Elicited and pre-existing anti-Neu5Gc antibodies differentially affect human endothelial cells transcriptome

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Abstract
Humans cannot synthesize N-glycolyneuraminic acid (Neu5Gc) but dietary Neu5Gc can be absorbed and deposited on endothelial cells (ECs) and diet-induced anti-Neu5Gc antibodies (Abs) develop early in human life. While the interaction of Neu5Gc and diet-induced anti-Neu5Gc Abs occurs in all normal individuals, endothelium activation by elicited anti-Neu5Gc Abs following a challenge with animal-derived materials, such as following xenotransplantation, had been postulated. Ten primary human EC preparations were cultured with affinity-purified anti-Neu5Gc Abs from human sera obtained before or after exposure to Neu5Gc-glycosylated rabbit IgGs (elicited Abs). RNAs of each EC preparation stimulated in various conditions by purified Abs were
INTRODUCTION

Humans cannot synthesize Neu5Gc or α1,3Gal endogenously, and as a result healthy sera contains high levels of antibodies directed against glycans containing a terminal Neu5Gc 1-3 or α1,3Gal 4 that are absent in humans but are present on glycoproteins and glycolipids of most non-human mammals. Besides being a potential obstacle to xenotransplantation or to safe clinical utilization of animal-derived biodevices such as biological heart valves or molecules, the presence of circulating anti-Neu5Gc antibodies (Abs) in all normal individuals creates a unique biological situation since the diet-derived Neu5Gc antigen can be absorbed and deposited in low amounts on endothelia and on some epithelia of healthy humans.

High titers of elicited anti-Neu5Gc Abs are also generated following exposure to animal-derived products or proteins, such as after polyclonal rabbit anti-human T-cell globulin (ATG) infusion. Such elicited anti-Neu5Gc Abs also display higher affinity and an extended repertoire of responses against new Neu5Gc epitopes than diet-derived Neu5Gc Abs, and some are not present before immunization, as demonstrated by sialoglycan microarrays. Thus, in addition to the expected deleterious interaction with the immunizing animal-derived material, such elicited anti-Neu5Gc Abs can also result in the formation of in situ immune complexes and chronic inflammation in the micro-vicinity of the recipients’ own Neu5Gc-positive endothelial cells (ECs). Indeed, in vitro experiments have reported that sera containing anti-Neu5Gc Abs can stimulate Neu5Gc-loaded ECs, favoring their interaction with blood mononucleated cells. Hence, it is possible that introducing Neu5Gc-containing animal-derived material products or proteins in humans (eg by xenotransplantation, bioprosthetic heart valves implantation, or therapeutic polyclonal Ig) could induce vascular inflammation. Nevertheless, a clear demonstration of clinical deleterious effects of diet-induced or elicited Neu5Gc Abs in humans is lacking despite working hypotheses and preliminary reports.

Here, we explored for the first time the effect of affinity-purified anti-Neu5Gc Abs, obtained before (diet-derived, pre-existing) and after immunization with ATG rabbit IgGs (elicited), on the whole transcriptome of 10 human primary EC preparations loaded with Neu5Gc onto surface membranes as in physiological conditions. Contrary to prior hypotheses, these in vitro experiments do not suggest that elicited anti-Neu5Gc Abs skew the EC transcriptome to an inflammatory profile compared with the diet-derived anti-Neu5Gc Abs that are normally found in the EC microenvironment.

KEYWORDS
anti-Neu5Gc antibodies, endothelial cells, N-glycolyneuraminic acid (Neu5Gc), sialic acid, xenotransplantation

METHODS

2.1 Study approval

Sera obtained from the START study were used in accordance with the Helsinki declaration and Institutional Review Board of Tel Aviv University and INSERM Université de Nantes. All participants or their parents provided written informed consent for using the sera for the experiments performed in this paper. Authorization to harvest artery from brain-death organ donors was obtained from the French "Agence de la BioMedicine" (agreement PFS08-017).
2.2 | Procurement of anti-Neu5Gc Abs

The START study (Study of Thymoglobulin to ARrest Type 1 diabetes; http://www.type1diabetestrial.org/) is a placebo-controlled phase II clinical trial which evaluated the effect of Thymoglobulin® (rabbit anti-thymocyte globulins, ATG) in patients with recent-onset type 1 diabetes (Table S1 and Reference 15 for details). Anti-Neu5Gc Abs purified from sera harvested before and after ATG treatment will be referred to as “pre-existing” and as “elicited” Abs, respectively.

