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Orthodox vs paradox: The roles of glycomics, genetics and beyond in immunity, immune disorders and glycomedicine

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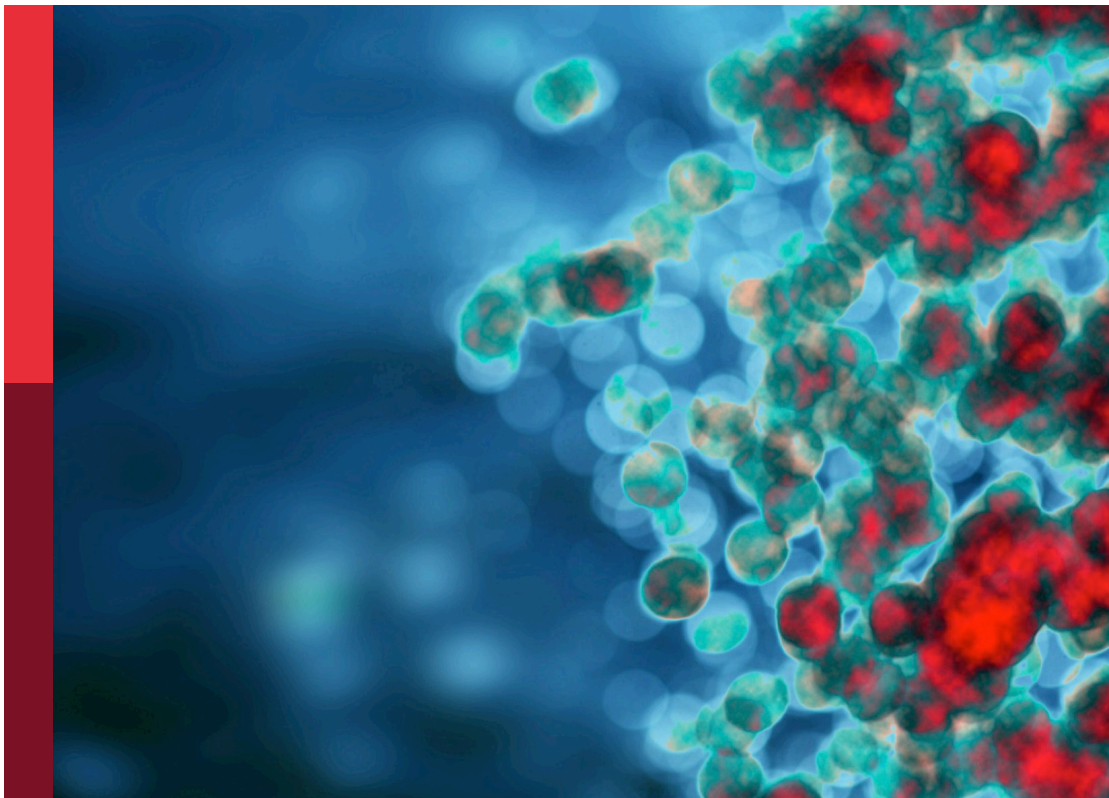

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Orthodox vs paradox: The roles of glycomics, genetics and beyond in immunity, immune disorders and glycomedicine

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Editorial: Orthodox vs paradox: the roles of glycomics, genetics and beyond in immunity, immune disorders and glycomedicine

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KEYWORDS

paracentral dogma, sugar codes, glycomics, immunity, immune disorders, glycomedicine

Editorial on the Research Topic

Orthodox vs paradox: the roles of glycomics, genetics and beyond in immunity, immune disorders and glycomedicine

The traditional 'central dogma' describes the flow of genetic information from DNA to RNA to protein. This process highlights the critical role of genes in living organisms. Nonetheless, ongoing immunological research coming to realize that emerging disciplines like glycomics and epigenetics are challenging the traditional viewpoint and extending the boundaries of the 'central dogma'. This pivotal development has led to profound shifts in our understanding of how the immune system functions. Hence, one might wonder if there exists a 'paracentral dogma' that can offer answers to these revolutionary discoveries by taking sugars as the 3rd life codes after nucleic acids and proteins, the 1st and 2nd of life codes for cellular materiality (1, 2).

The emergence of glycomics, particularly in the field of immunology, has revealed the biological functions of glycans and their key roles in the immune system (3). The richness and intricacy of glycans endow the immune system with extraordinary diversity and adaptability, impacting crucial processes within immune cells, encompassing signaling, interactions, and adhesion. Such innovative discoveries provide new perspectives for immunological research and bridge glycomics and immunology as well as genetics and epigenetics, contributing to a deeper insight into the functioning of the immune system (4).

Genetics and epigenetics play an integral role in immune-related disease research. Along with the co- and post-translation modifications, genetic variants significantly affect the functioning of the immune system, leading to the occurrence and progression of immune-related diseases. Investigating the relationship between genetic/epigenetics and immune diseases has become an important part of unraveling the mysteries of immunology. Intensive research in this area has provided us with key information on the diversity of the immune system and the omics basis of immune-related diseases (5).

This Research Topic, therefore, aims to integrate glycomics and genetics knowledge for a more comprehensive and in-depth understanding of the function and regulation of the immune system. This unique insight will contribute to the development of innovative disease therapy and vaccination. This Research Topic brings together important research findings from distinguished researchers and scientists globally. With this Research Topic, we hope to offer readers more opportunities to learn about the latest research and cutting-edge advances in this domain. We believe that we can open up new avenues for future research in glycomedicine and life sciences through such interdisciplinary collaboration and knowledge sharing.

In this Research Topic, we are pleased to present one Brief Research Report, four Review articles, and six Original Research articles. A diverse array of pivotal subtopics is explored:

- Synergistic regulation of Notch signaling by different O-glycans promotes hematopoiesis (Tanwar and Stanley).
- Review breakthrough of glycobiology in the 21st century (Mahara et al.)
- Blood DNA methylation marks discriminate Chagas cardiomyopathy disease clinical forms (Brochet et al.)
- Effects of low-calorie and different weight-maintenance diets on IgG glycome composition (Deriš et al.)
- The IgG glycome of SARS-CoV-2 infected individuals reflects disease course and severity (Siekman et al.)
- Identification and validation of IgG N-glycosylation biomarkers of esophageal carcinoma (Pan et al.)
- Glucose metabolism and glycosylation links gut microbiota to autoimmune diseases (Wang et al.)
- Alterations of m6A RNA methylation regulators contribute to autophagy and immune infiltration in primary Sjögren's syndrome (Cheng et al.)
- N-glycosylation and inflammation; the not-so-sweet relation (Radovani and Gudelj)
- Glycometabolism reprogramming of glial cells in central nervous system: Novel target for neuropathic pain (Kong et al.)
- Cytokines in the immune microenvironment change the glycosylation of IgG by regulating intracellular glycosyltransferases (Cao et al.)

These articles focus on the latest advancements in the field of glycomics, genetics, immunology and glycomedicine, addressing topics including:

- Glycobiology and the immune system: how glycobiology interacts with the immune system and how they work together to influence health and disease.
- DNA methylation and disease: how DNA methylation plays a role in different clinical cases, especially in the identification of Chagas cardiomyopathy disease.
- Diet, disease and IgG glycome composition: how diet affects the IgG glycome composition and its implications for disease.
- Inflammation, autoimmune disease, and glucose metabolism: the link between autoimmune disease, inflammation, and glucose metabolism, with particular reference to interactions with gut microbiota.
- RNA methylation, autophagy and immune infiltration: how RNA methylation affects autophagy and immune infiltration, especially in primary Sjögren's syndrome.
- Cytokines and glycosylation in the immune microenvironment: how cytokines in the immune microenvironment affect IgG glycosylation by regulating glycosyltransferases.

Echoing the presuppositions of the 'paracentral dogma-supporting the central dogma with sugar codes", this Research Topic not only broadens the scope of central dogma, but also lays a solid foundation for future research in immunology and glycomedicine. We further contribute to the development of emerging disciplines by offering insights into the interrelationships between glycobiology, immunology and genetics. We expect that this Research Topic will stimulate more collaborations among these fields and lead to new breakthroughs and innovations for the scientific community. This series of articles will enable us to promote 'glycomedicine' and it's role in maintaining health and addressing immune diseases (1, 6, 7). Relevant findings will shed valuable light on the realization of preventive, predictive, and precision medicine.

Author contributions

WW: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Cytokines in the Immune Microenvironment Change the Glycosylation of IgG by Regulating Intracellular Glycosyltransferases

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Cao Y, Song Z, Guo Z, Zhao X, Gong Y, Zhao K, Qu C, Huang Y, Li Y, Gao Y, Zhang J and Guo X (2022) Cytokines in the Immune Microenvironment Change the Glycosylation of IgG by Regulating Intracellular Glycosyltransferases. Front. Immunol. 12:724379. doi: 10.3389/fimmu.2021.724379

Background: Changes in IgG glycosylation, as a novel pathological feature, are observed in various autoimmune diseases (AIDs). The glycosylation patterns of IgG play a critical role in regulating the biological function and stability of IgG involved in the pathophysiology of many AIDs. However, the intracellular regulatory mechanisms underlying the effects of disturbances in various cytokines on IgG glycosylation are poorly understood. Thus, we investigated the regulatory effects of elevated cytokines in AIDs on intracellular IgG glycosylation within B cells.

Methods: First, we established a controlled primary culture system *in vitro* to differentiate human $CD19^+$ B cells into antibody-secreting cells (ASCs). Then, the IgG concentrations in the supernatants were measured by enzyme-linked immunoassay (ELISA) under IFN-γ, TNF-α, IL-21, IL-17A, BAFF, or APRIL stimulation. Next, the glycosylation levels of IgG under different stimuli were compared *via* a lectin microarray. The fine carbohydrate structures of IgG were confirmed by matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight-mass spectrometry (MALDI-TOF-MS). Finally, the expression of glycosyltransferases and glycosidases in B cells under stimulation with several cytokines was detected by real-time PCR and western blotting.

Results: We found that cytokines significantly promoted IgG production *in vitro* and led to considerably different IgG glycan patterns. Specifically, the results of lectin microarray showed the galactose level of IgG was increased by IFN-γ stimulation ($p<0.05$), and the sialylation of IgG was increased by IL-21 and IL-17A ($p<0.05$). The MALDI-TOF-MS data showed that the frequency of agalactosylation was decreased by IFN-γ with the increased frequency of mono-galactosylation and decreased frequency of digalactosylation, accompanied by upregulation of β-1,4-galactosyltransferase 1. Both frequencies of mono-sialylated and disialylated N-glycans were increased by IL-21 and IL-17A with decreased frequency of asialylation, and the expression of β-galactoside α-2,6-sialyltransferase 1 was upregulated by IL-21 and IL-17A.

Conclusion: Abnormally elevated cytokines in the microenvironment regulates IgG glycan patterns by regulating intracellular glycosyltransferases in human B cells.

Keywords: IgG, glycosylation, cytokines, B cell, glycosyltransferase, autoimmune diseases

INTRODUCTION

The appearance of autoantibodies in circulation, which are mainly IgG, is a characteristic of autoimmune diseases (AIDs). Autoantibodies can mediate immune responses against autoantigens, leading to inflammation and destruction by antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (1–3).

IgG is a glycoprotein secreted by antibody-secreting cells (ASCs) and is composed of a crystallizable fragment (Fc) responsible for triggering effector functions and Fab arms responsible for antigen binding. Two conserved repertoires of N-linked glycans, each composed of a biantennary heptasaccharide core structure and optional terminal glycans, are attached to the Fc tail *via* the asparagine residue at position 297 (Asn297) (**Figure 1A**). Glycosylation patterns differ between the Fab and Fc regions, and the former contains glycans with high sialylation (up to 93%) (4). The glycan patterns of IgG vary widely among different immune states (5, 6), and they expand the functional repertoires of IgG. In the literature, the level of IgG glycans lacking a galactose residue (G0-IgG) is remarkably correlated with the disease activity of rheumatoid arthritis (RA) (7, 8). In our previous study, we found that the glycosylation levels of TgAb IgG were increased in patients with Hashimoto's thyroiditis (HT) compared to healthy donors (9, 10). Glycan patterns are not templated but remarkably dynamic and govern the biological functions of IgG by affecting its affinity to Fcγ receptors and C1q (11–14), leading to a wide range of immune responses (15). Therefore, carbohydrate structures are critical for modulating the biological functions of IgG in the execution phase of the immune response, and an investigation of the mechanisms underlying the effects of changes in IgG glycosylation could shed new light on the pathogenesis and progression of AIDs.

As shown in **Figure 1B**, the processing pathway of the N-linked glycan structure occurs in a strictly sequential manner by two major enzyme families, namely, glycosyltransferases and glycosylhydrolases (16). It has been reported that multiple factors, such as interleukin-21 (17, 18), antigens (19), nucleotide sugar precursors in culture medium (20), and activated platelets (21), are involved in dynamically regulating the glycosylation of IgG. However, studies on the regulatory mechanism of glycosylation have mainly focused on recombinant IgG in antibody-producing cell lines based on genetic engineering (22, 23) and extracellular modification of serum IgG treated with soluble enzymes (24, 25), and little is known about the effects of cytokines in the microenvironment on changes in the IgG glycan profile and glycosylation enzymes during the differentiation of B lymphocytes.

Elevations of various cytokines in both circulation and the site of inflammation have been reported in AIDs (15, 26). An imbalance between T helper (Th)1 cells/Th2 cells and Th17 cells/Treg cells was reported to play a critical role in the breakdown of immune tolerance and prompt the development of AIDs with local production of cytokines (including IFN-γ, TNF-α, IL-21, and IL-17A), including RA, systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), and autoimmune thyroid diseases (AITD) (27–32). In addition, B-cell-activating factor (BAFF, formerly BLyS) and a proliferation-inducing ligand (APRIL) were reported to be overexpressed in AIDs, which is a survival tactic that supports autoreactive B cells and may represent a critical event that disrupts the immune tolerance of B cells (33–36). The contributions of these elevated cytokines to IgG glycosylation and glycan processing enzymes in B cells remains poorly characterized.

Thus, we established a two-step *in vitro* B cell differentiation system and explored whether various cytokines commonly elevated in AIDs contribute to changes in the IgG glycosylation pattern by regulating the expression of intracellular glycosylation enzymes in B cells.

METHODS

Two-Step *In Vitro* B Cell Differentiation System

This study complied with the Declaration of Helsinki, was approved by the Medical Ethics Committee of Peking University First Hospital and was conducted in accordance with approved guidelines. All participants provided written informed consent (2021–318).

To study the effects of cytokines in the thyroid microenvironment on IgG glycosylation, we first set up a two-step *in vitro* differentiation culture system. Peripheral blood mononuclear cells (PBMCs) from healthy donors were separated by density gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare Life Sciences, USA). Then, $CD19^+$ B lymphocytes were purified by positive selection using magnetic cell separation (CD19 MicroBeads, Human; Miltenyi Biotech, Germany). The purity of the $CD20^+CD19^+$ B cell isolates was above 98% according to flow cytometry (**Figure 2A**).

$CD19^+$ B cells were cultured in X-VIVO 15 serum-free medium (Lonza, Switzerland) supplemented with 100 U/mL penicillin, 100 U/mL streptomycin (Gibco, USA), 5 μg/mL human holo-transferrin (Sigma-Aldrich, USA), and 5 μg/mL insulin (Sigma-Aldrich, USA). It is known that the differentiation of ASCs in response to T cell-dependent antigens requires costimulatory signals to activate B cell receptors, CD40 ligands, and cytokines. Thus, we used the three essential signals to mimic T cell-dependent B cell activation and transformation. $CD19^+$ B cells were cultured at 1×10^6 cells/mL in 12-well plates and activated with 5 μg/mL

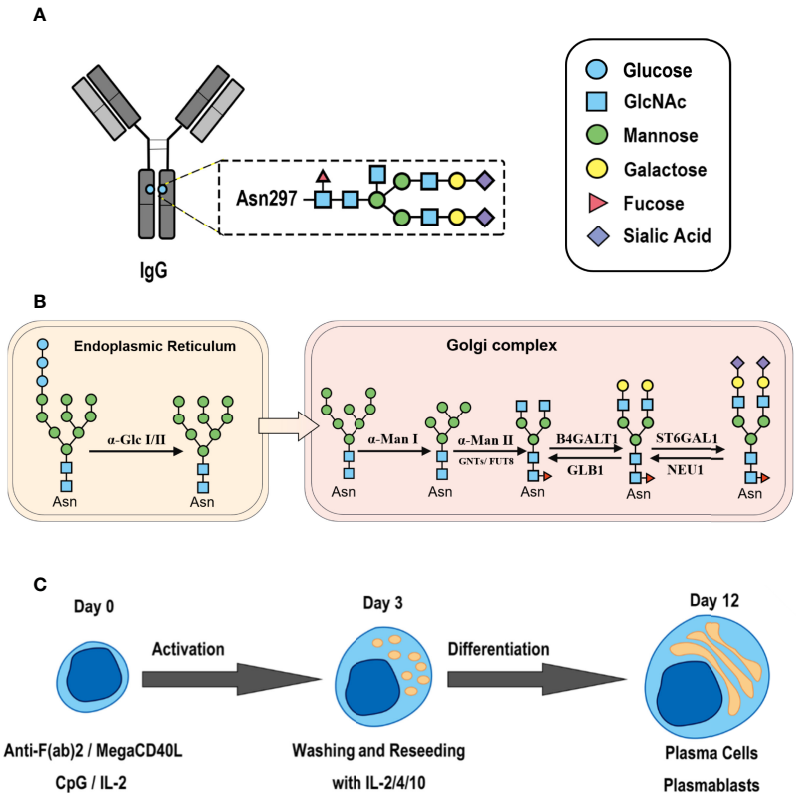


FIGURE 1 | Synthesis of N-glycan in IgG and the two-step differentiation system of B cells. **(A)** Conserved repertoire of an N-linked glycan attached to the Fc domain of IgG at Asn297, which has a biantennary core heptasaccharide consisting of a chain with two N-acetylglucosamines (GlcNAc) and a mannose, followed by two mannose branches and a further GlcNAc following each mannose. The optional residues of a core fucose, a bisecting GlcNAc, one or two galactoses, and sialic acids can attach to the core structures to enrich the structural diversity of IgG. **(B)** Sythesis of N-glycan in B cells. Various α-mannosyltransferases (ALGs) catalyze the synthesis of triantennary $Glc_3Man_9GlcNAc_2$ glycans in the lumen of the endoplasmic reticulum (ER). Then, α-glucosidases I and II remove α-glucose from the sugar chain to form the high-mannose type Glc_3Man_{5-9}. After transfer into the Golgi complex, α-mannosidase I trims mannose residues from the N-glycan to form $Man_5GlcNAc_2$, which is the core structure of hybrid-type N-glycans. Next, α-mannosidase II removes the two α-mannoses from the glycan chain, and two GlcNAc sequences are catalyzed by N-acetylglucosaminyltransferases (GNTs) to form the biantennary heptasaccharide core structure $Man_3GlcNAc_4$. Then, α-1,6-fucosyltransferase 8 (FUT8) catalyzes the addition of fucose to the core structure. β-1,4-Galactosyltransferase 1 (B4GALT1) and β-galactosidase (GLB1) are responsible for the addition and removal of galactose. β-Galactoside α-2,6-sialyltransferase 1 (ST6GAL1) and sialidase-1 (NEU1) transfer and cleave sialic acid to/from oligosaccharides. **(C)** B cell *in vitro* differentiation system. First, B cells were activated by anti-$F(ab')_2$, MegaCD40 L, CpG ODN, and IL-2 for 3 days and then washed and reseeded with IL-2, IL-4, and IL-10 to help activate B cell differentiation into antibody-secreting cells (ASCs) for up to 12 days.

