for proper localization and function of membrane proteins (35). Although we have demonstrated that rituximab does not affect the expression of vinculin, podocin, or nephrin, it would be interesting to determine if rituximab and SMPDL-3b affect slit diaphragm protein membrane localization, which is considered essential for podocyte function. It is interesting to note that rituximab-mediated preservation of the podocytes' actin cytoskeleton was associated with protection from apoptosis in CD20-SMPDL-3b+ human podocytes, which is opposite from what has been described in CD20+ cells (36).

In conclusion, we have demonstrated that rituximab treatment of high-risk FSGS patients is associated with lower incidence of post-transplant proteinuria and could directly protect podocytes in a SMPDL-3b-dependent manner. The retrospective nature of the clinical analysis is one limitation to our study. The smaller number of living donors in the rituximab group, which could be perceived as a confounding variable (37), did not impact the significance of the observed rituximab protective effect. This study offers a strong rationale to the design of a prospective, randomized trial for rituximab-mediated prevention of recurrent FSGS in high-risk patients. Furthermore, our experimental data suggest a new pathogenic mechanism for recurrent FSGS and are an example of how agents developed for certain clinical conditions might translate into novel indications.

MATERIALS AND METHODS

Immunofluorescence and immunoperoxidase

Ten millimeter-thick frozen sections of normal human kidneys from deceased donors were utilized to test CD20 (goat polyclonal anti-human CD20, Santa Cruz Biotechnology), SMPDL-3b (rabbit polyclonal anti-SMPDL-3b, GeneWay), and synaptopodin (mouse monoclonal anti-synaptopodin, Byodesign International) expression in podocytes. Immunoperoxidase was used to evaluate SMPDL-3b expression in fixed kidney sections from transplanted patients with focal segmental glomerulosclerosis (FSGS) that were sequentially incubated with the primary rabbit IgG polyclonal anti-SMPDL-3b antibody, with the secondary biotinylated antibody (Zymed, HistoLine, Milan, Italy) and with the peroxidase-labeled streptavidin (Zymed). Peroxidase activity was detected with 3,5-diaminobenzidine (Sigma). Sections were dehydrated and mounted in Bio Mount (Bio Optica, Milano, Italy). Specificity of antibody labelling was demonstrated by the lack of staining after substituting proper control immunoglobulins (Zymed) for the primary antibody. The number of stained podocytes was then quantified. After manual selection of glomeruli as regions of interest (ROI), a color threshold procedure allowed ROI highlighting of the staining in gray mode, and the software was programmed to automatically calculate the number of stained cells as described (38). Images were acquired by a Zeiss Axioscope 40FL microscope, equipped with AxioCam MRc5 digital video camera; recorded using AxioVision software 4.3; and analyzed by the AxioVision analysis module (Carl Zeiss SpA, Arese, Italy) (38).

In order to determine rituximab binding to normal human podocytes from kidney tissue sections, we fluorescently labeled rituximab, a nonspecific IgG1, and a monoclonal antibody directed against CD20 that does not recognize the common epitope between CD20 and SMPDL-3b (IF5 antibody, gift of Dr. Press OW, University of North Carolina, Chapel Hill, NC) with FITC using the Thermo Scientific Labeling kit (Rockford, IL). Frozen normal human kidney sections were incubated with FITC-rituximab (10 µg/ml) or with the same concentration of FITC-IgG1 (Invitrogen) or of FITC-IF5. As rituximab has been shown to recognize a peptide sequence that belongs to SMPDL-3b (SLWPKWLEAIQ), this recognizable peptide sequence was synthesized for blocking experiments (Biosynthesis Inc., Lewisville, TX). Preincubation of FITC-rituximab with a 10 molar excess of SLWPKWLEAIQ to rituximab was utilized to verify rituximab binding to SMPDL-3b. An irrelevant peptide sequence was used as a control (WKLSPEQILAW). The same antibody concentrations were used to detect rituximab binding in cultured human podocytes with Raji cells as a positive control. Because preliminary data failed to detect FITC-rituximab binding to podocytes, signal amplification with an HRP-conjugated secondary antibody (Invitrogen) was performed.

