

T-Lymphocyte Activation Markers As Predictors Of Responsiveness To Rituximab Among Patients With Focal Segmental Glomerulosclerosis

Supplementary Information

This contains information on detailed methodology.

Detailed Methods

Study Design

Ethics approval was obtained from the 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-NKF-NUH Children's Kidney Centre, National University Hospital, Singapore, between October 2009 and February 2014 were included in this study. Patients presented between the ages of 1 to 18 years with either SDNS or SRNS. SDNS was defined as inability to taper off prednisolone therapy without relapse or relapse within 14 days of its discontinuation. SRNS was defined as failure to achieve complete remission defined by loss of proteinuria (urinary total protein $<0.3 \text{ g}/1.73\text{m}^2/\text{day}$ or urine protein:creatinine ratio $<0.02 \text{ g}/\text{mmol}$ or $<1+$ protein on dipstick for three consecutive days) despite therapy with prednisolone at $60 \text{ mg}/\text{m}^2/\text{day}$ for 8 weeks (1). Initial non-response was defined as resistance at the onset of nephrotic syndrome, and late non-response was defined as resistance in a previously steroid-dependent patient. Clinical indications for rituximab therapy in SRNS patients were lack of remission despite achieving high therapeutic trough levels of CNI (either cyclosporine $200\text{-}250\mu\text{g}/\text{L}$ or tacrolimus $10\text{-}12\mu\text{g}/\text{L}$) and addition of mycophenolate; disease relapse following prolonged ($>$ two years) CNI therapy; or biopsy evidence of nephrotoxicity secondary to CNI. Clinical indications for rituximab therapy in SDNS patients were lack of steroid-sparing effect (inability

to sustain remission at a prednisolone dose of $\leq 0.5\text{mg/kg}$ alternate day) despite treatment with oral cyclophosphamide (2mg/kg per day for 12 weeks) and CNI therapy; or prolonged CNI therapy with evidence of nephrotoxicity.

Exclusion criteria included patients with the following: estimated glomerular filtration rate (eGFR) $< 60\text{ ml/min per } 1.73\text{m}^2$; infantile onset of nephrotic syndrome; nephrotic syndrome secondary to chronic infections such as hepatitis B, hepatitis C or human immunodeficiency virus, systemic lupus erythematosus, Henoch-Schönlein purpura, IgA nephropathy, membranoproliferative glomerulonephritis 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 375mg/m^2 fortnightly to a maximum of four doses according to our Center's clinical protocol. Two doses of rituximab were administered if the patient was in remission at the time rituximab was given, whereas four doses were given if in relapse. All patients were monitored for infusion-related reactions and screened at each visit for infections. Daily testing of urine for protein using Albustix[®] was performed at baseline and following rituximab therapy. Clinical investigations and immunological subset monitoring were performed at baseline, 14 days, one-, three- and six-months post-rituximab administration and if the patient relapsed. CD19 subsets were performed monthly until recovery of B-cells. CD19 recovery was defined as CD19 peripheral blood count $\geq 10/\mu\text{l}$. Response to rituximab was defined as resolution of proteinuria (urine protein:creatinine $< 0.02\text{g/mmol}$ or urine protein excretion $< 0.3\text{g}/1.73\text{m}^2/\text{day}$) and ability to wean off prednisolone and CNI at three months after the last dose of rituximab. After completion of the rituximab treatment, all patients were placed on our Center's routine immunosuppressive tapering protocol. In patients who had achieved remission, prednisolone was tapered to alternate day dosing, and subsequently decreased by 5 mg every week until cessation within three months. Two weeks after

administration of rituximab, CNI dose was also decreased by 50% and then tapered progressively till cessation at three months. All patients were continued on mycophenolate mofetil at 600mg/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 gender-matched healthy controls were recruited from the General Nephrology Clinic and comprised patients who had non-glomerular disorders such as mild vesicoureteric reflux and duplex kidneys, with normal renal function and no evidence of albuminuria or urinary tract infection.

Immunological Subset Monitoring

The following mouse IgG1, κ isotype control and mouse anti-human monoclonal antibodies (mAbs) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA): Fluorescein isothiocyanate (FITC)-conjugated IgG1, CD3, CD8, CD19, CD25, CD45RA, HLA-DR, FAS and OX40; phycoerythrin (PE)-conjugated IgG1, CD16, CD45RO, CD56, CD69, CD154, CTLA-4 and ICOS; peridin chlorophyll protein (PerCP)-conjugated IgG1, CD3, CD4 and CD19; allophycocyanin (APC)-conjugated IgG1, CD3 and CD25; V450-conjugated CD3; and V500-conjugated mouse IgG1 and CD8. Lymphocyte subset staining was performed by the lysed whole blood method. Briefly, for every 50 μ l of blood, 10 μ l of respective fluorochrome-conjugated mAb was added, and incubated for 30 minutes at room temperature. Background staining was carried out using isotype-matched control mAb. The red blood cells were then lysed with 1 ml of FACS™ Lysing Solution (Becton Dickinson). The cells were washed twice with 1x phosphate buffered saline (1x PBS), re-suspended in 1% paraformaldehyde and analyzed on flow cytometry (Becton Dickinson). Data analysis for determination of percentage of positive cells and geometric mean fluorescence was performed using CellQuest™ Pro software (Becton Dickinson).

T-Cell Activation

Blood samples were diluted in an equal volume of RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) and stimulated with 1 µg/ml of ionomycin (Sigma-Aldrich, St. Louis, MO, USA), 20 ng/ml of PMA (Sigma-Aldrich) and 6 µM monensin sodium (Sigma-Aldrich) to inhibit intracellular transport, and incubated for four hours at 37°C with 5% CO₂. Surface expression of CD154 and CD69 on T-cells was assayed using the lysed whole blood method as described above.

Intracellular production of interferon-gamma (IFN-γ) and interleukin-2 (IL-2) by CD3 cells was also performed. Briefly, for every 100 µl of stimulated mixture, red blood cells were lysed with 1 ml of FACS™ Lysing Solution followed by permeabilizing of cells with Permeabilizing Solution 2 (Becton Dickinson). The cells were then washed with wash buffer (PBS containing 0.5% bovine serum albumin and 0.1% sodium azide). Cells were subsequently stained with FITC-conjugated CD3 and PE-conjugated IFN-γ or IL-2 mAb (Becton Dickinson) for 30 minutes at room temperature. The cells were then washed twice with wash buffer and re-suspended in 1% paraformaldehyde and analyzed on flow cytometry.

Foxp3 Analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using lymphocyte separation medium (Lonza, Basel, Switzerland). The PE-conjugated Foxp3 antibody reagent kit from eBioscience (San Diego, CA) was used for intracellular Foxp3 staining according to the manufacturer's instructions.

Statistical Analysis

Results were analyzed using the Statistical Package for Social Sciences version 22.0. Comparison of the immunological subsets prior to rituximab therapy between FSGS rituximab responders, FSGS rituximab non-responders, 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. Fisher's 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, gender, histological subtype, steroid-resistance, prior non-corticosteroid immunosuppression and duration, remission status at time of rituximab administration, eGFR and number of rituximab doses.

Paired analysis of immunological subsets prior- and six months post-rituximab therapy was done using Wilcoxon signed rank test. Receiver-operating characteristic (ROC) 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 to 1).

References

1. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. *Kidney Int* 3: 1-150, 2013