

T Lymphocyte Activation Markers as Predictors of Responsiveness to Rituximab among Patients with FSGS

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Abstract

Background and objectives Rituximab is used with variable success in difficult FSGS. Because B cell depletion significantly affects T cell function, we characterized T cell subsets in patients with FSGS to determine if an immunologic signature predictive of favorable response to rituximab could be identified.

Design, setting, participants, & measurements Twenty-two consecutive patients with FSGS (median age =14.4 years old; range =6.2–25.0 years old) and age of onset of nephrotic syndrome 1–18 years old receiving rituximab for clinical indications between October of 2009 and February of 2014 were studied. Indications for rituximab were lack of sustained remission despite calcineurin inhibitors (CNIs) and mycophenolate in steroid-resistant patients and lack of steroid-sparing effect with cyclophosphamide and CNI or CNI toxicity in steroid-dependent patients. Exclusion criteria were infantile onset, known genetic mutations, and secondary causes. Rituximab (375 mg/m²) was given fortnightly up to a maximum of four doses. Immunologic subset monitoring was performed at baseline and regular intervals until relapse. Median follow-up duration postrituximab was 26.7 months (range =6.5–66.5 months). Baseline immunologic subsets were examined for association with rituximab response defined as resolution of proteinuria with discontinuation of prednisolone and CNI 3 months postrituximab.

Results Twelve patients (54.5%) responded to rituximab. Mitogen-stimulated CD154⁺CD4⁺CD3⁺ subset before rituximab was significantly lower in FSGS responders compared with nonresponders (54.9%±28.1% versus 78.9%±16.4%; $P=0.03$). IFN- γ ⁺CD3⁺ and IL-2⁺CD3⁺ were similarly decreased in responders compared with nonresponders (0.6%±0.8% versus 7.5%±6.1%; $P=0.003$ and 0.2%±0.5% versus 4.0%±4.7%; $P<0.01$, respectively). Recovery of all three activation subsets occurred 6 months postrituximab treatment (CD154⁺CD4⁺CD3⁺, 74.8%±17.2%; IFN- γ ⁺CD3⁺, 7.1%±7.7%; and IL-2⁺CD3⁺, 7.9%±10.9%; $P<0.01$). Receiver-operating characteristic analysis using optimal cutoff values showed that activated CD154⁺CD4⁺CD3⁺ <83.3% (area under the curve [AUC], 0.81; 95% confidence interval [95% CI], 0.61 to 1.00), IFN- γ ⁺CD3⁺ <2.5% (AUC, 0.90; 95% CI, 0.75 to 1.00), and IL-2⁺CD3⁺ <0.3% (AUC, 0.78; 95% CI, 0.57 to 0.98) were good predictors of rituximab response.

Conclusions We have identified prognostic markers that define a subset of patients with FSGS bearing an immunologic signature representing hyporesponsiveness to T cell stimulation and therefore, who respond better to rituximab.

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Introduction

Primary FSGS is one of the important histologic classes of glomerular disease presenting with nephrotic proteinuria, because it is often steroid resistant and associated with rapid progression to end stage renal failure in 50% over a period of 3–8 years (1). Patients who responded to second-line treatment with calcineurin inhibitors (CNIs) generally fared better, with 70% renal survival over a 5-year period compared with 20% in historical controls who were resistant to both steroids and cyclophosphamide (2).

The use of rituximab in refractory idiopathic nephrotic syndrome gained increasing popularity after the initial report by Benz *et al.* (3), who used this drug successfully in a patient with steroid-dependent

nephrotic syndrome (SDNS) and idiopathic thrombocytopenia purpura. Subsequently, several other case series showed beneficial effect in patients with SDNS in terms of achieving complete remission or the ability to decrease immunosuppression (4–12).

The efficacy of rituximab in patients with steroid-resistant nephrotic syndrome (SRNS), however, seemed to be more dismal (4,13–15). In an open label, randomized trial comparing rituximab therapy with steroids and CNI in 31 patients with SRNS, including 19 with FSGS, no demonstrable benefit was seen in patients treated with rituximab (15). Similarly, a study by Fernandez-Fresnedo *et al.* (16) in adult patients with steroid-resistant FSGS showed partial response to rituximab in three of eight patients, one of which was transient.

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The underlying reason behind this apparent varied response to rituximab may be the heterogeneous basis of this disease in the studied patient populations. Induction of remission in patients with FSGS treated with rituximab suggests a possible role of B cells either directly or indirectly through B cell-T cell interactions in modulating an immune cascade, resulting in podocyte injury and proteinuria. We, therefore, hypothesized that there exists a specific subset of patients with FSGS identified by an immunologic signature who would respond favorably to rituximab.

The efficacy of rituximab in T cell-mediated diseases suggests that rituximab can alter the T cell subpopulations, activation, or function (17). The effects of rituximab extend beyond B cell depletion and may involve altering the phenotype and function of the remaining B cell subsets (18), normalization of T cell abnormalities (19), and promoting expansion of the regulatory T cell population (20,21). The possibility of T cell involvement in FSGS was suggested by an earlier study on renal biopsy tissue, where IL-2 receptor- α mRNA transcripts were detected in 14 of 20 patients with FSGS (22). However, a T helper 2-predominant T cell infiltrate accompanied by elaboration of macrophage-associated cytokines has been described in a Buffalo/Mna rat model of proteinuria with corresponding histologic FSGS lesions (23). The same group further showed amelioration of proteinuria in Buffalo/Mna rats with regression of FSGS lesions by CD4⁺CD25⁺ T lymphocyte transfer (24), highlighting the potential therapeutic role of T regulatory cells in FSGS.

