Rituximab Targets Podocytes in Recurrent Focal Segmental Glomerulosclerosis

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Focal segmental glomerulosclerosis (FSGS) is a glomerular disease characterized by proteinuria, progression to end-stage renal disease, and recurrence of proteinuria after kidney transplantation in about one-third of patients. It has been suggested that rituximab might treat recurrent FSGS through an unknown mechanism. Rituximab not only recognizes CD20 on B lymphocytes, but might also bind sphingomyelin phosphodiesterase acid-like 3b (SMPDL-3b) protein and regulate 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. SMPDL-3b protein, ASMase activity, and cytoskeleton remodeling were studied in cultured normal human podocytes that had been exposed to patient sera with or without rituximab. Rituximab treatment was associated with lower incidence of posttransplant proteinuria and stabilization of glomerular filtration rate. The number of SMPDL-3b+ podocytes in postreperfusion biopsies was reduced in patients who developed recurrent FSGS. Rituximab partially prevented SMPDL-3b and ASMase down-regulation that was observed in podocytes treated with the sera of patients with recurrent FSGS. Overexpression of SMPDL-3b or treatment with rituximab was able to prevent disruption of the actin cytoskeleton and podocyte apoptosis induced by patient sera. This effect was diminished in cultured podocytes where 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; among the primary forms, several genetic mutations of proteins expressed in podocytes have been shown to cause FSGS (1). Podocytes and their foot processes compose 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, requiring dialysis or kidney transplantation (1). Recurrence of FSGS after transplantation occurs in about 30 to 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 on B lymphocytes that has several applications in treating nephrological conditions, including acute allograft rejection and steroid-resistant nephrotic syndrome (7). Two patients with post-transplant lymphoproliferative disorders and concomitant recurrent FSGS who 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 a B lymphocyte–independent mechanism of rituximab action. The screening of a phage display peptide library revealed a possible cross-reactivity of rituximab with sphingomyelin phosphodiesterase acid-like 3b (SMPDL-3b) protein (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. Rituximab might therefore 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).

Here, we found that the number of SMPDL-3b+ podocytes in postreperfusion biopsies is reduced in patients who later experience recurrent FSGS. Serum collected in the pretransplant setting from these patients who would ultimately develop recurrent FSGS was used to culture normal human podocytes and caused a down-regulation of SMPDL-3b protein and ASMase activity in vitro, a phenomenon that was prevented by rituximab. Podocytes exposed in vitro to the sera of affected patients...
experienced a marked disruption of the actin cytoskeleton, but 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) (Table 1). The patient population consisted of individuals who had received one dose of rituximab (375 mg/m²) within 24 hours of kidney transplant (rituximab group, n = 27) and individuals who did not receive rituximab (historical control group, n = 14) (Table 1). Nine of 14 patients in the historical group underwent a kidney biopsy after transplantation, and 5 of these 9 (56%) showed histological evidence of recurrent FSGS. Eleven of the 27 patients in the rituximab treatment group were biopsied for either rising creatinine or proteinuria, and 5 of these 11 (45%) showed histological evidence of recurrent FSGS.

To assess the effect of rituximab on peripheral B lymphocytes, we determined the amount of CD19, which is expressed during all stages of B cell differentiation (except plasma cells), in cells obtained from peripheral blood using flow cytometry. FSGS recurrence was defined as a spot urine protein/creatinine ratio of >3.5 g/g during the first 3 months after transplantation.

**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 who did not receive rituximab, but did receive the standard induction treatment, and 27 patients who received one dose of rituximab (375 mg/m²) within 24 hours of kidney transplant. CD19+ cells decreased significantly in rituximab-treated patients 3 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.

