

# Anti-Gal and Anti-Neu5Gc Responses in Nonimmunosuppressed Patients After Treatment With Rabbit Antithymocyte Polyclonal IgGs

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**Background.** Polyclonal antihuman thymocyte rabbit IgGs (antithymocyte globulin [ATG]) are popular immunosuppressive drugs used to prevent or treat organ or bone-marrow allograft rejection, graft versus host disease, and autoimmune diseases. However, animal-derived glycoproteins are also strongly immunogenic and rabbit ATG induces serum sickness disease in almost all patients without additional immunosuppressive drugs, as seen in the Study of Thymoglobulin to arrest Type 1 Diabetes (START) trial of ATG therapy in new-onset type 1 diabetes. **Methods.** Using enzyme-linked immunosorbent assay, we analyzed serial sera from the START study to decipher the various anti-ATG specificities developed by the patients in this study: antitotal ATG, but also antigalactose- $\alpha$ 1-3-galactose (Gal) and anti-Neu5Gc antibodies, 2 xenocarbohydrate epitopes present on rabbit IgG glycans and lacking in humans. **Results.** We show that diabetic patients have substantial levels of preexisting antibodies of the 3 specificities, before infusion, but of similar levels as healthy individuals. ATG treatment resulted in highly significant increases of both IgM (for anti-ATG and anti-Neu5Gc) and IgG (for anti-ATG, -Gal, and -Neu5Gc), peaking at 1 month and still detectable 1 year postinfusion. **Conclusions.** Treatment with rabbit polyclonal IgGs in the absence of additional immunosuppression results in a vigorous response against Gal and Neu5Gc epitopes, contributing to an inflammatory environment that may compromise the efficacy of ATG therapy. The results also suggest using IgGs lacking these major xenoantigens may improve safety and efficacy of ATG treatment.

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Rabbit polyclonal antihuman T cell IgGs, such as antithymocyte globulin (ATG) (Thymoglobulin), are popular immunosuppressive treatments,<sup>1,2</sup> notably used in the field of solid organ transplantation. Animal-derived (eg, rabbit or equine) polyclonal IgGs display xenoantigens resulting from differences in peptide sequences and posttranslational molecular motifs. These motifs include glycans branched on

the conserved canonical 297 asparagine on the IgG Fc fragment<sup>3,4</sup> or N- or O-branched sugars on the IgG Fab hyper-variable region.<sup>3,4</sup> Immunization against animal-derived IgGs may be associated with severe side effects as shown by the high incidence of serum sickness disease (SSD) in young patients with type 1 diabetes (T1D) treated with ATG.<sup>5</sup> There is an apparent gradient of SSD incidence after ATG administration

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A.S., G.E., J.R., and L.L.B. organized and performed the experiments, analyzed the data, prepared the figures and wrote the article. N.L. performed the statistical analyses and prepared the figures. A.N. provided the cytidine monophosphate acetyl hydroxylase KO mice and reviewed the article. S.B. contributed to find the funding for the experiments and reviewed the article. K.M.H., M.R.E., and S.E.G. directed the START study, prepared the patients samples, analyzed the clinical data, and reviewed the article. J.-P.S. directed the project and wrote the article.

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according to the strength of additional immunosuppression (IS), ranging to an almost 100% incidence in patients without IS,<sup>5</sup> to 25-30% with moderate IS regimens,<sup>6,7</sup> and to less than 10% with powerful modern IS in kidney transplant recipients.<sup>8</sup> Such strong immunogenicity may preclude efficient use of ATG in indications other than IS, such as in the prevention or treatment of infectious diseases,<sup>9</sup> because the recipient immune response may rapidly produce neutralizing antidrug antibodies. In addition, the occurrence of SSD may undermine the hoped-for efficacy of ATG therapy in autoimmunity or organ transplantation, due to an inflammatory environment, generation of immune complexes, and reduced drug bioavailability in the absence of additional immunosuppressive and anti-inflammatory agents. Moreover, several lines of evidence indicate that preexisting or acquired antixenoglycans can also have immediate<sup>10</sup> or delayed toxicity.<sup>8</sup> Early biochemical studies of the major antigenic determinants of ATG stressed the role of “heterophilic” epitopes, and particularly of the Neu5Gc antigen, which was described as the “serum-sickness antigen.”<sup>11-14</sup>

