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Antithymocyte Globulin Antibody Titer Congruent With Kidney Transplantation: Analysis of Incidence, Outcomes, Cost, and Alternative Targets

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Background. Rabbit antithymocyte globulin (rATG) use for immunosuppression induction is widespread but is contraindicated by the presence of anti-rATG antibodies. This study reports the incidence of positive anti-rATG antibody titers in patients before and after renal transplant and evaluates associated outcomes and costs. In addition, it will correlate CD40L and interleukin (IL)-21 with anti-rATG antibody titers. **Methods.** Clinical and billing records from the Indiana University Transplant Laboratory were reviewed for positive versus negative anti-rATG antibody titers, graft survival, and 7-day readmission costs between 2004 and 2018. Serum from patients with positive and negative rATG antibody titers were quantitated for CD40L and IL-21 by enzyme-linked immunosorbent assay. **Results.** On average, between 2004 and May 2018, 163 kidney transplants per year were performed. Anti-rATG antibody titers were ordered for 17 patients/year, of which 18.2% were positive at 1:100 titer either pre- or post-transplant. Time to graft loss correlated with a positive rATG titer at time of readmission. Moreover, second kidney transplant increased the anti-rATG positive rate. A weak correlation was observed between anti-rATG titer and recipient age. Seven-day readmission treatment costs were significantly lower in patients with positive anti-rATG titer. IL-21 and CD40L were significantly greater in patients with positive anti-rATG titers after transplant when compared with negative anti rATG patients. **Conclusions.** Positive anti-rATG antibody titer is associated with a significant negative impact on outcomes. Monitoring of anti-rATG antibody titer is recommended to optimize treatment options in patients, especially in the setting of second transplants. Elucidation of the mechanisms associated with positive anti-rATG antibody is required. IL-21 and CD40L are potential targets for future study.

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Antibody-mediated rejection (AMR) is a significant complication after kidney transplantation that carries a poor prognosis.[1] Approximately 10% of kidney transplant patients experience AMR. Of which 30% will experience graft loss as a consequence. To prevent AMR, rabbit antithymocyte globulin (rATG) induction immunosuppression is widely used to eliminate T helper cells, decrease donor-specific antigen (DSA) antibody titers, and reduce B-cell differentiation to plasma cells.[2-4] However, antibodies against rATG can negate its therapeutic purpose.[5] This is particularly important when deliberating follow-up rATG to combat suspected AMR.

The primary objective of this study was to identify the incidence of positive anti-rATG antibody titers in renal transplant recipients at the Indiana University Health Transplant program between 2004 and 2018. The secondary objective was to determine correlating factors, outcomes, and cost associated with positive anti-rATG antibody titer. The final objective was to investigate immunologic regulators of antibody production to identify targets for future investigations directed to reduce/

prevent positive anti-rATG antibody titer in renal transplant recipients.

rATG is a mixture of polyclonal rATGs that interact with immune response antigens, adhesion molecules, and cell-trafficking molecules resulting in rapid T-cell and B-cell depletion through complement-dependent cell lysis and apoptotic cell death in lymphoid tissues.[6] rATG is prepared by immunizing pathogen-free rabbits with a cell suspension of human thymic tissue. After immunization, the serum is harvested and immunoglobulins against thymocytes are isolated and purified.[7] Lymphocyte depletion occurs rapidly following the administration of rATG, within 2–3 hours and recovers gradually after treatment. T-cell counts begin to return toward baseline after about 10 days.[8] By 3 months, approximately 40% of patients recover >50% of the initial lymphocyte counts but disruption of subsets and CD4 T cells counts can be long-lasting.[9]

At Indiana University Hospital renal transplant program, anti-rATG antibody titers are performed on patients readmitted with suspected acute or chronic renal rejection. The purpose is to determine the applicability of repeated rATG treatment. While this approach will avoid contraindicated treatment, the question of how to prevent positive anti-rATG antibody titers and how best to treat patients with positive anti-ATG antibody titers remains.

One strategy to prevent positive anti rATG antibody titers is to further limit the maturation of B cells to antibody-producing plasma cells.[10] In renal recipients, B-cell populations are significantly reduced by rATG. However, B-cell levels are not eliminated entirely and do recover in patients, despite maintenance immunosuppression.[11] For example, studies have shown that renal transplantation long-term outcomes are impacted by DSA which elicit AMR through B-cell differentiation to plasma cells despite use of rATG.[12] Suggesting that B-cell disruption is not always successful. Therefore, reducing antibody production by reducing the generation of antibody-producing plasma cells by a non-rATG protocol may be advantageous.