Cell culture and transfection

Normal human podocytes were cultured as described (39). Briefly, normal human podocytes were immortalized with a thermosensitive SV40 construct that would allow for cell proliferation at 33 °C and for cell differentiation after 14 days of thermoshift at 37.5 °C. Stable podocyte cell lines expressing either green fluorescent protein (GFP)-labeled SMPDL-3b, GFP alone, siSMPDL-3b built on pGFP-V-RS vector (Origene) (TTGTGGAACGCCTGACCAAGCTCATCAGA) or a non targeting siRNA (scramble oligo) were developed by transfection with Fugene reagents (Roche) or electroporation (Mammo Zapper cloning gun, Tritech Research, Los Angeles, CA) followed by cell sorting (Supplemental Methods). Clones were derived via limiting dilution, expanded and selected for gene-downregulation efficiency by RT-PCR and by Western blotting. Pre-transplant sera were obtained from 22 patients with FSGS and 5 age matched controls and stored at -20°C.

Normal human podocytes were exposed to a serum concentration of 4% in RPMI medium for 24 hours at 37 °C. For rituximab treatment (Genentech Inc., San Francisco, CA), cells were pretreated with rituximab (100 µg/ml) for 30 minutes prior to sera exposure as described for lymphoma cells (19). Both a nonspecific IgG and a monoclonal antibody directed against human IL-8 (a gift of Genentech) were used as controls for rituximab treatment. Data shown as controls (CTRL) were treated with 100 µg/ml of the anti-IL8 antibody.

Polymerase Chain Reactions (PCR)

Both standard and real-time quantitative PCR assays (Applied Biosystems, Foster City, CA) were performed for CD20, SMPDL-3b, GAPDH, 18S and actin. For standard PCR, the following primers were designed: CD20 transcript variant 1, 5'-GCAGCAACGGAGAGAAAACTC and 3'-TTCCTGGAAGAAGGCAAAGA; CD20 transcript variant 3 5'-GCTGCCATTCTGGAATGAT and 3'-TTCCTGGAAGAAGGCAAAGA; SMPDL-3b, 5'-CTATACCAGCAATGCGCTGA and 3'-GAGAAGACGCAAAACAAGGC; actin, 5'-TCATGAGGTAGTCCGTCAGG and 3'-TCTAGGCACCAAGGTGTG; GAPDH, 5'-GTCAGTGGTGGACCTGACCT and 3'-GTCAACGGTACATCTGGGA. SMPDL-3b and 18S ribosomal RNA expression were evaluated using the 7500 Real-Time-PCR System (Hs 00205522_m1 and Hs 99999901_s1, Applied Biosystems, Foster City, CA). Relative quantification between different samples was determined as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct$ affected sample $-\Delta Ct$ unaffected sample).

Western blotting, and immunoprecipitation

Cell lysates were collected in lysis buffer in the presence of protease and phosphatase inhibitors (Bio-Rad). After protein quantification with a Detergent Compatible Assay (DCA protein assay, Bio-Rad), an equal amount of protein was loaded onto 4-20% SDS-PAGE gels (Lonza) and transferred to nitrocellulose membranes (Bio-Rad). Western blottings for SMPDL-3b, ASMase, actin, were performed with the utilization of the following primary antibodies: Rabbit polyclonal anti SMPDL-3b (Genway Biotech, Inc., San Diego, CA), a Goat polyclonal anti-ASMase (Santa Cruz, CA), a mouse monoclonal anti-nephrin (gift of Dr. Trygvason, Karolinska, Sweden), a rabbit polyclonal anti-podocin (gift of Dr. Mundel, MGH, Boston), a mouse monoclonal anti-vinculin (Sigma, 1:3000), and a mouse monoclonal anti B-actin (ABCAM Inc., Cambridge, MA). For immunoprecipitation experiments, HEK 293 cells were transfected with GFP-SMPDL-3b and collected in radioimmunoprecipitation assay (RIPA) buffer. After protein extraction using incubation on ice for 30 minutes followed by a centrifugation at 14.000rpm for 15 min at 4°C, 2 mg of protein lysate were incubated with 2 µg of preclearing beads (Santa Cruz) and then immunoprecipitated with 4 µg rituximab or anti-IL8 monoclonal antibody adsorbed on a mixture of protein A sepharose beads and protein G agarose beads (1:1; Sigma). Immunoprecipitates were eluted in Laemmli buffer and used for western blotting.