Humans differ from most mammals with respect to 2 gene loss-of-function mutations that affect the shape of oligosaccharides of glycoproteins and glycolipids such as sphingolipids: the Galactosyl-Transferase-1 (GT1) gene<sup>15</sup> encoding an  $\alpha$ 1-3-galactosyl transferase that catalyzes branching of galactose residues, and the cytidine monophosphate acetyl hydroxylase (CMAH) gene,<sup>16</sup> a hydroxylase generating the Neu5Gc species of neuraminic acid.<sup>17</sup> Almost all human sera exhibit “natural” antibodies against the 2 carbohydrate antigens galactose- $\alpha$ 1-3-galactose (Gal) and Neu5Gc. Neu5Gc conformational epitopes induce a prolonged IgG response in patients who have received engineered pig skin for the treatment of burns.<sup>18</sup> Rabbit IgGs that display Neu5Gc as well as, to a lesser extent, Gal, can also immunize strongly immunosuppressed patients such as kidney recipients under a combination of calcineurin inhibitor, mycophenolate mofetyl and steroids.<sup>8</sup> Although preexisting antibodies and evoked early immune responses against rabbit IgGs may result in immune complexes able to trigger SSD (the most clinically assessable form of immune complex disease), the presence of Neu5Gc of dietary origin on human endothelia and epithelia<sup>8,19,20</sup> also potentially contributes to other types of clinical conditions related to possible in situ “planted”<sup>21</sup> immune complex diseases with a potential for chronic activation of vessel walls.<sup>8,22</sup> Indeed, deciphering the fine specificities of the immune response against rabbit IgGs will allow to further characterize the effect of these antibodies on human endothelial cells, with possible direct consequences on graft recipients in the context of transplantation.

In this study, we took advantage of the unique samples from the randomized Study of Thymoglobulin to arrest Type 1 Diabetes (START) trial, which studied the effect of ATG in young adults with recently diagnosed T1D<sup>5</sup> receiving only minimal early IS. We show that these patients mount a vigorous humoral response against both xenopeptide and xenoglycan motifs and we report extremely high levels of IgGs against Neu5Gc in some individuals. Our observation could impact the use of animal-derived polyclonal IgGs in other diseases, such as infectious diseases, as well as the use of animal-derived bio-devices, such as biological heart valves, which contain the same antigens.

## MATERIALS AND METHODS

### Patient and Healthy Individual Samples

Sera were obtained from patients in the START study (<http://www.type1diabetestrial.org/>). START was a randomized, placebo-controlled, phase II clinical trial, with participants from 11 clinical centers in the United States, which evaluated the effect of Thymoglobulin (Genzyme) in patients with recent-onset T1D.<sup>5</sup> Briefly, patients with T1D within 100 days of diagnosis, aged 12 to 35 years, were randomized to ATG or placebo. Table 1 summarizes the major characteristics of the population tested. An independent data and safety monitoring board (DSMB) conducted regular safety reviews. The protocols and consent documents were approved by independent institutional review boards. All participants or their parents provided written informed consent, and those younger than 18 years provided assent.<sup>5</sup> Clinical data recorded in the START study were anonymously available for correlation with the tested anti-ATG antibody titers obtained in the present study. The patients in the ATG group received systemic steroids during drug infusions and after onset of SSD. The placebo group did not receive glucocorticoids. Aliquots (0.5 mL) of serum of ATG- or placebo-treated patients, stored at  $-80^{\circ}\text{C}$ , corresponding to pre-ATG (day 0) or to post-ATG infusion blood samples (at 1, 3, 6, and twelve months) were shipped to the Inserm U1064 Laboratory (Nantes, France), coded and anonymous, according to a co-signed TrialShare Sample Request document (Sample Sharing Agreement, Version 1.6, rev 9.15.14).

For healthy individuals, serum samples were part of a collection obtained from the regional blood bank of the “Etablissement Français du Sang- EFS,” as described previously.<sup>8</sup> Serum samples were coded and anonymous, under an ethical EFS agreement after informed consent, and stored at  $-80^{\circ}\text{C}$ . The samples from the healthy individuals were matched for age ( $\pm 2$  years) and sex with the START samples, and were used to compare preinfusion anti-ATG, anti-Gal, and anti-Neu5Gc antibody titers of the diabetic patients with the values of healthy individuals.