Antibody production is modulated by integrated signals from antigen-presenting cells and helper T cells.[13] In particular, T follicular helper cells play a crucial role in AMR, because they help naïve B cells to differentiate into memory B cells and alloantibody-producing plasma cells within germinal centers. In this way, they contribute to the induction of DSA antibodies, which are responsible for the humoral immune response to the allograft.[12] A literature search focused on the maturation of B cells identified CD40L and interleukin (IL)-21 as key. In particular, studies show CD40L and IL-21 as key signaling molecules involved in T follicular helper cell–associated B-cell differentiation (Figure 1).[14] Moreover, anti-CD40L antibodies are used to prevent AMR in models of xenotransplantation.[15] As CD40L and IL-21 are important in anti-DSA antibody production, it is possible that they are also important in anti-rATG antibodies production and are a potential target for intervention.

IL-21 is a member of the IL cytokine family and has pleiotropic biological effects on lymphoid and myeloid cells via IL-21 receptor on T cells and B cells. It is mainly synthesized and secreted by activated CD4(+) T cells and natural killer[16] T cells. As an immunoregulatory factor, IL-21 and IL-21 receptor play important roles in the development and progression of various autoimmune diseases, including lupus erythematosus and osteoarthritis.[17,18] Consequently, modulation of IL-21 synthesis and signaling may be successful to abate solid organ rejection.
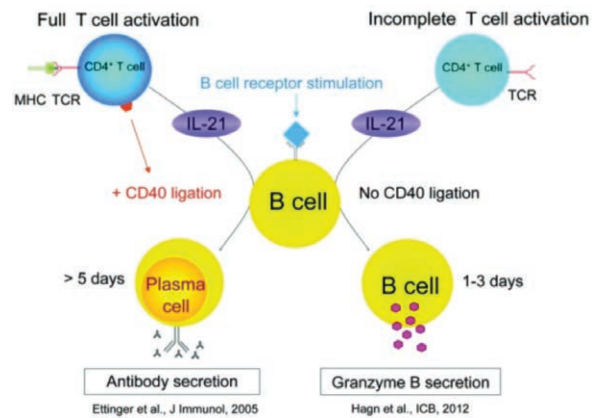
FIGURE 1. CD40 ligand regulates IL-21–induced differentiation of B cells into either plasma cells or granzyme B-secreting cells. B cells that are activated by specific BCR crosslinking associated with CD40L negative T helper cells and IL-21 differentiate into GrB-secreting B cells (right). In contrast, B cells activated by antigen-specific CD4+ T cells that express both IL-21 and CD40 ligand differentiate activated B cells into plasma cells (left). BCR, B-cell receptor; GrB, granzyme B; IL, interleukin. (Reproduced from Hagn and Jahrsdörfer,[14] no modifications, open-access license https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3518509.)

CD40L (also known as CD154) and its receptor CD40 belongs to the Tumor Necrosis Factor:Tumor Necrosis Factor Receptor family.[19] CD40 is expressed on B cells, macrophages, and dendritic cells and is important for generation of long-lived plasma cells and memory B cells.[20-22] CD40L is expressed by immune cells, endothelial cells, and activated platelets.[23-25] Targeting of CD40L, via the use of statins, has been suggested as a treatment of carcinogenesis.[26] To examine the potential of targeting IL-21 and CD40L for the prevention of renal rejection in patients with positive anti-rATG antibody titers, we quantitated both in serum collected at the time of readmission for suspected AMR in patients with either positive or negative anti-rATG titer.

MATERIALS AND METHODS

Patient Population

Data and serum samples collected from patients receiving renal transplants at the Indiana University Health Organ Transplant Unit (IUH-OTU) between January 1, 2004, and May 31, 2018, were reviewed. The standard of care for renal transplant recipients includes collection and storage of serum immediately before transplant and perioperative administration of rabbit antithymocyte immunoglobulin (rATG).