2.3 | Affinity purification of anti-Neu5Gc antibodies

Anti-Neu5Gc Abs were purified on sequential affinity columns as described. Seven patients’ sera that had the highest titers of anti-Neu5Gc IgG one month following the rabbit ATG treatment were obtained from the START study (Gitelman et al, 2013) as described and used in accordance with the Helsinki declaration and Tel Aviv University Institutional Review Board. The sera of all seven patients before ATG treatment (representing pre-existing diet-derived anti-Neu5Gc Abs) were pooled, and antibodies were affinity-purified on sequential columns containing Neu5Ac-glycoproteins then Neu5Gc-glycoproteins, followed by elution with Neu5Gc (yield 32.3 µg of pooled 10 mL sera), as described. Similarly, anti-Neu5Gc Abs were affinity-purified from sera of the same patients at their peak response at one month after ATG treatment (representing drug elicited anti-Neu5Gc Abs; yield 466.3 µg of pooled 11.75 mL sera), as described. Glycan microarray analysis confirmed that the affinity-purified antibodies from each of the seven patient sera analyzed individually maintained their unique serum-repertoire patterns and were specific for Neu5Gc, and lack reactivity against Neu5Ac.

For ECs stimulation experiments, affinity-purified Abs of pre-ATG infusion (pre-existing) and post-ATG infusion Abs (elicited) were pooled. As expected, the same specific

![Figure 1](image_url)
**FIGURE 2** FACS staining of pre-stimulation EC culture by polyclonal chicken anti-Neu5Gc IgY following Neu5Gc loading in medium containing 10% FCS. FACS diagrams of chicken anti-Neu5Gc IgY staining of the 10 primary EC cell lines. Staining was performed after culture in 10% FCS and immediately before incubation with pre-existing or elicited affinity-purified anti-Neu5Gc Abs (see Methods). Blue diagrams are controls, where secondary A647-anti-chicken IgY was used alone, and red is the histogram of co-staining, with anti-CD31 and chicken anti-Neu5Gc IgY. See Figure 1 for comparison with Neu5Gc loading observed on ECs freshly harvested from aorta.
pattern of anti-Neu5Gc reactivity was confirmed by glycan microarray on the pooled affinity-purified anti-Neu5Gc Abs (Figure S2). The dosage of anti-Neu5Gc Abs was performed using an ELISA, as described.\textsuperscript{16}

\section*{2.4 Human endothelial cells culture}

Human arterial endothelial cells (ECs) were isolated from unused large artery (abdominal aorta or iliac arteries) pieces harvested from brain-dead organ donors (agreement PFS08-017 of the French regulatory institution “Agence de la BioMedicine”), as previously described.\textsuperscript{17} Neu5Gc levels on each EC before culture was evaluated (Figure 1), as described previously.\textsuperscript{8} Our quality controls for EC culture ensure that cell cultures used in the present study are pure (100\% CD31-expressing EC) and not activated based on the lack of expression of E-selectin and VCAM-1. Comparison of in vitro Neu5Gc loading of ECs and freshly harvested aortic ECs is detailed in Figure 2 and in the Results section.

\section*{2.5 Fresh human aorta endothelial cells}

Aortic patches of 5-10 cm length were placed in cold PBS immediately after organ harvesting from deceased donors (agreement #: PFS08-017). The aortic patch was soaked in a 50-mL tube containing collagenase A at 1 mg/mL (Roche Diagnostics), for 45 minutes at 37°C and cut to expose the aortic lumen. Endothelium layer cells were gently harvested from the aortic lumen using a cell scraper, centrifuged, and re-suspended in PBS. Cells were first stained either with a polyclonal chicken anti-Neu5Gc IgY or a purified chicken IgY isotype control (both from BioLegend), revealed by Alexa Fluor 647-conjugated anti-IgY secondary Ab, and stained with a PE-conjugated mouse anti-human CD31 (BD Bioscience) to label endothelial cells. Further analysis was performed using an LSR II flow cytometer (n = 10 000-50 000 recorded events, depending on aorta patches size). Cells were washed with PBS after each step, and no animal-derived products were used in order to avoid external Neu5Gc contamination. In addition, comparison of Neu5Gc loading of primary EC preparations was assessed using incubation with 10\% FCS with or without 3 mmol/L of Neu5Gc, as described.\textsuperscript{10}

\section*{2.6 FACS staining of pre-stimulation EC culture by polyclonal chicken anti-Neu5Gc IgY}

FACS diagrams of chicken anti-Neu5Gc IgY staining of the 10 primary EC cell lines. Anti-Neu5Gc staining was performed after culture in 10\% FCS and immediately before incubation with pre-existing or elicited affinity-purified anti-Neu5Gc Abs.

\section*{2.7 Endothelial cell stimulation}

Stimulation of ECs was done in 12-well plates, in 150 µl/well of Neu5Gc-free medium in the various conditions. Two final concentrations of affinity-purified anti-Neu5Gc Abs were tested: (a) 1.5 ng/µL for the pre- and post-ATG immunization samples, mimicking the average concentration of anti-Neu5Gc Abs in normal individual sera\textsuperscript{18} measured by the same ELISA\textsuperscript{15}; and (b) an additional concentration of 9 ng/µL, for elicited antibodies which can reach high concentrations in vivo.\textsuperscript{7}