**TABLE 1.**

**Main baseline and follow-up clinical characteristics, patient information, and demographic variables of the START study participants used in this article**

	Treatment group (n = 38)	Placebo group (n = 20)
Age, y	19.4 (6.6)	20.5 (7.0)
12-21	26 (68%)	12 (60%)
22-35	12 (32%)	8 (40%)
Men	24 (63%)	11 (55%)
Ethnic origin		
White	32 (84%)	17 (85%)
Nonwhite	6 (16%)	3 (15%)
Body-mass index	22.8 (3.4)	24.4 (3.4)
Days since diagnosis	69.0 (21.0)	76.5 (18.0)
Baseline 2-h C-peptide AUC, pmol/mL	0.857 (0.371)	0.932 (0.502)

Data are mean (SD) or n (%).

## Measurement of Antirabbit IgG and IgM Antibodies

### Total Antirabbit Antibodies

Quantification of human serum IgGs against rabbit IgGs was adapted from Prin-Mathieu et al.<sup>23</sup> Plates (NUNC Maxisorp; NUNC AB) were coated overnight with ATG (1 µg/mL) in 50 mM sodium carbonate-bicarbonate buffer (pH 9). Wells were blocked for 2 hours at 37°C with phosphate-buffered saline (PBS), 0.05% Tween20 (Sigma-Aldrich), and 1% Ovalbumin (PBSTO, Sigma-Aldrich). Human serum samples diluted 1:1000 to 1:80,000 in PBS 0.05% Tween20 (PBST) were added and incubated for 2 hours at 37°C, before the addition of a horseradish peroxidase (HRP)-donkey antihuman IgG (H + L) (1:5,000, 709-035-149, Jackson ImmunoResearch) for 1 hour at 37°C, and development using TMB substrate (Sigma-Aldrich). For standard curves, wells were coated with serial dilutions of human polyclonal IgGs (concentrations starting at 400 ng/mL; Privigen, CSL Berhing, SA). Quantification of human serum IgM against ATG used serum dilutions of 1:100, a HRP-goat antihuman IgM (µ-chain-specific) secondary antibody (1:1,000, A0420, Sigma-Aldrich) and purified human IgMs (I8260, Sigma-Aldrich) standard, with an initial concentration of 400 ng/mL.

### Anti-Neu5Gc Antibodies

IgG and IgM anti-Neu5Gc antibodies were quantified using an enzyme-linked immunosorbent assay (ELISA) and mouse serum proteins as coating antigens in the ELISA plate.<sup>24</sup> Briefly, the plates were coated overnight at 4°C with wild-type mouse serum at 1 µg/well diluted in 50 µL of coating buffer. Diluted human sera were preincubated for 2 hours at 4°C with CMAH-KO mouse serum diluted 1:4,000 in PBSTO. After washing and blocking, pretreated human sera were added to the plates for 2 hours at room temperature. IgGs and IgMs were detected as described previously.<sup>18</sup> Standards for IgGs and IgMs were as described above for antirabbit antibodies. CMAH-KO mice sera were prescreened for absence of potential antibodies cross-reacting with human serum determinants, as described in Padler-Karavani et al,<sup>24</sup> and checked for Neu5Gc negativity using the Neu5Gc-specific chicken IgY antibody (Biolegend, San Diego, CA) and secondary HRP-coupled anti-IgY antibody (Abcam, Cambridge, UK).<sup>24</sup>

### Anti-Gal Antibodies

The IgG and IgM anti-Gal ELISA was adapted from Buonomano et al.<sup>25</sup> Plates were coated with Gal1-3Gal-polyacrylamide conjugate (5 µg/mL, PAA-Bdi; Lectinity, Moscow, Russia) overnight at 4°C, and blocked with PBS 0.5% fish gelatin (Sigma-Aldrich) for 2 hours at 37°C. Human sera (at 1:1,000, 1:2,000, and 1:4,000 in PBST) were incubated for 2 hours at 37°C. A rabbit antihuman IgG and an HRP-goat antirabbit antibody (both at 1:2,000; Jackson ImmunoResearch) were used as secondary antibodies. Standards for IgGs and IgMs were as described above for antirabbit antibodies.

### Measurement of Circulating ATG Levels

Circulating ATG levels were measured using the Rabbit IgG ELISA Quantitation Set (Bethyl Laboratories Inc.), according to the manufacturer's specifications.