Hence, the objective of this study was to characterize a distinct T cell immunologic profile in pediatric patients with FSGS that could predict response to rituximab. This was achieved by examining the total lymphocyte population, T cell activation and regulatory markers after B cell interaction as well as activated T cell cytokine production. We subsequently compared the potential predictive marker(s) in FSGS rituximab responders with those in patients with minimal change nephrotic syndrome (MCNS) in relapse and in healthy controls so as to confirm the uniqueness of these marker(s) in patients with FSGS responding to rituximab.

Materials and Methods

Study Design

Ethics approval was obtained from the National Healthcare Group institutional review board. Informed consent/assent was obtained from all subjects and/or their parents. Twenty-two consecutive patients with biopsy-proven FSGS and clinical indications for rituximab therapy seen at the Shaw-National Kidney Foundation-National University Hospital Children's Kidney Centre, National University Hospital, Singapore, between October of 2009 and February of 2014 were included in this study. Patients presented between the ages of 1 and 18 years old with either SDNS or SRNS. Definitions of steroid responsiveness are listed in Supplemental Material. Clinical indications for rituximab therapy in patients with SRNS were lack of remission despite achieving high therapeutic trough levels of CNI (either cyclosporin at 200–250 μ g/L or tacrolimus at 10–12 μ g/L) and addition of mycophenolate, disease relapse after prolonged (>2 years) CNI therapy, or biopsy evidence of nephrotoxicity secondary to CNI. Clinical indications

for rituximab therapy in patients with SDNS were lack of steroid-sparing effect (inability to sustain remission at a prednisolone dose of ≤ 0.5 mg/kg on alternate days) despite treatment with oral cyclophosphamide (2 mg/kg per day for 12 weeks) and CNI therapy or prolonged CNI therapy with evidence of nephrotoxicity.

Exclusion criteria included patients with the following: eGFR <60 ml/min per 1.73 m²; infantile onset of nephrotic syndrome; nephrotic syndrome secondary to chronic infections, such as hepatitis B, hepatitis C, HIV, SLE, Henoch-Schönlein purpura, IgA nephropathy, membranoproliferative GN, or membranous nephropathy; current or previous therapy for tuberculosis; and presence of mutations in *WT1*, *NPHS1*, *NPHS2*, and *TRPC6*.

Rituximab was administered at a dose of 375 mg/m² fortnightly to a maximum of four doses according to our center's clinical protocol (Supplemental Material). Daily testing of urine for protein using Albustix (Bayer, Leverkusen, Germany) was performed at baseline and after rituximab therapy. Clinical investigations and immunologic subset monitoring were performed at baseline, 14 days, and 1, 3, and 6 months postrituximab administration and if the patient relapsed. CD19 subsets were performed monthly until recovery of B cells (CD19 peripheral blood count $\geq 10/\mu$ l). Response to rituximab was defined as resolution of proteinuria (urine protein-to-creatinine ratio <0.02 g/mmol or urine protein excretion <0.3 g/1.73 m² per day) and ability to wean off prednisolone and CNI at 3 months after the last dose of rituximab. The immunosuppressive tapering protocol, which was part of routine clinical care, is detailed in Supplemental Material. All patients were continued on mycophenolate mofetil at 600 mg/m² per dose twice daily.

As a baseline comparison for lymphocyte subset analysis, 22 patients with biopsy-proven MCNS in relapse were also recruited as patient controls. Thirty age- and sex-matched healthy controls were recruited from the General Nephrology Clinic and were patients who had nonglomerular disorders, such as mild vesicoureteric reflux and duplex kidneys, with normal renal function and no evidence of albuminuria or urinary tract infection.

Immunologic Subset and T Cell Activation Monitoring

Lymphocyte subset staining was performed using the lysed whole-blood method. For T cell activation, blood samples were diluted in an equal volume of RPMI 1640 Medium (Gibco, Carlsbad, CA) and stimulated with ionomycin (1 μ g/ml), PMA (20 ng/ml), and monensin sodium (6 μ M) for 4 hours. Detailed descriptions of the protocol are in Supplemental Material.

Foxp3 Analyses

PBMCs were isolated using lymphocyte separation medium. The Foxp3 Antibody Reagent Kit (eBioscience, San Diego, CA) was used for Foxp3 staining according to the manufacturer's instructions.

Statistical Analyses

Results were analyzed using the Statistical Package for Social Sciences, version 22.0 (SPSS Inc., Chicago, IL). Comparison of the immunologic subsets before

rituximab therapy between FSGS rituximab responders, FSGS rituximab nonresponders, patients with MCNS in relapse, and controls was done using the Mann–Whitney *U* test. A *P* value <0.05 was considered statistically significant, and Bonferroni correction was carried out for multiple subset comparisons. The Fisher exact test was used to compare categorical variables between groups. Multivariate analysis was performed using binary logistic regression to examine the association between response to rituximab and clinical indicators, including age at diagnosis, sex, histologic subtype, steroid resistance, prior noncorticosteroid immunosuppression and duration, remission status at the time of rituximab administration, eGFR, and number of rituximab doses.

Paired analysis of immunologic subsets before and 6 months after rituximab therapy was done using the Wilcoxon signed rank test. Receiver–operating characteristic curve analysis was used to determine utility of the individual subsets for prognostication of response to rituximab therapy. Binary logistic regression was performed to evaluate the combined utility of the significant subsets to predict response to rituximab therapy, deriving a predictor score (range =0–1).