PBS buffer was used as a control, since free Neu5Gc would not completely inhibit the diverse reactivities of such antibodies given the potential complexity of Neu5Gc-antigens on EC. In addition, recent data suggest that Neu5Gc itself may have a direct biological effect on human cells.\textsuperscript{19} Details of ECs stimulation procedures are given in the legend of Table S2. When mentioned, human pooled sera (dilution of 1:2, 50\% of well volume) or TNFα (500 U/mL) were added to the culture medium. The respective volumes of reagents in the culture wells with pooled sera in the culture trays were calculated to not modify the diet-derived anti-Neu5Gc Abs concentration in the culture and only slightly increased the concentration of diet-derived anti-Neu5Gc Abs in the culture with elicited Abs. Exposure to anti-Neu5Gc Abs used for quantitative PCR (qPCR), measurement of apoptosis, propidium iodide exclusion, and supernatant harvestings was slightly different as the sera from the second month post-ATG and the rest of the first month post-ATG samples had to be pooled for antibodies affinity purification, as described above in the affinity-purification section. Finally, as pre-ATG sera contained much lower levels of anti-Neu5Gc Abs compared with the ATG-elicited samples (Table S1, ~39-fold higher in elicited samples), sera from patients randomized in the placebo group of the START study\textsuperscript{15} were also affinity-purified to complement the lack of pre-ATG sera in the patients randomized to receive ATG. Supernatants from these cultures were analyzed by Luminex assay to measure cytokines and chemokines.

\section*{2.8 RNA extraction, quality control, and sequencing}

Total RNA was isolated using TRIZOL method (Life Technologies SAS) combined with RNeasy mini kit procedure (Qiagen). RNA quality was assessed using the Agilent 6000 Pico kit as instructed by the manufacturer. All RNA samples yield maximum quality with the RNA integrity number (RIN) above 9. Smart-seq2 libraries were prepared by the Broad Technology Labs and sequenced by the Broad Genomics Platform according to the Smart-seq2 protocol\textsuperscript{20} with some modifications\textsuperscript{21} on an Illumina NextSeq 500, and data are presented in Table S3. Data were separated by barcode and aligned utilizing STAR v2.4.2a\textsuperscript{22} and gene-level abundance was estimated using tximport package.\textsuperscript{23} TMM (trimmed mean of M-values) normalization was performed using edgeR in R v3.3.2.\textsuperscript{24}

\section*{2.9 Statistics}

Identification of differential genes was performed with the limma-voom function.\textsuperscript{25} Genes were considered as differentially expressed with Benjamini and Hochberg adjusted p-value < 5\% and fold change (FC) >1.5. For gene expression representation, principal component
analysis (PCA) and clustering were performed using ade4/adeigraphics26 heatmap3 packages, respectively. The biological significance of selected genes was assessed using the R clusterProfiler package.27 Gene ontology (GO) categories enriched with a Benjamini and Hochberg adjusted P-value <5% and with at least five represented genes were selected. RNA sequencing data can be accessed under GEO accession number GSE (GSE117146; [NCBI tracking system #19238656]). The REVIGO tool was used to summarize GO enrichment,28 and the resulting GO networks were visualized using Cytoscape v.3.6.1.29

2.10 | Quantitative PCR (qPCR)

qPCR from 5 primary EC preparation cultures (two of the 10 preparations and three new ones) were performed as previously described8 (Table S4). HPRT was used to normalize the amounts of RNA. Relative expression was calculated according to the $2^{-\Delta\Delta C_T}$ method, in which the reference represents 1-fold expression.

2.11 | Measurement of cytokines and chemokines in supernatants

The level of 12 cytokines and chemokines (Table S5) was measured at the CIMNA platform (CHU de Nantes) using a luminex kit (ProcartaPlex 12-plex, Life Technologies) following the manufacturer’s instructions.

3 | RESULTS

Figure S1 and legend provide the global flowchart of the designed experiments.

3.1 | Neu5Gc loading of fresh living aorta endothelial cells

Figure 1A shows representative examples of flow cytometry images of seven experiments where freshly harvested aortic ECs were stained with polyclonal chicken anti-Neu5Gc IgY. ECs were gated on CD31-positive cells. Two examples are shown with positive staining and two with negative staining. Three out of the seven preparations were considered negative (~57% Neu5Gc-positive freshly harvested aortic ECs). Comparative staining of a human primary EC line following incubation in a culture milieu with 10% FCS, or supplemented with 3 mmol/L of Neu5Gc are shown in Figure 1B. Using 3 mmol/L of free Neu5Gc in the culture media results in extra-physiological Neu5Gc loading (Figure 1B). Figure 1C shows mass spectrometry analyses of three freshly harvested aortic EC preparations, and these experiments were performed twice with identical results. In the two experiments, all three EC preparations seemed to be Neu5Gc-positive, although one was very weak. Preparations #050517 and #191217 had enough ECs to also be studied via flow cytometry and were confirmed to be Neu5Gc-positive by both methods.