The immunosuppression induction protocol consisted of 3 equal doses of rATG (total dose=6 mg/kg) beginning preoperatively with standard premedication given immediately before its administration: solumedrol (500 [first dose], 250 [second dose], and 120 mg [third dose]), acetaminophen (650 mg), and diphenhydramine (25 mg). Maintenance immunosuppression was primarily tacrolimus monotherapy, although some early recipients did receive a short steroid taper with steroid withdrawal within 3 months of transplantation. Recipients also received a single dose of rituximab between the first and second doses of rATG.[27]

Suspected Renal Rejection

In patients who presented with symptoms of acute or chronic renal rejection (decreased renal function, general discomfort, uneasiness, or ill feeling, pain, or swelling in the area

of the organ, fever, and flu-like symptoms, including chills, body aches, nausea, cough, and shortness of breath), serum samples were tested for anti-rATG antibody titers (1:100, 1:500, and 1:1000) and compared with serum samples collected pre-transplant and stored within the IUH transplant immunology (TRIM) sample repository.

Database

From IUH TRIM laboratory records, a database of renal transplant recipients who had anti-rATG antibody titers quantitated at the time of transplant and following readmission for suspected rejection between 2004 and 2018 was created using Excel. The database included age, gender, date of transplants, patient survival, graft survival, number of transplants, lymphocyte populations, rATG antibody titers (at transplant and at suspected rejection), and cytomegalovirus (CMV) virus status. There was no selection bias, other than the availability of clinical follow-up for clinical correlates.

Anti-rATG Enzyme-Linked Immunosorbent Assay

Performed by the TRIM laboratory using an enzyme-linked immunosorbent assay (ELISA) assay developed in-house. The TRIM laboratory is accredited with the College of American Pathologists (CAP #1678922) and CMS Clinical laboratory Improvement Amendment certified (CLIA#15D0689426) to perform clinical testing. Serum samples are diluted (1:100, 1:500, and 1:1000) and incubated (quadruplicate) in 96-well plates coated with rabbit thymoglobulin for 45 minutes at room temperature. After triplicate washing with phosphate buffered saline (PBS), antibody binding was revealed with goat anti-human immunoglobulin G-peroxidase conjugate incubation for 45 minutes at room temperature. After triplicate washing with PBS, peroxidase substrate was added. After 20 minutes, the reaction was stopped with 1N sulphuric acid. Plates were read at 460 nm. Quality control included known positive, known negative, and plate control (no thymoglobulin). Quality control data were recorded in Levey Jennings charts. Positive, negative, and plate control limits equal 2 standard deviations of average from previous 12 months analysis. Positive titer determined as absorbance greater than double that of plate negative. Positive titer was ≤1:100. There are 4 potential resultant categories for comparison based on positive or negative anti-rATG antibody titer either before or after transplant. Group 1: negative pre-transplant and negative post-transplant. Group 2: negative pre-transplant and positive post-transplant. Group 3: positive pre-transplant and negative post-transplant. Group 4: positive pre-transplant and positive post-transplant.

Markers of Anti-rATG Antibody

To investigate the pathways associated with positive anti-rATG titer serum samples collected from patients with positive and negative anti-rATG antibodies were identified from the database. Based on power analysis following preliminary analysis of CD40 ligand (CD40L) and IL-21, 13 patients tested positive for anti-rATG antibodies were matched with 13 patients who have tested negative. Matching was based on similar age, gender, blood type, and renal transplantation. Thirteen positive and negatives patients were matched and underwent CD40 ligand and IL-21 ELISA testing from commercially available kits.

CD40 Ligand ELISA

Human CD40L was quantitated in serum samples collected after transplant, at time of suspected rejection using

commercial ELISA (ThermoFisher, IL). Standards were prepared using the standard concentrate that was reconstituted using standard diluent. The stock solution is 2000 pg/mL and served as the start of a serial dilution with the following concentration serving as the standards: 1000, 500, 250, 125, 62.5, 31.2, 15.6, and 0 pg/mL served as the blank which is just standard diluent. Detection reagents A and B were diluted 100-fold to their working concentration with assay diluents A and B. Wash solution was diluted to a 1× concentration from the 30× stock concentration. Samples were prepared at the suggested 10-fold dilution in PBS. Samples and standards (100 µL) were pipetted into wells, covered with a plate sealer, and incubated for 1 hour at 37°C. Liquid was removed from the wells and not washed. 100 µL of detection reagent A was added to the wells and incubated for 1 hour at 37°C. Solution was aspirated from the well and washed with 350 µL of previously prepared wash solution 3 times. Detection reagent B (100 µL) is added to each well and incubated for 30 minutes at 37°C. Aspiration/wash process was repeated for a total of 5 times. Substrate solution (90 µL) was added to each well and incubated for 10–20 minutes at 37°C. Fifty microliters of stop solution and absorbance were measured at 450 nm.

