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Quantitative and qualitative changes in anti-Neu5Gc antibody response following rabbit anti-thymocyte IgG induction in kidney allograft recipients

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Abstract

Antibodies of non-human mammals are glycosylated with carbohydrate antigens, such as galactose-α-1-3-galactose (α-Gal) and *N*-glycolylneuraminic acid (Neu5Gc). These non-human carbohydrate antigens are highly immunogenic in humans due to loss-of-function mutations of the key genes involved in their synthesis. Such immunogenic carbohydrates are expressed on therapeutic polyclonal rabbit anti-human T-cell IgGs (anti-thymocyte globulin; ATG), the most popular induction treatment in allograft recipients. To decipher the quantitative and qualitative response against these antigens in immunosuppressed patients, particularly against Neu5Gc, which may induce endothelial inflammation in both the graft and the host. We report a prospective study of the antibody response against α-Gal and Neu5Gc-containing glycans following rabbit ATG induction compared to controls. We show a drop in the overall levels of anti-Neu5Gc antibodies at 6 and 12 months post-graft compared to the pre-existing levels due to the major early immunosuppression. However, in contrast, in a cross-sectional study there was a highly significant increase in anti-Neu5Gc IgGs levels at 6 months post-graft in the ATG-treated compared to non-treated patients($P = 0.007$), with a clear hierarchy favouring anti-Neu5Gc over anti-Gal response. A sialoglycan microarray

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analysis revealed that the increased anti-Neu5Gc IgG response was still highly diverse against multiple different Neu5Gc-containing glycans. Furthermore, some of the ATG-treated patients developed a shift in their anti-Neu5Gc IgG repertoire compared with the baseline, recognizing different patterns of Neu5Gc-glycans. In contrast to Gal, Neu5Gc epitopes remain antigenic in severely immunosuppressed patients, who also develop an anti-Neu5Gc repertoire shift. The clinical implications of these observations are discussed.

1 | INTRODUCTION

Evolution has shaped the antigen patterns between humans and other mammals, and these patterns trigger vigorous immune responses when animal-derived molecules or tissues are introduced in humans.[1–4] Indeed, Humans and Old-World primates no longer express the galactose-α-1-3-galactose (α-Gal) epitope following a loss-of-function mutation of GGTA1 nor the Neu5Gc epitope following another mutation that affected the cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH) encoding gene.[1,2] All humans develop anti-Gal antibodies in response to the expression of α-Gal in the gut microbiota.[5,6] Anti-Neu5Gc antibodies are induced in the first year of life[7] following the consumption of dairy and red meat and human adults exhibit circulating anti-Neu5Gc antibodies of various isotypes directed against diverse Neu5Gc-containing epitopes due to dietary exposure.[8] In addition, in humans, Neu5Gc can be absorbed from a Neu5Gc-containing diet and then be detected, in small quantities, on some epithelial and endothelial cells.[9–11]

This peculiarity creates, at least theoretically, conditions of in situ immune complex diseases and tissue inflammation.[4,12]

Beside these pre-existing diet-induced anti-Neu5Gc antibodies, elicited anti-Neu5Gc antibodies can also result from exposure to animal-derived products, such as biotherapeutics,[13] engineered devices,[14] and cellular[15] or vascularized xenografts.[12,16–18] Non-immunosuppressed patients with severe burns treated by engineered pig skin dressings develop a long-lasting increase in their anti-Neu5Gc antibody levels.[14] Similarly, an intravenous (IV) injection of rabbit anti-human thymocyte IgGs, which display α-Gal and Neu5Gc carbohydrate antigens detectable by mass spectrometry analysis,[19,20] also induce a vigorous immune response against α-Gal and Neu5Gc antigens in non-immunosuppressed patients causing serum sickness in almost all cases.[21,22] In contrast, kidney recipients receiving ATG as an induction treatment in association with a cocktail of modern immunosuppressive agents develop a late anti-Neu5Gc response, and this response is stronger in patients who develop an early serum sickness disease (SSD).[13] Here, we investigated the early kinetics and nature of ATG-immunization in ATG-treated kidney recipients on prospectively harvested sera, and compared it with graft recipients who had not received ATG. Furthermore, we used sialoglycan microarrays to address possible repertoire changes of the anti-Neu5Gc specificities that are likely elicited by the highly diverse immunogenic Neu5Gc-glycans epitopes[8] and that may be a basis of possible inflammatory processes of patients endothelial cells in the long range.