<table>
<thead>
<tr>
<th></th>
<th>Controls (no rituximab), n = 14</th>
<th>Treated (rituximab), n = 27</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>12.3 ± 5.2</td>
<td>15.0 ± 5.5</td>
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<tr>
<td>Race (W/B)</td>
<td>9/5 (64%/36%)</td>
<td>14/13 (52%/48%)</td>
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<tr>
<td>Gender (M/F)</td>
<td>6/8 (43%/57%)</td>
<td>9/18 (33%/67%)</td>
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<td>Time to ESRD (mean year ± SD)</td>
<td>3.3 ± 2.1</td>
<td>3.4 ± 2.0</td>
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<td>Donor (LD/DD)</td>
<td>9/5 (64% LD)</td>
<td>4/23 (15% LD)</td>
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<td>Donor age (mean ± SD)</td>
<td>31.3 ± 9.4</td>
<td>24.7 ± 14.6</td>
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<td>Nephrotic proteinuria within 1 month</td>
<td>7/7 (50% Y)</td>
<td>16/11 (59% Y)</td>
<td>0.74</td>
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<td>Plasmapheresis within 1 month</td>
<td>10 (71%)</td>
<td>8 (30%)</td>
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<tr>
<td>CD19 count (mean ± SD)</td>
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<tr>
<td>Week 0</td>
<td>412 ± 223</td>
<td>360 ± 223</td>
<td>0.43</td>
</tr>
<tr>
<td>Week 0.5</td>
<td>327 ± 290</td>
<td>107 ± 115</td>
<td>0.015</td>
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<td>Week 1</td>
<td>472 ± 437</td>
<td>45 ± 31</td>
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<td>Week 2</td>
<td>559 ± 526</td>
<td>16 ± 23</td>
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<td>Week 3</td>
<td>729 ± 670</td>
<td>5 ± 6</td>
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<td>Week 4</td>
<td>630 ± 384</td>
<td>6 ± 7</td>
<td>0.00020</td>
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<td>ΔeGFR (mean versus 1-month baseline ± SD)</td>
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<tr>
<td>3 months</td>
<td>−18.0 ± 16.9</td>
<td>−1.3 ± 14.6</td>
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<td>6 months</td>
<td>−19.0 ± 19.8</td>
<td>−5.3 ± 18.4</td>
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<tr>
<td>12 months</td>
<td>−26.9 ± 26.7</td>
<td>−20.3 ± 27.3</td>
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Percent graft survival

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<th>Controls (no rituximab), n = 14</th>
<th>Treated (rituximab), n = 27</th>
<th>P</th>
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<tbody>
<tr>
<td>6 months</td>
<td>92.9%</td>
<td>100%</td>
<td>0.17</td>
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<tr>
<td>12 months</td>
<td>85.7%</td>
<td>95.8%</td>
<td>0.26</td>
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**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 who did not receive rituximab, but did receive the standard induction treatment, and 27 patients who received one dose of rituximab (375 mg/m²) within 24 hours of kidney transplant. CD19+ cells decreased significantly in rituximab-treated patients 3 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 in estimated glomerular filtration rate (ΔeGFR) at 3 and 6 months after transplant (from baseline at 1 month) were significantly higher in the control group than in the rituximab group. There was no statistically significant difference in graft survival between groups at 6 and 12 months. Fisher’s exact test was used for categorical variables race, gender, donor (LD/DD), nephrectomy (Y/N), nephrotic proteinuria, and plasmapheresis. Log-rank test was used for survival analysis (6 and 12 months). All other variables were compared with the Wilcoxon rank-sum test, and all P values result from the comparison of historical control and rituximab-treated patients. B, black; DD, deceased donor; F, female; LD, living donor; M, male; W, white.
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 protein/creatinine ratio of <3.5 g/g during the first 30 days after transplantation and no need for plasmapheresis were classified in the nonrecurrent group. Changes in renal function from baseline were evaluated at 3 and 6 months using the differences of estimated glomerular filtration rate (ΔeGFR) from the baseline posttransplantation value at 1 month (22, 23). For those patients with native kidney proteinuria at time of transplantation (21.4% in the historical group; 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 to 3 weeks later. Two patients from the historical group (2 of 14; 14%) and 3 patients from the rituximab group (3 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 end-stage renal disease (Table 1). There was a higher percentage of deceased donors in the rituximab group, reflecting the change in United Network for Organ Sharing policy, allowing pediatric patients a higher priority. Complete depletion of CD19+ cells was observed in rituximab-treated patients after 2 weeks of treatment. 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. Similarly, rituximab administration was associated with a decreased prescription for plasmapheresis. Whereas