Results

Baseline Characteristics of Patients with FSGS Receiving Rituximab

Twenty-two patients with biopsy-proven FSGS (median age =14.4 years old; range =6.2–25.0 years old) were given rituximab therapy and followed up for a median duration of 26.7 months (range =6.5–66.6 months). Of these, 12 patients (54.5%) responded to treatment, defined as resolution of proteinuria and ability to wean off prednisolone and CNI at 3 months after the last dose of rituximab. There were no significant differences in terms of age at diagnosis, sex, histologic subtype, steroid resistance, prior noncorticosteroid immunosuppression and duration, remission status at time of rituximab administration, eGFR, or number of rituximab doses given between responders and nonresponders using both univariate and multivariate analyses (Table 1).

Responders and nonresponders showed similar median B cell recovery times of 7.2 months (range =4.4–10.0 months) and 7.0 months (range =5.5–32.7 months), respectively (*P*=0.77). Of the 12 complete responders, ten had completed ≥12 months of follow-up; three of the ten complete responders (30.0%) relapsed within 11 months of

Table 1. Baseline clinical characteristics of responders and nonresponders to rituximab therapy in patients with FSGS

Variables	FSGS Rituximab		Univariate <i>P</i> Value	Multivariate <i>P</i> Value
	Responders, <i>n</i> =12	Nonresponders, <i>n</i> =10		
Age at diagnosis, yr	2.6 (1.6, 3.7)	3.9 (2.1, 7.5)	0.07	0.26
Age at time of rituximab therapy, yr	13.8 (9.8, 16.3)	15.4 (10.3, 21.9)	0.32	NA
Duration of prior immunosuppression, yr	11.7 (6.6, 14.5)	10.7 (6.9, 14.6)	0.90	0.20
Men	11 (91.7%)	5 (50.0%)	0.06	0.33
Duration of follow-up, mo	20.9 (11.1, 47.6)	46.2 (6.8, 66.4)	0.29	NA
Histologic subtype				
Perihilar	2 (16.7%)	2 (20.0%)	0.57	0.38
Cellular	0 (0.0%)	1 (10.0%)		
Tip variant	1 (8.3%)	0 (0.0%)		
Collapsing	1 (8.3%)	0 (0.0%)		
Not otherwise specified	8 (66.7%)	7 (70.0%)		
Nephrotic status at time of initiation of rituximab			0.23	0.98
Remission	7 (58.3%)	3 (30.0%)		
Nonremission	5 (41.7%)	7 (70.0%)		
Prior steroid response			0.59	0.73
Steroid resistance	9 (75.0%)	9 (90.0%)		
<i>Early</i>	8 (88.9%)	3 (33.3%)		
<i>Late</i>	1 (11.1%)	6 (66.7%)		
Steroid dependence	3 (25.0%)	1 (10.0%)		
Prior noncorticosteroid immunosuppressants				
Cyclophosphamide	8 (66.6%)	6 (60.0%)	>0.99	0.35
Calcineurin inhibitors	12 (100.0%)	10 (100.0%)	NA	NA
Mycophenolate mofetil	10 (83.3%)	7 (70.0%)	0.63	0.41
eGFR, ml/min per 1.73 m ²	76.7 (65.0, 111.2)	75.4 (57.0, 92.2)	0.55	0.53
No. of rituximab doses given	2.0 (2.0, 3.5)	3.0 (2.0, 4.0)	0.42	0.79

Data are presented as *n* (%) unless otherwise indicated. Nonparametric data are presented as median (25th percentile, 75th percentile). NA, not applicable.

rituximab therapy (median =281 days; range =262–317 days), all of whom showed B cell recovery.

Rituximab was generally well tolerated without serious adverse effects. Five patients experienced chest tightness and pruritus when the infusion rate was increased to >50 mg/h but were subsequently able to complete the infusion with slower rates.

Immunologic Characteristics of Patients with FSGS Receiving Rituximab

The baseline immunologic subsets of prerituximab therapy between FSGS rituximab responders and nonresponders are outlined in Table 2. There was no difference in the baseline B cells, T cells, and natural killer cells as well as unstimulated helper T cell (CD4⁺CD3⁺), cytotoxic T cell (CD8⁺CD3⁺), and regulatory T cell (Foxp3⁺CD25⁺CD4⁺CD3⁺) subsets in FSGS rituximab responders and nonresponders in terms of both percentage and absolute cell counts (Table 2). Although the percentage of HLA-DR⁺CD4⁺CD3⁺ was lower in responders (4.2%±1.9%) compared with nonresponders (8.5%±5.8%) (Table 2), this was not statistically significant after applying Bonferroni correction ($P=0.62$). At 6 months postrituximab therapy, there was a significant decrease in percentage and absolute cell counts of CD19 along with an increase in spontaneous activation markers ICOS⁺CD8⁺CD3⁺ and FAS⁺ICOS⁺CD8⁺CD3⁺ in both FSGS rituximab responders and nonresponders compared with baseline.

Hyporesponsive T Cell Phenotype in FSGS Responders to Rituximab

The *in vitro* response of T cells to activation was studied after PMA and ionomycin stimulation, and all samples expressed high levels of CD69⁺CD3⁺ (>72.8%) after stimulation, indicating that T cells were sufficiently activated. As shown in Table 3, the stimulated CD154⁺CD4⁺CD3⁺ subset was significantly lower in responders (54.9%±28.1%) than nonresponders (78.9%±16.4%; $P=0.03$). Concomitant with this muted activation response, IFN- γ ⁺CD3⁺ and IL-2⁺CD3⁺ subsets were also significantly lower in the responders than nonresponders (0.6%±0.8% versus 7.5%±6.1%; $P=0.003$ and 0.2%±0.5% versus 4.0%±4.7%; $P<0.01$, respectively). After applying Bonferroni correction for the number of activated subsets being analyzed, the differences between responders and nonresponders remained significant only for IFN- γ ⁺CD3⁺ and IL-2⁺CD3⁺ ($P=0.01$ and $P=0.03$, respectively).