2 | METHODS

2.1 | Patients

A total of 60 patients from the Institute of Clinical and Experimental Medicine (IKEM, Prague, Czech Republic) were included in the study between November 2014 and June 2015: A total of 40 patients were recipients of a first kidney graft, 14 received a second transplantation and six had a history of multiple kidney transplantations (Figure 1). Eight patients had multiple courses of ATG treatment and thus were also studied to explore the eventual shift of anti-Neu5Gc antibody repertoire following single or multiple courses of ATG induction. Patients' clinical characteristics and maintenance immunosuppression regimen are listed in Table 1. All patients received an induction treatment with Thymoglobulin® (ATG, Genzyme, Saint-Germain-en-Laye, France), at a mean total dose of 5 mg/kg, administered over five days.

As recommended,[23] the indication of ATG was restricted to recipients with at-risk clinical profile in terms of immunization, recipients of expanded-criteria donors of kidney grafts or re-transplantations. Therefore, the patients that had not received ATG differed in some pre-graft characteristics. Two non-ATG-treated cohorts comprised of 15 patients who received Simulect® (Basiliximab, anti-IL2R monoclonal antibody) induction and 15 patients with no induction treatment. Patients of control groups were matched to patients in the ATG cohort by recipient age and gender. However, while rabbit ATG was given as induction to patients with presence of PRA > 20%, anti-

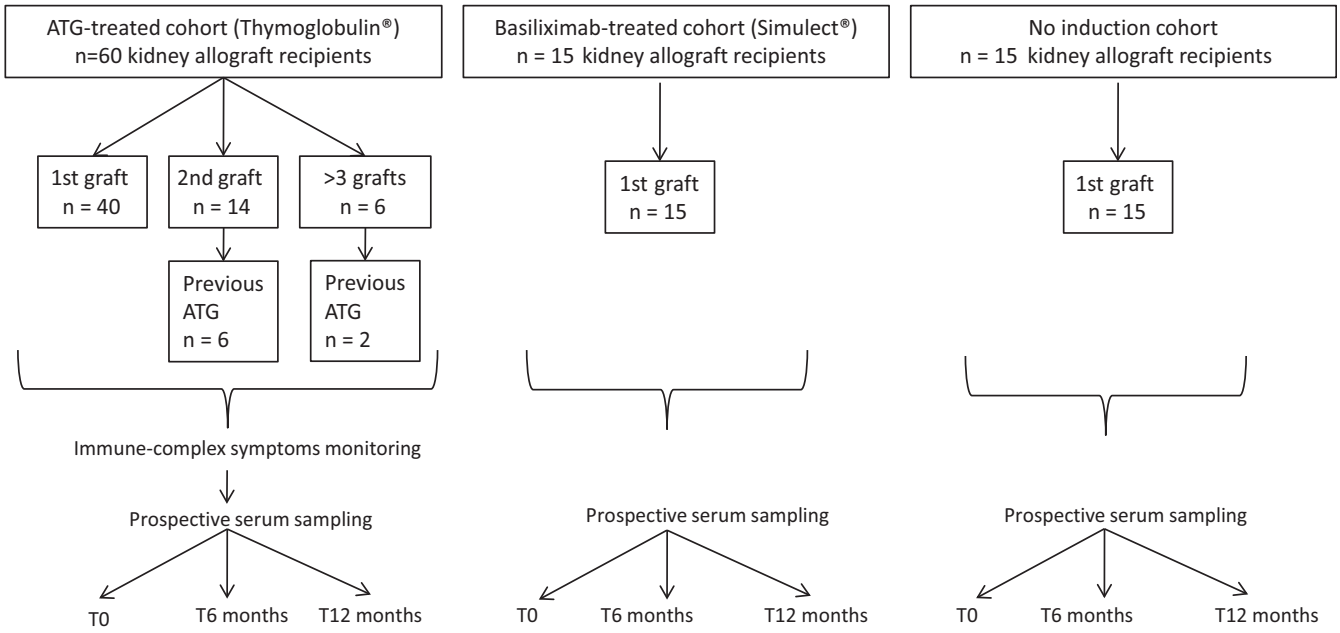

FIGURE 1 Flow chart of the study cohorts: serum sampling strategy and time-points