$F(ab')_2$ fragment goat anti-human IgM (Jackson ImmunoResearch Laboratories, USA), 200 ng/mL recombinant MEGACD40L protein (soluble, human) (ENZO Life Sciences, USA), 2.5 μg/mL CpG oligodeoxynucleotide 2006 (*In vivo*Gen, USA), and 100 U/mL recombinant IL-2 and then incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 3 days. After 3 days of stimulation, the activated B cells were seeded into 96-well plates at 5×10^5 cells/mL and cultured with 50 U/mL IL-2, 50 ng/mL IL-10, and 20 ng/mL IL-4 as the control culture conditions. The differentiation efficiency of ASCs was confirmed by flow cytometry.

B cells were washed and resuspended in phosphate-buffered saline (PBS) and incubated with 7-aminoactinomycin D (7-AAD) for 20 min at room temperature (RT) to exclude dead cells. Single-cell suspensions were then washed and stained with optimal dilutions of FITC-CD19, PE-CD20, PerCP-CD38, BV510-CD27 (BD, USA), and APC-CD138 in the dark for 30 min at room temperature (RT). Isotype-matched antibodies were used in all procedures as negative controls. The above antibodies were obtained from Biolegend, Inc., unless otherwise indicated. Then, the cells were washed for further flow cytometry analysis with a FACSCanto II flow cytometer and FlowJo software (Version 10, FlowJo, USA).

Stimulation of B Cells *In Vitro*

The sorted B cells were cultured to differentiate into ASCs under control culture conditions, and IFN-γ, TNF-α, IL-21, IL-17A, BAFF, and APRIL (Peprotech, USA) were individually added to the control culture system in a concentration gradient during the differentiation period. The culture supernatants at day 12 were collected and stored at -80°C. At the same time, the cells were harvested, washed, and stored at -80°C for further experiments. Experiments involving stimulation with each cytokine were performed at least three times with different donors in three replicates.

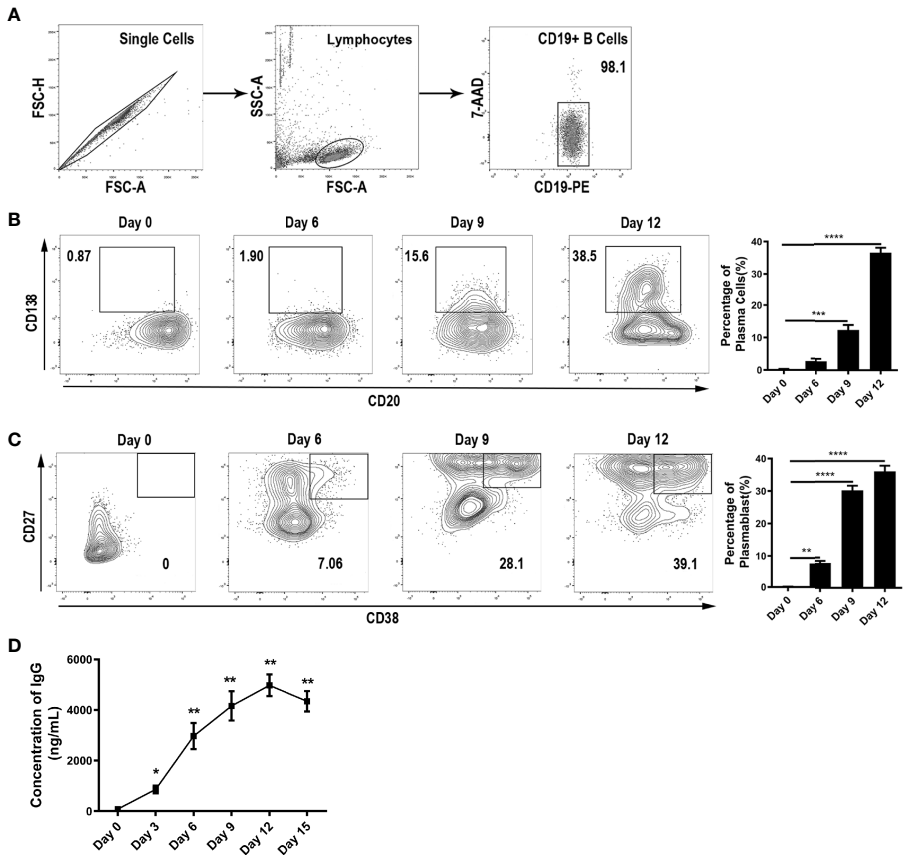


FIGURE 2 | B cell differentiation into ASCs *in vitro*. **(A)** Purity of B cells after sorting by immunomagnetic beads. **(B–C)** Differentiation of B cells into ASCs was monitored by flow cytometry *via* the surface expression of CD138, CD20, CD27, and CD38 on Day 0, Day 6, Day 9, and Day 12. All events were gated by the 7-AAD^{neg} population. The population of CD138^{+}CD20^{low} represented plasma cells, and the CD27^{high}CD38^{+} population represented plasmablasts. The bar charts shown on the right indicate the percentage of ASCs along with the stimulation time. The error bars represent the SD. *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001. **(D)** Measurement of IgG in the culture medium was carried out continuously for up to 15 days. The error bars represent the standard deviation (SD). *p < 0.05 vs. Day 0, **p < 0.001 vs. Day 0.

Measurement of IgG in Supernatants by ELISA

The concentrations of total IgG in the supernatants were measured by enzyme-linked immunoassay (ELISA). Briefly, anti-human IgG (1:400 dilution; Abcam, UK) antibodies were precoated onto a plain 96-well plate at 4°C overnight. Then, the culture samples and IgG standards (Sigma-Aldrich, USA) were incubated with capture antibodies after blocking with 3% bovine serum albumin (BSA) at 37°C for 1 h. Diluted horseradish peroxidase (HRP)-conjugated detection antibody (1:2,000 dilution; Abcam, UK) was added to each well after washing to remove unbound protein, and the plate was maintained at 37°C for 1 h. After washing, hydrogen peroxide and o-phenylenediamine (OPD, Sigma-Aldrich, USA) in citrate–phosphate buffer (pH=5.0) were used as substrate solutions to react with HRP, and the absorbance at 492 nm was recorded with a microplate reader. The concentrations of IgG were determined according to the standard curve of serial dilutions.

Detection of IgG Glycosylation by High-Density Lectin Microarrays

We used commercial lectin microarrays from BCBIO (Guangzhou, Guangdong, China) to detect the glycosylation of IgG in culture supernatants under different stimuli and controls. The details of the lectin microarrays are shown in **Table S1**. The specificities and sources of lectins used in the microarray have been reported in a previous study (37). The microarray was blocked with 50 mM ethanolamine in borate buffer (pH=8.0), and then the IgG concentrations of all samples were adjusted to 5.1 μg/mL with PBST buffer (PBS buffer with 0.05% TWEEN-20) for microarray detection. Alexa Fluor 647-conjugated goat anti-human IgG (H+L) cross-adsorbed antibody (Invitrogen, CA, USA) was oxidized by 20 mM sodium periodate and hybridized with the microarray for 1 h at RT. A Lux Scan 10K-A scanner (CapitalBio Corporation, China) was used to scan the microarray at 10-μm resolution with preadjusted parameters as follows: a 700-photomultiplier tube (PMT) and a power of 75 for the Cy5 channel.

The lectin microarray images were converted to numerical format using LuxScan 3.0 software.

The signal-to-noise ratio (S/N) of each lectin spot was obtained for further screening and statistical analysis. Each lectin on the microarray was present in triplicate, and the signal intensities from replicate lectin measurements in which the coefficient of variation was less than 30% were accepted and averaged. For the negative control in each microarray, the 95% confidence interval of the S/N was 0.8-1.2. Consequently, detectable signals were retained only if the S/N was greater than or equal to 1.2, and any undetected signal was set as 1.0 (37).

Purification of IgG From Culture Supernatants

Culture supernatants were filtered through a 0.22 μm filter (Millipore, USA), and then Hitrap Protein G HP (1 mL, GE Healthcare, USA) was used to purify IgG according to a previously published procedure (9). Briefly, culture samples stimulated with various cytokines were pumped into the affinity column, and unbound proteins were removed using five column volumes of binding buffer (0.02 MTris, pH=7.2). Bound IgG was eluted with elution buffer (0.1 M glycine, pH=2.7), and the eluate was neutralized to pH 7.2 immediately. Then, IgG samples were desalted and exchanged in PBS buffer using a PD-10 Desalting Column (GE Healthcare, USA) according to the manufacturer's instructions, and the IgG solutions were concentrated using a 10-kD ultrafiltration tube (Millipore, USA). The purified IgG samples were stored at -80°C until further use.

N-Glycosylation Profile Analysis of Purified IgG Using MALDI-TOF-MS

The N-glycans of purified IgG in the supernatants were released by PNGase F glycosidase (New England Biolabs, MA) according to our previous protocol (9). Briefly, 40 μg purified IgG sample was added to glycoprotein denaturing buffer (5% SDS, 0.4 M dithiothreitol) and heated at 100°C for 10 minutes. Then, the denatured sample was added to 10% Nonidet P 40 (NP-40) and incubated with 5 μL PNGase F at 37°C for 3 h. The released glycans of IgG were purified by UniElut graphitized carbon solid phase extraction (SPE) columns (Acchrom Technologies Co., Ltd., China). The sample was then lyophilized for the following procedure.

The glycan samples were permethylated and purified according to a previously reported study (38). Briefly, lyophilized samples were mixed with sodium hydroxide slurry in dimethyl sulfoxide and methyl iodide and vortexed for 20 min at RT. After adding chloroform and deionized water (DI water, 18.2 MΩ·cm), the samples were mixed thoroughly and centrifuged. Afterward, the upper aqueous layer was removed, and the chloroform layer was lyophilized. Permethylated glycans were dissolved in 50% methanol, purified on a C18 Sep-Pak 96-well cartridge (Waters, Milford, MA) and lyophilized again. Afterward, the derivatized glycans were dissolved in 10 μL methanol. One microliter of a matrix solution containing 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) was mixed with 1 μL of derivatized glycan, added to a μfocus matrix-assisted laser desorption/ionization (MALDI) plate target (900 μm, 384 circles, HST), and then air-dried at RT. MALDI-mass spectrometry (MALDI-MS) data were obtained in positive mode with a power setting of 130, a mass range from m/z 1000 to 4000U, and 250 shots per sample using a MALDI-time of flight (MALDI-TOF) mass spectrometer (Shimadzu Axima Resonance). All detected glycan peaks are listed in **Table 1**. The frequency of derived glycosylation traits was calculated as described in **Table S2** according to the previous studies (39, 40), including fucosylated N-glycans (F), agalactosylated N-glycans (G0), mono-galactosylated N-glycans (G1), digalactosylated N-glycans (G2), asialylated N-glycans (S0), mono-sialylated N-glycans (S1), disialylated N-glycans (S2), and bisecting GlcNAc (B).

Identification of Glycosylation in F(ab')$_2$ and Fc Fragments by Lectin Blotting Under Control Culture Conditions

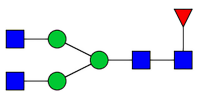
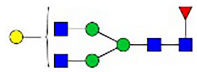
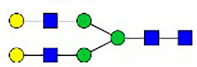
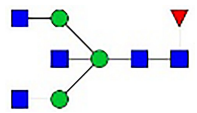
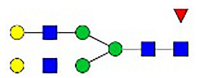
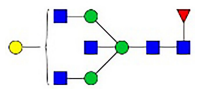
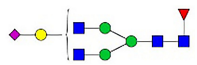
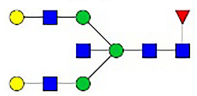
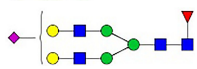
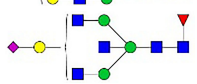
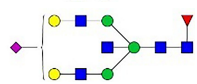
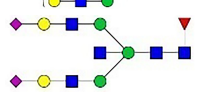
First, the purified IgG under control conditions was adjusted to a concentration of 1 μg/μL with digestion buffer (50 mM sodium phosphate, 150 mM NaCl, pH=6.6). Immunoglobulin-degrading enzyme from *Streptococcus pyogenes* (IdeS protease, Promega, USA) was added to the IgG samples at a ratio of 1:1 (1 μg IgG/1 unit IdeS Protease), and then the samples were incubated at 37°C for 60 minutes. The mixture of F(ab')$_2$ and Fc fragments from IgG was immediately placed on ice. Then, the digestion efficiency of IdeS protease was determined by nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples of F(ab')$_2$ and Fc fragments were diluted to a concentration of 0.5 μg/μL in nonreducing loading buffer (Beyotime, China) containing 5% SDS. Furthermore, the samples were denatured by heating at 70°C for 5 min. A total of 2 μg IgG sample per well was loaded for nonreducing SDS-PAGE with 4-12% ExpressPlus™-PAGE gels (GenScript, USA). The gels were washed with deionized water three times, stained with BeyoBlue™ Coomassie Blue Super Fast Staining Solution (Beyotime, China) and photographed with GBOX-Chemi XT4 (Syngene, UK).

We used *Sambucus nigra lectin* (SNA) to identify glycosylated fragments, since both IgG Fab and Fc fragments contain various percentages of sialylation (4). Electrophoresis was performed on 4-12% ExpressPlus™-PAGE gels (GenScript, USA) to separate the F(ab')$_2$ and Fc fragments, and then the samples were transferred to BioTrace™ NT nitrocellulose (NC) membranes (Pall Corporation, USA), which were blocked with 3% BSA at 4°C overnight. Biotinylated SNA lectin (1:1,000 dilution, Vector Laboratories, USA) was incubated with the NC membranes at RT for 2 h. Streptavidin peroxidase was diluted 1:2,000 in 3% BSA for 1 h at RT. SuperSignal West Femto maximum-sensitivity substrate (Thermo Scientific, USA) was used to visualize the bands, and the membrane was scanned by GBOX-Chemi XT4 (Syngene, UK).

Measurement of Glycosylation Enzymes by Quantitative Real-Time PCR

Cytokines that significantly altered the glycosylation of IgG were selected for coculture with B cells, and then the mRNA

TABLE 1 | The fine structures of 12 IgG N-glycans detected by MALDI-QIT-TOF-MS.

No.	Caculated $[M+Na]^+$ m/z[1]	Detected $[M+Na]^+$ m/z	Structure Abbreviation	Glycan Structure[2]	Relative Indensity (%)						
					Control	IFN-γ	TNF-α	IL-21	IL-17A	BAFF	APRIL
1	1835.9	1835.69	G0F		18.1	15.4	13.5	15.1	15.5	16.9	12.7
2	2040.0	2039.75	G1F		40.8	35.9	25.7	26.8	31.5	41.2	50.4
3	2070.0	2069.75	G2		-[3]	0.4	0.4	0.2	0.5	–	–
4	2081.1	2080.8	G0FN		6.3	3.8	6.5	4.9	3.6	6.6	3.4
5	2244.1	2243.79	G2F		24.6	8.2	6.1	6.4	6.4	12	10.6
6	2285.2	2284.88	G1FN		5.9	35	43	39	35.8	21.2	21.9
7	2401.2	2400.86	G1FS1		–	–	0.7	0.9	0.6	–	–
8	2489.3	2488.93	G2FN		–	0.8	0.5	0.2	–	0.9	0.6
9	2605.3	2604.88	G2FS1		4.2	0.3	2.9	5.5	4.4	0.8	0.3
1-	2646.3	2645.96	G1FNS1		–	–	–	0.2	0.1	–	–
11	2850.4	2850.05	G2FNS1		–	–	0.4	0.4	0.5	0.2	–
12	3211.6	3211.11	G2FNS2		–	–	–	0.4	1	–	–

[1]*Permethylated mass value;*
[2]*Glycan structural features: blue squares, N-acetylglucosamine (N); red triangles, fucose (F); green circles, mannose (M); yellow circles, galactose (G); purple rhombus, sialic acid (S).*
[3]*The undetected N-glycan signals were represented as "-".*

expression of glycosylation enzymes in B cells was detected by real-time PCR. Total RNA was extracted from stimulated B cells by TRIzol reagent (Life Technologies, USA) and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real-time PCR was carried out with a 7500 Fast Real-Time PCR System using SYBR Green Master Mix (Applied Biosystems, USA). The optimal primer sequences were obtained from the Primer-Blast tool from NCBI (National Center for Biotechnology Information, National Institutes of Health, http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer Bank (http://pga.mgh.harvard.edu/primerbank/), as listed in **Table 2**. Experiments involving stimulation with each cytokine and control conditions were performed with at least three batches from different donors in three replicates.

Measurement of Glycosylation Enzymes by Western Blotting

B cells were harvested and lysed on ice for 15 min in RIPA lysis buffer (Pulilai Gene Technology, Beijing) supplemented with a protease inhibitor cocktail (MedChemExpress, USA). Cell lysates (20 μg per sample) were denatured and subjected to SDS-PAGE (10%), followed by transfer to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk and subsequently immunoblotted with anti-B4GALT1 antibody (1:250 dilution; Abcam), anti-GLB1 antibody (1:1000 dilution; Abcam), anti-CD75/ST6GAL1 antibody (1:1,000 dilution; Abcam), anti-sialidase-1 (NEU1) antibody (1:2,000 dilution; Abcam), and anti-GAPDH antibody (1:2,000 dilution, TransGen Biotech, China) overnight at 4°C. HRP-conjugated secondary antibodies were added for incubation, and then blots were developed with

TABLE 2 | Primer sequences for the glycosylation enzymes.

Gene name	Forward Primer	Reverse Primer
ST6GAL1	5′- AACTCTCAGTTGGTTACCACAGA-3′	5′- GGTGCAGCTTACGATAAGTCTT-3′
NEU1	5′- CTTTGCTGAGGCGAGGAAAAT-3′	5′- TTGACAATGAACGCTGTAGGAG-3′
B4GALT1	5′- CCAGGCGGGAGACACTATATT-3′	5′- CACCTGTACGCATTATGGTCAT-3′
GLB1	5′- GTGCTGTACCGGACAACACTT-3′	5′- ATCACATTGTTTCGCTCAAGGA-3′
GAPDH	5′-GGAGCGAGATCCCTCCAAAAT-3′	5′- GGCTGTTGTCATACTTCTCATGG-3′

Sequences of the glycosylation enzyme primers used in the study for real-time quantitative PCR detection.

SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA). ImageJ software (developed at the National Institutes of Health) was used to quantify the relative staining intensity. Each experiment was conducted with at least three batches from different donors.