**Fig. 1.** CD20 is not expressed in human podocytes, and SMPDL-3b is expressed in podocyte lipid rafts. (A) Immunofluorescence staining for CD20 (top, green) or SMPDL-3b (bottom, green) and synaptopodin (SYNPO) (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 was used as a negative control. Scale bars, 25 μm. (B) FITC-rituximab (FITC-RITUX; 10 μg/ml) binds to normal kidney sections. Binding of FITC-rituximab can be blocked by preincubation 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 sections at 10 μg/ml. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). (C) Immunoperoxidase amplification staining of rituximab (10 μg/ml) binding to normal human podocytes (shown in brown) can be prevented by preincubation with the SMPDL-3b–blocking peptide. Binding of rituximab to Raji cells, which express CD20, was used as positive control. Scale bars, 5 μm. (D) Cultured differentiated human podocytes do not express CD20, as assessed by mRNA expression levels for both transcript variant 1 [v1, 200 base pairs (bp)] and variant 3 (v3, 240 bp) using standard RT-PCR in cultured differentiated human podocytes (Podo). Raji cell line was the positive control. Scale bars, 5 μm. (E) CD20 protein expression was also analyzed in both cultured differentiated human podocytes and Raji cells by Western blotting (WB). A unique band of 35 kD was observed in Raji cells only. (F) PCR and Western blot analyses for SMPDL-3b in cultured differentiated human podocytes. Both isoforms (40 and 50 kD) were identified. Actin was used as housekeeping gene. (G) Membrane extracts (ME) were separated from cytosolic extracts (CE) of cultured HEK293 cells and differentiated human podocytes. MEs were identified as EGFR-1* and CEs were identified as AKT*. The SMPDL-3b 50-kD isoform localized predominantly at the plasma membrane in both HEK293 cells and podocytes. (H) Lipid raft (+) fractions were separated from nonlipid raft (−) fractions to determine whether the 50-kD SMPDL-3b isoform localizes specifically to the lipid raft microdomains. Flotillin-1 (48 kD) was used as a specific raft fraction marker. (I) Lysates of HEK293 cells transfected with GFP–SMPDL-3b were immunoprecipitated either with rituximab or with a monoclonal antibody directed against IL-8 (αIL-8). Both the initial lysate and the immunoprecipitation (IP) eluate were tested for the presence of SMPDL-3b.
control patients experienced a loss of eGFR, the eGFR remained unchanged at 6 months in rituximab-treated patients (Table 1). 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 posttransplant lymphoproliferative disorders complicating single-dose rituximab infusion were observed.

**Rituximab binds to SMPDL-3b in podocyte lipid rafts**

We first investigated whether 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). Podocytes express B7-1/CD80 in proteinuric but not normal states (24); we therefore
tested CD20 expression in postreperfusion 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 either healthy or diseased conditions (fig. S1). Conversely, SMPDL-3b was expressed in the glomeruli and was partially colocalized with synaptopodin (Fig. 1A). Fluorescently labeled rituximab bound to podocytes in kidney tissue sections (Fig. 1B). This binding could be prevented by preincubation of the antibody with a linear SMPDL-3b–blocking peptide (18). A nonspecific immunoglobulin G1 (IgG1) and an anti-CD20 monoclonal antibody that is unable to recognize SMPDL-3b (IF5) did not bind to podocytes in tissue sections (Fig. 1B).

Rituximab was also able to bind to differentiated human podocytes in culture (Fig. 1C). This binding was prevented by the SMPDL-3b–blocking peptide (Fig. 1C), thus 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 kD) (Fig. 1D) or the 35-kD CD20 protein (Fig. 1E) when compared to Raji lymphoma cells as positive controls. However, SMPDL-3b was expressed in human podocytes, as determined by reverse transcription–polymerase chain reaction (RT-PCR) and Western blot (Fig. 1F).