**FIGURE 3** Transcriptomic changes induced by pre-existing and ATG-elicited anti-Neu5Gc Abs compared to incubation with anti-Neu5Gc-free milieu untreated cells. A, Comparison of the gene expression profiles induced by pre-existing or elicited anti-Neu5Gc Abs vs untreated ECs. Principal component analysis (PCA) was based on 12,208 expressed genes and shows a clear distinction between untreated cells compared to cells treated with both types of purified anti-Neu5Gc Abs. B, The Venn diagram exhibits the numbers of differentially expressed genes between untreated cells in anti-Neu5Gc-free milieu (n = 9) vs cells treated with pre-existing (n = 10) (967 genes) or ATG-elicited (n = 10) (875 genes) anti-Neu5Gc Abs using the limma-voom function25 with Benjamini and Hochberg adjusted P-value < 5% and FC > 1.5. Down- and up-regulated genes are denoted in green and red, respectively. C, Heatmap of the jointly 736 up-regulated genes following stimulation by both pre-existing and elicited anti-Neu5Gc Abs. Down- and up-regulated genes are colored in blue and yellow, respectively.)
3.2 FACS staining of pre-stimulation EC culture by polyclonal chicken anti-Neu5Gc IgY following Neu5Gc loading in medium containing 10% FCS

Histological analyses of formalin-fixed autopsy samples of various tissues, including placenta, brain, or kidney, have shown faint staining of microvascular endothelial lining by chicken anti-Neu5Gc IgY.10 In our attempt to define, as closely as possible, the basal Neu5Gc pattern of living ECs described above, the levels of Neu5Gc loading of the culture expanded living EC preparation were analyzed immediately before incubation with chicken anti-Neu5Gc IgY. FACS diagrams of chicken anti-Neu5Gc IgY staining of the propagated 10 primary EC cell lines are shown in Figure 2. Blue diagrams are controls, where secondary A647-anti-chicken IgY was used alone, and red is the histogram of co-staining with anti-CD31 and chicken anti-Neu5Gc IgY. This analysis confirmed that the staining patterns are close to those of freshly harvested aorta ECs (See Figure 1 for comparison with various Neu5Gc loading methods of primary cell lines and loading observed on ECs freshly harvested from aorta). These propagated aorta ECs, expressing physiological levels of Neu5Gc, were used for ECs stimulation with affinity-purified human anti-Neu5Gc Abs.

3.3 Anti-Neu5Gc Abs generate a modified gene expression profile of ECs

Human EC expressing physiological levels of Neu5Gc were exposed to affinity-purified anti-Neu5Gc Abs or control buffer without antibodies, and then, RNA was extracted and subjected to RNASeq analysis. Principal component analysis (PCA) based on the 12,208 expressed genes (Figure 3A) shows a clear distinction between untreated ECs and ECs exposed to either affinity-purified human diet-derived or elicited anti-Neu5Gc Abs. In addition, EC incubation with pre-existing anti-Neu5Gc Abs, at 1.5 ng/µL, results in a significant alteration of expression of 967 genes (adjusted P-value < 5%), mostly up-regulated (96.3% >1.5 FC) as compared to anti-Neu5Gc-free milieu (Figure 3B). As a normal component of ECs microenvironment, this result suggests that pre-existing anti-Neu5Gc Abs are likely providing signals contributing to normal ECs basic biology. A roughly similar number of DE genes was also observed following incubation with elicited anti-Neu5Gc Abs, with 96.7% of up-regulated transcripts (>1.5 FC; Figure 3B).

As expected, since affinity-purified preparation of elicited anti-Neu5Gc Abs also contained pre-existing, diet-derived Abs, a large set of 739 transcripts was similarly affected following exposure to diet-derived pre-existing and elicited anti-Neu5Gc Abs stimulation. However, 364 transcripts (305 up- and 59 down-regulated) were differentially expressed between the two experimental conditions. A first set of 110 transcripts was over-accumulated following stimulation with elicited anti-Neu5Gc Abs only. Figure S3 shows the predominant DE transcript networks within this set of 110 DE transcripts that identified two major groups, related mostly to basic cellular functions rather than to an EC activation pattern: RNA splicing or processing (CASC3, SRSF4, FBL), DNA replication and repair (CDT1, ERCC2, MCM2), mitochondrial function (OPA3, SCO1, TACO1), ubiquitination process (UNKL, UBE2E2, TMEM189), or cell cycle (RAD51). The second set of 228 DE genes (195 up-regulated, Figure S4) related only to displays of networks of interacting master genes involving NFKB1, NFE2L2, PPAR6, IL8, ICAM1, or the TNFR family. The significant GOs and hierarchy of the 195 up-regulated DE transcripts are shown in Figure S5. Thus, pre-existing diet-derived anti-Neu5Gc Abs mobilize important clusters of genes involved in EC biology, including cytokine signaling pathways, apoptosis, NF-kappaB, G-protein coupled signaling, and chemokine-mediated pathways.