Subcellular fractionation and lipid raft isolation

Plasma membranes and cytosolic fractions were separated from HEK cells and from cultured human podocytes with a Subcellular Protein Fractionation Kit (Thermo Scientific). Epidermal growth factor receptor-1 (EGFR1, Rabbit monoclonal anti-human, 1:1000, Cell Signaling) was utilized as a plasma membrane marker, and AKT (Rabbit monoclonal anti-human, 1:1000, Cell Signaling) as a cytosolic marker.

For lipid raft isolation, lysates were centrifuged at 1000× g for 10 min. The resulting supernatant was collected and mixed with equal volume of 70% OptiPrep in basic buffer (20 mM Tris-HCl, pH 7.8, 250 mM sucrose), placed on the bottom of the Ultra-clear tube (Sigma), where 5 ml of 30%, 2 ml of 5% and 1 ml of 0% basic buffer were then added.

Gradients were centrifuged for 4 h at 170,000× g (37100 rpm), and the appropriate fraction transferred to a protein concentration unit (Centricon-10, 2ml capacity) and spun at less than 5000 g for 100 min at 4 degree. Fraction concentrates were collected for SDS-PAGE gel and Western blotting for flotillin-1 (raft domain marker, rabbit polyclonal anti-human, 1:200, Santa Cruz Biotechnology) and SMPDL-3b was performed (40).

Acid sphingomyelinase activity

Acid sphingomyelinase (ASMase) activity was measured in differentiated podocytes that were exposed to 4% patient sera for 24 hours in the presence or absence of rituximab (100 µg/ml). Lysates were collected in acetate buffer (50mM sodium acetate pH 5.0) and subjected to freeze/thaw (−80 °C) without the addition of protease or phosphatase inhibitors. ASMase activity was measured with a two step procedure using the Amplex Red Sphingomyelinase kit (Molecular Probes/Invitrogen Corporation, Eugene, OR), and was normalized for protein content. Fluorescence was measured with a fluorescence microplate reader using ex/em 544/590 nm.

Quantitative determination of stress fibers

Podocytes were plated on glass chamber slides and treated with rituximab and patient sera as described above. After incubation in the presence of patients sera for 6, 12, or 24 hours, cells were fixed in 4% paraformaldehyde, incubated with rhodamine phalloidin (Invitrogen) in 0.01% Triton-X for 30 min at 37 °C, and mounted for analysis. Twenty fields at 40× magnification were quantitatively evaluated using fluorescence microscopy for the loss of actin filaments across the cytoplasm (defined as disruption of stress fibers and expressed as percentage of cells over total cells).

Apoptosis

Podocytes were incubated in the presence of 4% pooled normal human serum (n=5), or serum from patients with non-recurrent FSGS (n=10), or recurrent FSGS (n=12). Cells were pretreated with rituximab or with anti-IL8 (100 µg/ml for 30 minutes) prior to exposure to serum. The percentage of cells positive for Annexin V was determined at 6 and 24 hours by flow cytometry after cell staining with the Vybrant apoptosis assay kit (Invitrogen).

Statistical analysis

Categorical variables were compared by chi-squared test and Fisher's exact test, when appropriate. Continuous variables were compared using two-tailed Student's *t*-test or Wilcoxon rank-sum test. Graft survival was calculated using Kaplan-Meier method and compared by log-rank test. Comparison of the number of SMPDL-3b+ cells in kidney biopsies in recurrent and non-recurrent FSGS patients was performed with unpaired Student's *t*-test. For experimental studies, one-way ANOVA was used and the results represent the mean and standard deviation of 4 to 8 independent experiments. When one-way ANOVA showed statistical significance, results were compared using *t*-test after Tukey's correction for multiple comparisons. Results were considered statistically significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CD20 is not expressed in human podocytes, while SMPDL-3b is expressed in podocyte lipid rafts