Interleukin-21 ELISA

Human IL-21 was quantitated in serum samples from patients after transplant, at time of suspected rejection using commercial ELISA (ThermoFisher, IL). Assay diluent B is diluted 5-fold with distilled water before use. Serum samples are diluted 40-fold with assay diluent C. Standards are produced by diluting the standard protein with 400 µL of assay diluent C which produced a 50 ng/mL stock solution. Using the stock solution, a serial dilution was created 0–8000 pg/mL. Samples and standards (100 µL) are added to each well and incubated at room temperature for 2.5 hours. Solution is then discarded and washed with 300 µL 4 times. The prepared 1× detection antibody (100 µL) is added to each well and incubated for 1 hour at room temperature. Detection antibody solution is discarded and the wells are washed 4 times. Next, 100 µL of Horse Radish Peroxidase-streptavidin is added to each well and incubated for 45 minutes at room temperature. Horse Radish Peroxidase-streptavidin solution is discarded and plate is washed 4 times. 3,3',5,5'-tetramethylbenzidine—1 substrate reagent (100 µL) —is added to each well and incubated for 30 minutes at room temperature in the dark. Finally, 50 µL of stop solution is added to each well and absorbance is read at 450 nm.

Statistical Analysis of Database
Cox Proportional Hazards Model

Relationships among the length of time on dialysis, anti-rATG, and time to rejection were performed. Length of time on dialysis and anti-rATG antibody result post-transplant were used as explanatory variables. Time until rejection was used as the time variable, with rejection being the end event. This test used all positive assays for individuals following their first transplant who were tested for anti-rATG antibody titers and had known dialysis times and times until rejection (n = 22). Negative assays meeting those criteria were used dating back to 2011 (n = 90). Age, recipient immunoglobulin G status, number of transplants, and anti-rATG antibody titer results following transplant were tested for correlation with time to rejection using a Pearson correlation test. Dialysis time

was excluded from these tests due to no effect on rejection. Second transplants were treated as a separate observation from first transplants. All patients in the gathered data with complete sets of this information were used (n = 132).

Partial Least Square Regression

Was used to evaluate (1) lymphocyte subtypes before transplant as an explanatory variable for time until graft rejection, and (2) lymphocyte subtypes before transplant as an explanatory variable for anti-rATG antibodies titer. Pearson correlation test was used to examine (1) relationship between time before rejection and anti-rATG antibody titer, (2) relationship between anti-rATG titer before rATG and at time of rejection, and (3) correlation between second transplants and anti-rATG titer.

RESULTS

Data Cohort

Between 2004 and 2018, 2278 patients received kidney transplants. Of these, anti-rATG antibody titer quantification was ordered for 241 patients (10.5%). Forty-four patients had a titer >1:100 (positive) (22.3%). Clinical follow-up and outcomes were available for 112 patients, 22 positives and 90 negatives.

The frequency of anti-rATG assay requests is low because it is only ordered/captured in our database when patients present to the IUH transplant unit outpatient unit with suspected renal rejection. Patients who receive their transplant at IUH but follow-up care else were not included.

Anti-rATG Titers in Renal Transplant Recipients Presenting With Renal Rejection

Anti-rATG antibody titers were extracted from patient records. Recipients were transplanted at Indiana University Hospital between January 2004 and May 2018. On average, 160 renal transplants were performed per year. Anti-rATG antibody titer quantification was requested by transplant nephrologists and performed by the IUH TRIM laboratory on recipients per year. Results were recorded at serum samples collected at 2 time points: (1) before transplantation, and (2) at the time of suspected rejection (post). There were 4

potential outcomes: (1) 80.4% were negative pre-transplant and negative post-transplant (–/–) (2) 0% was negative pre-transplant and positive post-transplant (–/+), (3) 8.9% were positive pre-transplant and positive post-transplant (+/+), and (4) 10.7% were positive pre-transplant and negative post-transplant (+/–).

Patients receiving a second kidney where either (1) 62.5% negative pre-transplant and post-transplant or (2) 37.5% negative pre-transplant and positive post-transplant. There were 4 patients who had anti-rATG antibody titers calculated after both renal transplants. Hundred percentage of these patients were negative for anti-rATG antibodies after the first transplant but 50% were positive after the second transplant. There was no evidence of deviation from standard of care immunosuppression or noncompliance between the groups at first or second transplant.