Similar analyses were performed on the 736 transcripts shared by the EC transcriptome following exposure to either pre-existing or elicited anti-Neu5Gc Abs. Figure S6 shows the three main clusters of transcripts with DE > 3-fold. The first set involved genes encoding mitochondrial structures (MRPS26, MROL12, and MROL4). The second set encompasses genes involved in ribosomes, processome, or exosome components (RRS1, RRP1, UTP18, EXOSC4) or in other basic cell function (TRMT6A, SURF6, NOA1, NOC4L, WDR 18, FKBP4). The third set included genes involved in apoptosis (TRADD, FADD, GADD45) or in cell metabolism (CDKN2A, DUSP, JUN, or DVL2), suggesting again a contribution to EC global metabolism and biology following exposure to pre-existing anti-Neu5Gc Abs, that are a normal component of EC microenvironment. There was no dose-response effect in DE gene numbers and fold changes between the elicited anti-Neu5Gc Abs at 1.5 ng/µL vs 9 ng/µL in the stimulation culture milieu, suggesting that the 1.5 ng/µL is already at a saturating concentration (Figure 3B). There was also no correlation between levels of DE induced by anti-Neu5Gc Abs and the levels of surface Neu5Gc on cultured ECs before stimulation (Figure 2).

The effect of activation of the same primary ECs by TNFα, used for comparison to identify inflammatory patterns in these EC activation experiments, is detailed in Figures S7 and S8 and corresponding legends.

3.4 Post-immunization, elicited anti-Neu5Gc Abs differentially modulate the EC transcriptome compared with pre-existing, diet-derived, anti-Neu5Gc Abs

We then directly compared the patterns resulting from ECs incubation with pre-existing anti-Neu5Gc Abs, the normal ECs microenvironment, or with elicited anti-Neu5Gc Abs. Elicited Abs were associated with significant DE of a set of 73 genes, 66 being down-regulated (Figure 4A and 4B). The down-regulation also occurred with a higher concentration of elicited anti-Neu5Gc (9 ng/µL) (Figure 4B). The GO analysis of these 73 DE genes (Figure 4E) identified clusters with documented connection to cytokines response, MAPkinase cascades, chemotaxis, and integrins. The gene networks (Figure 4C and 4D) identified among these 66 down-regulated genes (such as CD83, IL8, the transcription factor NFKB1, MAPK or the
ICAM1 integrin), suggest a potential specific biological role of elicited anti-Neu5Gc Abs which are not normal components of the EC environment. Altogether, these data do not support the notion that elicited anti-Neu5Gc Abs skew the ECs transcriptome profile toward an inflammatory one, at least in these in vitro conditions. Furthermore, elicited anti-Neu5Gc Abs appear to down regulate several master genes involved in important pathways of ECs activation, which is consistent with the absence of detected vascular complications in
**FIGURE 4** Modulation of transcript patterns induced by elicited anti-Neu5Gc Abs. A, Seventy-three modulated genes (adjusted $P$-value < 5% and FC > 1.5) by elicited (n = 10) compared with pre-existing anti-Neu5Gc Abs (n = 10) are represented in the supervised hierarchical clustering (down- and up-regulated genes are denoted in blue and yellow, respectively). B, Histograms representing fold change expression of these 73 genes comparing elicited, at the dose of 1.5 ng/µL (left) or at 9 ng/µL (right), compared with pre-existing anti-Neu5Gc Abs. Genes are ordered according to the comparison of 1.5 ng/µL elicited to pre-existing anti-Neu5Gc Abs. C, Gene network from the 73 genes using the STRING database (https://string-db.org/). The line thickness indicates the confidence of predicted protein interaction. D, Significant enriched GO (adjusted p-value < 5%) are summarized using REVIGO to build a comprehensive GO network. Enrichment is denoted according the blue scale. E, The 20th significant enriched GOs clusters are represented according to number of enriched genes and colorized according to adjusted $P$-value. The x-axis represents the number of enriched genes per GO.

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**FIGURE 5** Modulation of transcript expression pattern by elicited anti-Neu5Gc Abs in the presence of complement. A, The Venn diagram exhibits numbers of differential genes (adjusted $P$-value < 5% and FC > 1.5) following incubation with of 1.5 and 9 ng/µL of elicited anti-Neu5Gc antibodies together with complement (n = 10 and 9, respectively) compared with complement alone (n = 10). Number of down- and up-regulated transcripts are written in green and red, respectively. B, Heatmap of the 523 down-regulated genes by incubation with 9 ng/µL of elicited anti-Neu5Gc Abs with complement compared with complement alone, highlighting a dose effect when ECs were incubated with elicited anti-Neu5Gc Abs at 1.5 ng/µL vs 9 ng/µL. Down- and up-regulated genes are denoted in blue and yellow, respectively. C, GO clusters generated by stimulation of ECs with elicited anti-Neu5Gc Abs at 9 ng/µL with complement vs untreated ECs. The x-axis represents the number of enriched genes per each GO.
patients who were immunized with ATG and developed elicited anti-Neu5Gc Abs, even at very high titers.  