(A) Immunofluorescence staining for CD20 (top, green) or SMPDL-3b (bottom, green) and synaptopodin (both, red) is shown on frozen tissue sections of a normal human kidney. Human lymph node tissue was used as a positive control for CD20. An irrelevant IgG served as negative control. Images were acquired at 63× magnification. (B)- FITC-rituximab (FITC-RITUX; 10 µg/ml) binds to normal kidney sections. Binding of FITC-RITUX can be blocked by pre-incubation with an SMPDL-3b blocking peptide. FITC-IgG1 and FITC-IF5 anti-CD20 monoclonal antibodies were used as negative controls and did not show any binding to human kidney section at 10 µg/mL. (C) Immunoperoxidase amplification staining of rituximab (10 µg/ml) binding to normal human podocytes (shown in brown) can be prevented by pre-incubation with the SMPDL-3b blocking peptide. Binding of rituximab to Raji cells, which express CD20, was used as positive control (63×1.7× magnification was utilized). (D) Cultured, differentiated human podocytes do not express CD20, as assessed by mRNA expression levels for both transcript variant 1 (v1, 200 bp) and 3 (v3, 240 bp) using standard RT-PCR in cultured differentiated human podocytes (Podo). Raji cell line was the positive control. (E) CD20 protein expression was also analyzed in both cultured differentiated human podocytes and Raji cells by Western blotting. A unique band of 35 kDa was observed in Raji cells, but did not appear in differentiated human podocytes. (F) PCR and Western blot analyses for SMPDL-3b in cultured differentiated human podocytes. Both isoforms 1 (50 kDa) and 2 (40 kDa) could be identified. Actin was utilized as housekeeping gene. (G) Cultured HEK293 cells and differentiated human podocytes were utilized to separate membrane extracts (ME) from cytosolic extracts (CE). ME were identified as EGFR-1+ and CE as AKT+. SMPDL-3b 50 kDa isoform localizes predominantly at the plasma membrane. SMPDL-3b (50 kDa) was only identified in the membrane extract from human podocytes. The 40 kDa isoform was not detected in any fraction, in either podocytes or HEK293 cells. (H) Lipid raft (+) were separated from non-lipid raft (-) fractions to

determine if SMPDL-3b localizes specifically to the lipid raft microdomains. Flotillin-1 (48 kDa) was used as a specific raft fraction marker. **(I)** Lysates of HEK293 cells transfected with GFP-SMPDL-3b were immunoprecipitated with either rituximab (Rit) or with a monoclonal antibody directed against IL8 (α IL8). Both the initial lysates and the immunoprecipitation eluates (IP) were tested for the presence of SMPDL-3b.

Figure 2. Rituximab prevents the downregulation of SMPDL-3b in recurrent FSGS

(A) Low and high power images (at 63 $\times$ and 63 $\times$ 1.7 $\times$ magnification) of immunoperoxidase staining for SMPDL-3b (red) and synaptopodin (brown) in post-reperfusion biopsies of patients with recurrent (REC) and non-recurrent (NON-REC) FSGS. Black arrows point to podocytes. (B) Number of SMPDL-3b+ podocytes per glomerulus, as evaluated by SMPDL-3b and synaptopodin labeling in post-reperfusion kidney biopsies from patients that later on developed recurrent (REC) disease (n=8) and patients that did not develop clinical recurrence (NON-REC) (n=12). All kidney biopsies were obtained prior to initiation of treatment with rituximab. An average of 13 $\pm$ 4 glomeruli per patient were analyzed. * p <0.05, unpaired Student's t -test. (C) Regulation of podocyte SMPDL-3b mRNA expression by normal (NHS), non-recurrent (NON-REC) FSGS, and recurrent (REC) FSGS human sera (n=4) and by rituximab. * p <0.05, ** p <0.01 by one-way ANOVA. (D) The amount of SMPDL-3b protein is normalized to actin in human podocytes treated with normal (NHS, n=5), REC (n=12), NON-REC (n=10) human sera and exposed to rituximab. * p <0.05, ** p <0.01 by one-way ANOVA. (E) Western blot for SMPDL-3b protein of normal podocytes cultured with sera from consecutive non-recurrent (n=4) and recurrent (n=4) FSGS patients in the presence or absence of rituximab. (F) The amount of 52 and 54 kDa ASMAse protein is normalized to actin in human podocytes that were exposed to normal (NHS) (n=5), non-recurrent (NON-REC) FSGS (n=10), and recurrent (REC) FSGS (n=12) human sera in the presence or absence of rituximab. * p <0.05, ** p <0.01 by one-way ANOVA (G) ASMAse activity per μ g of total lysate protein as evaluated by enzyme-linked immunosorbent assay. ** p <0.01 by one-way ANOVA.