Statistical Analysis

All analyses were performed using Prism 8 software (GraphPad, USA). All experiments were performed at least three times from different donors with three replicates. Statistical analysis was performed with an unpaired Student's t-test or one-way analysis of variance (ANOVA), as appropriate. A p value less than 0.05 was considered statistically significant.

RESULTS

Differentiation of B Lymphocytes Into ASCs *In Vitro*

Unlike previous studies on PBMCs, we cultured human primary B cells and used a two-step *in vitro* differentiation culture system to simulate the microenvironment of AIDs (**Figure 1C**). In this system, we found that up to 38.5% and 39.1% of $CD19^+$ B cells had differentiated into plasma cells and plasmablasts, respectively, at day 12 (**Figures 2B, C**). We determined day 12 as the harvest day (**Figure 2D**) under optimized culture conditions (**Figures S1A–G**), which were applied as the control culture conditions in the subsequent experiments.

Production of IgG in Culture Supernatants Under Different Cytokines

We found that the addition of IFN-γ, TNF-α, IL-21, IL-17A, BAFF, and APRIL to the control culture medium could promote the secretion of IgG (p<0.001). IL-21 increased the yield of IgG in a concentration-dependent manner (**Figure 3C**). The production of IgG in response to the other cytokines varied according to the concentration, presenting an inverted U-shaped curve (**Figures 3A, B, D–F**). The following cytokine concentrations were selected for subsequent experiments according to the peak yield of IgG: 2.5 ng/mL for IFN-g, 50 ng/mL for TNF-a, 50 ng/mL for IL-21, 20 ng/mL for IL-17A, 50 ng/mL for BAFF, and 3.13 ng/mL for APRIL.

Glycosylation Patterns of IgG Detected by Lectin Microarray

As shown in **Figure 4A**, the glycosylation patterns of IgG in the supernatants showed considerable differences under stimulation

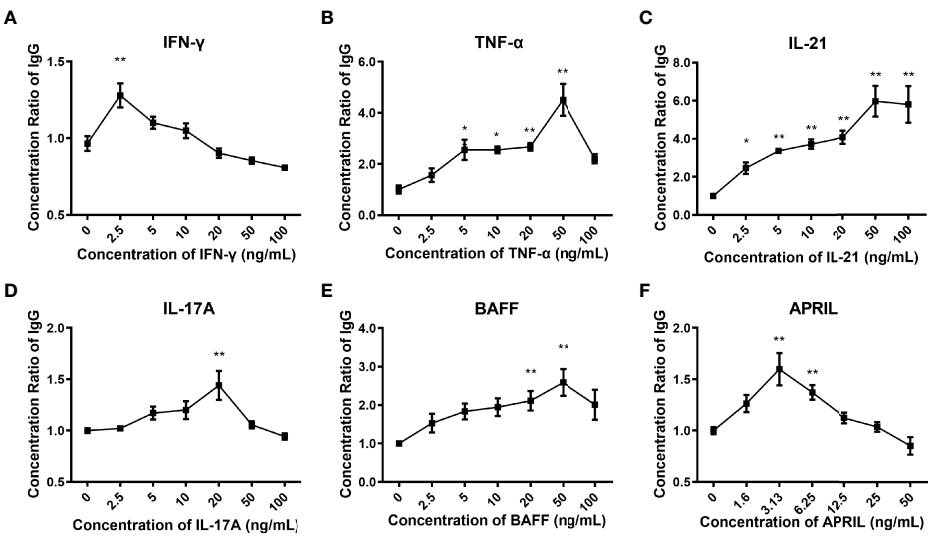


FIGURE 3 | Production of IgG in the culture supernatants. **(A–F)** Concentration ratios of IgG in the culture supernatants stimulated by different concentrations of IFN-γ, TNF-α, IL-21, IL-17A, BAFF, and APRIL. The concentration ratio of IgG was calculated as the concentration of IgG under the specific stimulation divided by the basic concentration of IgG under the control condition. The error bars represent the SEM. *p < 0.05 vs. 0 ng/ml group, **p < 0.001 vs. 0 ng/ml group.

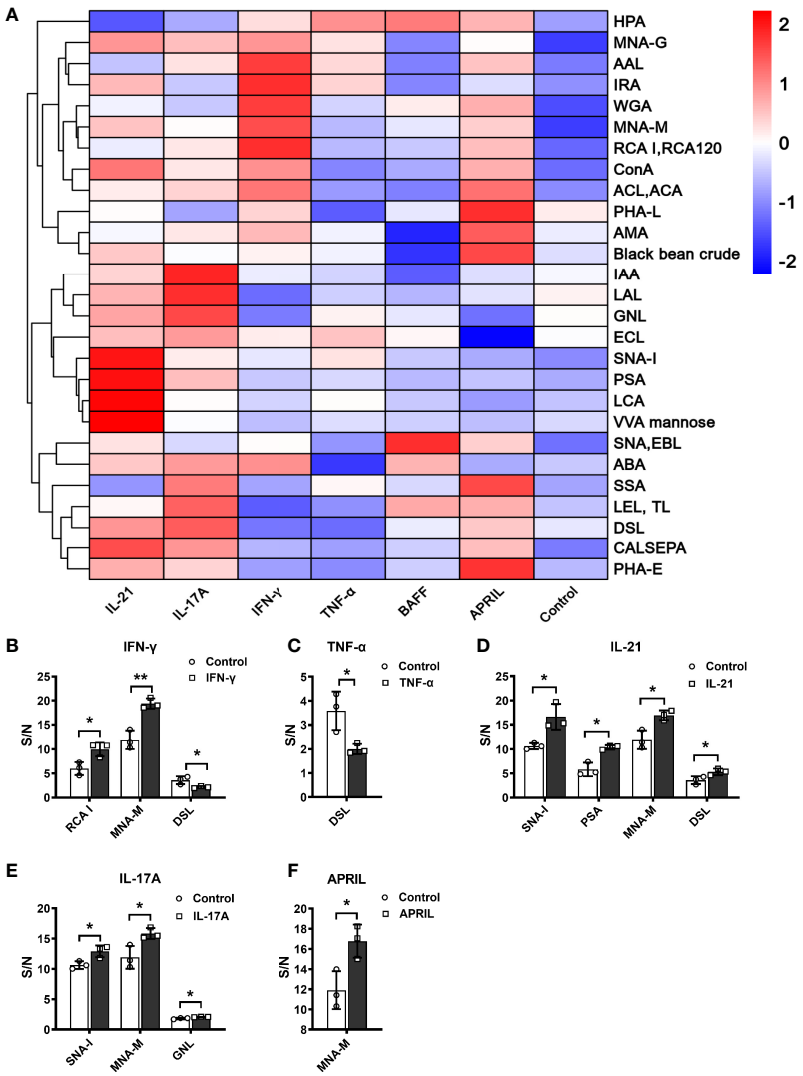


FIGURE 4 | S/N of detectable lectins for IgG under different cytokine stimulations. **(A)** Clustered heatmap of lectin IgG binding profiles. Detectable lectins were defined as those with (1) an S/N > 1.2 for each lectin spot and (2) an S/N coefficient of variation (measured in triplicate) less than 30%. The detectable lectins are listed on the left vertical axis, and the samples under different stimuli are indicated along the horizontal axis, including IFN-γ, TNF-α, IL-21, IL-17A, BAFF, and APRIL. Each square represents the S/N of a lectin binding pattern on IgG. The values of S/N were transformed by min–max normalization. The color bar represents the scale—red indicates a higher S/N, and blue indicates a lower S/N. **(B–F)** S/N of IgG with significant changes under stimulation by IFN-γ, TNF-α, IL-21, IL-17A, and APRIL *in vitro*. The binding specificities of lectins were SNA-I, SNA, and EBL for terminal Neu5Acα2–6/sialic acid (SA); RCA-I, ECL, PHA-L, PHA-E, MNA-G, ACL, ACA, and ABA for galactose (Gal); and DSL, LEL, TL, and WGA for N-acetylglucosamine (GlcNAc). The error bars represent the SD. *p < 0.05, **p < 0.001.

with different cytokines. The recognition specificities and sources of the lectins are listed in **Table 3**. Each cytokine played a unique role in regulating the glycosylation profile of IgG. Specifically, IFN-γ upregulated the levels of galactose (RCA-I p<0.05) and mannose (MNA-M p<0.05) and decreased the level of GlcNAc (DSL, p<0.05) (**Figure 4B**). TNF-α decreased the level of GlcNAc (DSL, all p<0.05) (**Figure 4C**). IL-21 significantly increased the levels of sialic acid (SNA-I p<0.05), mannose (PSA p<0.05, MNA-M p<0.05), and GlcNAc (DSL p<0.05) (**Figure 4D**). The addition of IL-17A increased the levels of sialic acid (SNA-I p<0.05) and mannose (MNA-M, GNL all p<0.05) (**Figure 4E**). APRIL significantly increased the mannosylation of IgG (MNA-M, p<0.05) (**Figure 4F**). The addition of BAFF showed no influence on IgG glycosylation. In short, the level of galactose was upregulated by IFN-γ; sialylation of IgG was upregulated by IL-21 and IL-17A; mannosylation of IgG was upregulated by IFN-γ, IL-21, IL-17A, and APRIL; and the level of GlcNAc was regulated by IFN-γ, TNF-α, and IL-21. All changes of glycosylation on IgG stimulated by various cytokines in lectin microarray are listed in **Table S3**.

TABLE 3 | The recognition specificity and source of lectin.

Glycan	Full name	Abbr.	Preferred sugar	Source
Sialic acid	*Sambucus nigra* (elderberry bark)	SNA-I	α-2,6 linked sialic acid residues	4
	Sambucus nigra lectin	SNA, EBL	Neu5Acα6Gal/GalNAc	2
	Salvia sclarea	SSA	NeuAc	4
Galactose	*Morniga G lectin* (black elderberry)	MNA-G	Gal	4
	Ricinus communis agglutinin I	RCA I	Gal	4
	Phaseolus vulgaris leucoagglutinin	PHA-L	Galβ4GlcNAcβ6 (GlcNAcβ2 Manα3) Manα3	1
	Phaseolus vulgaris Erythroagglutinin	PHA-E	Galβ4GlcNAcβ2 (Galβ4GlcNAcβ6) Man	4
	Erythrina cristagalli Lectin	ECL	Galβ4GlcNAc (Terminal)	4
	Agaricus bisporus Lectin (Mushroom)	ABA	Galβ3GalNAc	4
	Amaranthus caudatus lectin	ACL,ACA	Galβ3GalNAc	4
Fucose	*Aleuria aurantia lectin*	AAL	Fucα6GlcNAc	2
	Laburnum anagyroides lectin (gold chain)	LAL	α-Me-L-Fucose among monosaccharides	4
	Lens Culinaris Agglutinin	LCA	Fucose linked α(1,6) to core GlcNAc of N-linked glycopeptides	3
Mannose	*Pisum sativum agglutinin*	PSA	αMan,αGlc	4
	Morniga M lectin (black elderberry)	MNA-M	Man	4
	Galanthus nivalis (snowdrop) *lectin*	GNL	αMan	2
	Canavalia ensiformis, jack bean	ConA	αMan,αGlc	4
	Vicia villosa Lectin (Hairy Vetch, Mannose Specific)	VVA Man	Man	4
	Calystega sepiem Lectin (Hedge Bindweed Rhizomes)	CALSEPA	Man	4
GlcNAc	*Datura stramonium lectin*	DSL	(GlcNAc)2-4	3
	Triticum vulgare lectin (wheat germ)	WGA	GlcNAcβ4GlcNAc)1-4	1
	Lycopersicon esculentum lectin	LEL,TL	GlcNAc (prefer trimer and tetramer)	3
GalNAc	*Helix pomation Lectin* (Snail)	HPA	GalNAc	1
	Iris hybrid Lectin (Dutch Iris)	IRA	GalNAc	4
	Phaseolus vulgaris sp. *Lectin*	Black bean crude	GalNAc> galactose, sialic acid	4
	Iberis amara Lectin	IAA	GalNAc	4

Detailed information on the detectable lectins is listed above.

The lectins used in this study were obtained from four different sources: 1, Molecular Probes, Inc.; 2, Irwin J. Goldstein's group; 3, Vector Laboratories, Inc.; and 4, EY Laboratories, Inc. Sugar abbreviations: Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; Neu5Ac, N-acetylneuraminic acid (sialic acid).

N-Glycan Structure Analysis by MALDI-TOF-MS

Since galactose, sialic acid, mannose, and GlcNAc were attached to the heptasaccharide core structure in various manners, the fine structure of various glycoforms could not be distinguished by lectin microarray. MALDI-TOF-MS was further performed to confirm the carbohydrate structures of glycans released from IgG. A total of 12 distinct N-glycan structures were identified and annotated with the proposed structures shown in **Table 1**. The relative intensities of derived glycosylation traits are listed in **Table 4**. Though the increased level of mannose was detected by lectin microarray, we found that all detectable glycoforms were complex-type N-glycans (**Figure 5**). This could be associated with the sample preparation causing a great loss, the amounts of terminal mannose-containing N-glycoform was too low to be detected by MALDI-TOF-MS. An overlap of 6 N-glycan peaks was shown in the control and stimulation groups (G0F m/z 1835.69, G1F m/z 2039.75, G0FN m/z 2080.8, G2F m/z 2243.79, G1FN m/z 2284.88, and G2FS1 m/z 2604.88).

More detectable N-glycoforms containing galactose were observed in IFN-γ group than in control group, including G1F (m/z 2039.75), G2 (m/z 2069.75), G2F (m/z 2243.79), G1FN (m/z 2284.88), G2FN (m/z 2488.93), and G2FS1 (m/z 2604.88) (**Figure 5B**). We found a decreased frequency of G0 in the IFN-γ group (19.2%) compared to the control group (24.4%), and a higher frequency of G1 in the IFN-γ group (70.9%) than in the control condition (46.7%). This result indicated that the increased frequency of G1 may contribute to the increased

TABLE 4 | Relative intensity of derived glycosylation traits.

Derived traits	Relative Intensity (%)						
	Control	IFN-γ	TNF-α	IL-21	IL-17A	BAFF	APRIL
Fucosylated N-glycans (F)	99.9	99.4	99.3	99.8	99.4	99.8	99.9
Agalactosylated N-glycans (G0)	24.4	19.2	20.0	20.0	19.1	23.5	16.1
Mono-galactosylated N-glycans (G1)	46.7	70.9	69.4	66.9	68	62.4	72.3
Digalactosylated N-glycans (G2)	28.8	9.7	10.3	13.1	12.8	13.9	11.5
Asialylated N-glycans (S0)	95.7	99.5	95.7	92.6	93.3	98.8	99.6
Mono-sialylated N-glycans (S1)	4.2	0.3	4.0	7.0	5.6	1.0	0.3
Disialylated N-glycans (S2)	0	0	0	0.4	1.0	0	0
Bisecting GlcNAc (B)	12.2	39.6	50.4	45.1	41.0	28.9	25.9

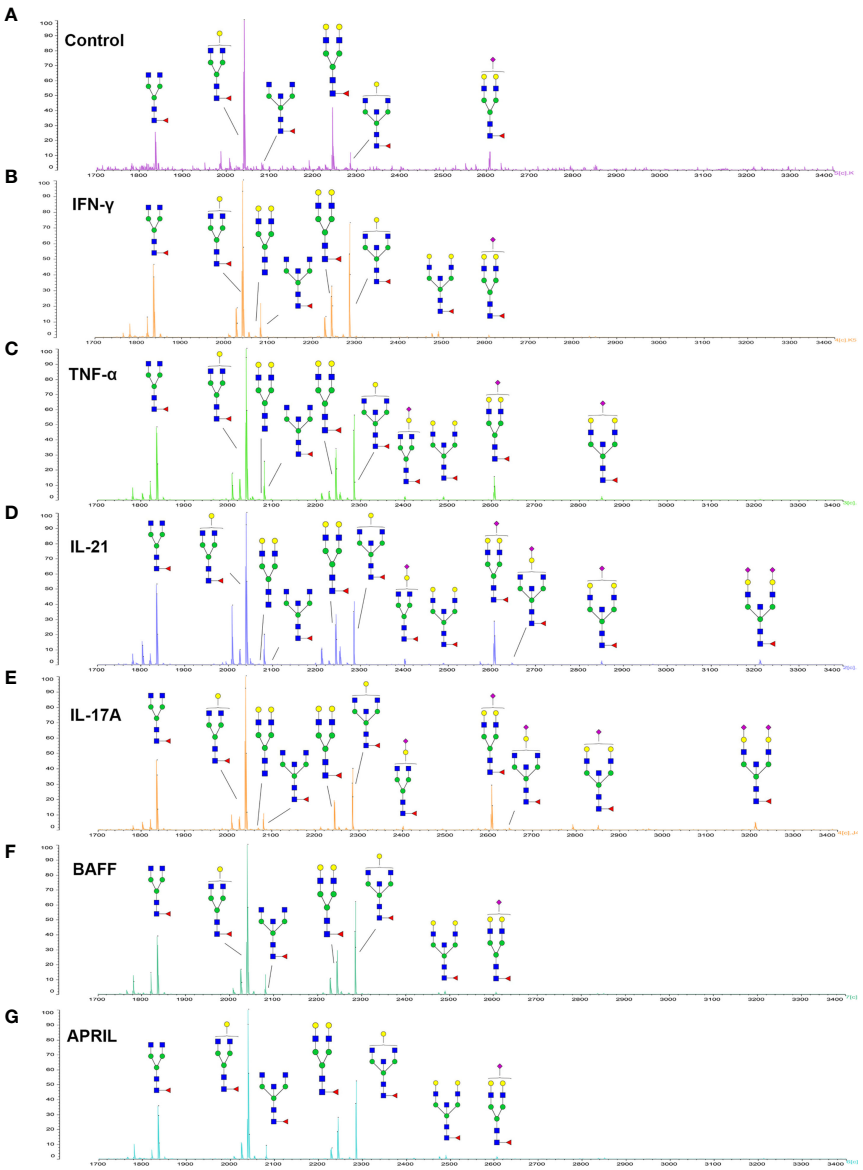


FIGURE 5 | MALDI-TOF-MS spectrum of N-glycans from purified IgG stimulated by cytokines. **(A–G)** Glycosylation profiles of purified IgG from the culture medium under control conditions and stimulation with IFN-γ, TNF-α, IL-21, IL-17A, BAFF, and APRIL detected by MALDI-TOF-MS at m/z 1700–3400.

binding of IgG to RCA-I, which recognized galactose in lectin microarray.

The addition of IL-21 and IL-17A enriched sialylated N-glycan structures (including G1FS1 m/z 2400.86, G2FS1 m/z 2645.96, G2FNS1 m/z 2850.05, and G2FNS2 m/z 3211.11) (**Figures 5D, E**). We found that the frequency of S0 was decreased by IL-21 and IL-17A compared to the control condition (92.6% in the IL-21 group; 93.3% in the IL-17A group; 95.7% in the control group). And the frequencies of both S1 (7.0% in the IL-21 group; 5.6% in the IL-17A group; 4.2% in the control group) and S2 (0.4% in the IL-21 group; 1.0% in the IL-17A group; undetected in the control group) were higher in IL-21 and IL-17A groups, which was consistent with the data in lectin microarray.