## Statistical Analyses

Mixed model for repeated measures was applied to compare between treatment groups and various time points. Comparisons of baseline levels in the START participants with healthy volunteers were performed using unpaired *t* tests. Correlations among IgG and IgM levels for the different antibody specificities and between antibodies and IL-6, IL-10, and C-peptide areas under the curve (AUCs) were analyzed using a Pearson correlation test. IL-6, IL-10, and all the antibody levels were log-transformed before they were fitted into the statistical analyses.

## RESULTS

### Preexisting and Elicited Antirabbit IgG Responses

As previously demonstrated, rabbit IgGs display Neu5Gc and Gal xenoantigens, detectable by mass spectrometry.<sup>8</sup> Here, antibodies against these 2 epitopes, as well as against the global rabbit IgG molecules, were measured before and at various time points after ATG infusion.

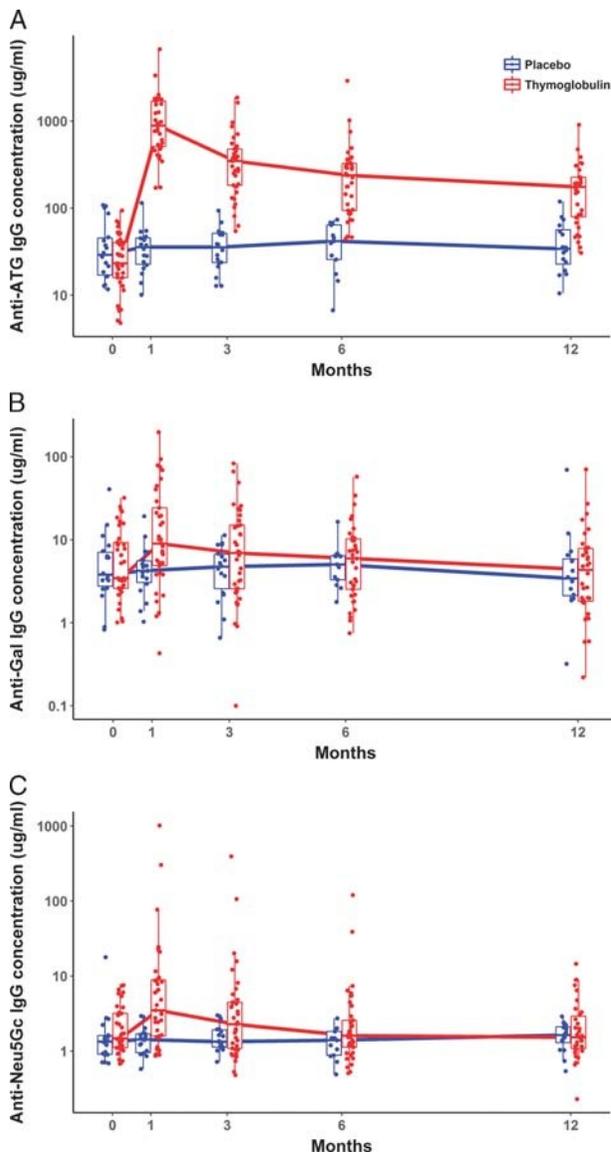
### Antitotal Rabbit IgGs

Baseline levels of preexisting anti-ATG antibodies were assessed based on the entire START cohort (ie pre-ATG samples). All patients had detectable levels of antirabbit IgGs before treatment, with a median of 29.1 µg/mL (interquartile range (IQR) from 16.9 to 45.3 µg/mL) for the placebo group (n = 18) and 23.2 µg/mL (IQR from 15.9 to 40.4 µg/mL) in the ATG-treated group (n = 37).

We analyzed the response against rabbit IgGs (post-infusion samples) for the START patients treated with study drug compared to those having received the placebo (saline), using a paired comparison within each group (Figure 1A). ATG-treated patients developed a vigorous anti-ATG IgG response peaking at 1 month (median of 886.9 µg/mL, IQR from 510.5 to 1696.7 µg/mL, *P* < 0.001), which slowly decreased thereafter, with significantly elevated titers up to 1 year after treatment when comparing to baseline (median 175.5 µg/mL, IQR from 79.6 to 226.9 µg/mL, *P* < 0.001). **Figure S1A, SDC** (<http://links.lww.com/TP/B406>) shows the individual data of the upper quartile of anti-ATG concentration from ATG-treated patients during the first year after treatment. This additional representation highlights the variation of the levels of elicited antibodies for individual patients, by joining the points within the study follow-up. No significant differences were observed in the placebo-treated group.