Human Leucocyte Antigen (HLA) antibodies, or recipients of expanded-criteria donors kidney grafts or re-transplantations, Basiliximab or no induction was given to first kidney transplant recipients at a low risk. All patients on the waiting list were screened routinely for the presence of PRA by a complement-dependent cytotoxicity test. The specificity of HLA antibodies was analysed by LABScreen Mixed and Single Antigen (SAB) class I and class II beads (One Lambda Inc) using the Luminex 200 flow analyzer (One Lambda Inc) and the HLA Fusion software (version no.2).

All patients signed informed consent. The study protocol was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine (A 13-02-01 (83/13)).

2.2 | Measurement of anti-Neu5Gc antibodies, anti-Gal antibodies and total anti-rabbit IgG antibodies (anti-ATG)

Three serum samples were serially and prospectively collected at pre-transplantation (T0), 6 months (T6) and 12 months (T12) following transplantation (Flow chart in Figure 1) for these three cohorts. The tests were carried out while blinded. The study fulfilled the ethical rule of the University of Prague, Tel Aviv and Nantes in term of informed consent and anonymous processing of data.

Anti-Neu5Gc IgG was quantified using an ELISA originally described by Padler-Karavani V. et al[24] Anti-Gal was quantified using ELISA according from Buonomano et al[25] The quantification of the human serum IgGs against rabbit IgGs (Thymoglobulin®, Sanofi-Genzyme, Cambridge, Massachusetts, USA) was done according from Prin-Mathieu et al[26] The titration curve used was the same as for the anti-Neu5Gc assay.

2.3 | Sialoglycan arrays

Sialoglycan microarrays were fabricated (Version 5.0), developed and analysed as described[27] (list and structure of glycans available in Table S1 and Appendix S1).

2.4 | Statistical analyses

For the analysis of clinical data, as described in Table 1, continuous variables were analysed using a Kruskal-Wallis test and the categorical data were compared using a Pearson's chi square test. For ELISA analysis, comparisons between time-points for patients of the same group were performed using paired *t* tests, while comparisons between groups (ATG induction vs Basiliximab induction vs without induction) were performed using non-parametric Mann-Whitney tests. In the sialoglycan array analysis, two-tailed unpaired student's *t* test was used for group comparison.

3 | RESULTS

3.1 | Main clinical events

Although the study was not devoted to compare the clinical outcome between the different groups, the main post-transplant events are briefly provided below. In the entire ATG-treated group (n = 60), post-ATG clinical events were observed in two patients with good graft outcome who presented skin urticarial rashes. One was a first transplantation;

TABLE 1 Demographic and clinical characteristics of the cohorts

	ATG	Basiliximab	No induction	*P* value
Number of patients (n)	52[a]	15	15	
Recipient age, years[#]	52 [32; 76]	53 [39; 64]	56 [28; 63]	0.956
Recipient gender, male, n (%)	34 (65.4%)	8 (53.3%)	7 (46.7%)	0.366
Dialysis vintage, months[#]	30.6 [0; 97.2]	24 [7; 56.3]	21.1 [2; 50.2]	0.356
Donor age, years[#]	57 [19; 75]	64 [47; 75]	47 [22; 69]	0.001
Retransplantation	14 (25%)	0	0	0.012
Cold ischaemia, hours[#]	15.5 [6; 28]	17.4 [14; 21]	15.2 [11; 21]	0.115
HLA mismatches[#]	3 [0; 6]	4 [2; 5]	3 [2; 6]	0.129
PRA max[#]	18 [0; 100]	12 [0; 44]	4 [0; 22]	0.042
Maintenance immunosuppression				0.099
Tacrolimus/MMF/steroids	49	15	14	
Cyclosporine A/MMF/steroids	0	0	1	
Everolimus/MMF/steroids	3	0	0	
Creatinine (μmol/L) at 1 y[#]	135 [71;306]	162 [94; 288]	129 [73; 260]	0.230
Rejections, n (%)				0.507
Antibody-mediated rejections	9	2	1	
T-cell–mediated rejections	4	3	1	
PRA values >10	34	9	5	
PRA values >20	29	8	2	