We further identified bisecting GlcNAc glycoforms by MALDI-TOF-MS, including G0FN (m/z 2080.8), G1FN (m/z 2284.88), G2FN (m/z 2488.93), G1FNS1 (m/z 2850.05), and G2FNS2 (m/z 3211.11). We found that the relative intensity of bisection glycoforms was higher in the stimulation groups (39.6% in the IFN-γ group; 50.4% in the TNF-α group; 45.1% in the IL-21 group; 41.0% in the IL-17A group; 28.9% in the BAFF group; 25.9% in the APRIL group) than in the control group (12.2%).

Combined with the results of fine structural analysis by MALDI-TOF-MS and the quantification of lectin microarray,

we finally confirmed that the proportion of galactose-containing glycans, especially G1, was increased by IFN-γ and that the frequency of sialylated glycoforms was increased by IL-21 and IL-17A.

Regulation of Glycosylation Enzymes by Different Cytokines

We further explored changes in the mRNA and protein levels of galactose- and sialic acid-related glycosyltransferases and glycosylhydrolases under stimulation with IFN-γ, IL-21, and IL-17A. The main functions and details of the glycosylation enzymes are shown in **Table S4**.

Elevated of IgG galactosylation was observed when B cells were stimulated with IFN-γ. Both the mRNA (1.45-fold) and protein levels of β-1,4-galactosyltransferase 1 (B4GALT1, 2.18-fold) were increased in the IFN-γ group compared with the control group, while no changes were found in either the mRNA or protein levels of β-galactosidase (GLB1) between the IFN-γ and control groups (**Figures 6A, B**). As shown in **Figure 1B**, the absence of sialic acid may result in the exposure of galactose residues. We found no significant changes in the expression of ST6GAL1 and NEU1 in B cells after stimulation with IFN-γ (**Figures 6A, B**) at the transcriptional and translational levels. This result indicated that the increased galactosylation regulated by IFN-γ resulted from changes in B4GALT1 rather than the expression of ST6GAL1 and NEU1.

For sialic acid, we found that IL-21 significantly upregulated the mRNA expression of β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) by 1.25-fold and sialidase-1 (NEU1) by 1.2-fold (**Figure 6C**). The protein expression of ST6GAL1 was significantly increased to 1.33-fold, and no significant difference in NEU1 protein expression was detected after the addition of IL-21. The addition of IL-17A resulted in upregulation of ST6GAL1 mRNA and protein expression by 1.42-fold and 1.92-fold, respectively, and a 1.6-fold downregulation of NEU1 mRNA expression (**Figure 6E**) with no significant difference in NEU1 protein expression (**Figures 6D, F**). Our data showed that changes in galactose and sialic acid resulted from upregulation of glycosyltransferases rather than glycosylhydrolases.

DISCUSSION

Glycosylation, as a posttranslational modification of IgG, is critical for the modulation of the immune response to inflammation, including ADCC (41), CDC (42), antibody-dependent cellular phagocytosis (ADCP) (43), and antigen binding (4), and may act as a 'switch' to control the pathogenicity of IgG (15). In our study, we found that cytokines in the microenvironment promoted IgG secretion and changed the N-glycan patterns of IgG. Meanwhile, the expression of B4GALT1 and ST6GAL1 was upregulated to meet the demands for IgG glycosylation, including the increased amount of IgG and the level of sialylation and galactosylation. This study may provide a new perspective for the control of the IgG evolutionary direction to prevent the development of AIDs.

Accumulating evidence has identified a characteristic microenvironment with abnormally elevated cytokines and molecules in AID patients. In our research, the changes in

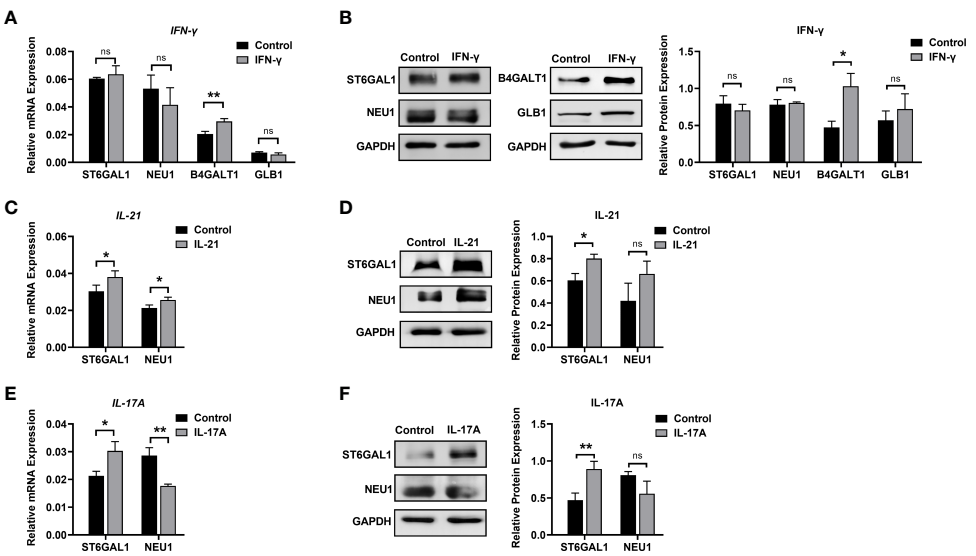


FIGURE 6 | Changes in glycosylation enzymes in B cells under stimulation. **(A)** mRNA expression of B4GALT1, GLB1, ST6GAL1, and NEU1 under stimulation with IFN-γ. **(B)** Protein expression of B4GALT1, GLB1, ST6GAL1, and NEU1 under stimulation by IFN-γ. **(C, E)** mRNA expression of ST6GAL1 and NEU1 under stimulation by IL-21 and IL-17A, respectively. **(D, F)** Protein expression of ST6GAL1 and NEU1 under stimulation by IL-21 and IL-17A, respectively. Total RNA was extracted from activated B cells after 12 h of stimulation. Protein was also collected from activated B cells after 72 h of stimulation. The mRNA and protein expression of glycosylation enzymes was normalized to the GAPDH mRNA and GAPDH protein relative staining intensity. The error bars represent the SD. ns means no significance, $^{*}p < 0.05$, $^{**}p < 0.001$.

glycosylation profiles of secreted IgG under stimulation with six cytokines during the differentiation phase were confirmed by lectin microarray and MALDI-TOF-MS. Data of lectin microarray showed that IFN-γ significantly increased the proportion of galactose-containing glycans, especially the G1 N-glycans detected by MALDI-TOF-MS. In addition, the upregulation of B4GALT1 after IFN-γ treatment further confirmed the changes of galactosylation of IgG. IL-21 and IL-17A significantly increased sialylation of IgG detected by lectin microarray and confirmed by mass spectrum, accompanied by upregulation of ST6GAL1. The discrepancy in GlcNAc levels between lectin microarray and MALDI-TOF-MS may result from the combined effect of bisecting and biantennary GlcNAc. MALDI-TOF-MS identified ab increased frequency of bisecting glycans in the stimulation groups compared to control group, which indicated that cytokines may regulate the synthesis of bisecting GlcNAc in IgG. The above results highlight that the intracellular glycosylation-regulating mechanisms of cytokines are complex and independent, forming a multiple regulatory network. In the literature, studies have indicated that the intracellular glycosylation regulatory effects of cytokines on different cell types (44–46) and species (17, 18) have discrepant results. In addition, the regulation of IgG glycosylation *in vivo* and *in vitro* differs since extrinsic glycosylation plays a critical role in the homeostasis of IgG (21, 47, 48). Changes in IgG glycosylation are dynamically but precisely regulated throughout the course of disease, and the combined influences of complex *in vivo* immunologic environments on human B cells and IgG glycans remain poorly defined and should be further studied.

Glycosylation may be introduced in variable domains of the IgG Fab fragment. Fab glycans showed a high percentage of sialic acid (up to 93%) compared to Fc N-glycans (49), since the spatial structure of the Fab fragment was more accessible to glycosyltransferases. To determine whether Fab glycosylation occurred under our two-step culture condition *in vitro*, SNA (which specifically recognizes sialic acid) lectin blotting was performed to determine the glycosylation of the $F(ab')_2$ fragment. We found that under control condition glycosylation in the $F(ab')_2$ portion was quite low compared to that in the Fc fragment. However, we found that the relative intensity of G2FNS2 glycoform, which was reported to be presented in Fab glycosylation (40, 50), was increased by IL-21 and IL-17A. And in previous study (17), the G2FNS2 glycoform was undetected in Fc glycan profile of IgG after IL-21 stimulation of B cells, which indicated that introduced glycans may exist in variable domain of IgG. In addition, the elevated mannosylation of IgG with unified concentration was also observed in IL-21 and IL-17A groups with no detection of high mannose or hybrid type by MALDI-TOF-MS, which indicated that the increased mannose levels of IgG may result from the introduced glycans in Fab fragment of IgG. Above findings indicated that cytokines may play a role in regulating introduced glycosylation in Fab fragment of IgG during the differentiation period of B cell response.

Upregulated levels of galactose and sialic acid in IgG Fc may affect the biological function of IgG (15). Changes in Fc glycoforms might be involved in disease onset as the trigger to control the final immune response of IgG. Several studies observed that terminal galactosylation increases the affinity to FcγRIIIa and complement component 1q (C1q), leading to enhancement of ADCC and CDC activity (41, 42). Sialylation of IgG has been reported to decrease its affinity to FcγRIIIa to reduce ADCC activity but enhance ADCP activity (15) and prolong the half-life of IgG in serum (51). In short, the regulatory effects of cytokines on IgG glycosylation are multifactorial and complex. According to the results in our study, some cytokines such as IFN-γ trigger the pathogenicity of IgG by remodeling the carbohydrate structure of IgG, while other cytokines, such as IL-21 and IL-17A, endow IgG with dual roles of pro- and anti-inflammatory glycan patterns. Cytokines in the microenvironment dynamically and accurately regulate the immune responses of IgG *in vivo*.

In contrast to extracellular IgG glycosylation, we focused on the effects of cytokines on IgG glycoforms and further confirmed that the increased levels of galactose and sialic acid in IgG were upregulated by B4GALT1 and ST6GALT1 in human B cells, which complemented the intracellular regulatory mechanisms of diverse IgG glycosylation. The regulatory effects of cytokines on glycosylation enzymes are examined in limited studies and are poorly understood and controversial. For example, IFN-γ increases sialylated N-glycan structures due to upregulated mRNA levels of sialyltransferases (ST6GAL1) in human bone marrow-derived mesenchymal stromal cells (MSCs) to influence migration and survival (52). However, another study reported that IFN-γ decreased the expression of ST6GAL1 in LPS-induced B cell cultures (44). In our study, we found that the addition of IFN-γ showed no significant regulatory effect on ST6GAL1. These discrepancies might result from the different activation modes under different disease contexts in various studies, which indicated that the expression of glycosyltransferases was regulated by a variety of factors and involved in controlling the biological functions of functional proteins in different cell models.

This study had several limitations. First, glycan peaks with low intensity were undetected in MALDI-TOF-MS, which may result from the limited amount of IgG secreted *in vitro*, and the intricate sample preparation process of IgG purification, desalting, and N-glycan release caused high sample loss. However, the obtained glycoforms have proven the complex effect of cytokines on IgG glycosylation profiles. Second, the limited differentiation efficiency of our culture conditions *in vitro* resulted in the obtained IgG being insufficient to perform the functional experiments. Another notable limitation was that our research only confirmed the multiple regulation of single cytokines on IgG glycosylation; thus, the integrated effects of cytokine combinations on IgG glycosylation should be detected in B cells from patients with AIDs in future.

In conclusion, we proved that cytokines in the microenvironment promoted the secretion of IgG and altered the carbohydrate structures of IgG by regulating the expression of intracellular glycosylation enzymes in B cells *in vitro*. Our study revealed the interconnected complexity of glycan regulatory networks in the microenvironment, which is an essential

complement of the intracellular regulation mechanisms of IgG glycosylation.

DATA AVAILABILITY STATEMENT

The original datasets analyzed in the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YC, Conceptualization, methodology, formal analysis, investigation, resources, writing - original draft, and project administration. ZG, methodology and formal analysis. ZS, methodology, formal analysis, and investigation. XZ and YH, investigation and resources. YGo, conceptualization and methodology. KZ, methodology and investigation. CQ, conceptualization and investigation. YL, conceptualization and formal analysis. YGa, conceptualization, project administration, writing - review and editing, and funding acquisition. JZ and XG, conceptualization, and supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.724379/full#supplementary-material

Supplementary Figure 1 | Determination of the optimal culture conditions. **(A–D)** Concentrations of IgG in the supernatant when primary B cells were treated with stimuli in a concentration gradient during the activation phase. **(E–G)** Concentrations of IgG in the supernatant when primary B cells were treated with stimuli in a concentration gradient during the differentiation period. The two-step *in vitro* B cell differentiation system was optimized by concentration gradient treatment with all stimuli. IgG secretion in the culture medium on day 12 was determined by ELISA. The error bars represent the SD. *$p<0.05$ vs. 0 ng/ml group, **$p<0.001$ vs. 0 ng/ml group.

Supplementary Figure 2 | Confirmation of glycosylation in IgG $F(ab')_2$ and Fc fragments. **(A)** Nonreducing SDS-PAGE gel stained with Coomassie brilliant blue, confirming that the full-length IgG molecule (left lane, 180 KD) was digested into $F(ab')_2$ (right lane, 130 KD) and the Fc fragment (right lane, 30 KD) by IdeS protease. **(B)** SNA lectin blotting was performed to confirm the glycosylation of IdeS protease-treated purified IgG. IgG obtained under control conditions was digested into $F(ab')_2$ and Fc fragments. Three biological repetitions were performed.

Supplementary Figure 3 | Percentage of CD19+ B cells in peripheral blood mononuclear cells (PBMCs). **(A)** Histrogram of PBMC from healthy donor detected by flow cytometry. **(B)** Percentage of CD19+ B cells in PBMCs. The bars show the means, and the error bars show the SD.

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REVIEW
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Glycometabolism Reprogramming of Glial Cells in Central Nervous System: Novel Target for Neuropathic Pain

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Neuropathic pain is characterized by hyperalgesia and allodynia. Inflammatory response is conducive to tissue recovery upon nerve injury, but persistent and exaggerated inflammation is detrimental and participates in neuropathic pain. Synaptic transmission in the nociceptive pathway, and particularly the balance between facilitation and inhibition, could be affected by inflammation, which in turn is regulated by glial cells. Importantly, glycometabolism exerts a vital role in the inflammatory process. Glycometabolism reprogramming of inflammatory cells in neuropathic pain is characterized by impaired oxidative phosphorylation in mitochondria and enhanced glycolysis. These changes induce phenotypic transition of inflammatory cells to promote neural inflammation and oxidative stress in peripheral and central nervous system. Accumulation of lactate in synaptic microenvironment also contributes to synaptic remodeling and central sensitization. Previous studies mainly focused on the glycometabolism reprogramming in peripheral inflammatory cells such as macrophage or lymphocyte, little attention was paid to the regulation effects of glycometabolism reprogramming on the inflammatory responses in glial cells. This review summarizes the evidences for glycometabolism reprogramming in peripheral inflammatory cells, and presents a small quantity of present studies on glycometabolism in glial cells, expecting to promote the exploration in glycometabolism in glial cells of neuropathic pain.

Keywords: inflammation, glycolysis, microglia, astrocyte, synapse, neuropathic pain, glycometabolism reprogramming

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INTRODUCTION

Neuropathic pain is caused by a lesion/disease of the somatosensory system, and is estimated to affect 7%-10% of the general population (1, 2). The pathogenesis of neuropathic pain is complex, and involves the entire nociceptive pathway (primary afferent nerves, spinal cord, brain, and descending pathways) as well as glial cells.

The pathological basis of neuropathic pain is hyperalgesia and allodynia caused by synaptic remodeling in the nociceptive pathway. Chronic nerve injury promotes the release of pro-inflammatory cytokines to activate intracellular signal transduction pathways, and to disturb the balance between facilitation and inhibition in pain signal transduction. A variety of animal models for neuropathic pain (e.g., chronic constriction injury and spinal nerve ligation) have been developed based on persistent nerve injury (3). Recent studies identified abnormal glycometabolism in neurons and the supporting glial cells upon chronic nerve injury (4). Under normal oxygen-rich conditions, pyruvate enters the tricarboxylic acid (TCA) cycle for oxidative phosphorylation into CO_2 and NADH in the central nervous system. Under hypoxic conditions, however, pyruvate is converted into lactate and NAD^+ through anaerobic glycolysis. Anaerobic glycolysis has low efficiency in energy production than oxidative phosphorylation, but is the preferred metabolic pathway in the active phase of cell proliferation (5).

Glial cells are implicated in a variety of neurophysiological processes, including neuronal development, synaptic remodeling, and neuropathic pain (6). Glial cells have been shown to participate in chronic pain *via* multiple mechanisms, including regulating glutamate concentration in synaptic cleft through glutamate transporters (7), controlling the release of neurotransmitters (8), altering stability of the synaptic microenvironment (9), and modifying inter-neuronal communications (10). When activated by inflammation, glial cells switch from oxidative phosphorylation to preferentially use glycolysis as energy source, with accompanying changes in pentose phosphate pathway, amino hexanoic acid and glutamine hydrolysis pathway (11). Metabolic intermediates produced in glycometabolism reprogramming also provide substrates for other biosynthetic pathway in cell growth and differentiation, and could regulate a variety of intracellular signaling pathways at both the transcriptional and post-transcriptional levels. To some extent, these effects determine the fate of neurons or glial cells (12).

This review summarizes the neuropathology of neuropathic pain, regulation of phenotypic transition and pain sensitization by glycometabolism reprogramming of glial cells under chronic nerve injury, and the influences of glycometabolism reprogramming on synaptic plasticity and neuronal excitability. The viewpoints are helpful in exploring the crucial roles of glycometabolism reprogramming of glial cells in the development of neuropathic pain and providing potential targets for the intervention of neuropathic pain.

NEUROPATHOLOGY OF NEUROPATHIC PAIN

Nociceptive stimuli are converted into electrochemical signals by pain receptors and transmitted to the spinal cord *via* primary afferent neurons. As the station of signal regulation and integration, the spinal cord sends pain signals to the brain through the upward projection fibers. The upward signal transmission is regulated by downward signals through the spinal cord to effectors *via* efferent neurons (13). Changes in any part of this nociceptive pathway can lead to allodynia or hyperalgesia.