3.5 Addition of pooled sera as source of complement to elicited anti-Neu5Gc Abs affects the EC transcriptome

As previous studies performed on unpurified Abs suggested a complement-dependent effect of anti-Neu5Gc Abs in vitro, the effect of adding complement to anti-Neu5Gc Abs upon exposure to ECs was tested. There was no difference of propidium iodide staining of ECs following incubation with both types of anti-Neu5Gc Abs, with or without complement, indicating the absence of cytotoxicity. Serum alone was used as a source of complement in the culture medium; however, it also provides multifactorial signals (e.g., growth factors). Exposure of ECs to serum complement induced a substantial regulation of 1200 genes; 31% of the up-regulated genes were shared with the TNFα-induced pattern, and 616 transcripts were also shared with the patterns triggered by elicited anti-Neu5Gc Abs alone (not shown). Thus, the patterns associated with the presence of serum or anti-Neu5Gc Abs cannot be simply interpreted as a specific “inflammatory” signature, on the basis of an overlap with the DE of the genes observed following addition of TNFα to the culture. Rather, this overlapping DE of the genes seems to contribute to the basal profiling of the ECs transcriptome in the experimental conditions.

Of note, in contrast to elicited anti-Neu5Gc Abs alone on untreated ECs (Figure 3), addition of serum actually restored a dose-dependent effect of the elicited anti-Neu5Gc Abs on gene expression (Figure 5). Indeed, 33 and 523 transcripts were differentially expressed.

**Figure 6** qPCR measurement of selected ECs transcripts following incubation of primary EC cell lines with various preparation of affinity-purified anti-Neu5Gc Abs. A, Levels of transcripts following incubation with pre-existing and elicited anti-Neu5Gc Abs on the panel of selected transcripts. B, Effect of addition to pre-existing or elicited anti-Neu5Gc Abs at 9 ng/μL on the transcriptome of ECs incubated with serum alone as a complement provider. C, The qPCR assessment of DE genes involved in EC/leukocytes adhesion process following exposure to 9 ng/μL of elicited anti-Neu5Gc Abs. The nature of the anti-Neu5Gc Abs tested in qPCR presented some differences with the anti-Neu5Gc Abs utilized in sequencing experiments. See Methods section for details (n = 4-5, *P < 0.05, **P < 0.01, paired t test)
to a significant extent following incubation at a concentration of 1.5 ng/µL vs 9 ng/µL, respectively, of elicited anti-Neu5Gc Abs in the presence of complement compared with complement alone (Figure 5A and 5B). However, the most striking observation was that the combination of elicited anti-Neu5Gc at 9 ng/µL with complement resulted in a down-regulation of most of the transcripts differentially affected by the complement alone (Figure 5A and 5B). Figure 5C shows also that the GO clusters generated by the stimulation of ECs with elicited anti-Neu5Gc at 9 ng/µL with complement vs untreated ECs do not show patterns skewed toward more inflammation; in addition, the down-regulation effect reported was not restricted to elicited Abs (see below).

Figure S9 shows the complex gene networks of the down-regulated genes with FC above 2-fold, involving a variety of master genes such as JUN, RAF, or mediators of apoptosis following addition of pooled sera to elicited anti-Neu5Gc Abs. Restricting the analysis to down-regulation with a DE > 3-fold (Figure S9, right panel) narrows down the networks to two major clusters. The first cluster predominantly involves apoptosis and is centered by TRADD (TNFR1-associated death domain protein) and FADD (Fas-associating protein with death domain) responsible for the apoptotic function of Fas and TNFR1, and by PPM1D which induces autophagy. The second network involves RCN1, a member of the CREC family, also regulating stress-induced apoptosis and Cytochrome c-1, indispensable for intact activity of
3.6 | qPCR validation of anti-Neu5Gc Abs effect on ECs

Table S4 shows the transcripts tested by qPCR. The experimental conditions for qPCR were limited by the available amounts of purified anti-Neu5Gc Abs and, to some extent, differ from those noted above (see Methods section). The rationale for choosing the tested transcripts was to validate: (a) the effect of anti-Neu5Gc Abs on untreated ECs; (b) the down-regulation of some transcripts accumulated following stimulation by pre-existing anti-Neu5Gc Abs compared with elicited Abs; (c) the down-regulation effect of adding anti-Neu5Gc Abs to complement on some genes with a >3-fold DE; and (d) the DE of some genes previously found up-regulated by anti-Neu5Gc Abs containing sera, and involved in adhesion of EC to leukocytes.

Possibly due to qualitative differences in the reagents used in the qPCR experiments and the limited number of EC preparations tested, Figure 6A shows that the qPCR only partially validated the increase observed by RNA sequencing. While BAD, DUSP1, ICAM, and DAPK3 transcripts were substantially increased following incubation with pre-existing anti-Neu5Gc Abs, the other tested transcripts were not significantly modified. Figure 6A also shows that only ICAM1 was strongly down-regulated by elicited anti-Neu5Gc Abs compared with pre-existing anti-Neu5Gc Abs, with the three other selected transcripts (ID1, NFkB1, and CXCL8) not being modified. However, in keeping with the sequencing data, Figure 6B shows that despite large value distribution, there is a down-regulation of the average RNA levels of all the eight examined transcripts following the presence of anti-Neu5Gc Abs and sera in the culture medium, compared with sera alone. This effect was not restricted to elicited anti-Neu5Gc Abs. Figure 6C shows the effect of anti-Neu5Gc Abs on three transcripts involved in endothelial cell adhesion following culture with 9 ng/µL Abs showing only a marginal difference between pre-existing and elicited anti-Neu5Gc Abs at the highest concentration.