Cell membrane extract (ME) from lysed differentiated human podocytes and SMPDL-3b–transfected human embryonic kidney (HEK) 293 cells were separated, revealing a predominant plasma membrane localization of the SMPDL-3b 50-kD isoform (Fig. 1G). The 40-kD isoform was not detected in any fraction, in either podocytes or HEK293 cells. Lipid raft separation from podocyte and HEK293 cell lysates showed a unique localization of SMPDL-3b to flotillin-1–positive lipid raft fractions (Fig. 1H), thus 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-8 (IL-8) monoclonal antibody control. Two bands at about 70 and 80 kD were observed in cell lysates, corresponding to the expression of GFP-tagged SMPDL-3b isoforms (Fig. 1I), whereas a single band was detected in the eluate from the rituximab immunoprecipitate at 80 kD, indicating that rituximab binds the 50-kD isoform.

**SMPDL-3b is down-regulated in podocytes in postreperfusion biopsies of patients who develop recurrent FSGS**

Postreperfusion kidney biopsies obtained before rituximab infusion were available from 12 patients with nonrecurrent FSGS and 8 patients with recurrent FSGS. A mean of 13 glomeruli per patient biopsy was analyzed. The number of SMPDL-3b synaptopodin cells per glomerulus was lower in biopsies from patients with recurrent disease when compared with nonrecurrent biopsies (Fig. 2, A and B).

**Rituximab prevents SMPDL-3b and ASMase down-regulation 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 nonrecurrent (n = 10) FSGS. A significant down-regulation of SMPDL-3b mRNA (Fig. 2C) and protein (50-kD isoform) (Fig. 2D) was observed when cultured with recurrent FSGS serum and compared to normal human serum (NHS). Conversely, SMPDL-3b mRNA (P = 0.08, Fig. 2C) and protein (P = 0.23, Fig. 2D) were not significantly down-regulated when podocytes were cultured in the presence of serum from patients with nonrecurrent FSGS when compared to NHS (Fig. 2, C and D). The down-regulation of SMPDL-3b in podocytes induced by sera from recurrent FSGS patients was prevented by rituximab; 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 sera from normal subjects (n = 5) or from patients with either recurrent (n = 12) or nonrecurrent (n = 10) FSGS. A significant down-regulation of SMPDL-3b mRNA (Fig. 2C) and protein (50-kD isoform) (Fig. 2D) was observed when cultured with recurrent FSGS serum and compared to normal human serum (NHS). Conversely, SMPDL-3b mRNA (P = 0.08, Fig. 2C) and protein (P = 0.23, Fig. 2D) were not significantly down-regulated when podocytes were cultured in the presence of serum from patients with nonrecurrent FSGS when compared to NHS (Fig. 2, C and D). The down-regulation of SMPDL-3b in podocytes induced by sera from recurrent FSGS patients was prevented by rituximab; 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 sera from normal subjects (n = 5) or from patients with either recurrent (n = 12) or nonrecurrent (n = 10) FSGS. A significant down-regulation of SMPDL-3b mRNA (Fig. 2C) and protein (50-kD isoform) (Fig. 2D) was observed when cultured with recurrent FSGS serum and compared to normal human serum (NHS). Conversely, SMPDL-3b mRNA (P = 0.08, Fig. 2C) and protein (P = 0.23, Fig. 2D) were not significantly down-regulated when podocytes were cultured in the presence of serum from patients with nonrecurrent FSGS when compared to NHS (Fig. 2, C and D). The down-regulation of SMPDL-3b in podocytes induced by sera from recurrent FSGS patients was prevented by rituximab; 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 sera from normal subjects (n = 5) or from patients with either recurrent (n = 12) or nonrecurrent (n = 10) FSGS. A significant down-regulation of SMPDL-3b mRNA (Fig. 2C) and protein (50-kD isoform) (Fig. 2D) was observed when cultured with recurrent FSGS serum and compared to normal human serum (NHS). Conversely, SMPDL-3b mRNA (P = 0.08, Fig. 2C) and protein (P = 0.23, Fig. 2D) were not significantly down-regulated when podocytes were cultured in the presence of serum from patients with nonrecurrent FSGS when compared to NHS (Fig. 2, C and D). The down-regulation of SMPDL-3b in podocytes induced by sera from recurrent FSGS patients was prevented by rituximab; 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 sera from normal subjects (n = 5) or from patients with either recurrent (n = 12) or nonrecurrent (n = 10) FSGS. A significant down-regulation of SMPDL-3b mRNA (Fig. 2C) and protein (50-kD isoform) (Fig. 2D) was observed when cultured with recurrent FSGS serum and compared to normal human serum (NHS). Conversely, SMPDL-3b mRNA (P = 0.08, Fig. 2C) and protein (P = 0.23, Fig. 2D) were not significantly down-regulated when podocytes were cultured in the presence of serum from patients with nonrecurr...
with serum from patients with nonrecurrent FSGS or NHS. These data suggest that rituximab binding to SMPDL-3b might prevent its degradation that would otherwise occur in recurrent FSGS. A Western blot with both nonrecurrent (n = 4) and recurrent (n = 4) consecutive patients is shown, which highlights the variability in SMPDL-3b expression in normal podocytes exposed to the sera of different patients, at baseline or in response to rituximab (Fig. 2E).