We subsequently compared the expression of these three subsets between FSGS rituximab responders and patients with MCNS in relapse as well as healthy controls (Table 3). FSGS rituximab responders had no difference in the expression of stimulated CD154⁺CD4⁺CD3⁺ compared with patients with MCNS in relapse (64.2%±23.5%); however, this expression was significantly lower compared with those in controls (75.7%±13.2%; $P<0.01$). However, the expressions of IFN- γ ⁺CD3⁺ and IL-2⁺CD3⁺ subsets in FSGS rituximab responders were significantly lower compared with those in patients with MCNS in relapse (7.8%±11.2%; $P<0.01$ and 4.3%±7.4%; $P=0.04$, respectively) as well as controls (12.8%±11.2%; $P<0.001$ and 6.9%±8.4%; $P=0.002$, respectively).

We followed the temporal trends of these three activation markers in FSGS rituximab responders. Compared with

pretherapy levels in FSGS rituximab responders, pairwise analysis showed that, 6 months after rituximab therapy, stimulated CD154⁺CD4⁺CD3⁺, IFN- γ ⁺CD3⁺, and IL-2⁺CD3⁺ subsets were significantly higher (74.8%±17.2%; $P<0.01$; 7.1%±7.7%; $P<0.01$; and 7.9%±10.9%; $P=0.01$, respectively) (Figure 1), approaching the levels in controls. All 12 FSGS rituximab responders were still in remission 6 months postrituximab therapy, although in three of them (25.0%), B cells had recovered. Stimulated CD154⁺CD4⁺CD3⁺, IFN- γ ⁺CD3⁺, and IL-2⁺CD3⁺ in FSGS rituximab nonresponders remained unchanged 6 months post-therapy compared with pretherapy (78.4%±13.3%; $P=0.88$; 7.2%±6.7%; $P=0.80$; and 3.4%±4.8%; $P=0.77$, respectively) (Table 3). B cell recovery was seen in three of the ten FSGS rituximab nonresponders (30.0%) at 6 months postrituximab therapy. There was no correlation between B cell recovery and improvement in these three markers at 6 months postrituximab therapy.

Immunologic Subsets as Predictors of Rituximab Response in Patients with FSGS

To identify the markers that might provide prognostic value in predicting response to rituximab in patients with FSGS, we performed receiver–operating characteristic analysis (Table 4). At baseline, these three activation markers, CD154⁺CD4⁺CD3⁺ (area under the curve [AUC], 0.81; 95% confidence interval [95% CI], 0.61 to 1.00), IFN- γ ⁺CD3⁺ (AUC, 0.90; 95% CI, 0.75 to 1.00), and IL-2⁺CD3⁺ (AUC, 0.78; 95% CI, 0.57 to 0.98), when optimal cutoff values were selected, were good predictors of response to rituximab. Of the three subsets, IFN- γ ⁺CD3⁺ provided the most superior discriminatory performance characteristics: when the threshold was determined at <2.5%, IFN- γ ⁺CD3⁺ predicted response to rituximab with a sensitivity of 100.0% and a specificity of 80.0% (positive predictive value =85.7% and negative predictive value [NPV] =100.0%). The other two subsets fared well, with excellent sensitivity and specificity for predicting response to rituximab with the discriminatory thresholds for CD154⁺CD4⁺CD3⁺ of <83.3% (91.7% and 70.0%, respectively) and IL-2⁺CD3⁺ of <0.3% (75.0% and 80.0%, respectively).

We then derived a score to determine the combined utility of these three significant subsets (Table 4). The combined score maintained the optimal AUC of 0.90. Using a threshold score of 0.5, the sensitivity, specificity, positive predictive value, and NPV were 100.0%, 80.0%, 85.7%, and 100.0% respectively. Hence, evaluation of the combined score yielded no incremental value in predictor performance characteristics compared with the best individual biomarker (IFN- γ ⁺CD3⁺) alone.

Discussion

This study represents, to our knowledge, the first attempt to provide a basic description of the immunologic profile before rituximab therapy in a well characterized group of patients with FSGS to delineate an immunologic signature that may predict response to rituximab therapy. A recent study of 28 pediatric patients with frequently relapsing steroid-sensitive nephrotic syndrome showed that delayed reconstitution of switched memory B cells

Table 2. Immunologic subsets prirituximab and 6 months postrituximab therapy in FSGS rituximab responders and nonresponders