Chronic nerve injury could produce structural changes in the spinal projection area of afferent neurons. In physiological conditions, peripheral C fibers mainly project to the substantia gelatinosa (lamina II) of spinal cord to transmit chronic pain signals, whereas the Aδ fibers mainly project to lamina I and III to transmit acute pain signals. Tactile information is mainly transmitted by Aβ fibers that project to lamina III and IV. In neuropathic pain, chronic nerve injury induces abnormal projection of the Aβ fibers to neurons in lamina I and III to form additional neural circuits for hyperalgesia and allodynia. Chronic nerve injury also induces actin cytoskeleton remodeling, thus changing the density and length of dendritic spines of the neurons in spinal cord (14, 15). Rho/Rac molecules in the GTPase superfamily could transmit pain signals to intracellular actin cytoskeleton through neurotransmitters, and promote the generation of dendritic spines and ultimately structural communication among neurons. Selective inhibition of Rac1 protein has been shown to attenuate hyperalgesia and reduce the changes in dendritic spines in an animal model of neuropathic pain (16). Chronic nerve injury also promotes autophagy and apoptosis in the inhibitory γ-aminobutyric acid (GABA) interneurons, and reduces the activity of GABA synthase and glutamic acid decarboxylase (GAD), ultimately leading to neuronal disinhibition (17). As the supporting cells of neurons, glial cells are exquisitely sensitive to microenvironment changes. Activated glial cells secrete a variety of substances to promote interneuron sensitization. Intrathecal injection of a microglia activation inhibitor has been shown to attenuate hyperalgesia in a neuropathic pain model by reducing the expression of inflammatory factors in spinal microenvironment (18). Chronic nerve injury also affects a variety of other signaling molecules in central nervous system, including growth factors, neurotransmitters, intracellular second messengers, nuclear transcription factors and membrane receptors. Central sensitization seems to be the result of complex interaction among these mechanisms and disturbed balance between excitatory and inhibitory synapses (19) (**Figure 1**).

Synaptic plasticity is implicated in the development and ageing of the central nervous system, as well as the pathophysiology of a number of diseases, including Alzheimer's disease and neuropathic pain. Synaptic plasticity can be categorized into functional (changes in information transmission) or structural (changes in information storage). Examples of functional synaptic plasticity included long-term potentiation (LTP; strengthening of synaptic connection) and long-term depression (LTD; weakening of synaptic connection). LTP is typically occurs in large synapses and dendritic spines whereas LTD tends to occur in small synapses (20). Upon repeated input of nociceptive signals from peripheral nerves to the spinal neurons, excitatory postsynaptic currents (EPSC) mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) on postsynaptic membrane tend to increase over time. This process requires synergistic activation of the N-methyl-D-aspartate (NMDA) receptor,

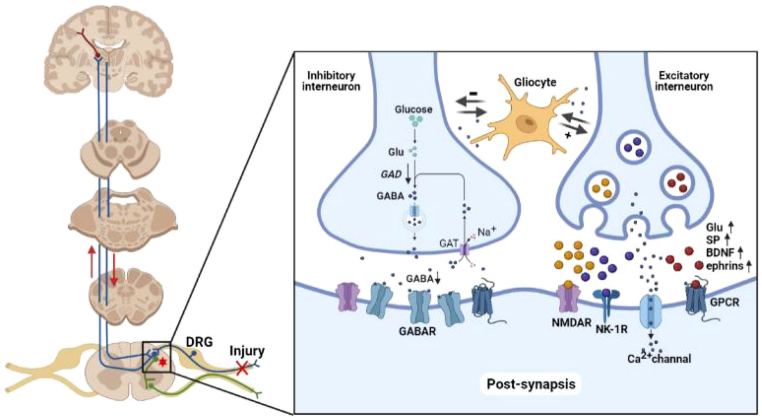

FIGURE 1 | Neuropathology of neuropathic pain. Peripheral nociceptive stimuli are converted into electrochemical signals by pain receptors and transmitted to the brain through the spinal cord *via* upward projection fibers. The spinal cord also relays downward signal to regulate the nociceptive signal transmission. The imbalance of inhibitory/excitatory interneurons forms the basis of neuropathic pain. In inhibitory interneurons, GABA is synthesized from glutamate by GAD and released into the synaptic cleft. GABA in the synaptic cleft is taken up by interneurons *via* GABA transporters in a Na^+-dependent mechanism. Chronic nerve injury reduces the activity of GABA synthase and GAD, ultimately resulting in disinhibition. Chronic nerve injury enhances the synthesis of glutamate, SP, BDNF and ephrins in excitatory interneurons. These neurotransmitters enhance the spontaneous excitatory postsynaptic currents mediated by AMPAR, NMDAR, NK-1R, and Ca^{2+} channel on postsynaptic membrane. Glu, glutamate; GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid; GAT, GABA transporter; SP, substance P; BDNF, brain derived neurotrophic factor; NMDAR, N-methyl-D-aspartate receptor; NK-1R, neurokinin 1 receptor; GPCR, G protein-coupled receptor.

neurokinin 1 (NK1) receptor and low-threshold T-type Ca^{2+} channel (21). Chronic pain is mainly transmitted to the interneurons in lamina II of spinal cord through C fibers. Chronic depolarizing stimulation removes the conformational block of voltage-dependent Mg^{2+} channel inside the NMDA receptor of the postsynaptic membrane, and enhance the sensitivity of neurons to subsequent stimulation. Chronic stimulation of C fibers also promotes the platform currents of L-type Ca^{2+} channel on spinal neurons to further enhance neuronal excitability (22). This complex process involves both excitatory and inhibitory transmitters, as well as neuromodulators such as substance P (SP) and brain derived neurotrophic factor (BDNF), G protein-coupled receptors (GPCR), NK-1 and tyrosine kinase B (TrkB) (23) (**Figure 1**).

GLYCOMETABOLISM REPROGRAMMIN IN GLIAL CELLS

Glucose is the main source of energy in central nervous system. Glucose enters neurons mainly actively through glucose transporter (Glut) on cell membrane (24). Pyruvate, the final product of glycolytic process, enters the TCA cycle in mitochondria under normal oxygen-rich conditions but is converted to lactate under hypoxic conditions. Pyruvate generates 32 ATP molecules through the TCA cycle and electron transport chain in mitochondria and only 2 ATP molecules upon conversion to lactate by lactate dehydrogenase (LDH) (25). Pyruvate dehydrogenase (PDH) is a rate-limiting enzyme in the TCA cycle, and is regulated (inhibited) *via* phosphorylation by pyruvate dehydrogenase kinase (PDK). When PDH is phosphorylated, pyruvate is shunted from the TCA cycle to anaerobic glycolysis (26). Four human PDK subtypes have been identified. PDK_1 is activated in anoxic environment; PDK_2 is activated upon acetyl-CoA and NADH accumulation; PDK_3 is active in high-ATP environment; PDK_4 plays a vital role upon starvation. Increased PDK_2 and PDK_4 expression has been found in spinal cord neurons in a diabetic model for neuralgia, and double knockout of PDK_2 and PDK_4 could alleviate hyperalgesia *via* inhibiting synaptic accumulation of pro-inflammatory factors and lactate and subsequent changes of ion channel permeability in neurons as well as glial cells (27). In primary culture of spinal neurons, exogenous lactate increases the permeability of cell membrane and alters the electrophysiological properties of synapses by facilitating calcium influx through calcium ion channels. The PDK inhibitor dichloroacetate and LDH inhibitor FX11 partially alleviate hyperalgesia in diabetic neuralgia model, providing a potential target for treatment of diabetic neuralgia (28).

Chronic Nerve Injury Promotes Microglia Activation by Enhancing Glycolysis

Microglia and astrocytes play critical roles in neuroinflammation (29). Microglia are resident immune cells in the central nervous system, and could activate inflammasome, NF-κB and other inflammatory signaling pathways; in contrast, astrocytes are mainly involved in regulating the integrity and permeability of blood-brain barrier (30). In the resting state, microglia primarily rely on oxidative phosphorylation of glucose as energy source. Upon activation, microglia shift from the TCA cycle to anaerobic glycolysis (31) *via* multiple mechanisms, including increased expression of pro-inflammatory factors and accumulation of advanced glycation end products (AGEs) (32). Microglia activation also promotes autophagy and apoptosis of neurons.

Short-term exposure to β amyloid shifts microglia from oxidative phosphorylation to glycolysis *via* the mammalian target of rapamycin/hypoxia inducible factor-1α (mTOR/HIF-1α) pathway (33). Long-term exposure to β amyloid, however, attenuated both glycolysis and oxidative phosphorylation, and reduced the responsiveness of microglia to noxious stimuli. In a mouse model for Alzheimer's disease, exogenous interferon-γ (IFN-γ) attenuated neurological deficits by attenuating the stimulation of β amyloid to microglia through promoting glycolysis by activating mTOR pathway (33). In further studies, mice with TREM-2 knockout in Alzheimer's disease showed decreased mTOR pathway activity, impaired glycolysis and increased neuronal autophagy (34). Enhanced glycolysis in activated microglia has also been noted in patients with multiple sclerosis, an autoimmune disease (35), and has been explored as potential target in the treatment of multiple sclerosis (36).

In addition to increased glycolysis at the expense of TAC cycle, glucometabolic reprogramming in microglia also features enhanced glutamine hydrolysis and pentose phosphate pathway. All together, these metabolic changes lead to the accumulation of a variety of intermediates, including phosphoenolpyruvic acid (PEP), succinate, citric acid, methylene succinic acid, α-ketoglutaric acid, lactate and 2-hydroxyglutaric acid, which in turns alters the acid-base balance in microenvironment, promotes transcription of pro-inflammatory factors and activates inflammatory signaling pathways to change the inflammatory phenotypes of both microglia and peripheral immune cells (37, 38). Upon activation of T cells by chronic nerve injury, PEP accumulation interferes with Ca^{2+} signaling and promotes the inflammatory cascade (39). When T cell receptor (TCR) is activated by antigen, cell membrane permeability increases and Ca^{2+} enters the cytoplasm to activate a number of signaling pathways. PEP accumulation by glycolysis inhibits Ca^{2+} channels in endoplasmic reticulum, thus preventing Ca^{2+} from entering into the Ca^{2+} reservoir in endoplasmic reticulum. These changes increase Ca^{2+} concentration in cytoplasm, further activating inflammatory pathways to maintain the activated state of peripheral immune cells and promoting the transcription of pro-inflammatory factors. PEP accumulation produces similar effects in macrophages, including the induction of M1 polarization and increased expression of pro-inflammatory factors (40). The responses of resident microglia to the glycolysis metabolites are also characterized by a shift towards the inflammatory phenotype (41) (**Figure 2**).

Chronic Nerve Injury Promotes Lactate Transfer Between Astrocytes and Neurons

Astrocytes are also part of the resident immune system in the central nervous system, and could release cytokines or chemokines upon activation. Similar to glial cells, astrocytes also undergo glycometabolism reprogramming during the development of neuropathic pain (42). A1 astrocytes are characterized by activation of the classical complement cascade, which in turn promotes neuropathic pain by disrupting the stability and function of synaptic structures. In contrast, A2 astrocytes are characterized by upregulation of neurotrophic factors, which in turn play protective roles during the process of neurodegeneration (43).

Recent *in vivo* and *vitro* studies have demonstrated that the astrocyte-neuron lactate shuttle (ANLS) is a crucial additional source of energy for neurons, especially under stress and continuous neuronal stimulation (44, 45). Lactate produced in astrocytes can be transported out of cells and enters neurons by monocarboxylate transporters (MCTs). MCTs of different subtypes have cell-specific distribution. MCT_1 and MCT_4 have low affinity for lactate and are mainly distributed on glial cells to mediate the outward transport of lactate, whereas the high-affinity MCT_2 is mainly distributed on neurons and mediate the uptake of lactate. In comparison to the very low glycogen storage capacity in neurons, astrocytes have high glycogen storage. Upon sensing enhanced energy requirements by surrounding neurons, astrocytes increase lactate production through glycolysis to provide metabolic substrates for neurons (46, 47). In comparison to neurons, astrocytes also express higher levels of 2,6-phosphofructo-2-kinase 2 ($PFKFB_2$) and $PFKFB_3$, and could thus provide energy to neurons *via* activated glycolysis (44). Accumulated glutamate in synapses upon chronic nerve injury enters astrocytes *via* glutamate transporters (GLT), increases intracellular Na^+ concentration and activates Na^+-K^+-ATPase on cell membrane of astrocytes. This in turn promotes glucose uptake and induces glycolysis. Anaerobic glycolysis can also produce ATP, which is used for *de novo* glutamate synthesis, glutamate transfer between astrocytes and neurons, and the maintenance of Na^+-K^+-ATPase function (48). Dependence of neurons on lactate of astrocyte origin varies among different species, and disruption of glycolysis in astrocytes has been shown to result in loss of neurons in drosophila (49). Metabolic dependence of neurons on lactate of astrocyte origin, and relevance to neuropathic pain are illustrated in **Figure 2**.

Lactate Participates in the Synaptic Connections Between Neurons

Under normal conditions, lactate concentration is 10 to 50 times higher than pyruvate, and can be released into blood to provide energy for tissues and organs except in the central nervous system. Neurons only use glucose from the blood for energy metabolism in physiological condition, whereas glial cells could provide additional energy source for neurons under stress. Both *in vitro* and *in vivo* studies have shown that lactate concentration gradient from astrocytes to neurons plays an important role in neuronal stability, apoptosis and energy metabolism (25, 50). The lactate concentration gradient from astrocytes to neurons could be seen with two-photon microscopy (51). Lactate in astrocytes upregulates learning-related genes and produce intercellular connectivity changes in interneurons (52). Exposure to lipopolysaccharide or interferon increases lactate shuttle between microglia and neurons, indicating that microglia could also provide lactate for neurons under stress (53). Glutamine and glutamate that enter into astrocytes from synaptic cleft could enter the TCA cycle. Since the blood-brain

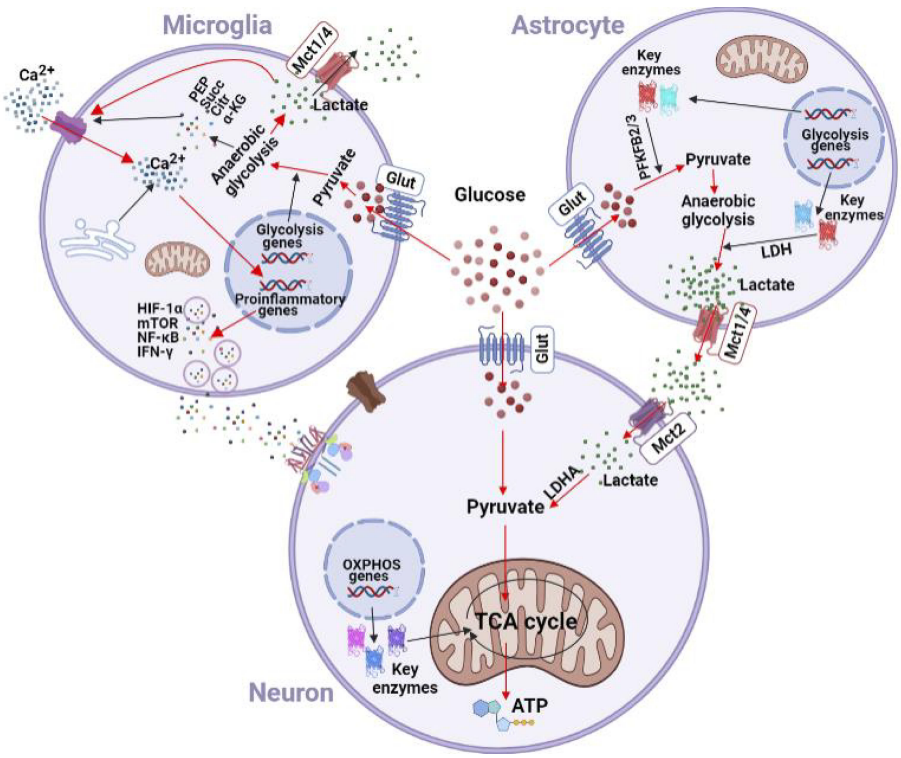

FIGURE 2 | Effects of chronic nerve injury on glycometabolism in microglia and astrocytes. Glycolysis is enhanced in microglia under hypoxic and inflammatory insults. Enhanced glycolysis leads to accumulation of lactate, PEP, succinate, citric acid, and α-KG. Lactate is transferred into synpatic microenvironment by the lactate shuttle to facilitate the Ca^{2+} channel in cytomembrane and inhibit the Ca^{2+} channel in the endoplasmic reticulum. The resulting increase of Ca^{2+} concentration in cytoplasm activates inflammatory pathways (e.g., NF-κB, HIF-1α, mTOR, and IFN-γ) to promote the transcription of pro-inflammatory factors in microglia. Astrocytes express high levels of $PFKFB_{2/3}$, and could metabolize glucose into pyruvate. Upon chronic nerve injury, glutamate is taken up by astrocytes in a Na^+-dependent mechanism, which in turn increases intracellular Na^+ concentration and activates Na^+-K^+-ATPase on the cell membrane to promote glucose uptake and induce anaerobic glycolysis. Lactate is then transferred out of astrocytes *via* Mct1/4, and enters neurons *via* Mct2. Glut, glucose transporter; Mct, monocarboxylate transporter; PEP, phosphoenolpyruvic acid; Succ, succinate; Citr, citric acid; α-KG, α-ketoglutarate; HIF-1α, hypoxia inducible factor-1α; mTOR, mammalian target of rapamycin; IFN-γ, interferon-γ; PFKFB, 2, 6-phosphofructo-2-kinase; LDH, lactate dehydrogenase; LDHA, lactate dehydrogenase A; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation.

barrier is impervious to lactate, astrocyte-neuron lactate shuttle is critical for neuronal survival, memory and synaptic remodeling under hypoxic conditions or inflammation (54, 55) (**Figure 2**).

GLYCOLYSIS AND PAIN SENSITIZATION

Glycometabolism reprogramming is critical in pain sensitization. Reciprocally, transformation of glycometabolism is subject to epigenetic regulation by intermediates and co-factors, thus forming a feedback regulation system between glycometabolism and pain sensitization (56). Glycometabolism usually affects the expression of pain related genes by regulating substrates required for gene modification. By disrupting the NAD^+/NADH balance, glycometabolism transformation can alter the function of histone deacetylase since the catalytic process of acetylase requires a certain concentration of NAD^+. Histone deacetylase has been implicated in pain signal transmission and regulation (57). For example, lactate may have opposite roles in different stages of the inflammatory process (58). In the early stage of macrophages activation in peripheral nervous system, lactate accumulation is generated by glycolysis conversion to promote histone acetylation, which in turn upregulate anti-inflammatory genes. In a sense, lactate serves as a feedback signal that switches macrophage to anti-inflammatory phenotype in the development of inflammation. Lactate dehydrogenase A (LDHA), an enzyme that catalyzes the conversion between pyruvate and lactate, regulates the expression of IFN-γ in T cells *via* acetyl-CoA. In $CD4^+$ T cells, acetyl-CoA is mainly used for histone acetylation of IFN-γ promoters, thereby promoting T cell differentiation into Th1 subsets (59). Reduction of acetyl-CoA levels by ATP-citrate lyase (ACLY) knockout reduces the expression of key enzymes in glycolysis (e.g., hexokinase 2, PFKFB and LDHA), whereas exogenous lactate reduces the effects of ACLY knockout on glycolysis (60). Under low glucose conditions, exogenous lactate enters $CD8^+$ T cells and is converted into acetyl-CoA to increase IFN-γ expression (61). Succinate has been shown to inhibit the DNA methylase TET family genes in Treg cell subsets to alter cell proliferation (62). Changes in the intermediates in glycolysis in

macrophages, T cells and other immune cells have been implicated in the abnormal excitability in primary sensory nerve fibers and dorsal root ganglion during pain sensitization and synaptic plasticity. Parallel glycometabolism reprogramming has also been found in spinal cord, where the nociceptive signal is integrated (63).