ASMase protein level and activity were also analyzed in human podocyte cultures. Exposure of normal podocytes to nonrecurrent 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-kD isoforms) in podocytes treated with sera from patients with recurrent FSGS compared to normal and nonrecurrent sera treatments (Fig. 2F). Rituximab was able to prevent this down-regulation, primarily by preserving the 52-kD ASMase isoform. Rituximab also preserved ASMase enzymatic activity at normal levels in podocytes treated with serum from recurrent FSGS patients (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 overexpression prevent the serum-induced loss of podocyte stress fibers**

When human podocytes were cultured overnight in the presence of recurrent FSGS patient serum (n = 12), a marked disruption of stress fibers was observed by the quantitative confocal image analysis of phalloidin-stained podocytes (Fig. 3, A and B). Nonrecurrent FSGS sera (n = 10) did not significantly affect podocyte cytoskeleton remodeling when compared to NHS (P = 0.16, Fig. 3B). We also noted a correlation (R² = 0.59) between the loss of stress fibers and the urinary protein/creatinine ratio after transplantation (Fig. 3C), which suggests that disruption of stress fibers in vitro could be used as a prediction bioassay for recurrence of proteinuria after transplantation in FSGS patients. Rituximab prevented podocyte apoptosis in NHS, but not in nonrecurrent sera. *P < 0.05; **P < 0.01 by one-way ANOVA. Data are means ± SD. (G) Proposed disease mechanism, including rituximab activity. Although SMPDL-3b deficiency is not sufficient to cause actin remodeling and proteinuria in podocytes, SMPDL-3b down-regulation after exposure to FSGS patient sera renders podocytes more susceptible to actin remodeling, which is caused by a variety of permeability factors. Rituximab partially preserves disruption of stress fibers through stabilization of SMPDL-3b.
SMPDL-3b overexpression also protected against the disruption of stress fibers by recurrent FSGS sera (Fig. 3E). Confirmation of successful SMPDL-3b overexpression in SMPDL-3b–transfected podocytes 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 either decreased or internalized after exposure to nephrotic human sera (25, 26). These data suggest that recurrent FSGS sera might affect podocyte actin cytoskeleton through a sphingolipid-dependent, slit diaphragm–independent mechanism. Additionally, the effect of recurrent FSGS sera on SMPDL-3b shown in Fig. 2 is likely upstream of actin cytoskeleton remodeling, because treatment with cytochalasin D (200 μM 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 gene expression was silenced (fig. S5). SMPDL-3b knockdown cell lines (siSMP) showed a conserved actin cytoskeleton when exposed to nonrecurrent FSGS human sera (n = 10) (Fig. 4, A and B), whereas exposure to recurrent FSGS sera (n = 12) resulted in a more profound disruption of actin fibers when compared with nontargeting (NT) control cells (Fig. 4B). Rituximab could not prevent the recurrent sera–induced disruption of stress fibers in siMP cells (P = 0.8; Fig. 4, A and B), thus 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 (Fig. 4C) and was amplified in siSMP cells. Disruption of stress fibers preceded the development of significant apoptosis: 24 hours after exposure to either nonrecurrent or recurrent sera, we observed a significant increase in apoptosis (Fig. 4D), which was amplified in siSMP cells. Although rituximab was able to partially prevent podocyte apoptosis induced by exposure to recurrent FSGS sera in control cells [NT small interfering RNA (siRNA)] (Fig. 4E), this was not observed in siSMP podocytes (P = 0.6 in recurrent sera–treated cells; Fig. 4F). These data suggest that SMPDL-3b deficiency is not sufficient to cause a phenotype in podocytes. However, down-regulation 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 appears to prevent podocyte actin remodeling through stabilization of SMPDL-3b; the proposed mechanism is shown in Fig. 4G.