### Anti-Gal IgGs

Anti-Gal IgGs were detectable in all patients before treatment (3.8 µg/mL, IQR from 2.8 to 7.1 µg/mL in the placebo group, 3.5 µg/mL, IQR from 2.6 to 9.2 µg/mL in the ATG-treated group, **Figure S2A, SDC**, <http://links.lww.com/TP/B406>), as expected.<sup>15</sup> However, these values were not different from those of control individuals (5.2 µg/mL, IQR from 1.9 to 7.7 µg/mL, n = 45), taken as a whole group or restricted to the age/sex-matched subgroup (**Figure S2C, SDC**, <http://links.lww.com/TP/B406>). In the ATG-treated patients, there was a highly significant increase of anti-Gal IgGs at 1 month posttreatment onset compared to baseline values (9 µg/mL, IQR from 4.9 to 24.2 µg/mL, *P* < 0.001, paired comparison, **Figure 1B** and **Figure S1B, SDC**, <http://links.lww.com/TP/B406> for data of individuals of the upper



**FIGURE 1.** Anti-ATG (A), anti-Gal (B) and anti-Neu5Gc (C) IgG titers in ATG- (red line) and placebo-treated (blue line) patients, before treatment (day 0) or at 1, 3, 6, and 12 months after injection, as assessed by ELISA. Concentrations are indicated in  $\mu\text{g}/\text{mL}$  for each antibody tested. Comparisons between groups were performed using an unpaired *t* test, whereas changes in each group were analyzed using a paired *t* test. In the ATG-treated group,  $P < 0.001$  at months 1, 3, 6, and 12 compared to baseline for anti-ATG IgG titers, and at month 1 compared with baseline for anti-Gal and anti-Neu5Gc IgG titers,  $P = 0.002$  at month 3 compared to baseline for anti-Neu5Gc IgG titers.

quartile). However, the other time points did not differ significantly from baseline. No difference was observed in the placebo-treated patients.

### Anti-Neu5Gc IgGs

First, the levels of basal anti-Neu5Gc IgGs were lower (1.34  $\mu\text{g}/\text{mL}$ , IQR from 0.9 to 1.6  $\mu\text{g}/\text{mL}$  in the placebo group, and 1.48  $\mu\text{g}/\text{mL}$ , IQR from 1.1 to 3.2  $\mu\text{g}/\text{mL}$  in the ATG-treated group) than that of anti-Gal IgGs in unprimed START patient cohorts. These values did not differ from those observed in the control healthy individuals (1.1  $\mu\text{g}/\text{mL}$ , IQR from 0.4 to 2.3  $\mu\text{g}/\text{mL}$ , Figure S2B and D, SDC,

<http://links.lww.com/TP/B406>). Second, the IgG response, also highly significant at 1 month compared with baseline, was characterized by the fact that a substantial fraction of the patients treated with ATG developed an extremely vigorous IgG response against Neu5Gc. Indeed, 19% of the ATG-treated patients exhibited greater than 20  $\mu\text{g}/\text{mL}$  of anti-Neu5Gc IgGs, and 3 patients had a concentration above 75  $\mu\text{g}/\text{mL}$ , with 1 patient over 1000  $\mu\text{g}/\text{mL}$  of anti-Neu5Gc antibodies at 1 month post-ATG infusion (median, 3.5  $\mu\text{g}/\text{mL}$ ; IQR, from 1.6 to 8.9  $\mu\text{g}/\text{mL}$ ;  $P < 0.001$ , Figure 1C and Figure S1C, SDC, <http://links.lww.com/TP/B406>, for data of individuals of the upper quartile). All these high values were validated with repeated assessments using varying dilutions and human IgG standard for a precise quantitation of the concentration of antibodies. No difference was observed in the placebo-treated diabetic patients.

### IgM Responses Against Rabbit IgG Antigens

Pre-ATG infusion baseline levels of IgM against rabbit antigens did not differ between the START patients and healthy individuals values (data not shown). However, ATG induced a vigorous IgM response against rabbit IgGs at 1 month after treatment (92.2  $\mu\text{g}/\text{mL}$ ; IQR, from 27.3 to 280.4  $\mu\text{g}/\text{mL}$ ;  $P < 0.001$ , Figure 2A). The increases in anti-Gal and anti-Neu5Gc IgMs were also significant at 1 month, with median values of 3.5  $\mu\text{g}/\text{mL}$  (IQR, from 2.8 to 6.7  $\mu\text{g}/\text{mL}$ ;  $P = 0.002$ ) and 0.65  $\mu\text{g}/\text{mL}$  (IQR, from 0.4 to 1.2  $\mu\text{g}/\text{mL}$ ,  $P < 0.001$ ), respectively (Figures 2B and C).