Effects of Lactate on Pain Sensitization

It is well known that lactate participates in various physiological processes as a signal molecule, and it may exhibit anti-inflammatory effects in some states. Lactate inhibits toll-like receptor induction of inflammasome and production of IL-1β *via* the GPR81-mediated suppression of innate immunity (64, 65). In dendritic cells, lactate accumulation drives the transformation of inflammatory phenotype by regulating the secretion of interleukin-10 (IL-10) (66). Lactate has been shown to inhibit the migration and cytotoxicity of $CD8^+$ T cells and promote the proliferation of Treg cells *via* its action on the key enzymes in glycolysis (67). In certain conditions, however, lactate accumulation could enhance the immune response and inflammatory cascade mediated by the NF-κB pathway in Th17 cells (68). Similarly, in endothelial cells, lactate accumulation stimulates the NF-κB/IL-8 pathway and induces the production of reactive oxygen species (ROS), resulting in increased cytomembrane permeability and blood-brain barrier disruption (69). Lactate has also been implicated in ROS production in myogenic cells under stress conditions and could up-regulate the expression of multiple genes related to oxidative stress and pro-inflammatory activities (70). The inflammatory effects and oxidative stress induced by lactate in periphery contribute to pain sensitization. In the brain and spinal cord, lactate accumulated under chronic nerve injury can enter neurons and microglia and serve as a substrate in the TCA cycle to generate both ATP and ROS. Immune regulatory factors and ROS cascades produced by microglia promote apoptosis and autophagy, change the stability and permeability of ion channels, and contribute to pain sensitization by depolarizing synaptic membrane and remodeling (31, 71).

Effects of Succinate on Pain Sensitization

Succinate could be accumulated upon TCA cycle disruption and enhanced glutamine decomposition. Accumulation of succinate in synovial macrophages in response to lipopolysaccharide (LPS) exposure directly inhibits M1 polarization caused by proline hydrolase, thereby promoting stable expression of HIF-1α and IL-1β (72), and inhibiting the transcription of anti-inflammatory cytokine IL-10 (73). Extracellular succinate binds to succinate receptor 1 (SUCNR1) to modulate pro-inflammatory/anti-inflammatory signal pathways in nociceptive neurons. Knockout of the SUCNR1 gene in myeloid cells in adipose tissue has been shown to promote the expression of pro-inflammatory genes in macrophages, adding support to the anti-inflammatory function of succinate (74). Extracellular succinate could produce pro-inflammatory action under different conditions. For example, extracellular succinate has been shown to promote the production of prostaglandin E2 in neural stem cells to exert pro-inflammatory effect (75). Succinate can affect macrophage migration and increase the secretion of pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α) and IL-1β, in dendritic cells (76). Succinate also indirectly enhances IFN-γ and TNF-α production in effector T cells during antigen presentation (77).

Effects of Citric Acid on Pain Sensitization

Citric acid is generated during the TCA cycle in mitochondria and transported to the cytoplasm by the citrate carrier (CIC) to participate in the synthesis of fatty acids. In the cytoplasm, citric acid is metabolized by ACLY to acetyl-CoA and oxaloacetate. This process also promotes ROS and nitric oxide (NO) synthesis. CIC knockout reduces citric acid level in the cytoplasm and the production of ROS, NO and prostaglandins to prevent transition to the pro-inflammatory phenotype (78). Citric acid is converted into cis-aconitate in mitochondria and transferred into cytoplasm for oxidative dealkylation to methylene succinic acid. Methylene succinic acid accumulates in large quantities in M1 macrophages and acquires anti-bacterial properties when the TCA cycle is disturbed (79). Disruption of methylene succinic acid synthesis using gene knockout techniques has been shown to induce the expression of pro-inflammatory factors (80). In general, methylene succinic acid exerts its anti-inflammatory effects primarily *via* two signal transduction pathways: one to directly inhibit succinate dehydrogenase to downregulate pro-inflammatory factors, and the other to activate the transcription factors, including nuclear factor E2-related factor 2 (Nrf2) and activating transcription factor 3 (ATF3), in macrophages (81). Through these mechanisms, citric acid participate in synaptic remodeling in pain sensitization.

Effects of Other Metabolic Intermediates on Pain Sensitization

Several other metabolic intermediates in glycolysis also contribute to pain sensitization. In peripheral monocytes, β-glucan increases cytoplasmic fumaric acid levels and reduce the activity of KDM5 demethylase and promotes cell migration (82). α-Ketoglutarate (α-KG) accumulation induces gene expression related to M2 polarization in macrophages through histone demethylation, promotes the transformation of macrophages to M2 phenotype and enhances their anti-inflammatory activity (83). α-KG seems to produce opposite action in T cells. The IL-2 signal pathway can increase α-KG accumulation in T cells to induce their differentiation into the Th1 subset. α-KG also increases the expression of pro-inflammatory and glycolysis genes through DNA methylation modification, which in turn promotes the glycolysis process in T cells (84). 2-HG, a structural analogue of α-KG, competitively inhibits α-KG-dependent histone demethylase and promotes histone hypermethylation. Increased production of 2-HG under hypoxic conditions increases the hypermethylation of histone and DNA by inhibiting URX and TET2 proteins to increase the expression of CD62L and CD127 and promote T cell differentiation into memory cells to induce synaptic plasticity and pain sensitization (85). Abnormal accumulation of S-adenosylmethionine (SAM) also alters the synaptic microenvironment. LPS stimulation of macrophages promotes

histone trimethylation by increasing SAM production and SAM/S-adenosine homocysteine ratio, resulting in the upregulation of pro-inflammatory genes. *In vivo* and *in vitro* studies have confirmed that reduced production of SAM caused by methionine deficiency could alter the inflammatory state of primary afferent neurons and dorsal root ganglion neurons by inhibiting T cell proliferation and cytokine production (86). In summary, accumulation of metabolic intermediates in glycolysis could alter the phenotype of immune and glial cells through different pathways to participate in the development of pain sensitization and synaptic plasticity.

EFFECTS OF GLYCOMETABOLISM REPROGRAMMING ON SYNAPTIC PLASTICITY

Microglia and Synaptic Plasticity

Microglia serve as sensors to detect changes in the microenvironment in the central nervous system (87). Through modifying synaptic pruning, microglia regulate experience-dependent plasticity in the barrel cortex and visual cortex after removal of monocular deprivation. Microglia in the resting state plays a vital role learning and memory. Upon infection, injury or stress, microglia migrate to the site of inflammation, assume an amoeboid shape and secrete cytokines, chemokines and ROS (88). Metabolic changes in neurons and glial cells upon injury or stress (e.g., lactate, succinate and citric acid) are important in the formation of synaptic plasticity in pain sensitization and neuropathic pain, as well as a variety of other central nervous system diseases (89).

Microglia are exquisitely sensitive to acidic metabolites. Exposure of microglia to exogenous lactate increases the mRNA of thioredoxin interacting protein (TXNIP) to accelerate neuronal apoptosis and autophagy. This process causes the irreversible changes in synaptic structure and function in the progression of chronic pain, vascular dementia and Alzheimer's disease (90). H^+ could act as a second messenger to regulate the activity of voltage-gated Ca^{2+} channels, NMDA and GABA receptors. Blocking NMDA receptors could alter the expression of genes related to lactate-induced LTP and LTD, suggesting NMDA receptors are key downstream signal molecules of lactate (91). In addition, lactate and ROS can also promote neuro-inflammation by activating NMDA receptors to mediate Ca^{2+} influx and the downstream signal cascade. The Src family kinases (SFKs) phosphorylate NMDA receptor subunits to promotes Zn^{2+} entry into cells through NMDA receptor channels to participate in the activation of the TrKBs/ERK pathway, and ultimately synaptic remodeling and learning/memory (92, 93). A negative correlation between pH and lactate levels in the synaptic microenvironment has been found in patients with schizophrenia or bipolar disorder (94). Overall, the effects of glycometabolism reprogramming in microglia on pH homeostasis in the synaptic microenvironment are important to synaptic plasticity and development of neuropathic pain.

Changes in the NAD^+/NADH ratio caused by glycometabolism reprogramming in microglia alter the redox status in synaptic microenvironment. Lactate accumulation increases NADH in both neurons and microglia, and upregulates the expression of a variety of genes, including BDNF, Arc and Zif268 to influence synaptic plasticity (95). Reduced NAD^+/NADH ratio activate NMDA receptors and increases Ca^{2+} influx to trigger downstream inflammatory cascade. Imbalanced NAD^+/NADH ratio also affects the levels of transcription factors, deacetylase activity and Ca^{2+} pathways in microglia (96, 97). The Ca^{2+} pathway is a critical link in the lactate signal process in glycometabolism reprogramming. Many other key pathways in synaptic plasticity, including the ERK/CREB pathway, dopamine D2 receptors, metabotropic glutamate receptors (mGluRs) and cannabinoid receptors, are regulated by Ca^{2+} (98). NAD^+-dependent sirtuin-1 signal pathway also participate in synaptic plasticity by regulating the expression of plasticity-related genes (e.g., BDNF, Arc and Zif268) and modifying dendritic morphology of neurons. To sum up, cellular redox state and particularly NAD^+/NADH ratio are key steps between glycometabolism reprogramming and synaptic plasticity (99) (**Figure 3**).

Astrocytes and Synaptic Plasticity

Recent studies indicated that lactate produced through glycogenolysis in astrocytes is critical for long-term memory formation. Disturbing the lactate shuttling between astrocytes and neurons impairs memory formation and consolidation, whereas exogenous lactate attenuates such effects. Astrocyte-neuron lactate shuttle in the spinal cord participates in the formation of abnormal neural circuits in persistent hyperalgesia in neuropathic pain (46). Repeated exposure to noxious stimuli result in substantial changes in astrocyte. Chronic peripheral nerve injury has been shown to promote the proliferation of astrocytes in the spinal cord, as evidenced by increased expression of astrocyte marker glial fibrillary acidic protein (GFAP). Activated astrocytes release a variety of inflammatory mediators and metabolites (e.g., TNF-α, IL-1β, ATP, glutamic acid and serine) to enhance spontaneous excitatory postsynaptic currents and reduce inhibitory interneuron activities, ultimately leading to central sensitization in neuropathic pain (100, 101). Chronic nerve injury has also been shown to increase glutamate concentration in the synaptic cleft *via* inhibition of glutamate transporters (7).

Synaptic sensitization largely depends on the activation of neuronal receptors and ion channels, intracellular signal transduction pathways and related gene expression. Central sensitization in neuropathic pain shares some common molecular mechanisms with LTP in hippocampus (102). For example, lactate transfer from astrocyte to neuron and subsequent NMDA receptor activation and downstream intracellular signal pathways could alter the synaptic plasticity in context of synaptic plasticity in the spinal cord as well as in LTP in the hippocampus in mice. Disrupting the astrocyte-neuron lactate shuttle has been shown to results in amnesia by impairing LTP in the hippocampus (103).

In the central nervous system, glycogen is stored primarily in astrocytes as a reservoir of energy source upon glucose deprivation. In contrast, glycogen store is practically absent in neurons. In comparison to adult brain, neurons in developing

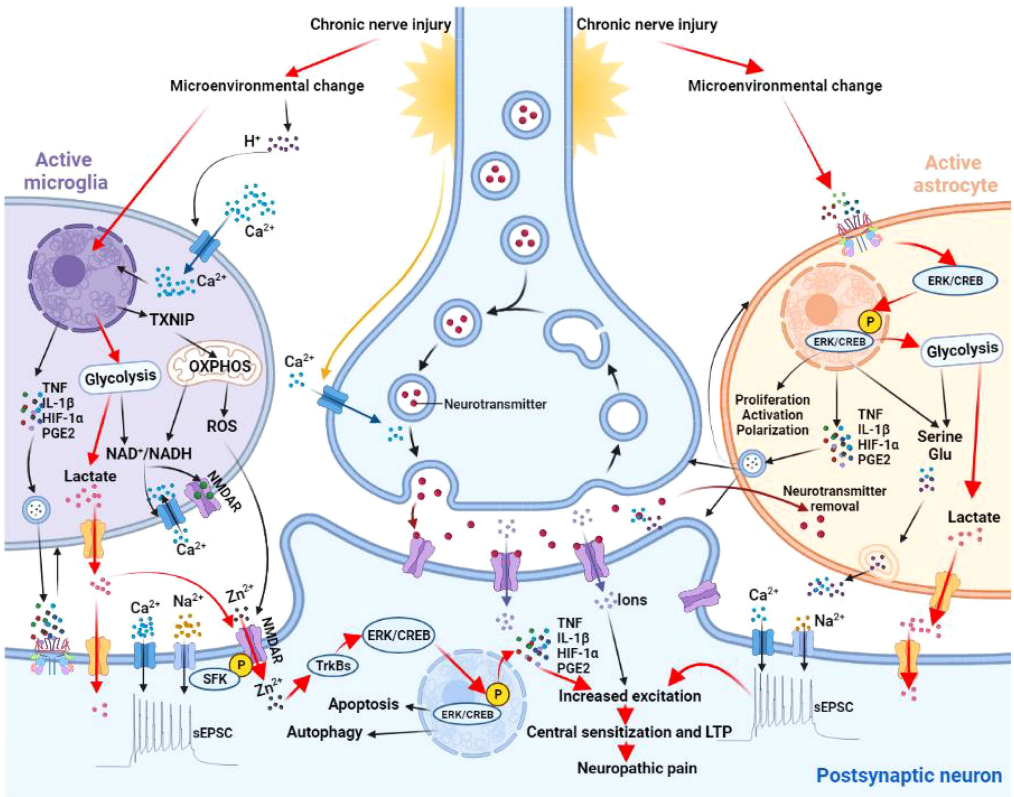

FIGURE 3 | Effects of microglia and astrocyte activation on synaptic plasticity. Microglia sense changes in synaptic microenvironment and H^+ accumulation activates a series of stress responses to shift microglia into activated state. These changes promote the transcription and expression of TXNIP, key enzymes of glycolysis and proinflammatory mediators (e.g.,TNF, IL-1β, HIF-1α, and PGE2). TXNIP is closely related to oxidative stress in producing ROS to increase cytomembrane permeability. Increased expression of key enzymes in glycolysis promotes lactate production and disrupts the balance of NAD^+/NADH. An imbalanced NAD^+/NADH ratio activates the NMDA receptor and increases Ca^{2+} influx to trigger downstream inflammatory cascade. Increased concentration of proinflammatory mediators in the vicinity of synapses promotes synaptic plasticity and polarization of microglia itself. Activation of NMDA receptors, Ca^{2+} influx and the downstream signaling cascade are regulated by SFKs and TrKB receptors. SFKs phosphorylates NMDA receptor subunits to promotes Zn^{2+} entry into postsynaptic neurons through NMDA receptor channels to activate the TrKBs/ERK pathway. Phosphorylation of TrKBs/ERK promotes autophagy, apoptosis, and neuroinflammation. Astrocytes also sense the changes in synaptic microenvironment and activates the ERK/CREB signaling. Upon entering the nucleus, *p*-ERK activates p-CREB to initiate related gene transcription and promotes proliferation, activation, and polarization. Phosphorylation of ERK/CREB also enhances glycolysis and proinflammatory mediators, leading to the accumulation of lactate, glutamate, and serine. All together, these changes increase the excitability of postsynaptic neurons and contribute to central sensitization in neuropathic pain. TXNIP, thioredoxin interacting protein; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; NMDAR, N-methyl-D-aspartate receptor; HIF-1α, hypoxia inducible factor-1α; TNF, tumor necrosis factor; IL-1β, interleukin-1β; PGE2, prostaglandin E2; SFK, Src family kinases; ERK, extracellular signal regulated kinase; CREB, cyclic AMP response element binding protein; LTP, long-term potentiation; Glu, glutamate; sEPSC, spontaneous excitatory postsynaptic current.

brain (rats less than 3 weeks of age) expresses less glucose transporters but more MCTs, indicating the crucial role of lactate in energy metabolism during development of the central nervous system (104). Intraventricular administration of selective inhibitors of MCT or phosphoenolpyruvate carboxykinase (a key enzyme in lactate metabolism) attenuates the proliferation and differentiation of neuronal precursor cells in newborn mice (105). Lactate in the general circulation can also affect synaptic function. Increased lactate in blood by strenuous exercise has been implicated in synaptic remodeling by stimulating the synthesis of vascular endothelial growth factor (VEGF) *via* hydroxy-carboxylic acid receptors on vascular endothelial cells (106, 107).

Plantar injection of complete Freund's adjuvant (CFA) increases lactate release in the anterior cingulate cortex; inhibiting glycolysis in anterior cingulate cortex, in contrast, alleviates CFA-induced chronic inflammatory pain (108). Knockout of MCT genes in neurons of the anterior cingulate cortex also alleviates CFA-induced inflammatory pain, indicating a prominent role of lactate transfer from astrocytes to neurons in central sensitization and neuropathic pain. Numerous studies have demonstrated a critical role of phosphorylated ERK and activation of the transcription factor cyclic AMP response element binding protein (CREB) in synaptic plasticity and central sensitization. Inhibition of glycolysis has been shown to block the ERK/CREB pathway.

Specifically, exogenous lactate may participate in synaptic remodeling and central sensitization in neuropathic pain by enhancing the phosphorylation of ERK and CREB (109, 110). Overall, compromised energy supply to neurons due to disruption of the lactate shuttle between astrocytes and neurons contribute significantly to synaptic plasticity and neuropathic pain *via* multiple mechanisms (111) (**Figure 3**).

CONCLUSION

Increasing evidence suggests important role of glycometabolism reprogramming in microglia and astrocytes in neuropathic pain. Glycometabolism reprogramming in microglia promotes the transformation of microglia to the pro-inflammatory phenotype and increases ROS production. The resulting changes in synaptic microenvironment are important pathological basis for pain sensitization. Astrocytes provide energy support to surrounding neurons *via* astrocyte-neuron lactate shuttle. In addition to a substrate for TAC cycle, lactate that enters the neurons from astrocytes also serves as a signal molecule to promote synaptic plasticity by regulating a variety of signaling pathways. In summary, a plethora of information showed that glycometabolism reprogramming of glial cells contribute to hyperalgesia and allodynia in neuropathic pain and represent potential targets for developing novel treatment for neuropathic pain.

AUTHOR CONTRIBUTIONS

EK, YL, and MD drafted the manuscript; TH, MY, JL, and XF designed and prepared the figures; HY conceived the study. All authors made significant contribution and approved the submitted version.