DISCUSSION

This study unveils a mechanism by which the monoclonal antibody rituximab might prevent recurrent FSGS after kidney transplantation through a direct regulation of podocyte function. This mechanism appears to be B lymphocyte–independent, which 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). Rituximab treatment has been associated with a decreased incidence of nephrotic-range proteinuria in a review of case series in patients with recurrent FSGS (27). 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 (28). 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 sera from patients with recurrent FSGS. Moreover, this demonstrates a role for SMPDL-3b in the modulation of actin remodeling in podocytes. A genetic disease characterized by excessive accumulation of sphingomyelin owing to a lack of ASMase activity (Niemann-Pick disease) causes glomerular pathology (29), which supports an important role of sphingomyelin metabolism in the pathogenesis of glomerulopathies. The fact that the down-regulation 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, which can be prevented by rituximab (Fig. 4G).

One of the major barriers to preventing recurrent proteinuria is the fact that the pathogenic mechanisms involved are yet to be identified. Although a circulating permeability factor has been described (30), the mechanisms responsible for podocyte damage remain unclear. The finding that the sera of patients with recurrent FSGS affect integrin-linked kinase activity (26) as well as SMPDL-3b and ASMase suggests that circulating factors might affect the activity of enzymes relevant to podocyte function. Our observation here that rituximab affects ASMase enzymatic activity suggests that rituximab might prevent proteinuria through enzymes in addition to those we have recently shown to be important in the pathogenesis of proteinuric kidney diseases (31, 32).

Patients treated with statins might be more resistant to the effect of rituximab (33), which further supports the possibility that rituximab function is highly dependent on cellular lipid content. Rituximab modifies the functional organization of lipid raft microdomains in B lymphoma cells, where it allows for proper localization and function of membrane proteins (34). Although we have demonstrated that rituximab does not affect the expression of vinculin, podocin, or nephrin, it would be interesting to determine whether rituximab and SMPDL-3b affect slit diaphragm protein membrane localization, which is considered essential for podocyte function. It is worth noting that rituximab-mediated preservation of the podocyte 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 (35).

We have demonstrated that rituximab treatment of high-risk FSGS patients is associated with lower incidence of posttransplant 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 (36), did not
affect 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

Patient population
This research protocol was approved by the institutional review board and ethics committee. From 2004 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 who did not receive rituximab (historical control group, 1999 to 2003). Progression to end-stage renal disease was comparable (Table 1). All patients received combined thymoglobulin (1 mg/kg, three to five doses) and daclizumab (1 mg/kg, two doses), as standard induction protocol for immunosuppression (37), and alentumab (0.4 mg/kg) induction for one patient in each group. Patients with delayed graft function received one to five additional doses of thymoglobulin (1 mg/kg). Tacrolimus (target trough level of 5 to 7 ng/ml), mycophenolate (500 mg, twice per day), and corticosteroids (methylprednisolone, 4 mg/day) were used (target trough level of 5 to 7 ng/ml), mycophenolate (500 mg, twice per day), and corticosteroids (methylprednisolone, 4 mg/day) were used for maintenance of immunosuppression. Biopsies obtained 1 to 2 hours after reperfusion, but before rituximab infusion, were available from 20 of 27 patients in the rituximab group. Additional biopsies were performed after transplantation when clinically indicated.