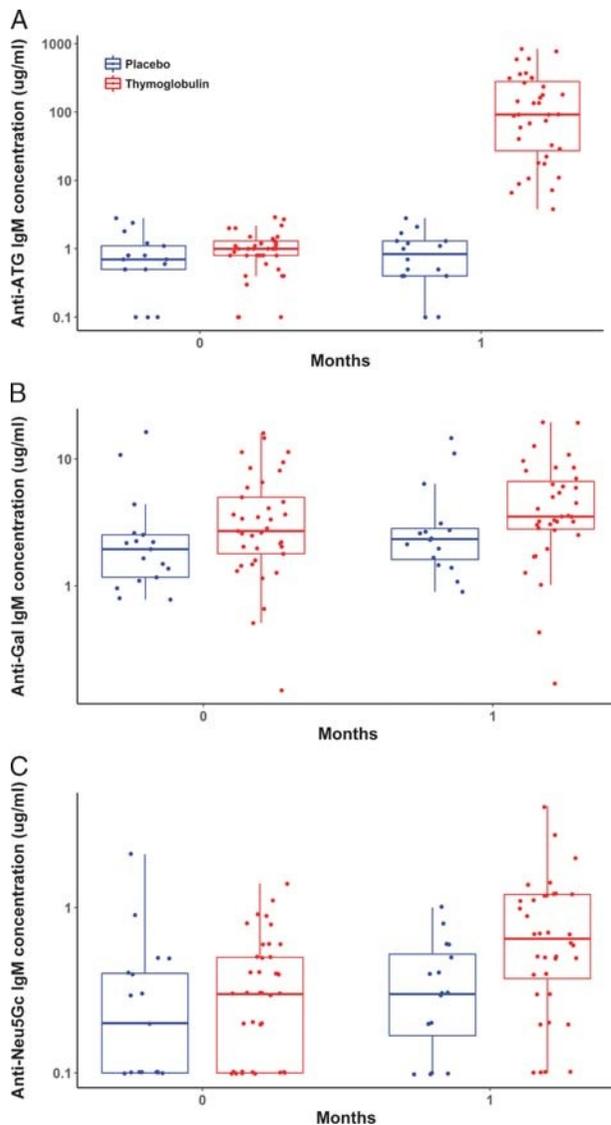
### Circulating ATG Levels

Circulating rabbit IgGs were quantified in serum samples from 9 patients at 1 month posttreatment. No ATG above background was detected at 1 month postinfusion (data not shown). This is in strong contrast to the situation previously observed in patients receiving the same ATG dose but with a potent concomitant immunosuppressive regimen.<sup>8</sup> This suggests that the recipient immune response against these xeno-IgGs heavily impacts the bioavailability of the ATG in the absence of additional IS.

### Correlation Between the Levels of Anti-ATG Antibody Specificities and Clinical and Biological Variables in the START Study

The upper panels of Figure S3, SDC (<http://links.lww.com/TP/B406>) show the correlations of IgG and IgM levels within the placebo-treated patients at 1 month. There was a significant correlation between IgG and IgM levels for anti-Gal ( $r^2 = 0.31$ ;  $P < 0.05$ , Figure S3B, SDC, <http://links.lww.com/TP/B406>) and anti-Neu5Gc ( $r^2 = 0.54$ ;  $P = 0.001$ , Figure S3C, SDC, <http://links.lww.com/TP/B406>), but not for total anti-ATG antibodies (Figure S3A, SDC, <http://links.lww.com/TP/B406>). The lower panels of Figure S3, SDC (<http://links.lww.com/TP/B406>) show the same correlations observed in the ATG-treated group. There was a significant correlation between IgG and IgM levels for anti-ATG ( $r^2 = 0.38$ ;  $P < 0.0001$ , Figure S3D, SDC, <http://links.lww.com/TP/B406>) and anti-Gal ( $r^2 = 0.16$ ;  $P < 0.05$ , Figure S3E, SDC, <http://links.lww.com/TP/B406>), but not for anti-Neu5Gc antibodies (Figure S3F, SDC, <http://links.lww.com/TP/B406>).