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REVIEW
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N-Glycosylation and Inflammation; the Not-So-Sweet Relation

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Chronic inflammation is the main feature of many long-term inflammatory diseases such as autoimmune diseases, metabolic disorders, and cancer. There is a growing number of studies in which alterations of N-glycosylation have been observed in many pathophysiological conditions, yet studies of the underlying mechanisms that precede N-glycome changes are still sparse. Proinflammatory cytokines have been shown to alter the substrate synthesis pathways as well as the expression of glycosyltransferases required for the biosynthesis of N-glycans. The resulting N-glycosylation changes can further contribute to disease pathogenesis through modulation of various aspects of immune cell processes, including those relevant to pathogen recognition and fine-tuning the inflammatory response. This review summarizes our current knowledge of inflammation-induced N-glycosylation changes, with a particular focus on specific subsets of immune cells of innate and adaptive immunity and how these changes affect their effector functions, cell interactions, and signal transduction.

Keywords: N-glycosylation, N-glycans, inflammation, immunity, cytokines, leukocytes, immunoglobulins

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INTRODUCTION

Inflammation is part of a complex biological tissue response triggered by infectious, traumatic, toxic, or autoimmune injury. In acute inflammation, a controlled inflammatory response usually results in restoration of homeostasis. However, persistent induction and dysregulation of inflammation may contribute to the development of chronic inflammatory diseases (1). Chronic inflammation is characterized by numerous systemic physiological and biochemical changes, most of which are mediated by abundantly secreted proinflammatory cytokines (**Figure 1**). They are the key molecules responsible for triggering the proinflammatory potential of innate and adaptive immunity, oftentimes leading to tissue destruction (2). Moreover, chronic inflammation is characterized by marked changes in glycosylation (3, 4). Glycosylation is one of the most common posttranslational modifications of proteins and plays an important role in a variety of biological functions, including protein stability and effector functions, intercellular interactions, signal transduction, and cell immunogenicity. The enzymatic processes of protein glycosylation normally occur in the endoplasmic reticulum (ER) and Golgi apparatus, but can also occur in the cytoplasm and nucleus. The glycan structures are covalently linked to the protein backbone *via* the nitrogen atom of the asparagine or the oxygen atom of the serine/threonine side chains, forming N-linked and O-linked glycoproteins, respectively. The core of N-linked glycans consists of two consecutive N-acetylglucosamines (GlcNAc) and three mannoses, which can be further extended and modified by various glycosyltransferases (GTs) and glycosidases to form oligomannose, complex, or hybrid

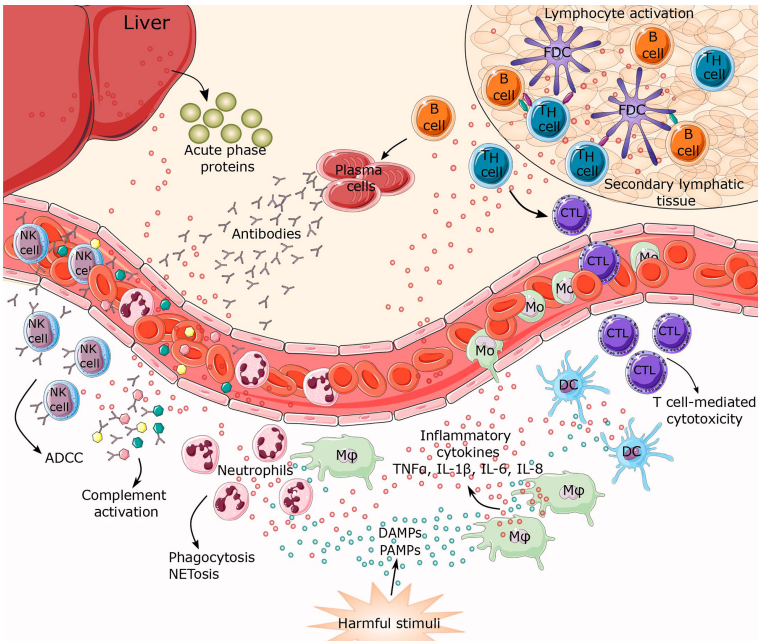

FIGURE 1 | Inflammatory response to harmful stimuli. When tissue or cellular damage occurs, danger-associated molecular patterns (DAMPS), pathogen associated molecular patterns (PAMPs) and myriad inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8) are released. These biomolecules can initiate activation of inflammatory pathways resulting in leukocyte recruitment of innate and adaptive immunity, thus establishing a highly coordinated network of many cell types. Activated macrophages, together with damaged endothelial cells, release factors that attract neutrophils and monocytes to the site of inflammation. This represents the first line of defense characterized mostly by phagocytosis and NETosis. Macrophages, together with mature dendritic cells (DCs), are specialized in exposing antigens to lymphocytes (T and B cells), thereby activating antigen-specific adaptive immunity. Lymphocyte differentiation leads to T cell-mediated cytotoxicity, antibody secretion, and antibody dependent cell cytotoxicity (ADCC). Simultaneously, cytokines trigger synthesis and secretion of acute phase proteins from the liver. CTL, cytotoxic T lymphocytes; FDC, follicular dendritic cells; Mφ, macrophage; Mo, monocyte; NK cell, natural killer cell.

N-glycans (**Figure 2**) (5). N-glycans are found on the surface of key entities involved in the inflammatory response, including endothelial adhesion molecules, immune cells of innate and adaptive immunity, and secreted immunoglobulins and acute phase proteins (APP). The composition of their N-glycans has been shown to be modulated by abundantly secreted proinflammatory cytokines, presumably by regulating the expression of GTs and affecting the substrate availability required for N-glycan biosynthesis. Overall, the changes in N-glycosylation observed in chronic inflammation are diverse but strongly dependent on the particular subset of immune cells. Affected features of N-glycan structure include changes in the number of antennae, changes in N-glycan structure composition, and diversification of saccharide bonds resulting in different ligand epitopes. Consequently, altered N-glycosylation can significantly affect leukocyte trafficking, trigger a shift toward more proinflammatory effector functions of leukocytes, and initiate proinflammatory transformation of secreted immunoglobulins and APPs, ultimately leading to the development of various inflammatory diseases. Therefore, the aim of this review is to summarize what is known about the relationship between N-glycosylation and chronic inflammation, proinflammatory cytokines, and consequently the development of inflammatory diseases.

ENDOTHELIUM

One of the main functions of the endothelium is transportation of immune cells to the site of inflammation. To successfully pass through the endothelium, immune cells undergo complex process which involves ligand dependent binding followed by surface rolling, adhesion, and finally transendothelial migration (6, 7). Each step in this cascade is dependent on interaction between endothelial adhesion molecules and their counterreceptors expressed on the surface of leukocytes. Key players in leukocyte transmigration process are selections, integrins, intercellular and vascular adhesion molecules (ICAMs and VCAMs), platelet endothelial cell adhesion molecules (PECAMs), and junctional adhesion molecules (JAMs) (8). The majority of the endothelial adhesion molecules are heavily N-glycosylated (9), which is crucial for successful leukocyte trafficking, as defined by the "zip code" hypothesis. In the circulation, leukocytes encounter various proteins and sugars expressed on the endothelial surfaces. Efficient leukocyte adhesion is achieved only when a specific combination of an adhesion molecule protein and N-glycan is expressed (10). Adhesion molecules are not normally expressed in resting cells, however their expression is upregulated in inflammation, *via* cytokine-induced signaling pathway, such as

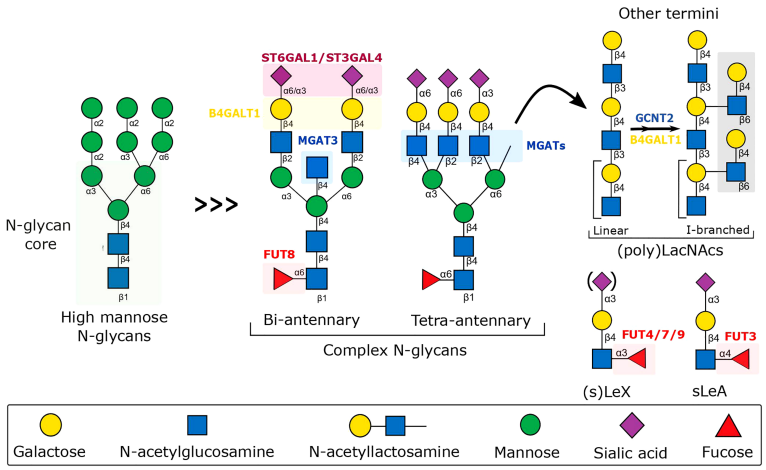

FIGURE 2 | Schematic representation of the biosynthesis of N-glycans involved in the fine-tuning of the immune response to inflammation. The schematic includes the major N-glycan structures found on the surface of endothelium, immune cells, and secreted molecules, along with the relevant glycosyltransferases, whose expression has been shown to be modulated by inflammatory cytokines, dramatically affecting glycan-dependent interactions important for leukocyte immune regulation. B4GALT1, Beta-1,4-Galactosyltransferase 1; FUT, Fucosyltransferase; GCNT2, Glucosaminyl (N-acetyl) Transferase 2; MGAT, N-acetylglucosaminyltransferase; ST6GAL4, Beta-Galactoside Alpha-2,3-Sialyltransferase 4; ST6GAL1, Beta-Galactoside Alpha-2,6-Sialyltransferase 1.

NF-κB (11–14). Additionally, the life cycle of N-glycans involved in the leukocyte trafficking is significantly controlled by inflammation (15–17). Since inflammation-dependent modulation of adhesion molecule expression and N-glycome biosynthesis is critical for the innate immune response, dysregulation of this axis may be crucial for the transition from an innate immune response to inflammatory disease.

Selectins

Selectins are calcium-dependent (C-type) lectins that recognize specific glycan residues as their ligands and mediate the adhesion of immune cells to the endothelium. To date, three members of the selectin family have been identified, P-selectin, E-selectin and L-selectin (18). L-selectin is highly expressed on hematopoietic stem cells and mature leukocytes, and is rapidly shed by proteolytic cleavage upon leukocyte activation (18–20). P- and E-selectin are known as "vascular selectins" because they can be expressed on endothelial cells. P-selectin is constitutively expressed by endothelial cells and platelets where they are stored in Weibel-Palade bodies and α-granules, respectively. Therefore, they can be translocated to the cell surface within minutes after a proinflammatory stimuli such as thrombin and histamine, making them the most important adhesive molecules in acute injury. On the other hand, E-selectin is not constitutively expressed by endothelial cells, but their expression is strongly upregulated by inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) through binding of NF-κB to regulatory domains in the E-selectin promoter. The latter is not possible in the case of P-selectin, as the P-selectin promoter in humans lacks binding sites for NF-κB (21, 22). Because of this property, E-selectin is considered the most important adhesive molecule involved in leukocyte capture in chronic inflammation. The involvement of selectins in the development of many acute and chronic inflammatory conditions is dependent on the selectin-ligand axis, with N-glycosylation playing an important role. The interaction required for leukocyte capture on the endothelial surface is dependent on the affinity of selectins for sialofucosylated glycan epitopes expressed on both endothelial and immune cells. These include sialyl Lewis x (sLex), sialyl Lewis a (sLea), and 6-sulfo sialyl Lewis x (6-sulfo sLex) epitopes, which are responsible for mediating leukocyte capture/rolling during inflammation and are relevant to the successful homing of lymphocytes to lymph nodes (15, 23, 24). E-selectin binds to sialofucosylated N-glycans on E-selectin ligand-1 (ESL-1) and CD44 glycovariant, hematopoietic cell E-/L-selectin ligand (HCELL), to support leukocyte extravasation to the site of inflammation (25–27). Interestingly, Pachón-Peña et al. demonstrated the potential use of glycoengineered HCELL on human adipose-derived mesenchymal stem cells (A-hMSCs) to direct their migration to sites of tissue injury/inflammation, thus enabling relevant immunomodulatory and regenerative functions (28). In addition, the sLex epitope found on APPs may modulate the binding of leukocytes to E-selectin (29, 30). In the case of L-selectin, Mitoma et al. demonstrated a critical role for 6-sulfo sLex-decorated N-glycans found on CD34, a major component of the L-selectin ligand, in the leukocyte trafficking in the high-endothelial venules (HEV) of the peripheral lymph node (17). Interestingly, Huopaniemi et al. showed that co-regulated expression of CMP-sialic acid and GDP-fucose transporters, essential for the synthesis of 6-sulfo sLex, occurs in inflammation, which is not common in physiological conditions. Therefore, it has been suggested that there must be an inflammation-induced transcriptional regulation for Golgi membrane transporters that support trafficking of substrates necessary for synthesis of 6-sulfo sLex N-glycans (31). Furthermore, sulfonation of sLex epitopes on N-glycans is

thought to be the result of N-acetylglucosamine-6-O-Sulfotransferase-1 (GlcNAc6ST-1) activity, but further studies are needed to uncover how this synthesis is regulated under physiological and pathological conditions (32). Moreover, Beta-Galactoside-Alpha-2,3-Sialyltransferase 4 (ST3GAL4) is the primary sialyltransferase regulating the synthesis of sLex epitopes in human myeloid leukocytes (33), the expression of which, together with the expression of 6-sulfo sLex, has been shown to be increased by TNFα in chronic lung disease. Thus, disruption of ST3GAL4 function in human myeloid cells may represent a potential target for anti-cell adhesion and anti-inflammatory therapy (16). In addition, fucosyltransferases such as FUT7 and FUT9 are involved in the synthesis of the Lex epitope, and $FUT7^{-}9^{-}$ dual knockdown has been demonstrated to significantly decrease the selectin-dependent interaction between leukocytes and endothelial cells (34). Interestingly, an *in vivo* study has shown that the cytokines IL-6 and/or IL-8 can induce a significant increase in the expression of alpha-1,3/4-fucosyltransferases in mucosal cells, contributing to an increased level of sLex epitopes and thus to dysregulated transendothelial migration. The latter might potentiate the persistent lung inflammation and tissue damage in cystic fibrosis (CF) (35).

ICAM-1 and VCAM-1

After leukocyte rolling and capture by selectins, firm endothelial adhesion of leukocytes is mediated by ICAM-1 and VCAM-1. They are members of the immunoglobulin supergene family that are expressed during chronic inflammation on vascular endothelium, lymphocytes, and macrophages (36). The expression of ICAM-1 and VCAM-1 is under the direct influence of proinflammatory cytokines, such as TNFα, which increase their levels on the endothelial surface (37). In response to inflammation, increased ICAM-1 and VCAM-1 cell surface levels enhance adhesive interactions with their ligands on leukocytes, Lymphocyte function-associated antigen 1/ Macrophage-1 antigen (LFA-1/MAC-1) and Very late antigen-1 (VLA-1), respectively (38, 39). N-glycosylation is a crucial factor that can significantly affect the ligand binding of ICAM-1 and VCAM-1 (38, 40–43). In general, the transition from the homeostatic to the inflammatory state of the endothelium is characterized by a decrease in N-glycan complexity and increased expression of high mannose and hybrid structures (44–47). This has been demonstrated to be a consequence of proinflammatory stimulation, possibly by inhibition of early mannose-trimming enzymes (α-mannosidase) (42). Not surprisingly, increased presence of high-mannose ICAM-1 (HM-ICAM-1) results in high-affinity leukocyte binding (38). In particular, this phenomenon is seen in $CD16^{+}$ proinflammatory monocytes, which have a higher affinity for HM-ICAM-1 molecules in atherosclerotic lesions compared with complex α-2,6-sialylated ICAM-1 (48, 49). However, this is not the only mechanism by which the pathological state is maintained in chronic inflammation, as far as N-glycosylation is concerned. In systemic lupus erythematosus (SLE), diabetes, and rheumatoid arthritis (RA), this is regulated by inhibition of galectin-mediated immunosuppressive prevention of ICAM-1/LFA-1 interaction (50), aberrant expression of ICAM-1 N-glycans due to high glucose (51), and activity of glycosyltransferases (52). Another possible candidate for inflammatory modulation is VCAM-1, where removal of α2,6-sialic acid increases leukocyte trafficking (41). The mechanism of action does not involve stronger binding with VLA-1 on leukocytes but with galectin-3 (Gal-3), which supports leukocyte adhesion (53).

PECAMs

The final step in the process of leukocyte extravasation is transendothelial migration (TEM). Adhesion molecules mainly associated with this phenomenon are PECAMs (54). PECAM-1 is a member of the immunoglobulin (Ig) superfamily selectively expressed on the surface of immune cells and is highly enriched at the intercellular interface of endothelial cells (54). The main mechanism responsible for interaction of PECAMs with leukocytes involves homophilic binding (55). As PECAM-1 is highly N-glycosylated, Lertkiatmongkol and her group showed that homophilic binding interactions of human PECAM-1 are supported by α2,3-linked, but inhibited by α2,6-linked sialic acid residues (56). This was previously demonstrated by Doring et al. who presented solid evidence for the importance of α2,3-linked sialic acid in leukocyte activation, adhesion, and recruitment to inflamed vessels (57). In agreement with this, it was later shown that a variety of proinflammatory cytokines secreted in chronic inflammation can downregulate the levels of the (extracellular) enzyme Beta-Galactoside Alpha-2,6-Sialyltransferase 1 (ST6GAL1), responsible for the synthesis of α2,6-linked sialic acid, and by that way maintain the inflammatory state (58, 59).

INNATE IMMUNITY

Innate immunity is the first line of defense against infection and consists of resistance mechanisms that are not specific to any pathogen. In any infection or tissue injury, inflammation is triggered when innate immune cells recognize molecular patterns that are foreign to a tissue, called pathogen-associated molecular patterns (PAMPs), and initiate a cascade of inflammatory responses. These innate immune cells include tissue-derived macrophages, natural killer cells (NK cells) and dendritic cells (DCs), as well as circulating leukocytes such as monocytes and neutrophils (60, 61). To communicate with other immune cells and exert their immunomodulatory functions, they often rely on N-glycans expressed on their surface and counterreceptors expressed by their binding partners. In this section, we present examples of altered N-glycosylation in innate immune cells due to chronic inflammation and show how this is reflected in the functionality of immune cells and consequently in the progression of various chronic inflammatory conditions.