Immunofluorescence and immunoperoxidase
Frozen sections (10-µm thick) of normal human kidneys from deceased donors were used 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, Biodesign International) expression in podocytes. Immunoperoxidase was used to evaluate SMPDL-3b expression in fixed kidney sections from transplanted patients with FSGS that were sequentially incubated with the anti–SMPDL-3b antibody, a secondary biotinylated antibody (Zymed, HistoLine), and peroxidase–labeled streptavidin (Zymed). Peroxidase activity was detected with 3,5-diaminobenzidine (Sigma). Sections were dehydrated and mounted in Bio Mount (Bio Optica). The number of stained podocytes was then quantified. After manual selection of glomeruli as regions of interest (ROIs), 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 with a Zeiss Axioscope 40FL microscope equipped with an AxioCam MRC5 digital video camera, recorded with AxioVision software 4.3, and analyzed with AxioVision analysis module (Carl Zeiss SpA) (38).

To determine rituximab binding to normal human podocytes from kidney tissue sections, we fluorescently labeled rituximab, a non-specific IgG1, and a monoclonal antibody directed against CD20 that does not recognize the common epitope between CD20 and SMPDL-3b (IF5 antibody, gift of O. W. Press, University of North Carolina, Chapel Hill, NC) with fluorescein isothiocyanate (FITC) using the Thermo Scientific Labeling kit. Frozen normal human kidney sections were incubated with FITC-rituximab (10 µg/ml) or with the same concentration of FITC-IgG1 (Invitrogen) or FITC-IF5. Because 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.). Preincubation of FITC-rituximab with a 10 molar excess of SLWPKWLEAIQ to rituximab verified rituximab binding to SMPDL-3b. A scrambled peptide sequence was used as a control (WKLSPQILAW). 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 a horseradish peroxidase (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 simian virus 40 (SV40) construct that would allow for cell proliferation at 33°C and for cell differentiation after 14 days of thermoshock at 37.5°C. Stable podocyte cell lines expressing GFP-labeled SMPDL-3b, GFP alone, siSMPDL-3b built on pGFP-V-RS vector (Origene) (TTGTTGAAAGCTGACCATCATCAGA), or an NT siRNA (scrambled oligo) were developed by transfection with FuGENE reagents (Roche) or electroporation (MammoZapper cloning gun, Tritiche Research) followed by cell sorting. Clones were derived via limiting dilution, expanded, and selected for gene downregulation efficiency by RT-PCR and Western blot. Pretransplant 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.), cells were pretreated with rituximab (100 µg/ml) for 30 min before 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. Controls were treated with anti–IL-8 (100 µg/ml).

Polymerase chain reaction
Both standard and real-time quantitative PCR assays (Applied Biosystems) were performed for CD20, SMPDL-3b, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 18S ribosomal RNA, and actin. For standard PCR, the following primers were designed: CD20 transcript variant 1, 5'-GCAAGCAACGGAGAAAAACTC and 3'-TTCTGGGAAGAAGGCAAGAGAAGAG; CD20 transcript variant 3, 5'-GCTGCCATTTCTGGGATG and 3'-TTCTCTGAGAGAAGGAAGCAGG; SMPDL-3b, 5'-CTATAACGCAATGGCCCTGA and 3'-GAGAAGACGAAAAAMGCAAGGC; actin, 5'-TCACTGGATGACTCGGAGG and 3'-TCTAGGCAAAATGGTGTG; GAPDH, 5'-GTCAGTGGTGAGCCCTGACCT and 3'-GTCAACGGTGATCATGCGGGA; SMPDL-3b and 18S ribosomal RNA expression were evaluated with the 7500 Real-Time PCR System (Applied Biosystems). Relative quantification between different samples was determined as 2−ΔΔCt (∆ΔCt = ∆Ct affected sample − ∆Ct unaffected sample).