Outcomes in Renal Transplant Recipients Presenting With Renal Rejection

Anti-rATG antibody titer and timing had a significant impact on the time to rejection. Recipients who were negative for anti-rATG antibodies before transplant and at the time of presentation (–/–), the time to graft loss was 747 days (95% CI, 567-1174). For recipients, positive pre-transplant and negative at readmission (+/–), the average time to graft loss was 540 days (95% CI, 76-652) (t test +/– versus –/–; $P < 0.22$). For recipients, positive pre-transplant and at time of readmission (+/+), the time to graft loss was 216.7 days (95% CI, 58-447) (t test +/+ versus –/–; $P < 0.04$) (Figure 2A).

Risk Factors Associated With Time to Rejection

There was no correlation between length of time on dialysis and time to rejection (Hazard Ratio, 1.000, $\chi2 = 0.001$). There was also no significant correlation associated with CMV status versus time to rejection (coefficient of variance = –0.0093; $P = 0.29$). There was no correlation between white blood cell counts and time to rejection (PLS $Q_2 = –0.059$). There was no correlation between recipient age and time to rejection (coefficient of variance = –0.005; $P = 0.951$). There was no correlation between number of transplants and time to rejection (coefficient of variance = 0.01; $P = 0.911$). There was a correlation
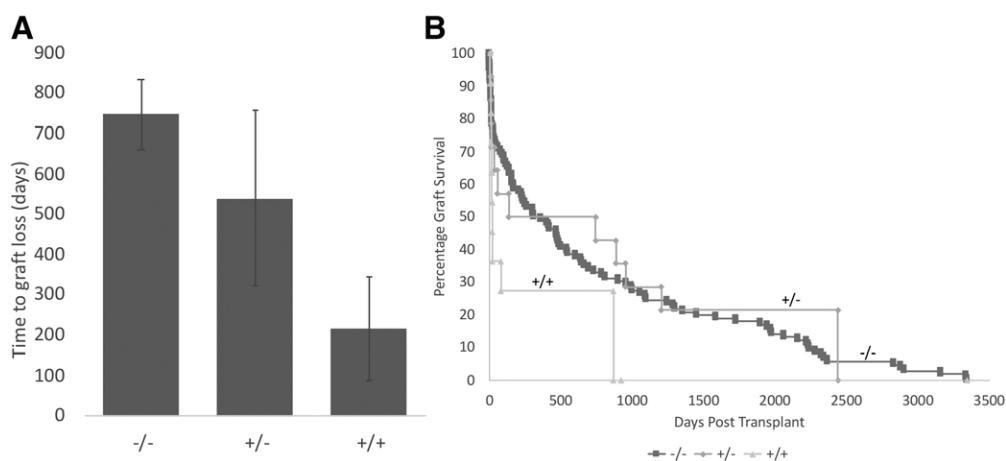
FIGURE 2. Graft survival associated with anti-rATG antibody titer. A, Time to graft loss from time of anti-rATG antibody assay was evaluated from clinical records for patients who were (1) negative at both pre-transplant and at time of presentation for renal rejection (–/–), (2) positive at both time of transplant and at time of presentation for renal rejection (+/+), and (3) positive at time of transplant but negative at time of presentation for renal rejection (+/–). Students t test –/– vs +/+; $P < 0.05$. Students t test (–/–) vs (+/–); $P = 0.22$. B, Renal graft survival Kaplan-Meier curve for each group. rATG, rabbit antithymocyte globulin.
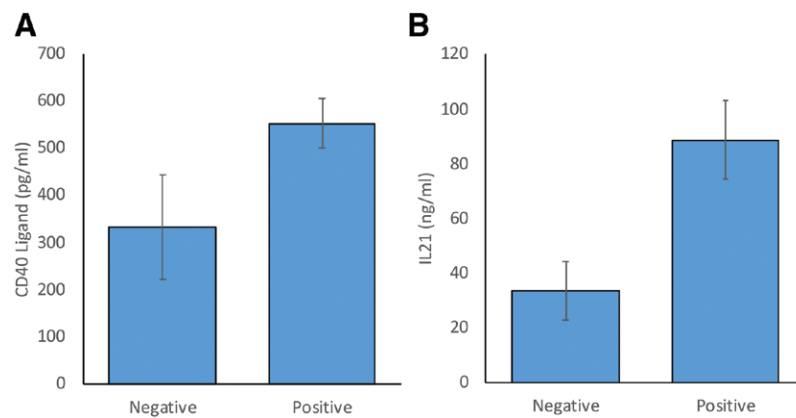
FIGURE 3. CD40L and IL-21 are significantly elevated contemporaneous with anti-rATG diagnosis. Serum CD40 ligand and IL-21 were quantified using commercially available ELISA-based assays. Levels were significantly increased contemporaneous with anti-rATG titer (t test; $P = 0.049$ and $P = 0.002$, respectively). ELISA, enzyme-linked immunosorbent assay; IL, interleukin; rATG, rabbit antithymocyte globulin.

between positive anti-rATG antibody titer and time to rejection, coefficient of variance (–0.224; $P = 0.01$) (Figure 2B).