3.7 | Analysis of supernatants from EC stimulations

To further validate the sequencing data, a panel of cytokines and chemokines, including a set of molecules previously reported to be produced by ECs following stimulation by serum containing anti-Neu5Gc Abs, and CXCL8 (Table S5) were tested. There was a significant release of all tested molecules following the combined presence of complement (human sera) together with anti-Neu5Gc Abs of both types at 9 ng/µL, compared with uncombined reagents (Figure 7A). Pre-existing anti-Neu5Gc Abs and human serum, two normal components of ECs in vivo, were also associated with some release of factors into the culture supernatant (such as for IL-1α, IL-12p40, MCP-2 and MIP-1α/b), with only traces of IL-10 detected. Interestingly, Fractalkine was the only factor also strongly released in the presence of 9 ng/µL of elicited anti-Neu5Gc Abs alone (Figure 7B).

4 | DISCUSSION

To the best of our knowledge, only two papers have addressed the biological effects of anti-Neu5Gc Abs on ECs in vitro. Both used unpurified full sera with (or without) anti-Neu5Gc Abs. Pham et al utilized umbilical cord ECs (HUVEC) with high concentrations of Neu5Gc. Here, the primary EC preparations that were subjected to stimulation were loaded with physiological Neu5Gc levels, that more closely resembled the levels observed in the Neu5Gc-positive fresh ECs. This is in contrast to the largely extra-physiological Neu5Gc-loading induced by 3 mmol/L free Neu5Gc in the culture media for 3 days, which generates MFI ~100-fold higher by FACS analysis (Figure 1). Finally, the key serum tested (S34) in the Pham et al study contained an unusually high level of anti-Neu5Gc Abs (47.9 ng/µL IgG), compared with the average level found in normal individuals (~2 ng/µL).

The aim of the present study was to analyze the modified transcriptome of human ECs (expressing Neu5Gc at a level mimicking that of fresh aortic ECs) after exposure to purified elicited anti-Neu5Gc Abs (used at a concentration close to the physiological one), in comparison with pre-existing diet-induced anti-Neu5Gc Abs. It should be pointed out that the latter were selected for comparison since in contrast to elicited anti-Neu5Gc Abs, these pre-existing diet-induced Abs, are normal components of human EC microenvironment.

We first show that exposure of ECs to anti-Neu5Gc Abs preparations results in distinct PCA and DE gene patterns involving a large number of DE transcripts. Such a feature is likely related to the high percentage of EC surface glycoproteins decorated with diverse Neu5Gc epitopes, thereby potentially triggering a large number of pathways. As pre-existing anti-Neu5Gc Abs are normally present in the EC microenvironment, such data suggest that they display signals that contribute to the basic EC biology in our experimental system.

We observed that, as a basal pattern, several clusters of DE were induced by pre-existing anti-Neu5Gc Abs and overlapped with GO patterns commonly associated with ECs activation. These findings are most likely related to the experimental context. Of note, the ECs were obtained after brain death and characterized by a metabolic storm with high levels of TNFα and were also cultured with growth factors required for their expansion; they were also not submitted to sheer stress regulation and thus do not authentically reflect the "homeostasis" profile of quiescent ECs.

Substantial differences were observed in the effect of elicited anti-Neu5Gc Abs compared with the basal situation. For instance, following ECs stimulation with elicited anti-Neu5Gc Abs, a set of 110 DE transcripts were over-expressed. GO analyses showed that the pattern differences following exposure to pre-existing anti-Neu5Gc Abs or anti-Neu5Gc-free milieu involve a wide variety of genes, including genes related to cell adhesion, apoptosis, and cytokine signaling pathways (Figure 4). In addition, elicited anti-Neu5Gc
harvesting and serum separation, or contact with polystyrene trays,34 of activation of the complement cascade already occur following blood following incubation with sera is likely multifactorial. Indeed, some levels of ECs. suggests that elicited anti‐Neu5Gc Abs do not trigger inflammation pertinently the normal environmental milieu of ECs), which further suggests that elicited anti‐Neu5Gc Abs do not trigger inflammation of ECs.