Western blot and immunoprecipitation
Cell lysates were collected in lysis buffer in the presence of protease and phosphatase inhibitors (Bio-Rad). After protein quantifica-
tion with a detergent compatible assay (DC protein assay, Bio-Rad), an equal amount of protein was loaded onto 4 to 20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Lanza) and transferred to nitrocellulose membranes (Bio-Rad). Western blotting for SMPDL-3b, ASMase, and actin was performed with the following primary antibodies: rabbit polyclonal anti–SMPDL-3b (Genway Biotech Inc., 1:1000), goat polyclonal anti-ASMase (Santa Cruz Biotechnology, 1:1000), mouse monoclonal anti-nephrin (gift of K. Trygvsason, Karolinska, Sweden, 1:2000), rabbit polyclonal anti-podocin (gift of P. Mundel, Massachusetts General Hospital, Boston, 1:500), mouse monoclonal anti-vinculin (Sigma, 1:3000), and mouse monoclonal anti-β-actin (Abcam Inc., 1:1000). For immunoprecipitation experiments, HEK293 cells were transfected with GFP–SMPDL-3b and collected in radioimmunoprecipitation assay buffer. After protein extraction (incubation on ice for 30 min followed by centrifugation at 14,000 rpm for 15 min at 4°C), 2 mg of protein lysate was incubated with 2 μg of precleaving beads (Santa Cruz Biotechnology) and then immunoprecipitated with 4 μg of rituximab or anti–IL-8 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 (EGFR-1; rabbit monoclonal anti-human, 1:1000, Cell Signaling) was used 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 an equal volume of 70% OptiPrep in basic buffer [20 mM tris-HCl (pH 7.8), 250 mM sucrose] and placed on the bottom of an 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 hours at 170,000 g (37,100 rpm). For animal experiments, HEK293 cells were transfected with GFP–SMPDL-3b and collected in radioimmunoprecipitation assay buffer. After protein extraction (incubation on ice for 30 min followed by centrifugation at 14,000 rpm for 15 min at 4°C), 2 mg of protein lysate was incubated with 2 μg of precleaving beads (Santa Cruz Biotechnology) and then immunoprecipitated with 4 μg of rituximab or anti–IL-8 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.

ASMase activity
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 (50 mM, 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 Sphinomyelinase kit (Molecular Probes/Invitrogen Corp.) and was normalized for protein content. Fluorescence was measured with a fluorescence microplate reader using excitation/emission wavelengths of 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 patient sera for 6, 12, or 24 hours, cells were fixed in 4% paraformaldehyde, incubated with rhodamine phalloidin (Invitrogen) in 0.01% Triton X-100 for 30 min at 37°C, and mounted for analysis. Twenty fields at ×40 magnification were quantitatively evaluated with 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 nonrecurrent (n = 10) or recurrent FSGS (n = 12). Cells were pretreated with rituximab or anti–IL-8 (100 μg/ml for 30 min) before exposure to serum. The percentage of cells positive for annexin V was determined at 6 and 24 hours by flow cytometry [fluorescence-activated cell sorting (FACS), Becton Dickinson] after cell staining with the Vybrant apoptosis assay kit (Invitrogen).

Statistical analysis
Categorical variables were compared by χ2 test and Fisher’s exact test, when appropriate. Continuous variables were compared with two-tailed Student’s t test or Wilcoxon rank-sum test. Survival analysis was calculated with Kaplan-Meier method and compared with the log-rank test. Comparison of the number of SMPDL-3b+ cells in kidney biopsies in recurrent and nonrecurrent FSGS patients was performed with unpaired Student’s t test. For experimental studies, the results represent the mean and SD of four to eight independent experiments and one-way analysis of variance (ANOVA) was used. When one-way ANOVA showed statistical significance, results were compared with t test after Tukey’s correction for multiple comparisons. Results were considered statistically significant at P < 0.05.

SUPPLEMENTARY MATERIAL
www.sciencetranslationalmedicine.org/cgi/content/full/3/85/85ra46/DC1
Fig. S1. CD20 is not expressed in glomeruli from patients with recurrent FSGS.
Fig. S2. Generation of SMPDL-3b knockdown 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 in podocytes.
Fig. S5. Generation of SMPDL-3b knockdown podocytes.
Reference


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