[a]Among the 60 patients included in this study, only the 52 patients with a first course of ATG were analysed in the table and selected for ELISA analysis.
[#]data are presented as medians [min;max]. PRA: Panel-Reactive Antibodies; Tx: Transplantation; MMF: Mycophenolate Mofetil. The continuous variables were compared by Kruskal-Wallis test, and the categorical data were compared with a Pearson's chi square test.

the other underwent a second graft and a second course of ATG. Three patients were treated by methylprednisone (MP) for a biopsy-confirmed T-cell–mediated rejection (TCMR). One patient had untreated vascular rejection with concomitant BK virus nephropathy. In five patients, early acute antibody-mediated rejections (ABMR) occurred within two months after transplantation with positive donor-specific HLA antibodies, and they were treated with a combination of plasmapheresis, IVIG, MP, bortezomib and rituximab. In the Basiliximab-treated group (n = 15), two patients developed TCMR within the first week after transplantation and were treated with MP. One patient had TCMR at 3 months protocol biopsy and was treated with MP and one patient had early acute ABMR and was treated with plasmapheresis and IVIG. In the "no induction" group (n = 15), one early TCMR treated by MP and one early TMA treated by plasmaphereses were observed.

3.2 | Serial comparison of anti-ATG antibody levels in the different groups at baseline, 6 months and 12 months

The kinetics of the global anti-ATG antibody concentrations were analysed by ELISA in the various serial sera from the ATG-treated group (first course of ATG only), comparing serially the basal antibody levels to the levels at 6 and 12 months post-ATG infusion in each patient, allowing a paired approach (Figure S1). There was a non-significant trend for decreased levels in the global anti-ATG IgG levels at T6 and T12 following ATG induction (Figure S1A).

More specifically, serial changes in anti-Neu5Gc and anti-Gal IgG levels (Figure S1B,C, respectively) showed a reduction in antibody levels at T6 (paired *t* test $P = 0.0003$ and $P < 0.0001$, respectively) compared to the pre-existing levels, a decrease still detectable at T12 ($P = 0.03$ and $P = 0.0005$, respectively). Comparisons of anti-ATG, anti-Gal and anti-Neu5Gc IgG titres were also performed on the patients without ATG as a whole, as well as independently in the two control subgroups of recipients with no induction (n = 15) or with Basiliximab (Simulect, n = 15) induction(Figure S2) owing the fact that Basiliximab does not carry Neu5Gc epitope.[28] There was an increase in anti-Gal IgG between T6 and T12 for the group without induction ($P = 0.03$, Figure S2E). For anti-Neu5Gc IgG levels, there was a decrease between T0 and T12 months in the group with Basiliximab induction ($P = 0.0098$, Figure S2D). Finally, anti-ATG IgG antibodies, were significantly decreased between T0 and T6 in patients without induction and with Basiliximab induction ($P = 0.002$ for

both groups), followed by an increase in titres between T6 and T12 (respectively, $P = 0.007$ and $P = 0.014$, Figure S2A,B). Altogether, this global decrease compared to the basal levels, which was not observed in non-immunosuppressed patients receiving ATG,[22] demonstrates the strong effect of immunosuppression on antibody responses against the various antigens on ATG.

3.3 | Transectional comparison of antibody levels

A transectional comparison was then performed at 6 months following transplantation, comparing patients with ATG induction (n = 52 first ATG course) to patients without ATG induction, either pooled (combining the Basiliximab induction (n = 15) and no induction (n = 15)) or separately. The T6 anti-ATG levels were heterogeneous, with 10 patients displaying no significant anti-ATG response, in contrast to the remaining patients (n = 42), whose T6 anti-ATG levels ranged from 0.1 to 26.7 μg/mL (with similar heterogeneity in the control group ranging from 0.04 to 38.4 μg/mL). There was no difference in the global anti-ATG or in anti-Gal IgGs levels between the groups at T6 (Figure 2A,C). In contrast, the ATG-treated patients had a highly significant increased anti-Neu5Gc IgG levels in comparison to no-ATG induction treatment at T6 ($P = 0.008$, Figure 2B). The difference remained significant for the comparison with the no-induction group ($P = 0.009$), but did not reach significance for the Basiliximab-treated group ($P = 0.13$, Figure S3). Finally, the differences remained significant between ATG-treated and no induction patients ($P = 0.017$) when the second grafts were excluded. Altogether, these data show a hierarchy in immunogenicity of Neu5Gc versus Gal in immunosuppressed patients, and indicate that even an immunosuppressed conditions, patient are exposed to higher levels of elicited anti-Neu5Gc IgGs for several months.