Immunologic Subsets	Subset, % (Absolute Count; Cells Per μ l)			
	FSGS Rituximab Responders, n=12		FSGS Rituximab Nonresponders, n=10	
	Prirituximab	6 mo Postrituximab	Prirituximab	6 mo Postrituximab
Lymphocytes				
CD19	12.1 $\pm$ 4.7 (432.6 $\pm$ 390.1)	1.7 $\pm$ 2.6 (34.7 $\pm$ 60.3) ^a	12.5 $\pm$ 4.4 (468.3 $\pm$ 260.3)	1.5 $\pm$ 1.6 (36.7 $\pm$ 39.0) ^a
CD3	69.9 $\pm$ 12.7 (2313.1 $\pm$ 1076.1)	78.9 $\pm$ 9.0 (1924.5 $\pm$ 773.9)	74.7 $\pm$ 6.0 (2686.7 $\pm$ 962.1)	81.0 $\pm$ 5.1 (2203.0 $\pm$ 461.3)
CD4 ⁺ CD3 ⁺	38.1 $\pm$ 9.4 (868.5 $\pm$ 387.5)	43.1 $\pm$ 7.3 (852.8 $\pm$ 340.3)	38.4 $\pm$ 9.1 (1062.2 $\pm$ 464.7)	41.2 $\pm$ 8.8 (908.7 $\pm$ 302.0)
CD8 ⁺ CD3 ⁺	24.6 $\pm$ 6.0 (599.9 $\pm$ 372.3)	28.0 $\pm$ 7.8 (614.1 $\pm$ 342.7)	29.4 $\pm$ 4.5 (795.6 $\pm$ 349.9)	31.2 $\pm$ 6.5 (704.2 $\pm$ 262.5)
CD16 ⁺ CD3 ⁺	11.4 $\pm$ 8.6 (373.6 $\pm$ 276.1)	11.1 $\pm$ 7.2 (246.0 $\pm$ 148.2)	8.0 $\pm$ 7.1 (256.4 $\pm$ 159.0)	11.7 $\pm$ 3.4 (323.1 $\pm$ 135.6)
CD56 ⁺ CD3 ⁺	12.3 $\pm$ 8.4 (390.7 $\pm$ 249.2)	13.0 $\pm$ 8.5 (290.3 $\pm$ 189.7)	8.4 $\pm$ 7.4 (266.8 $\pm$ 163.8)	12.8 $\pm$ 4.2 (352.0 $\pm$ 158.5)
Helper T cell (CD4⁺CD3⁺)				
CD45RO	55.3 $\pm$ 19.1 (508.9 $\pm$ 301.6)	55.3 $\pm$ 21.1 (453.9 $\pm$ 219.0)	59.7 $\pm$ 18.0 (542.4 $\pm$ 204.9)	51.0 $\pm$ 20.0 (446.8 $\pm$ 184.6)
CD45RA	38.3 $\pm$ 21.5 (475.4 $\pm$ 338.1)	39.5 $\pm$ 19.7 (353.5 $\pm$ 303.3)	38.6 $\pm$ 19.6 (294.7 $\pm$ 280.2)	40.8 $\pm$ 18.0 (398.6 $\pm$ 278.5)
CD25	35.0 $\pm$ 19.4 (304.3 $\pm$ 202.2)	28.7 $\pm$ 15.0 (245.2 $\pm$ 133.9)	34.4 $\pm$ 9.6 (352.2 $\pm$ 171.8)	31.1 $\pm$ 9.2 (264.5 $\pm$ 64.7)
CD45RO ⁺ CD25 ⁺	23.1 $\pm$ 13.8 (205.9 $\pm$ 154.1)	22.2 $\pm$ 13.1 (188.1 $\pm$ 117.2)	23.2 $\pm$ 7.2 (221.6 $\pm$ 84.9)	19.1 $\pm$ 10.0 (165.5 $\pm$ 83.9)
CD45RA ⁺ CD25 ⁺	2.7 $\pm$ 1.6 (31.6 $\pm$ 21.8)	3.4 $\pm$ 1.3 (29.8 $\pm$ 18.2)	5.3 $\pm$ 4.9 (50.0 $\pm$ 72.7)	4.5 $\pm$ 3.0 (43.3 $\pm$ 40.7)
FAS	53.8 $\pm$ 16.1 (491.2 $\pm$ 287.4)	54.4 $\pm$ 17.9 (449.1 $\pm$ 197.9)	53.5 $\pm$ 16.1 (512.8 $\pm$ 230.9)	46.9 $\pm$ 21.9 (415.4 $\pm$ 228.0)
ICOS	2.8 $\pm$ 2.3 (31.0 $\pm$ 30.7)	7.2 $\pm$ 5.5 (63.3 $\pm$ 49.7)	3.2 $\pm$ 2.7 (29.0 $\pm$ 32.7)	6.1 $\pm$ 3.7 (57.3 $\pm$ 38.9)
FAS ⁺ ICOS ⁺	2.5 $\pm$ 2.0 (27.7 $\pm$ 26.7)	6.7 $\pm$ 5.1 (58.6 $\pm$ 46.2)	3.0 $\pm$ 2.7 (27.1 $\pm$ 31.9)	4.2 $\pm$ 2.6 (41.6 $\pm$ 33.8)
OX40	7.6 $\pm$ 4.5 (70.3 $\pm$ 50.5)	8.5 $\pm$ 6.4 (75.7 $\pm$ 49.6)	6.5 $\pm$ 3.2 (72.5 $\pm$ 50.3)	6.5 $\pm$ 3.3 (54.3 $\pm$ 22.3)
CTLA4	0.2 $\pm$ 0.2 (7.4 $\pm$ 14.5)	0.5 $\pm$ 0.6 (4.1 $\pm$ 4.6)	1.5 $\pm$ 1.6 (7.9 $\pm$ 14.5)	1.1 $\pm$ 1.1 (12.1 $\pm$ 13.0)
OX40 ⁺ CTLA4 ⁺	0.1 $\pm$ 0.1 (1.1 $\pm$ 1.4)	0.1 $\pm$ 0.2 (1.2 $\pm$ 1.4)	0.1 $\pm$ 0.1 (0.6 $\pm$ 0.6)	0.1 $\pm$ 0.1 (1.3 $\pm$ 1.4)
HLA-DR	4.2 $\pm$ 1.9 (51.6 $\pm$ 52.8)	8.1 $\pm$ 4.4 (68.8 $\pm$ 34.2)	8.5 $\pm$ 5.8 (59.7 $\pm$ 39.6)	11.2 $\pm$ 5.2 (95.4 $\pm$ 45.1)
Cytotoxic T cell (CD8⁺CD3⁺)				
CD45RO	18.4 $\pm$ 12.9 (128.3 $\pm$ 113.2)	19.4 $\pm$ 11.9 (111.5 $\pm$ 80.8)	26.5 $\pm$ 12.0 (157.0 $\pm$ 104.2)	22.9 $\pm$ 15.5 (181.4 $\pm$ 161.1)
CD45RA	78.3 $\pm$ 11.5 (523.9 $\pm$ 292.0)	75.9 $\pm$ 9.6 (473.1 $\pm$ 293.8)	71.2 $\pm$ 11.4 (525.6 $\pm$ 324.2)	71.5 $\pm$ 12.5 (487.6 $\pm$ 157.4)
CD25	7.0 $\pm$ 7.1 (37.9 $\pm$ 35.6)	3.5 $\pm$ 2.5 (18.4 $\pm$ 15.3)	4.5 $\pm$ 2.7 (31.0 $\pm$ 15.7)	7.1 $\pm$ 13.1 (32.7 $\pm$ 36.0)
CD45RO ⁺ CD25 ⁺	3.4 $\pm$ 6.0 (18.9 $\pm$ 25.0)	1.9 $\pm$ 2.1 (8.3 $\pm$ 7.8)	2.4 $\pm$ 2.4 (12.2 $\pm$ 11.0)	1.3 $\pm$ 1.0 (10.2 $\pm$ 10.3)
CD45RA ⁺ CD25 ⁺	1.6 $\pm$ 1.4 (7.9 $\pm$ 6.2)	1.0 $\pm$ 0.5 (5.8 $\pm$ 4.4)	1.0 $\pm$ 0.7 (9.2 $\pm$ 7.8)	1.0 $\pm$ 0.9 (7.3 $\pm$ 8.4)
FAS	23.9 $\pm$ 13.4 (165.0 $\pm$ 121.4)	33.2 $\pm$ 12.1 (209.8 $\pm$ 115.7)	28.0 $\pm$ 11.3 (204.8 $\pm$ 145.6)	28.5 $\pm$ 15.9 (192.9 $\pm$ 136.5)
ICOS	0.4 $\pm$ 0.5 (2.9 $\pm$ 4.4)	2.2 $\pm$ 3.0 (12.4 $\pm$ 15.7) ^a	0.3 $\pm$ 0.3 (1.7 $\pm$ 2.4)	2.8 $\pm$ 4.6 (28.4 $\pm$ 56.2) ^a
FAS ⁺ ICOS ⁺	0.3 $\pm$ 0.3 (1.8 $\pm$ 2.0)	1.9 $\pm$ 2.6 (11.2 $\pm$ 14.9) ^a	0.2 $\pm$ 0.2 (1.1 $\pm$ 1.7)	1.1 $\pm$ 1.5 (8.8 $\pm$ 13.8) ^a
OX40	0.2 $\pm$ 0.2 (1.1 $\pm$ 1.2)	0.3 $\pm$ 0.3 (1.2 $\pm$ 1.0)	0.2 $\pm$ 0.3 (1.7 $\pm$ 2.6)	0.2 $\pm$ 0.1 (1.1 $\pm$ 1.0)
CTLA4	0.8 $\pm$ 1.5 (6.6 $\pm$ 12.3)	1.7 $\pm$ 3.1 (6.5 $\pm$ 8.4)	1.9 $\pm$ 2.0 (9.1 $\pm$ 8.7)	2.2 $\pm$ 2.9 (15.0 $\pm$ 18.1)
OX40 ⁺ CTLA4 ⁺	0.0 $\pm$ 0.1 (0.2 $\pm$ 0.3)	0.1 $\pm$ 0.1 (0.4 $\pm$ 0.4)	0.0 $\pm$ 0.0 (0.1 $\pm$ 0.2)	0.0 $\pm$ 0.0 (0.2 $\pm$ 0.3)
HLA-DR	10.0 $\pm$ 6.3 (69.1 $\pm$ 62.9)	16.4 $\pm$ 9.1 (114.7 $\pm$ 79.4)	15.5 $\pm$ 6.7 (119.7 $\pm$ 95.8)	24.1 $\pm$ 14.0 (187.9 $\pm$ 171.0)
Regulatory T cell (CD25⁺CD4⁺CD3⁺)				
Foxp3	4.1 $\pm$ 1.4 (12.4 $\pm$ 8.5)	6.0 $\pm$ 2.1 (15.5 $\pm$ 10.9)	4.1 $\pm$ 1.4 (12.9 $\pm$ 6.7)	5.1 $\pm$ 1.8 (13.1 $\pm$ 4.9)