Figure 3. Both rituximab and SMPDL-3b partially prevent the effect of recurrent FSGS sera on podocyte stress fibers

(A) Representative stress fiber confocal images (40× magnification) of normal human podocytes exposed to normal (NHS) (n=5), non-recurrent (NON-REC) FSGS (n=10), and recurrent (REC) FSGS (n=12) human sera. (B) The percentage of cells with disruption of stress fibers observed after exposure to NHS (n=5), NON-REC sera (n=10), and REC human sera (n=12) (***p<0.001 by one-way ANOVA). (C) Linear correlation between the percentage of cells with loss of stress fibers and the urine protein/creatinine ratio obtained from REC (red squares) and NON-REC (black squares) patients (n=22) in the first 30 days after transplantation ($R^2 = 0.59$; p<0.001). (D) Confocal images of stress fiber 40× magnification and bar graph analysis of normal human podocytes exposed to recurrent FSGS sera in the presence (REC+RITUX) or absence (REC) of rituximab. Rituximab protected the loss of stress fibers observed in stressed podocytes exposed to REC sera, but not NON-REC sera (**p<0.01 by one-way ANOVA). (E) Confocal images of stress fibers (40× magnification) and bar graph analysis of normal human podocytes exposed to REC sera transfected with an empty GFP vector (REC) or with a SMPDL-3b-GFP vector (REC + SMPDL-3b). SMPDL-3b overexpression protected the loss of stress fibers observed in podocytes exposed to REC sera (*p<0.05; ***p<0.001 by one-way ANOVA).

Figure 4. The renal-protective effect of rituximab in podocytes is mediated by SMPDL-3b (A) Confocal images (40× magnification) of normal human podocytes exposed to either non-recurrent FSGS (NON-REC, N=10) sera or recurrent FSGS (REC, N=12) sera in non-targeting (NT) control and siSMPDL-3b (siSMP) cell lines in the absence or presence of rituximab (+Ritux). The protective effect of rituximab was markedly reduced in siSMP podocytes. (B) Bar graph analysis of the disruption of stress fibers observed in NT and siSMP podocytes exposed to REC (n=12) or NON-REC (n=10) sera in the absence or presence of rituximab (+RITUX) (* $p < 0.05$ by one-way ANOVA). Rituximab did not prevent loss of stress fibers in siSMP podocytes ($p = 0.8$). (C) Disruption of stress fibers over time observed in NT control cells exposed to normal human sera (NHS) (closed triangles), REC sera (closed squares), or NON-REC sera (closed circles), and in siSMP cells exposed to NHS (open triangles), REC sera (open squares), and NON-REC sera (open circles) (* $p < 0.05$ when comparing by one-way ANOVA different time points to time 0; # $p < 0.05$ when comparing NT to siSMP cells exposed to REC sera). Error bars represent SD from four independent experiments performed with pooled sera from NHS (n=5), NON-REC (n=10), and REC (n=12) patients (D) Percentage of Annexin V+ cells in NT control cells exposed to NHS (solid triangles), REC sera (solid squares), or NON-REC sera (solid circles), and in siSMP cells exposed to NHS (open triangles), REC sera (open squares) and NON-REC sera (open circles) (* $p < 0.05$ when comparing by one-way ANOVA REC and NON-REC sera treated cells to NHS treated cells; # $p < 0.05$ when comparing NT to siSMP cells exposed to REC sera by one-way ANOVA). Error bars represent SD from four independent experiments performed with pooled sera from NHS (n=5), NON-REC (n=10), and REC (n=12) patients. (E and F) Bar graph analysis for Annexin V staining of four independent experiments performed at 24 hours with pooled sera from NHS (n=5), NON-REC (n=10), and REC (n=12) patients. Apoptosis determination was performed in both (E) NT control cells and in (F) siSMP cells. An increased cell death was observed in NON-REC sera treated podocytes ($p < 0.05$ by one-way ANOVA), but more so in REC sera treated