3.4 | Sialoglycan arrays

A limited number of patient serum samples were selected for an in-depth repertoire analysis using sialoglycan arrays. Eight control patients with no induction were compared to eight patients with ATG induction at T0 and T6 months post-transplantation. These eight patients were selected from the total cohort, with different levels of anti-Neu5Gc IgGs (high, medium or low total levels, ranging from 0.6 to 1.5 μg/mL at T6, and from 0.0 to 4.3 μg/mL at T12, by ELISA measurements), and with various clinical history. Two patients among them had received a second graft, and had a previous ATG course. Likewise, the no-induction control patients were selected to represent the variety of antibody levels observed at T6 after transplantation (from 0.0 to 38.4 μg/mL global anti-ATG and from 0.05 to 1.4 μg/mL anti-Neu5Gc IgG, by ELISA) and under the same conditions of immunosuppression. Changes in anti-Neu5Gc IgG repertoire were analysed through the differential binding of the antibodies on a panel of Neu5Gc-containing glycans or *N*-acetylneuraminic acid-(Neu5Ac)-containing glycans displayed on the arrays, before (T0) and 6 months after ATG (T6).

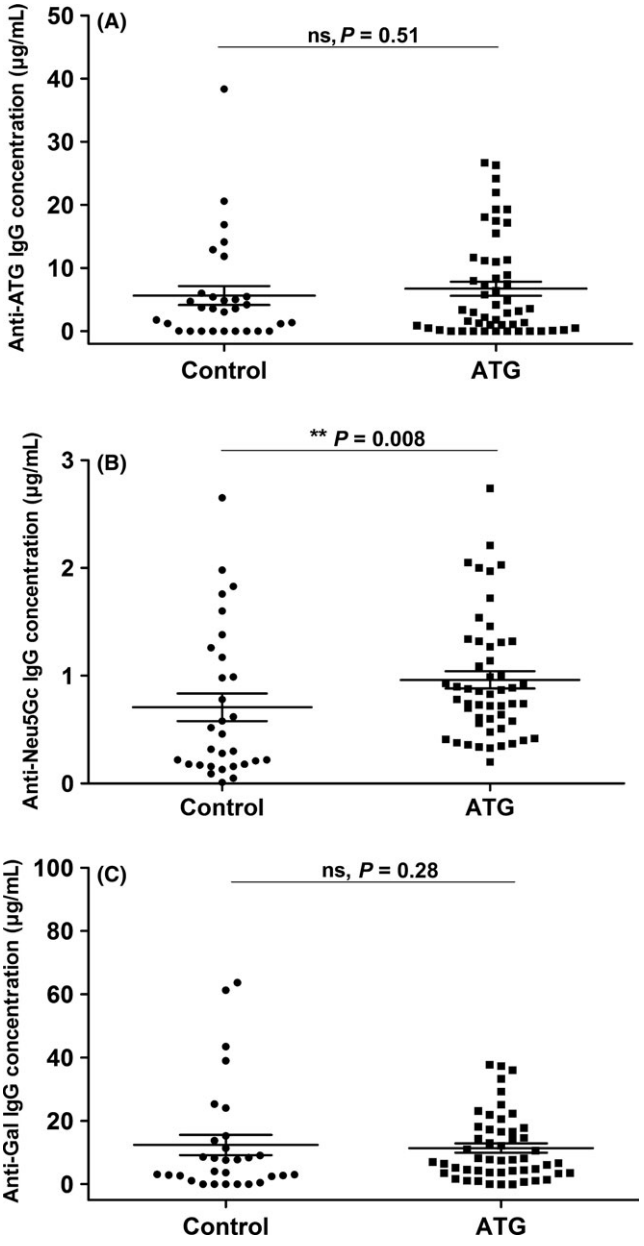

FIGURE 2 Anti-ATG (A), anti-Neu5Gc (B) and anti-Gal (C) IgG titres in the kidney allograft recipients with a first-time ATG induction (n = 52), or without ATG induction (controls including Basiliximab (Simulect) induction (n = 15) or no induction (n = 15)) at the 6-month post-transplantation time-point, as assessed by ELISA. The concentrations are indicated in μg/mL for each antibody tested. The comparisons between groups were performed using a non-parametric Mann-Whitney test. ns, not significant, $^{**}P < 0.005$