Results are expressed as mean $\pm$ SD.^aP<0.05; paired analysis comparing prirituximab and 6 months postrituximab therapy for both percentage and cell counts (Wilcoxon signed rank test).

Table 3. Stimulated T cell activation subsets prerituximab and 6 months postrituximab in FSGS rituximab responders and nonresponders compared with patients with minimal change nephrotic syndrome in relapse and healthy controls

Immunologic Subsets	Subset, %					
	FSGS Rituximab				MCNS in Relapse, <i>n</i> =22	Control, <i>n</i> =30
	FSGS Rituximab Responders, <i>n</i> =12		FSGS Rituximab Nonresponders, <i>n</i> =10			
	Prerituximab	6 mo Postrituximab	Prerituximab	6 mo Postrituximab		
T cell activation						
CD69 ⁺ CD3 ⁺	89.1±8.6	92.2±7.0	93.3±3.3	90.0±10.7	89.1±7.6	91.4±9.4
CD154 ⁺ CD4 ⁺ CD3 ⁺	54.9±28.1 ^a	74.8±17.2 ^b	78.9±16.4	78.4±13.3	64.2±23.5	75.7±13.2
IFN-γ ⁺ CD3 ⁺	0.6±0.8 ^{a,c,d}	7.1±7.7 ^b	7.5±6.1	7.2±6.7	7.8±11.2	12.8±11.2
IL-2 ⁺ CD3 ⁺	0.2±0.5 ^{a,c}	7.9±10.9 ^b	4.0±4.7	3.4±4.8	4.3±7.4	6.9±8.4
Results are expressed as mean±SD. MCNS, minimal change nephrotic syndrome. Comparison between groups was performed using the Mann–Whitney <i>U</i> Test with Bonferroni correction for multiple subset comparison.						
^a <i>P</i> <0.05: comparing FSGS rituximab responders and healthy controls.						
^b <i>P</i> <0.05: paired analysis comparing prerituximab and 6 months postrituximab therapy (Wilcoxon signed rank test).						
^c <i>P</i> <0.05: comparing FSGS rituximab responders and FSGS rituximab nonresponders.						
^d <i>P</i> <0.05: comparing FSGS rituximab responders and MCNS in relapse.						