Risk Factors Associated With Positive Anti-rATG Antibody Titer

There was no significant correlation between length of time on dialysis and positive anti-rATG antibody titer (Hazard Ratio = 1.000, $\chi 2 = 0.001$). There was a possible correlation between patient age at time of transplant and positive anti-rATG antibody titer (coefficient of variance = 0.106; $P = 0.228$). There was no significant correlation between CMV status and positive anti-rATG antibody titer (coefficient of variance = –0.63; $P = 0.47$). There was a possible correlation between number of transplants and positive anti-rATG antibody titer (coefficient of variance = –0.09; $P = 0.264$).

Positive Anti-rATG Antibody Rate Following Second Transplant

Between 1975 and May 2018, 40 patients received 2 renal transplants at IUH. First transplants were performed between May 1975 and January 2017. Second transplants were performed between May 1981 and March 2018. The average time between transplants was 3431 ± 412 days. Following the first transplant, 12.5% of patients had anti-rATG antibody titers quantified. The anti-rATG antibody-positive rate was 0%. Following the second transplant, 8 patients had anti-rATG antibody titers quantified at presentation for suspected rejection (20%). Three patients had a positive anti-rATG titer at presentation (37.5%). Four patients were in both groups (anti-rATG antibody titers were calculated after both first and second transplants). The anti-rATG antibody titer positive rate was 50% after the second transplant.

Serum IL-21

Serum IL-21 levels were quantitated in renal transplant recipients at the time of suspected AMR (N = 26). There were 2 groups. Group 1: recipients with a negative anti-rATG antibody titer before and after transplant (–/–) (N = 13), and group 2: recipients with a positive anti-rATG antibody titer before and after transplant (+/+) (N = 13). IL-21 was significantly greater in patients with positive anti-rATG antibody titer (88.5 ± 14.3 ng/mL) when compared with negative anti-rATG antibody patients (33.8 ± 10.7 ng/mL). Unpaired t test negative versus positive, $P = 0.002$ (Figure 3B).

Serum CD40 Ligand

Serum CD40L levels were quantitated in renal transplant recipients at the time of rejection (N = 26). There were 2 groups. Group 1: recipients with a negative anti-rATG titer before and after transplant (–/–) (N = 13), and group 2: recipients with a positive anti-rATG titer before and after transplant (+/+) (N = 13). Serum CD40L was significantly greater in patients with a positive anti-rATG titer (552 ± 76 pg/mL) when compared with negative anti-rATG controls (333 ± 54 pg/mL). Unpaired t test negative versus positive, $P = 0.049$ (Figure 3A).

Rejection Treatment Costs

To evaluate the financial burden associated with renal rejection in the presence of anti-rATG antibodies, 7-day total billable charges were extracted from clinical records for patients after anti-rATG antibody analysis (N = 13 positive/positive and N = 13 negative/negative). In patients with a negative anti-rATG antibody titer, pre- and post-transplant, the 7-day hospital costs was $43,284 ± $14,529 (range $21,130–74,961; Figure 4). In patients with a positive anti-rATG antibody titer, before and after transplant, the 7-day cost of treatment was $15,657 ± 8,501 (range $3,969–$33,700; Figure 4). There was no significant difference in costs between negative pre- and post-transplant patients and positive pre-transplant and negative post-transplant patients. This is because clinical treatment options are based on the post-transplant titer. None of the patients lost their kidney within the 7-day billing period monitored. That was one of the reasons the billing period was narrow. Consequently, costs associated with graft loss were not captured and therefore not included.

DISCUSSION

rATG is a powerful tool to encourage organ tolerance following kidney transplant. However, the development of anti-rATG antibodies limits its use in the context of acute and chronic renal rejection. Patients with anti-rATG antibodies have a significantly reduced graft survival. Moving forward, we are motivated to better understand the incidence, risk factors, and implications of anti-rATG antibody-positive titer to better counsels patients, improve patient stratification, and propose alternative targets for treatment.