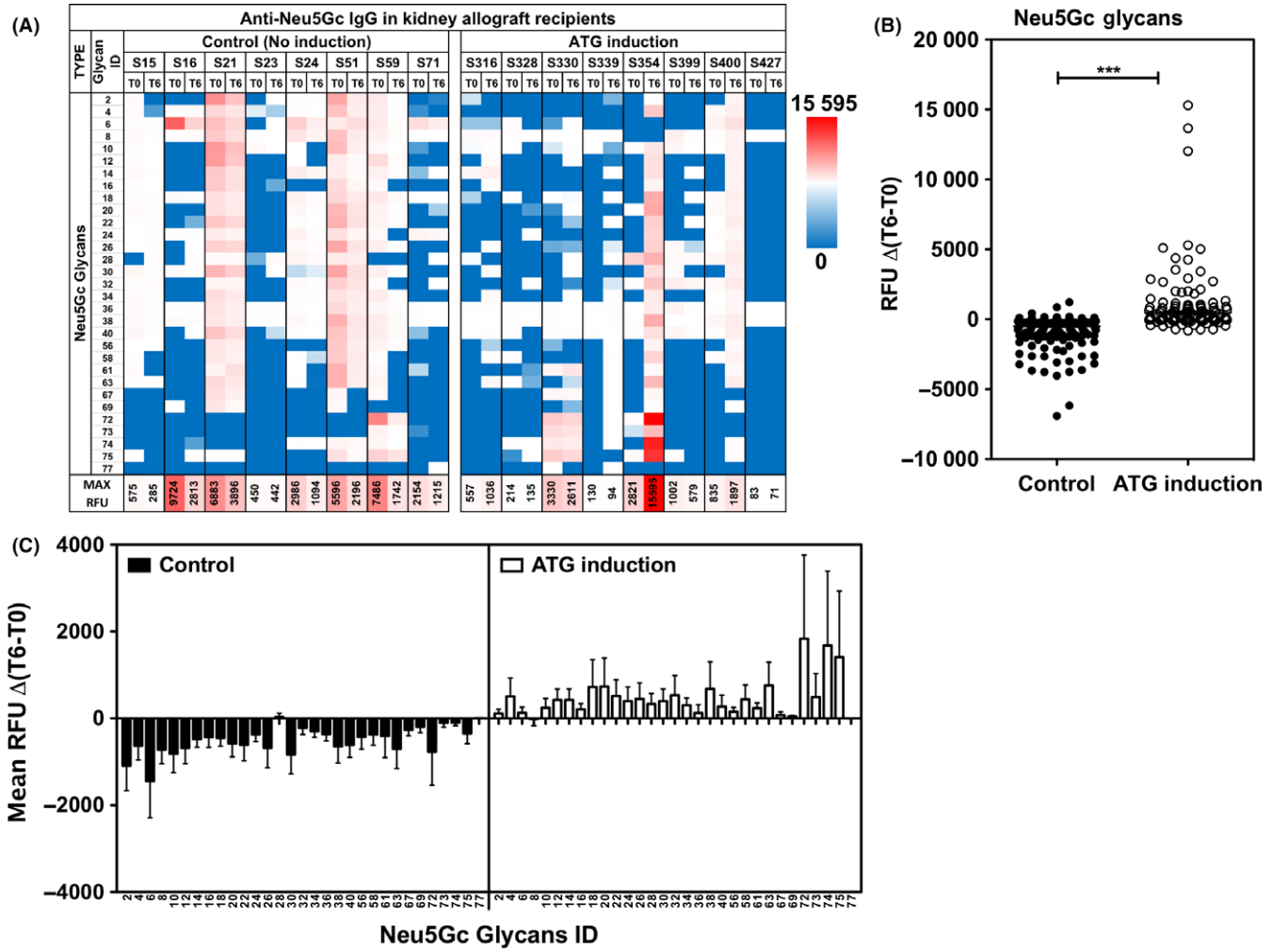

FIGURE 3 Analysis of the qualitative changes in anti-Neu5Gc specificities following ATG treatment. A, Sera samples at T0 and T6 from the 8 ATG-treated patients and eight controls with no-induction therapy were tested at a 1:100 dilution on the sialoglycan microarrays and were then detected by Cy3-anti-human IgG, Fcγ specific (1.5 μg/mL). The relative fluorescence units (RFU) of all the mono-sialylated Neu5Gc-glycans (Heatmap across all samples: red-white-blue represent maximum-50th percentile-minimum reactivity, respectively; minimum and maximum values are indicated in the Figure). The glycan structures and results of the array are detailed in Table S1. B, The difference in the anti-Neu5Gc IgG reactivity per glycan per patient between T6 and T0 (RFU at T6 per glycan minus RFU at T0 per glycan, in each serum sample). The negative RFU values for Δ(T6-T0) represent a decrease in reactivity, while positive RFU values for Δ(T6-T0) demonstrate an increase in reactivity, $^{***}P < 0.0001$, using a two-tailed unpaired Student's *t* test. C, The average difference in anti-Neu5Gc IgG reactivity per glycan across the patient/controls between T6 and T0 (the RFU at T6 per glycan minus the RFU at T0 per glycan, in each serum sample)

Figure 3A shows the overall reactivity restricted against diverse Neu5Gc-glycans in the ATG-treated or no-induction controls at T0 and T6. In both the controls and ATG-treated patients, a difference in the anti-Neu5Gc IgG repertoire was noticed between patterns at T0 and T6 (Figure 3A). However, a further qualitative analysis of these repertoire changes clearly differentiated between the ATG-treated and no-induction controls (Figure 3B,C). For this purpose, we computed the difference in anti-Neu5Gc IgG reactivity per glycan per patient between T6 and T0 (RFU at T6 per glycan minus RFU at T0 per glycan, in each serum sample). As shown in Figure 3B, whereas the anti-Neu5Gc IgG reactivity against diverse glycans decreased in the control group (negative values for RFUΔ(T6-T0)), there was a highly significant increase in the ATG-treated group (positive values for RFUΔ(T6-T0)). This was further corroborated by averaging these values per glycan across patients (Figure 3C). Thus, using a different method (binding to synthetic glycans by array versus ELISA screen against serum Neu5Gc-glycoproteins from wild-type mice), we also confirmed the results of the ELISA in the whole group and the increased response against Neu5Gc epitopes in the ATG-treated group, that is in contrast to a decreased response in the no-induction patients (Figure 3B,C). Changes in anti-Neu5Gc IgM repertoire were only observed in patient S354 at T6 (Figure S4A), a patient who also had a major shift in his anti-Neu5Gc IgG repertoire (Figure 3). Figure S4B,C also shows no increased anti-Neu5Gc IgM levels in the trans-sectional analysis.

3.5 | Clinical correlations

The clinical outcome of the patients with the highest baseline anti-Neu5Gc levels, the more pronounced anti-Neu5Gc repertoire shift or with the highest increased in ATG-elicited anti-Neu5Gc titres was then examined in detail.

One patient (318) out of the three patients with the highest pre-ATGanti-Neu5Gc titres presented an antibody-mediated rejection (ABMR) at 3 months. Despite the fact that this patient was broadly sensitized (peak PRA 42%), no donor-specific antibodies (DSA) were detected. One other patient (354) out of the three with the more pronounced anti-Neu5Gc IgG repertoire shift also presented an ABMR. Patient 354 (having also an IgM repertoire shift) was a lung transplant recipient with cystic fibrosis who had a former lung rejection episode treated by 150 mg of Thymoglobulin. This patient who subsequently developed a kidney failure (CNI toxicity), necessitated a kidney graft for which he received a second course of ATG of 150 mg (Cumulative dose: 3.9 g). Despite an ABMR with mild glomerulitis and borderline changes, no DSA were detected. Finally, none of the patients with the highest titres of elicited anti-Neu5Gc at six months had rejection and their protocol biopsies were normal. Although anecdotal, because these two cases without detectable DSA could suggest that anti-Neu5Gc may have contributed to some lesions, Table S2 gives the main clinical information with the histology Banff score, the anti-C4d staining and the level of pre-graft immunization. Of note, the renal function was not significantly altered at one year.