When analyzing antibody specificities at baseline and at 1 month after treatment, we found positive correlations which were statistically significant ( $P < 0.05$ ) between baseline anti-Neu5Gc and anti-Gal IgGs and IgMs in the ATG-treated



**FIGURE 2.** Anti-ATG (A), anti-Gal (B) and anti-Neu5Gc (C) IgM titers in ATG- (red line) and placebo-treated (blue line) patients, before treatment (day 0) and at 1 month after injection, as assessed by ELISA. Concentrations are indicated in  $\mu\text{g}/\text{mL}$  for each antibody tested. Comparisons between groups were performed using an unpaired *t* test, whereas changes in each group were analyzed using a paired *t* test.  $P < 0.001$  at month 1 compared to baseline for anti-ATG and anti-Neu5Gc IgM titers, and  $P = 0.002$  at month 1 compared with baseline for anti-Gal IgM titers, in the ATG-treated group.

group, which was retained at 1 month after treatment (Figure S4, SDC, <http://links.lww.com/TP/B406>). In this group, a positive correlation between anti-ATG and anti-Neu5Gc IgMs before and after treatment, as well as between anti-ATG and anti-Gal IgMs at baseline (not shown), were also found to be statistically significant ( $P < 0.05$ ). In the placebo group, statistically significant ( $P < 0.05$ ) correlations were also observed between the level of anti-ATG and anti-Neu5Gc at baseline and 1 month after treatment. We also found a significant correlation between anti-Neu5Gc and anti-Gal IgMs in the placebo group at 1 month only.

To assess the effect of cytokine release on antibody production, antibody titers were also analyzed for correlation with serum levels of IL-6 and IL-10 after onset of ATG infusions,<sup>1</sup> but showed no significant correlation (data not shown).

Finally, preservation of 2-hour C-peptide AUC baseline levels at month 24<sup>26</sup> did not correlate with any of the antibody level changes at 1 month after treatment from baseline.

## DISCUSSION

Animal-derived tissues or molecules are commonly used in modern medicine. Examples include acellular engineered heart valves, skin or tendons, or glycoproteins, such as polyclonal IgGs, used as immunosuppressive agents<sup>1</sup> or directed at toxins or severe infectious agents.<sup>9</sup> In this article, we show that rabbit IgGs elicit a strong humoral response against Gal and Neu5Gc glycan epitopes. Our data suggest that this response may shorten their circulating half-life and trigger a potentially toxic immune-complex disease in the absence of additional immunosuppressants. This vigorous anti-Gal and anti-Neu5Gc observed in patients primed by these xenoantigens also suggests that this response could affect the structure and function of other nonliving animal-derived products, such as biological heart valves<sup>27</sup> still expressing these epitopes after engineering.<sup>28</sup> These data suggest that IgGs from modified animals that do not express these xenoantigens could result in improved safety and efficacy for recipients of allografts requiring ATG induction treatment.

Unfortunately, a precise comparison of the respective quantities of the various antibody specificities cannot be accurately assessed, because the ELISAs are based on different reagents (including coating material, standards, and secondary antibodies). In the anti-ATG ELISA, the coated antigen is the same as the one injected to the patients, whereas in the case of quantification of anti-Gal and anti-Neu5Gc antibodies, the epitopes are either synthetic (for the anti-Gal assay) or different from the injected material (mouse sera for the anti-Neu5Gc assay). In this way, it was not possible to directly estimate the fraction of each specificity in the sera of the patients. However, the magnitude of the anti-ATG response observed in the patients from the START trial suggests that the anti-peptide antibodies may be a major component of the immune response.

The response against total rabbit IgGs at 1 month included an IgG response—in agreement with a memory-type response in patients who are already primed and with a substantial level of “natural” antibodies before treatment—but also comprised a strong IgM component, which was still present at 1 month. However at 1 month, the IgM response against Gal and Neu5Gc was almost lacking, suggesting a major memory component towards these 2 epitopes against which most humans are primed in the first year of life.<sup>15,29</sup> There was no statistical difference between the level of preexisting anti-ATG antibodies in patients from the START population compared to a cohort of normal individuals matched for age and sex. Although vigorous, the magnitude of the response is likely underestimated because of the concomitant presence of immune complexes of recipient antirabbit IgGs, attested to both by the occurrence of SSD in all but one START study participant<sup>5</sup> and by the absence of detectable circulating ATG at 1 month in our study.