Using clinical records pertaining to patients receiving renal transplants at the IUH between 2004 and 2018, we
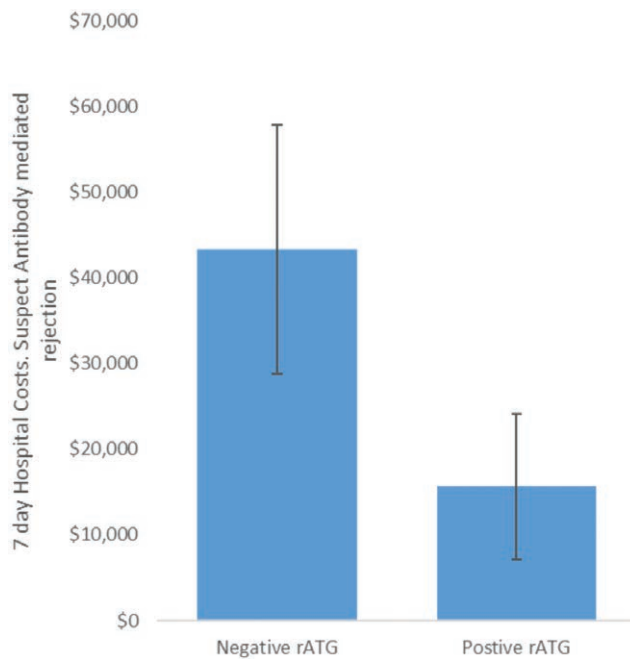
FIGURE 4. Seven-day hospital costs for treating renal rejection in patients with positive and negative anti-rATG antibody titers. Average 7-day hospital costs for patients with suspected antibody-mediated renal rejection postrenal transplant was pooled from billing records for patients who had tested positive or negative for anti-rATG at Indiana University Health renal transplant program between 2007 and 2018 (N = 13). Student's *t* test, $P < 0.01$. Chart 1. Anti-rATG antibody titer data. rATG, rabbit antithymocyte globulin.

report that the incidence of anti-rATG antibodies in patients, whom experience episodes of suspected renal rejection and were readmitted to IUH, to be 26%. Moreover, a reduction in the time to rejection was strongly associated with positive anti-rATG antibody titer. There was a possible correlation between age of recipient or number of transplants and positive anti-rATG antibody titer. In contrast, there was no correlation between anti-rATG antibody titer and either length of time on dialysis or CMV status. Moreover, positive anti-rATG titers were associated with a lower 7-day total billable events. This lower cost is due, in part, to the absence of rATG. The lack of difference in cost between –/– and +/– patients is because only post-transplant titers are reported. Therefore, both groups potentially will receive additional rATG. It is not possible to provide an accurate percentage of patients with positive rATG antibody titers for the entire transplant cohort at IUH as many patients who are transplanted at IUH receive follow-up care elsewhere. The number of patients receiving anti-rATG antibody quantitation was low because anti-rATG antibody titers are only ordered at time of suspected rejection and these rates are low. Interestingly, we did not observe patients that were negative pre-transplant and positive post-transplant (–/+). This is potentially an artifact due to the low sample size. We did observe patients whom were –/+ following liver and lung transplants. Demonstrating that rATG positive titers can manifest after solid organ transplant. Consequently, additional data are required. If the finding that patients with a negative anti-rATG titer pre-transplant do not develop anti-rATG antibodies in response to rATG, then an argument could be made to screen all kidney transplant patients pre-transplant. A negative titer pre-transplant would

negate the need for qualification at the time of suspected rejection. Moreover, such a finding would prompt studies into the unique characteristics of renal transplant and immunosuppression that differentiates it from lung and liver transplants. Any suggestion that all patients should receive anti-rATG antibody quantification as part of their presurgery work up is debatable. The assay is not commercially available and has been established in-house at IUH TRIM laboratory. Any decision would require a cost-benefit analysis, considering the cost of the assay and frequency of the event. We do not have access to all the financials for the patients and cost of renal rejection/graft loss and so such an analysis is beyond our expertise. The limited financial burden we have is presented to start the conversation and promote further studies.