podocytes ($p < 0.01$ by one-way ANOVA) when compared to NHS. Rituximab prevented podocyte apoptosis in NT cells, but not in siSMP cells ($*p < 0.05$, $**p < 0.01$ by one-way ANOVA). **(H)** Disease model. Although SMPDL-3b deficiency is not sufficient to cause actin remodeling and proteinuria in podocytes, SMPDL-3b downregulation after exposure to FSGS patient sera renders podocytes more susceptible to actin remodeling caused by a variety of permeability factors. Rituximab partially preserves disruption of stress fibers through stabilization of SMPDL-3b.

TABLE 1
Patient demographics and clinical outcome

Age, race, gender, nephrectomy of the native kidneys, time to end-stage renal disease (ESRD), and donor characteristics (living versus deceased) are shown for the 14 historical control patients that did not receive rituximab, but did receive the standard induction treatment, and the 27 patients that received one dose of rituximab (375 mg/m²) within 24 hours of transplant. CD19⁺ cells significantly decreased in rituximab-treated patients three days after infusion, and were almost undetectable 10 days after treatment. The incidence of recurrent nephrotic range proteinuria and the need for plasmapheresis between days 3 and 30 after transplantation were significantly lower in the rituximab group than in the control group. The changes of estimated glomerular filtration rate (Δ eGFR) at 3 and 6 months post-transplant (from baseline at 1 month) were significantly higher in the control group than the rituximab group. There was no statistically significant difference in graft survival between groups at 6 and 12 months.

	Controls (No rituximab) N=14	Treated (Rituximab) N=27	p value
Age (mean $\pm$ SD)	123 $\pm$ 5.2	150 $\pm$ 5.5	0.1650
Race (W/B)	9/5 (64%/36%)	14/13 (52%/48%)	0.5203
Gender (M/F)	6/8 (43%/57%)	9/18 (33%/67%)	0.7337
Time to ESRD (year)	33 $\pm$ 2.1	34 $\pm$ 2.0	0.7942
Donor (LD/DD)	9/5 (64%/36%)	4/23 (15%/85%)	0.0033 *
Donor age (mean $\pm$ SD)	313 $\pm$ 94	247 $\pm$ 146	0.1290
Nephrectomy (Y/N)	7/7 (50%/50%)	16/11 (59%/41%)	0.7417
Nephrotic proteinuria w/i 1 Mo	9 (64%)	7 (26%)	0.0229 *
Plasmapheresis w/i 1 Mo	10 (71%)	8 (30%)	0.0192 *
CD19 count			
Week 0	412 $\pm$ 223	360 $\pm$ 223	0.4344
Week 0-5	327 $\pm$ 290	107 $\pm$ 115	0.0145 *
Week 1	472 $\pm$ 437	45 $\pm$ 31	<
Week 2	559 $\pm$ 526	16 $\pm$ 23	<
Week 3	729 $\pm$ 670	5 $\pm$ 6	<
Week 4	630 $\pm$ 384	6 $\pm$ 7	<
Δ eGFR (vs. 1 mo nadir)			
3 months	-180 $\pm$ 169	-13 $\pm$ 146	0.0012 *
6 months	-190 $\pm$ 198	-53 $\pm$ 184	0.0075 *
12 months	-269 $\pm$ 267	-203 $\pm$ 273	0.3717
Graft survival			
6 months	92.9%	100%	0.1730
12 months	85.7%	95.8%	0.2639

Abbreviations: B, black; DD, deceased donor; F, female; LD, living donor; M, male; W, white.

* $p < 0.05$. Fisher's exact test was utilized for categorical variables: race, gender, donor (LD/DD), nephrectomy (Y/N), nephrotic proteinuria, and plasmapheresis. Log-rank test was utilized for survival analysis (6 months and 12 months). All other variables were compared using Wilcoxon rank-sum test.