The preinfusion levels of anti-Gal antibodies did not differ between diabetic patients and normal controls. Low amounts of Gal was unambiguously detected by mass spectrometry<sup>8</sup> and the immune response of the patients against this glycan epitope was strong. Of note, we were unable to record a

significant increase in anti-Gal in kidney graft recipients who received a similar ATG dose but were under efficient immunosuppressive treatment,<sup>8</sup> suggesting that a short course of additional IS drugs could strongly synergize with ATG in enhancing blockade of the autoimmune process in new-onset T1D. It is reasonable to speculate that the potential toxicity of immune complexes in the START study combined with the inflammation resulting from the post-ATG infusion cytokine release contributed to antagonizing a possible beneficial effect of the anti-T-cell polyclonal agent in the autoimmune process.<sup>5,26</sup> The recently published 2-year results of the START trial show ATG preserved peptide-C secretion in older patients,<sup>26</sup> which warrants further studies. However, we did not observe any correlation between the age of the patients of our cohort and the strength of their elicited antibody responses after ATG infusion (data not shown).

The reasons why some patients responded extremely vigorously to Neu5Gc is not well understood and we did not find a specific association with the baseline characteristics and other variables in the study participants when only the patients in the highest anti-Neu5Gc quartile were considered. We have no indication of the food habits of these patients, but the cohort size is too small to yield any relevant data on dietary Neu5Gc intake). In humans, Neu5Gc is present primarily on endothelial cells but also accumulates in atherosclerotic plaques, and anti-Neu5Gc antibodies induce vascular inflammation *in vitro*.<sup>22</sup> Taken together, these data support the notion that elicited anti-Neu5Gc antibodies may play a role in vascular inflammation in several conditions. Moreover, some patients from this cohort provide a source of anti-Neu5Gc antibodies with very high titers, which could be of use for the further analysis and functional characterization of the effect of these antibodies on human endothelial cells, with direct implications for solid organ transplant recipients.

As stated above, we speculate that the potential toxicity of immune complexes as well as the inflammation resulting from the cytokine release contributed to limiting the beneficial effect of the antithymocyte rabbit IgGs in the START trial. However, correlation of antibody levels with other variables of the START trial did not reveal significant associations between the levels of anti-ATG antibodies of any types and the levels of IL-6 or IL-10 related to the well-documented post-ATG cytokine storm.<sup>5</sup> Because all patients developed a clinically evident cytokine release syndrome and SSD, there was a likely global saturation of their biological effects, whatever the cytokine blood levels.

Patients with extremely high levels of anti-Neu5Gc IgGs at 1 month still had significantly more IgGs at 6 or 12 months, suggesting that this increase in titers and long-term exposure of tissues to anti-Neu5Gc may induce chronic activation of endothelial or epithelial cells displaying Neu5Gc of dietary origin.<sup>8,22</sup> Importantly, comparison of the patterns of Neu5Gc epitopes recognized before and after a stimulation by nondietary Neu5Gc-positive antigens, such as engineered acellular pig skin, has shown a long-term imprinting of newly recognized structures.<sup>18</sup> It is thus likely that the biological effects of “natural” and elicited antibodies (as after pig-skin dressing<sup>18</sup> or rabbit IgGs infusion<sup>8</sup>) are different and that exposure to an “elicited antibody repertoire” affects endothelial cell functions, for instance. We tested a possible correlation of the various anti-ATG titers with the preservation of baseline 2 hr

C-peptide AUC at month 24, but it was not significant, suggesting there was no additional toxic effect of anti-Neu5Gc antibodies on pancreatic islets. However, we recently showed that recipients of kidney allografts treated with ATG and having manifested SSD demonstrated persistent increases (>4 years) in anti-Neu5Gc (but not anti-Gal) antibodies, which were associated with poor long-term graft function.<sup>8</sup>

In conclusion, this study shows that the humoral immune response against antithymocyte rabbit IgGs is vigorous in the absence of concomitant IS and that the anti-Neu5Gc titers can reach extremely high levels, suggesting that this response could activate endothelial cells and potentially counteract the expected beneficial effects of such a treatment. Additional studies are needed to assess the potential long-term toxicity from elevated anti-Neu5Gc antibody levels, especially on patient endothelial cells of graft recipients.

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