The important number of genes with significantly altered DE following incubation with sera is likely multifactorial. Indeed, some levels of activation of the complement cascade already occur following blood harvesting and serum separation, or contact with polystyrene trays,34 and sera provide EC growth factors. Activation of complement, which is also inherent to the brain-death status of the EC donor,35 may have also primed the ECs.36‐38 The gene network analyses, stratified according transcripts DE following incubation with serum and elicited anti‐Neu5Gc Abs, show a down‐regulation of several important networks related to effectors of apoptosis (TRADD, FADD, and RCNI), autophagy (PPM1D), and mitochondrial function (Cytochrome c-1). There was also no concomitant significant DE of anti‐apoptosis genes (such as TNFAIP3, Bcl‐xL, HMOX1, or CD55 and other complement regulatory genes) described in EC accommodation to activation, such as by low levels of anti‐ABO Abs.39 In adult blood vessels, most ECs are relatively resistant to apoptosis,39 a property that may be reinforced by anti‐Neu5Gc Abs. If apoptotic ECs become pro‐coagulant,40 EC apoptosis in regressing vessels also allows remodeling to meet the changing requirements of the tissues they supply.41

Some differences between qPCR and RNA sequencing data may have several explanations. First, qPCR was performed using a limited number of EC preparations. In addition, as mentioned, due to the limited availability of sera harvested at the peak of elicited anti‐Neu5Gc Abs response,9 other batches of sera were used for purification of the anti‐Neu5Gc Abs used for qPCR. Nevertheless, the discrepancies from the PCR studies did not reveal any increase in RNA when there was no DE assessed by sequencing of the corresponding transcripts. qPCR also shows the down-regulation effect of anti‐Neu5Gc Abs on the transcriptome of ECs incubated with serum. Interestingly, the dosage of secreted soluble factors in the supernatants exhibited a contrasting profile to RNA pattern, with the former showing a significantly increased release of all the tested soluble factors by elicited anti‐Neu5Gc Abs at 9 ng/µL; this disconnection is likely related to complement. Indeed, C5a,37 as well as the membrane attack complex,42 can induce a rapid secretion of preformed inflammatory factors by ECs.33 Of note, Fractalkine (CX3CL1)43 was also released in the supernatant in the absence of complement and in the presence of elicited anti‐Neu5Gc Abs.

The reason that elicited anti‐Neu5Gc Abs do not result in a clear inflammatory profiling of the EC transcriptome remains hypothetical. Circulating anti‐Neu5Gc Abs show extremely large differential reactivities with multiple Neu5Gc‐containing glycans in an array format.44 One can speculate that such a huge epitope conformational diversity may result in a low concentration of Abs for a given EC surface membrane target. Such a situation combined with the yet unknown mechanisms shaped by evolution to cope with the condition resulting from the concomitant presence of natural anti‐Neu5Gc Abs and their target on EC (which most likely also benefits elicited Abs) may ultimately result in signals that remain below the activation threshold of ECs. To some extent, anti‐Neu5Gc Abs may thus behave as other "natural antibodies" found in all normal human sera, which react against a variety of autologous antigens. Such antibodies may have biological effects on autologous "target" cells that may be necessary for their homeostasis.45

Finally, interpretation of these in vitro data is in agreement with the current lack of evidence of anti‐Neu5Gc Abs‐induced vascular inflammatory in vivo in humans, and particularly when a very strong exposure to elicited anti‐Neu5Gc Abs was documented for several months,7 and in whom no vascular toxicity was reported.15,46 Furthermore, aggressive experimental conditions aimed at inducing a xenosialitis condition in the human‐like Cmoh‐KO mice did not result in noticeable vascular inflammation at the clinical and histological levels, although inflammatory markers were found in circulation.47

Our report presents several limitations. In particular, although we performed the exhaustive RNA sequencing on 80 EC samples, the scarcity of the elicited anti‐Neu5Gc Abs source available and the limited access to human arterial primary EC preparations allowed only a single time of incubation and restricted conditions of validations. A potential heterogeneity in the presence of Neu5Gc on freshly harvested ECs also suggests that conclusion from in vitro experimental conditions, using uniformly Neu5Gc‐loaded ECs, may not apply to all individuals in vivo. In addition, our analyses do not explore the possible effects related to binding of blood leukocytes through their Fc receptor to elicited anti‐Neu5Gc Abs on ECs and suggest caution in the interpretation of these in vitro data. Finally, our conclusions cannot be extended to the effect of anti‐Neu5Gc Abs on malignant cells, which commonly display a much larger Neu5Gc accumulation on these cells, far above that of ECs.48 Similarly, these data cannot directly be related to anti‐Neu5Gc Abs direct effects on xenotransplants5 or engineered animal biodoetic devices such as skin12 or biological heart valves.49

In conclusion, these data show that, compared with pre-existing anti‐Neu5Gc Abs, anti‐Neu5Gc Abs elicited by Neu5Gc‐positive animal‐derived glycoproteins do not skew the EC transcriptome toward a pro‐inflammatory profile in vitro. Accordingly, while restricted to the transcriptome analysis following in vitro stimulation, they do not support the concept that xenotransplantation, implantation of animal‐derived biodoetic devices (such as bioprosthetic heart valves), or infusion of glycosylated molecules (such as IgG) carry a risk of activation of the recipient own endothelial cells.
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CONFLICT OF INTEREST

Jean-Paul Soullilou and Cesare Galli are co-founders of Xenothera. All other authors have declared that no conflict of interest exists.

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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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