after rituximab treatment was protective against relapse (25). In contrast, the 22 patients in our study represented a difficult spectrum of nephrotic syndrome, where rituximab was indicated because of treatment refractoriness or CNI toxicity with evidence of increasing glomerulosclerosis on repeat renal biopsy.

The response rate to rituximab has been reported to range between 45% for steroid-resistant patients to 85%

for steroid-responsive patients (26). The response rate of 54.5% in this study compares favorably with the existing literature, particularly because 81.8% of our patients had steroid resistance during the course of the disease. The traditional clinical parameters did not provide useful prognostic information in terms of response to rituximab (Table 1). This was in contrast to the published study by Gulati *et al.* (4), in which patients with late steroid

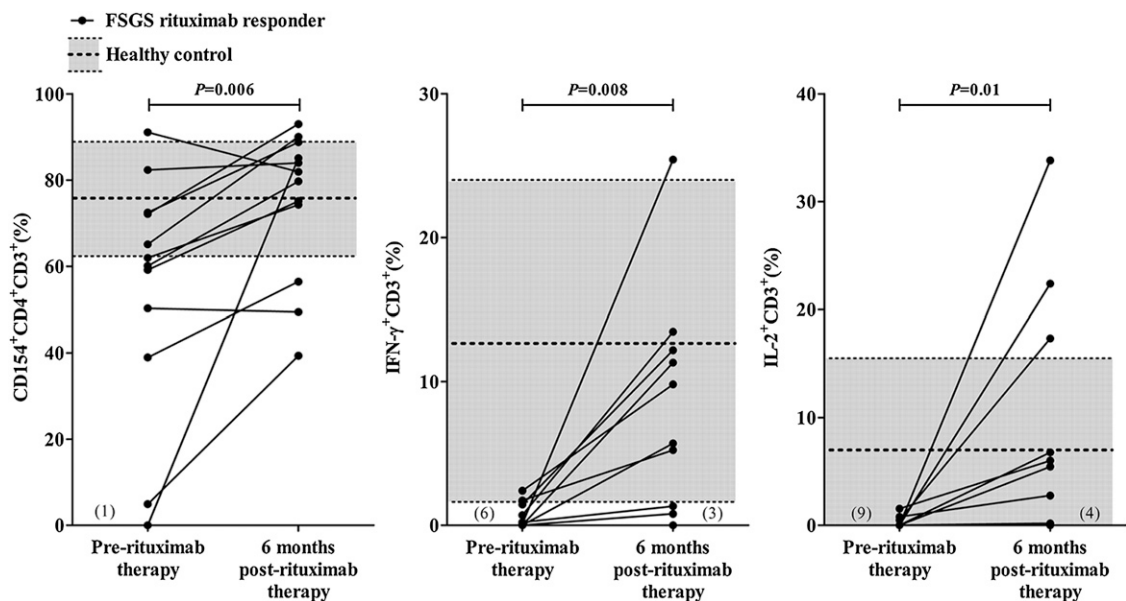


Figure 1. | Recovery of T cell function in FSGS rituximab responders after rituximab therapy. In all experiments, CD69⁺CD3⁺ expression was >72.8%, indicating that T cells were sufficiently activated. Stimulated CD154⁺CD4⁺CD3⁺, IFN-γ⁺CD3⁺, and IL-2⁺CD3⁺ subsets in FSGS rituximab responders were significantly higher 6 months postrituximab therapy compared with prerituximab therapy. Numbers in parentheses represent the numbers of patients with 0% of CD154, IFN-γ, or IL-2. The gray shaded region represents the mean±SD of healthy controls.

Table 4. Performance of significant stimulated subsets CD154⁺CD4⁺CD3⁺, IFN- γ ⁺CD3⁺, and IL-2⁺CD3⁺ as predictors of response to rituximab in patients with FSGS

Immunologic Subsets	AUC (95% CI)	Optimal Cutoff, % ^a	Sensitivity, %	Specificity, %	PPV, %	NPV, %	100% PPV Cutoff, %	100% NPV Cutoff, %
CD154 ⁺ CD4 ⁺ CD3 ⁺	0.81 (0.61 to 1.00)	<83.3	91.7	70.0	78.6	87.5	≤50.0	NA
IFN- γ ⁺ CD3 ⁺	0.90 (0.75 to 1.00)	<2.5	100.0	80.0	85.7	100.0	NA	≥2.5
IL-2 ⁺ CD3 ⁺	0.78 (0.57 to 0.98)	<0.3	75.0	80.0	72.7	81.8	NA	≥3.0
Combined score	0.90 (0.75 to 1.00)	≥0.5	100.0	80.0	85.7	100.0	NA	<0.5

AUC, area under the curve; 95% CI, 95% confidence interval; PPV, positive predictive value; NPV, negative predictive value; NA, not applicable.