To evaluate potential targets to prevent anti-rATG antibodies, we investigated 2 signaling molecules associated with DSA antibody-mediated rejection, IL-21, and CD40L after transplant and at the time of suspected rejection in patients that were either –/– or +/+. Patients whom were +/– were not included. It is anticipated that this group of patients will be included in future studies. IL-21 is a cytokine that plays a major role in stimulating the differentiation of B cells.[14] When human B cells are stimulated through the B-cell receptor, IL-21 induces minimal proliferation, IgD down-modulation, and small numbers of plasma cells.[14,28] Importantly, the effects of IL-21 on B-cell differentiation are crucially influenced by multiple T-cell–derived factors. Previous studies indicate that the IL-21–induced differentiation of plasma cells is strongly supported by CD4+ T-cell help, which includes CD40L. At the same time, CD40L strongly inhibits IL-21–induced granulocyte B expression by B cells. Fully activated CD4+ T cells (ie, receiving T cell receptor stimulation and costimulatory signals) express both IL-21 and CD40L, explaining their robust ability to favor the differentiation of plasma cells. In contrast, incompletely activated CD4+ T cells (receiving T cell receptor stimulation only) express IL-21 but not CD40L, making them potent inducers of granulocyte B production by B cells. In kidney recipients with a positive anti-rATG antibody titer, circulating IL-21 and CD40L were significantly increased when compared with patients with negative anti-rATG antibody titer. Consequently, targeting IL-21 and CD40L are potential targets to prevent the generation of antibodies to rATG and other DSA.

There are numerous reports relating to targeting IL-21 and CD40L for therapeutic benefit. For example, targeting IL-21 for therapy has been suggested to treat Crohn's disease, rheumatoid arthritis, and diabetes mellitus.[29-31] A review of US national library of medicine clinical trials database (www.clinicaltrials.gov) identified several clinical trials focused on the use of recombinant IL-21 to treat several types of cancer.[32-36] Inversely, there is a clinical trial using a recombinant anti-IL-21 monoclonal antibody in healthy subjects with rheumatoid arthritis. The study found that single doses (≤25 mg/kg IV; ≤4 mg/kg SC) were well tolerated and that the accumulation of IL-21–containing complexes suggests neutralization of IL-21.[37] In a similar manner, there are numerous clinical trials focused on the treating of cancer with CD40L[38-40] and anti-CD40L therapy has been the focus in the prevention of renal rejection in nonhuman primates[40] and as a potential novel treatment for autoimmune diseases.[41,42] In particular, anti-CD40L antibody lacking an Fc domain has been shown to inhibit CD40L-dependent immune responses without

thrombotic complications and effectively prevents nonhuman primate renal allograft rejection.[43,44]

We are cognizant to the possibility that reduced graft survival time observed in patients with a positive anti-rATG antibody titer is independent of the anti-rATG antibodies. For example, reduced survival time could be linked directly to the pathobiology associated with increased IL-21 levels. For example, studies have shown that increased IL-21 in T cells enhances cell cytotoxicity.[45] Moreover, IL-21 is also increased in other immune-mediated pathologies such as osteoarthritis.[17] However, IL-21 and CD40L levels are known to be unaffected by standard triple maintenance immunosuppression (calcineurin inhibitors + mycophenolate mofetil + steroids) typically used following renal transplant.[3] This evidence argues that high IL-21 and CD40L are potentially pathogenic for B-cell differentiation and antibody production.

At this time, we can only speculate regarding positive rATG titer in patients before administration of rATG. As rATG is primarily rabbit immunoglobulins, it is possible that patients with a positive anti-rATG titer have had previous exposure to rabbit immunoglobulins. In the alternative, they could be false positives in a similar manner to that seen with thyrotropin radioimmune assays.[46] Moreover, we are unable to categorically explain the observation of patients with positive pre-transplant anti-rATG antibody titer but a negative post-transplant anti-rATG antibody titer. An increase in anti-rATG titer following rATG treatment may be related to studies that show rATG induces complement-independent apoptosis of naive, activated, and plasma B cells.[47] Whatever the mechanism associating IL-21 and CD40L to renal graft loss the results of this study, in combination with works by others suggest that targeting IL-21 or CD40L signaling is a potential target for exploration to address anti-rATG antibodies in renal recipients.

Finally, additional studies are required to better understand the link between immune maintenance/protocol and anti-rATG antibodies titer. It is possible that immunosuppression combinations and patient compliance could be important. Studies have reported that monotherapy with tacrolimus is associated with higher rejection rates compared with 2 drug or 3 drug immunosuppression.[16] In a similar manner, differences in immunosuppression may manifest a risk factor for rATG induction.

CONCLUSIONS

Anti-rATG antibody-positive titer is associated with reduced outcomes for renal transplant recipient. Alternative targets to disrupt B-cell maturation pathways, such as IL-21 and CD40L, are potential areas for future studies.

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