4 | DISCUSSION

Animal-derived molecules[22] or engineered tissues[14] are highly immunogenic. ATG, a widely used polyclonal rabbit IgG preparation that expresses α-Gal and Neu5Gc-glycans according to a mass spectrometry analysis, induces a vigorous response against these two xeno-antigens in non-immunosuppressed patients.[21,22] However, as for allo-antibodies, the immune response against these foreign glycans was dramatically delayed and decreased by modern immunosuppressive cocktails, as recently described.[13] This previous study was, however, retrospective with no comparison to patients without ATG induction, and no analysis of the anti-Neu5Gc repertoire was done.

Here, we revisited the quantitative and qualitative response of kidney transplant recipients treated with or without an ATG-based induction with blood samples prospectively collected during the first year post-transplantation. Considering this response longitudinally (ie, compared to the pre-ATG antibody values), quantitative differences based on the ELISA showed a significantly decreased anti-Gal and anti-Neu5Gc IgG antibodies titres at 6 and 12 months post-treatment. This result suggested that the immunosuppressive drugs efficiently dampened the response against these glycans as for other antigens of the graft, such as HLA, for which a response occurs in only a few immunosuppressed patients.[29]

However, in the ATG-treated group there was a highly significant increased anti-Neu5Gc IgG levels. The difference remained significant even after excluding the patients who had several ATG courses, showing that a single ATG course is able to trigger Neu5Gc-immunization still detectable after 6 months. The difference was also significant when second graft recipients were excluded, ruling out a role of a high responder phenotype. This confirmed the hierarchy of response still favouring anti-Neu5Gc compared to anti-Gal in strongly immunosuppressed patients.[13]

The comparison with patients who received Basiliximab did not reach a significant difference, suggesting that despite the negative mass spectrometry patterns on Basiliximab,[28] traces of xeno-glycans[30,31] are immunogenic.

The diversity of Neu5Gc epitopes in glycans is enormous in contrast to α-Gal, generating a collection of epitopes with a terminal Neu5Gc deciphered using sialoglycan arrays.[27] In addition, whereas pre-existing anti-Neu5Gc antibodies (ie, diet induced) are present in all humans, ATG-elicited anti-Neu5Gc IgGs recognize new specificities and with higher affinities that could potentially induce a local inflammation, which had been coined "xenosialitis".[21,32–34] This inflammation may theoretically result from the presence, even at a low level, of the Neu5Gc antigen itself on endothelial cells.[9,11] The detailed analysis of the arrays performed on eight patients in this study showed a clear increase in the anti-Neu5Gc IgG repertoire and reactivity at 6 months post-ATG, in contrast to the controls. Despite the major immunosuppression, these data fit with those obtained in non-immunosuppressed patients.[21] These results thus sustain the possibility that ATG can extend the pre-existing anti-Neu5Gc antibodies' repertoire to new specificities, leaving xenosialitis conceivable. As mentioned above, tentatively analysing clinical correlation in some of potentially informative patients remained anecdotal in the context of this study devoted to a detailed analysis of the anti-Neu5Gc and Gal response. Nevertheless, two patients were diagnosed with ABMR without detectable DSA among the 6 who had either the highest baseline anti-Neu5Gc levels or the most pronounced repertoire shift. As several types of antibodies may contribute to ABMR in patients without DSA,[35] these two patients with well-characterized ABMR suggest to further explore the possibility that anti-Neu5Gc antibodies may contribute to endothelial inflammation. The two patients had creatinine maintained at the normal range at one year suggesting minor residual. Only further investigation based on large series and longer survey may establish a potential safety concern following induction with rabbit-derived IgG.

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CONFLICT OF INTEREST

JR, AS, GE, OD and DB are currently employees of Xenothera. J-MB and J-PS are cofounders of Xenothera. OV received a speaker´s fee from Sanofi.

AUTHOR CONTRIBUTIONS

J-PS, VPK and OV led the project, designed the experiments and wrote the manuscript. PH and JS organized the patient cohorts, collected the serum for the analysis and performed the clinical analysis of the cohort. JR, AS, L.B-AS, GE, HY, XC, J-MB and DB conducted the experiments and analysed the data. HY, XC and OD provided reagents and support. All authors reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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