^aOptimal cutoff refers to the best balance between sensitivity and specificity.

resistance seemed to respond better than those with early steroid resistance.

The baseline immunologic profile in our patients with FSGS did not show any difference in the helper T cell, cytotoxic T cell, and regulatory T cell subsets between the responders and nonresponders. However, FSGS rituximab responders had lower baseline levels of T cell activation shown by reduced expression of HLA-DR⁺CD4⁺CD3⁺, although this did not reach statistical significance. This was consistent with the significantly lower expression of *in vitro* mitogen-stimulated CD154⁺CD4⁺ expression in FSGS rituximab responders compared with those in both nonresponders and controls, and it was associated with significantly reduced mitogen-stimulated CD3 production of IFN- γ and IL-2. The baseline immunologic signature in our FSGS rituximab responders was also significantly different from that in patients with MCNS in relapse as shown in Table 3, indicating that the immunologic basis underlying the nephrotic syndrome is likely to be distinct in this group of FSGS rituximab responders compared with those in patients with MCNS, in whom increased mitogen-stimulated lymphocyte production of IL-2 and IFN- γ has been shown (27–31).

The hyporesponsiveness to T cell stimulation in our FSGS rituximab responders gives further credence to the hypothesis that persistent B cell interactions with T cells through either cytokine production or antigen-presenting cell activation render T cells incapable of full responses to *in vitro* stimulation. This could represent inhibition of T cell function or even a state of T cell exhaustion (32,33) that is corrected by B cell depletion. In fact, in the FSGS rituximab responders, we have shown recovery of T cell response to activation 6 months after rituximab treatment. These findings strengthen the evidence of an immunologic basis for rituximab treatment in FSGS in addition to the reported direct effect on the sphingomyelin phosphodiesterase acid-like 3b pathway in podocytes (34). However, FSGS rituximab nonresponders showed comparable T cell activation to that of controls, indicating a possible different pathogenesis for their disease that is not amenable to current immunosuppressive agents.

CD154 or CD40 ligand is a surface molecule expressed on activated CD4⁺ T cells mediating costimulation *via* antigen-presenting cells (B cells, dendritic cells, and macrophages) (35,36). Activated T cells expressing CD154 have been shown to produce IFN- γ and IL-2 (37). Hence, it is plausible that, in our cohort of FSGS rituximab responders who showed baseline (before rituximab) reduced mitogen-stimulated CD154, IFN- γ , and IL-2 expression, there exists defective T cell-B cell interaction associated with production of permeability factor(s) responsible for podocyte injury, resulting in massive proteinuria. The source of the permeability factor is unclear at this time. It is certainly possible that activated cytokine-producing B cells incite T cell activation and the eventual hyporesponsiveness that is depicted here. Depletion of B cells could potentially restore normal function of the T cells, promoting remission of the disease.

The role of regulatory B cells in immune-mediated diseases has been recognized in recent years (17).

Studies have shown that regulatory B cells can suppress inflammatory responses in experimental autoimmune encephalomyelitis, collagen-induced arthritis, and colitis (38–40). In patients with pemphigus vulgaris, depletion of B cells led to a significant reduction in the frequency of desmoglein-specific T cells producing IFN- γ or IL-4 (41). It is also possible that aberrant activation of B cells results in persistent cytokine production that constantly activates T cells. In this regard, recent data from patients with multiple sclerosis in relapse were associated with the demonstration of IL-6-producing B cells. Treatment with rituximab resulted in depletion of B cells, reduced IL-6 production, and remission of disease (42). Of note, multiple sclerosis is a disease traditionally regarded as T cell dependent; however, B cell depletion results in significant improvement in disease (43,44).

Our study has also shown that stimulated CD154⁺CD4⁺CD3⁺, IFN- γ ⁺CD3⁺, and IL-2⁺CD3⁺ were good predictive markers for response to rituximab therapy. IFN- γ ⁺CD3⁺ performed as the best individual marker with the most robust predictor characteristics. We have also shown that IFN- γ ⁺CD3⁺ performs well as a single subset and that complicated mathematical derivation of a combined risk score is unnecessary, because the latter confers no incremental discriminatory value and is impractical at the bedside or doctor's office.

In clinical practice, such a marker with 100% NPV is of great practical value, because it can identify which patients will not respond to rituximab and therefore, should not be unjustifiably exposed to the costs and risks of the treatment with little hope of receiving any therapeutic benefit from the exposure. We have shown that IFN- γ ⁺CD3⁺ $\geq$ 2.5% performs well as a predictor of lack of response to rituximab. Most patients seem to tolerate rituximab quite well (45); however, severe complications that are potentially fatal have been described, including progressive multifocal leukoencephalopathy (46) and pulmonary fibrosis (47). In addition, there is a lack of knowledge on the long-term immunologic outcome postrituximab therapy. Only if the risk-to-benefit ratio proves to be superior should rituximab be justified for the individual patient.

Although the discovery of novel immunologic biomarkers predicting responsiveness to rituximab therapy in patients with FSGS may seem encouraging, the applicability of the results of this study may be limited by its sample size and the uniqueness of the study population. We also recognize the limitations of overgeneralization of these preliminary findings on the basis of our single study population. There is, hence, a need to test the reproducibility of our findings in a separate large validation cohort. We are currently exploring the B cell subsets and cytokine production to better understand the immunologic basis for the efficacy of rituximab in this subset of patients with FSGS.

In conclusion, we have identified prognostic markers that could help in selecting a subset of patients with FSGS who are hyporesponsive to T cell mitogen and ionophore stimulation and therefore, may better respond to rituximab. A longitudinal follow-up study is currently underway to determine if any of these immunologic subsets are able to predict relapses after rituximab therapy.

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