Neutrophils

Neutrophils are polymorphonuclear leukocytes that have long been known to be key players in pathogen recognition and elimination in acute inflammation, but their role in chronic

inflammatory and autoimmune diseases, such as psoriasis, RA and SLE, has also been described (62, 63). Understanding the underlying mechanisms of neutrophil activation, migration, survival, and executive function may open new avenues for the treatment of chronic inflammation. N-glycosylation has been shown to contribute to important effector functions of neutrophils, such as extravasation, phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) (64) (**Figure 3**). On their surface, neutrophils express the N-glycosylated MAC-1 integrin, which consists of two subunits - CD11b and CD18. This complex is involved in the regulation of neutrophil trafficking and interaction with other immune cells (65). Kelm et al. performed an analysis of glycan epitopes expressed on the neutrophil MAC-1 surface and observed decreased sialylation together with an increase in the Lex motif and high mannose content in chronic inflammation. These changes were significant in inflammatory bowel disease (IBD) because blocking the terminal Lex motif reduces dysregulated transepithelial migration of neutrophils, presumably by inhibiting the binding of MAC-1 to ICAM-1 molecules expressed on the surface of the inflamed epithelium (66, 67). Moreover, the Lex motif expressed on MAC-1 mediates binding to DC-SIGN expressed on DCs, thus providing an indirect link between innate and adaptive immunity. Monocytes and macrophages also express MAC-1, but since they lack the Lex motif, this trait is exclusively dependent on neutrophils (68). Therefore, the Lex motif may represent a novel target for modulating inflammation in chronic diseases in which tissue damage is mediated by dysregulated neutrophil trafficking. After neutrophils accumulate at the site of inflammation, their immunomodulation depends on efficient degranulation, phagocytosis, and NET formation. Interestingly, granule glycoproteins show differential, stage-dependent glycosylation that decorates them with hyper-truncated chitobiose core, paucimannose and complex monoantennary N-glycans (64). Interestingly, it was recently demonstrated that N-acetyl-Beta-D-Hexosaminidase (Hex) enzyme is elevated in many inflammatory diseases (69–71) and catalyzes formation of paucimannosidic glycans found on neutrophilic granules (72). Those paucimannosidic N-glycoforms of human neutrophil elastase (HNE) show an enhanced ability to suppress the growth of *P. aeruginosa* (PA), presumably by bypassing interactions with its suppressive counter-binding partner, A1-antitripsyne (A1AT) (73). However, PA was demonstrated to uptake host's sialylated N-glycans, making these bacteria suitable binding partners for the inhibitory siglec-9 receptor expressed on neutrophils. As a result, neutrophils show reduced levels of reactive oxygen species (ROS) and released elastases which leads to reduced formation of NETs and increases survival of PA (74), eventually leading to chronic lung inflammation and

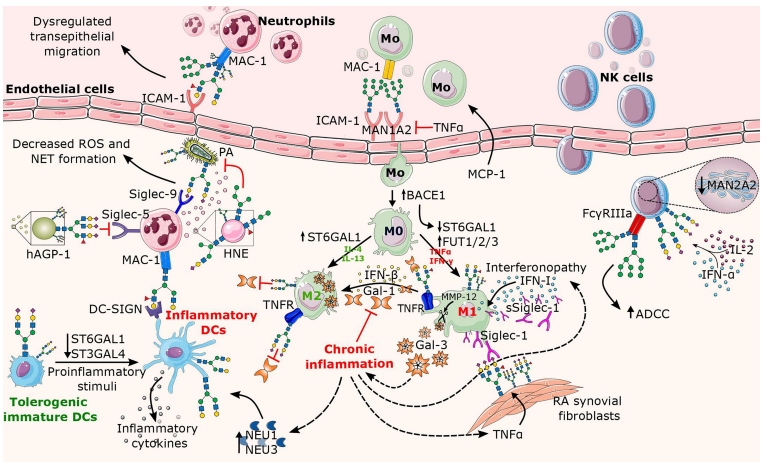

FIGURE 3 | Overview of altered N-glycosylation pathways in innate immune cells during chronic inflammation. The major factors contributing to the alterations in N-glycosylation are proinflammatory cytokines (e.g., TNFα, IL-2, IFN-α, IFN-γ) that are released in excess during inflammation. Here, the affected structural elements of N-glycans on the surface of innate leukocytes (neutrophils, macrophages, NK cells, and DCs) are shown along with their associated glycosyltransferases and glycosidases. In neutrophils, the increase of the Lex motif on the integrin MAC-1 leads to dysregulated neutrophil migration, whereas the binding of Lex decorated MAC-1 to DC-SIGN further triggers the activation of DCs. While neutrophilic granules (e.g., HNE) secreted by neutrophils carry truncated N-glycans, the presence of sialylated complex N-glycans and/or the sLex motif on Siglec counterbinding entities contributes to the inflammatory potential of neutrophils in a context-dependent manner. Proinflammatory cytokines enhance transport of monocytes and direct their differentiation into proinflammatory M1 macrophages, while contributing to the absence of sialylated N-glycans, cleavage of Gal-3, and increase in Siglec-1 expression. While surface-bound Siglec-1 is involved in the autoimmune response in rheumatoid arthritis (RA), soluble Siglec-1 is a marker in interferonopathy. In addition, the Gal-1/IFN-β feedback loop involved in termination of inflammation appears to be dysregulated in chronic inflammation. Similarly to macrophages, mature DCs also lack terminal sialic acids, plausibly due to inflammation-mediated decrease in sialyltransferase and/or increase in neuraminidase activity. As for NK cells, the presence of oligomannose N-glycans on FcγRIIIa significantly increases ADCC, whereas cytokine-induced increase in sialylation abrogates Siglec-9-dependent NK cell inhibition by cis-binding. BACE1, Beta-Site APP-Cleaving Enzyme 1; Gal, galectin; hAGP-1; hepatic α1-acid glycoprotein; HNE, human neutrophilic elastase; IFN, interferon; IL, interleukin; ICAM-1, intercellular adhesion molecule 1; MAC-1, macrophage-1 antigen; Man, Mannosidase; MMP-12, matrix metalloproteinase 12; MCP-1, monocyte chemoattractant protein-1; NEU, neuraminidase; NAGP-1, neutrophil α1-acid glycoprotein; PA, *Pseudomonas aeruginosa*.

tissue destruction as seen in CF patients susceptible to respiratory infections caused by PA. Therefore, further studies are needed to draw conclusions about neutrophil glycosylation role in CF pathogenesis and completely illuminate these processes. Another modulatory potential of neutrophil activity lies in alpha-1-acid glycoprotein (AGP-1). AGP-1 can stimulate neutrophil activation by inducing an increase in cytosolic calcium concentration through interactions with the neutrophil receptors siglec-5 and/or siglec-14, which preferentially bind α2-3 or α2-6 sialic acid. The latter is presumably true for acute inflammation as hyperfucosylation of AGP-1 in chronic inflammation leads to increased expression of sLex on AGP-1, a motif that is not a ligand for siglec-5 nor siglec-14 (75). Furthermore, in addition to the liver, AGP-1 is expressed by activated neutrophils. In contrast to the N-glycans expressed on hepatic AGP-1 (hAGP-1), neutrophil-expressed AGP-1 (nAGP-1) carries mainly high-mannose, nonsialylated, and mono-sialylated N-glycans (76). Taken together, these data may indicate that chronic inflammation in some cases attenuates neutrophil recruitment and activation in favor of other, more potent leukocytes.

Monocytes and Macrophages

Monocytes and tissue macrophages are part of the mononuclear phagocyte system, which plays a central role in inflammation through antigen presentation, phagocytosis, and cytokine-mediated immune modulation (77). These mononuclear leukocytes are considered hallmarks of the transition from acute to chronic inflammation, as their accumulation is the result of cytokine-induced neutrophil apoptosis and increased production of monocyte chemoattractant protein (MCP-1) (78). Not surprisingly, several studies have uncovered different mechanisms in which monocytes and macrophages are involved in the modulation and maintenance of chronic inflammation. In particular, these have been demonstrated in cardiovascular disease (CVD) (79), RA (80, 81), chronic obstructive pulmonary disease (COPD) (82), diabetes (83, 84), and IBD (85). In the last decade, N-glycosylation has gained much attention as a tool by which inflammation orchestrates the immune response of monocytes and macrophages. There are three main steps involved in the accumulation of macrophages in the inflamed environment: recruitment of monocytes from the circulation, differentiation into macrophages, and activation of macrophages at the site of inflammation (77). All three steps are under the direct influence of altered N-glycosylation (**Figure 3**). First, after monocytes are recruited by MCP-1 (83), their passage through the endothelial layer requires a complex system of interactions with heavily N-glycosylated adhesion molecules. Previous studies have shown that proinflammatory cytokines such as TNFα can upregulate the expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) (12, 13) and regulate their N-glycosylation (86). This was confirmed by Chacko et al., who further identified the mannosidases MAN1A2 and MAN1C1 as subjects of decreased expression by TNFα. These mannosidases catalyze the early removal of mannose, which is required for the conversion of high mannose to hybrid and subsequently complex N-glycans. Using THP-1 monocytes and PPARγ ligands, they also demonstrated that the dual function of TNFα, stimulation of adhesion molecules and regulation of their N-glycosylation, is controlled by independent pathways, underscoring the importance of high-mannose N-glycans for monocyte trafficking (87). Recently, Regal-McDonald and his team specified that MAC-1 receptors expressed on the intermediate, proinflammatory, subclass of monocytes ($CD14^+CD16^+$) have a higher affinity for HM-ICAM-1 compared with the classical subclass ($CD14^+CD16^-$). They also showed that the monocytes bind with higher affinity to HM-ICAM-1 than to α2,6-sialylated ICAM-1 (48, 49). After migration through the endothelium, monocytes differentiate into M0 macrophages, which can further polarize into different subclasses of macrophages stimulated by different cytokines - classically activated M1 macrophages, alternatively activated M2 macrophages, CD169 macrophages, or TCR macrophages (88). During differentiation from monocytes to macrophages, ST6GAL1 is downregulated, resulting in the loss of α2,6-sialic acid. Importantly, ST6GAL1 downregulation results from cleavage by Beta-Site APP-Cleaving Enzyme 1 (BACE1), which is dramatically upregulated during macrophage differentiation (89). This may also occur during differentiation into M1 macrophages, as it has already been shown that M2 macrophages, associated with anti-inflammatory effects, exhibit higher ST6GAL1 production compared with M1 (90). Moreover, α2,6-sialylation is included in the regulation of macrophage survival. Liu et al. showed that ST6GAL1 mediated α2,6-sialylation of TNFα death receptor 1 (TNFR1) expressed on primary macrophages inhibits apoptosis (91). This discovery further highlights the protective properties of α2,6-sialylation due to its promotion of proinflammatory M1 apoptosis, and survival of anti-inflammatory M2. In addition to sialylation, fucosylation also affects M1/M2 polarization in chronic inflammation. In RA, M1 macrophages were shown to express 5-10 times more fucosyltransferases (FUTs), catalyzing terminal and subterminal fucosylation (FUT1, FUT3, FUT7, and FUT9), than their monocyte progenitors, whereas this was not observed for FUT8, responsible for core fucosylation (92).Interestingly, terminal fucosylation is important for the synthesis of the sLex epitope, which is considered proinflammatory (93), whereas core fucosylation has more anti-inflammatory properties (94). Inhibition of terminal FUTs such as FUT1/2 leads to a shift in M1 differentiation toward M2 macrophages (92). This study was the first to highlight the potential of terminal fucosylation as a novel hallmark of inflammatory M1 macrophages. Another subclass of macrophages worth mentioning is $CD169^+$. These macrophages express high levels of CD169, also known as sialoadhesin or Siglec-1, and are strategically positioned at the entry site of lymphoid tissue, where they bind and remove pathogens from the lymphatic fluid and blood (95). In addition to "gatekeeper" $CD169^+$ macrophages that constitutively express Siglec-1, its expression can be upregulated in other tissue macrophages upon exposure to a type I interferon (IFN-I) (96). Siglec-1 belongs to the sialic acid-

binding immunoglobulin-like lectins (Siglecs) (97), preferentially binding to α2,3-linked sialic acids (98), and differing from other siglecs as it has a long extracellular region (17 Ig domains) that lacks intracellular signaling motifs (99). In their review, O'Neill et al. introduced Siglec-1 as a macrophage-specific marker of chronic inflammation and emphasized its contribution to cell-cell and cell-matrix interactions of macrophages in inflammation (100). Also, soluble Siglec-1 (sSIGLEC-1) has been presented as a marker of monocyte and macrophage activation as well as a marker of interferonopathy in SLE and other inflammatory disease (101). Recently, Wang et al. showed that there is a TNFα mediated reduction in α2,6-, but not α2,3-terminal sialylation in RA (59). The latter would support interactions of proinflammatory synovial fibroblasts with pathogenic macrophages *via* Siglec-1, whose expression is upregulated in macrophages in RA (59, 102). On the other hand, Tanno et al. showed decreased expression of Siglec-1 on alveolar macrophages in COPD, significantly reducing their phagocytic capabilities against microbial pathogens and thus maintaining the inflammatory state (103). This clearly demonstrates how inflammation alters glycosylation and glycan binding molecules to maintain inflammatory environment, rather than the other way around. Mesenchymal stem cells (MSCs) have recently gained much attention as potential macrophage immunomodulators in chronic inflammation. As mentioned previously, they can be α(1,3)-exofucosylated to express the sLex-decorated CD44 ligand (known as HCELL), allowing them to migrate to the site of inflammation (28, 104). Further evidence suggests that targets of MSC mediated immunosuppression include macrophages, as there is evidence of the ability of adipose derived MSCs to shift macrophages from the M1 to the M2 phenotype (105, 106). Whether binding of HCELL ligand to Siglec-1 expressed on macrophages may be a possible mechanism of immunosuppression, thereby stopping macrophage interaction with other immune cells, is a question that remains to be answered. Another family of glycan-binding proteins involved in macrophage-mediated immunomodulation are the galectins. Among the 15 galectins identified to date expressed by immune cells, galectin-1 (Gal-1) and galectin-3 (Gal-3) show significant expression in macrophages and monocytes (107, 108). Both Gal-1 and Gal-3 possess a conserved carbohydrate recognition domain (CRD) that recognizes glycans containing a terminal N-acetyllactosamine (Galβ1,4GlcNAc or LacNAc). However, the terminal α2,6-sialylation present on LacNAc prevents the binding of Gal-1, whereas this was not observed for Gal-3 (109). Since the contribution of Gal-1 and Gal-3 to immunomodulation has been discussed in great detail elsewhere (108, 110, 111), we were focused here only on recent discoveries regarding the interplay between these galectins and macrophages. According to several different studies, soluble Gal-3 is mainly associated with proinflammatory functions (112–114). However, Di Gregoli et al. in their recent work proposed a new mechanism in which high levels of circulating Gal-3 in inflammation could be a consequence of matrix metalloproteinase 12 (MMP-12) dependent cleavage of Gal-3 from the surface of macrophages. Moreover, Gal-3 negative macrophages tend to exhibit proinflammatory properties, whereas Gal-3 positive macrophages exhibit pro-resolving and profibrotic properties (115). The latter is consistent with previously published data that revealed a novel mechanism of alternative M2 macrophage activation through binding of Gal-3 to its heavily N-glycosylated CD98 membrane receptor while emphasizing the association between M2 macrophages and increased fibrosis (116). Thus, although Gal-3 does not show direct proinflammatory effects, it is still part of the axis in maintaining the inflammatory state. On the other hand, Gal-1 seems to be a "jack of all trades" in resolving inflammation (111). Regarding the macrophages, Gal-1 is known to promote the differentiation of macrophages into the M2 profile (111, 117, 118). Yaseen et al. recently uncovered a positive feedback loop involving interferon β (IFN-β) mediated expression of Gal-1 in proinflammatory macrophages, thereby promoting their reprogramming into a pro-resolving phenotype with high expression of IFN-β (119). However, the specific mechanism is still unknown. One possibility is autocrine stimulation of proinflammatory macrophages by binding self-expressed Gal-1 to N-glycan ligands on their surface that normally lack the inhibitory α2,6-linked sialic acid. The Gal-1/IFN-β feedback loop is thought to occur at the time of termination of acute inflammation, so any misstep (e.g., insufficient Gal-1 expression) could lead to the development of a chronic inflammatory state, as decreased Gal-1 has been observed in several chronic inflammatory states (120–122).

Dendritic Cells

DCs are antigen-presenting cells with the ability to take up antigens in the periphery and expose them to lymphocytes, thus bridging the gap between innate and adaptive immune responses (123). A specific subset of DCs derived from monocytes (Mo-DC) plays a key role in inflammation (124). The surface of Mo-DCs is covered with glycoproteins decorated predominantly with sialylated glycans (125). Sialylation of DCs is regulated during both differentiation and maturation, and has been found to significantly affect DC functions such as antigen uptake, phagocytosis, and T cell priming (126) (**Figure 3**). Immature Mo-DCs are often tolerogenic because they have high levels of α2,6- and α2,3-sialylated N-glycans that are recognized by inhibitory siglecs (127) and galectins (128), respectively. By binding to α2,3-sialic acid-decorated CD43/CD45 clusters expressed on DCs, Gal-1 has been shown to support differentiation of tolerogenic DCs, thereby promoting interleukin 10 (IL-10) mediated T cell tolerance and suppression of autoimmunity (129). However, DC maturation in the presence of proinflammatory stimuli results in significant downregulation of expression and activity of ST6GAL1 and ST3GAL4 (130, 131), which may cause phenotype switch to inflammatory DCs. In contrast to the strictly tolerogenic activity of Gal-1, there are conflicting data regarding the control of inflammatory and tolerogenic DC phenotypes mediated by Gal-3 (128, 132, 133), reflecting the fact that this is a context- and tissue-dependent phenomenon. Additionally, by regulating T cell differentiation, DCs may also indirectly contribute to

altered glycosylation of IgG molecules. Gringhuis et al. identified the molecular mechanism by which fucose specific triggering of DC-SIGN leads to increased Interleukin 27 (IL-27) expression by DCs, which promotes differentiation of T follicular helper (Tfh) cells known to affect IgG production by B cells (134, 135). Interestingly, DC-SIGN preferentially binds N-glycans with fucose incorporated into the Lex epitope (136), which is abundantly expressed by various immune cells during inflammation, suggesting another potential mechanism for maintaining the inflammatory state. While the presence of sialic acids has a tolerogenic effect on DCs, fully desialylated DCs exhibit a much more potent phenotype – high expression of major histocompatibility complex (MHC) molecules, secretion of inflammatory cytokines, phagocytosis, and activation of inflammatory T cells (137). Although the exact mechanism is still unclear, sialidases such as neuraminidase 1 and 3 (NEU1 and NEU3) are thought to contribute to the desialylation of DCs (138–140). This hypothesis is also supported by the fact that sialidases are abundant and involved in the pathology of many inflammatory diseases (141). However, Lübbers et al. have recently demonstrated an alternative pathway for the induction of tolerance by DCs independent of their sialylation status, driven by the immunoregulatory sialic acid-siglec axis. Specifically, binding of α2-3-sialic acid to Siglec-9 expressed on the surface of DCs alters metabolic pathways and cytokine signaling and reprograms DCs to enhance regulatory T cell/T helper type 1 (Treg : Th1) ratio balance (142). Collectively, these data highlight the importance of glycan recognition by DCs in controlling both inflammation and its resolution.

Natural Killer (NK) Cells

NK cells are known for their role in cell-mediated cytotoxicity and secretion of proinflammatory cytokines (143), which are critical for both the promotion of inflammation and immune regulation (144). The effector functions of NK cells are regulated by a series of activating and inhibitory receptors expressed on their surface, with glycosylation playing a key role in receptor-ligand recognition (**Figure 3**). FcγRIIIa (CD16a) is the most abundantly expressed activating receptor on circulating NK cells (145), and its role in antibody dependent cell mediated cytotoxicity (ADCC) is well established (146). While it is established that modulation of IgG N-glycome significantly affects its binding to FcγRIIIa (94), Several studies made observations that underscore the importance of N-glycosylation of FcγRIIIa for IgG binding affinity. Tremendous increase in binding affinity of proinflammatory afucosylated IgG was observed when oligomannose N-glycans were present on FcγRIIIa (147, 148), which correlated with decreased expression of α-mannosidase in NK cells (149). Furthermore, higher levels of sialylated complex N-glycans on FcγRIIIa were shown to correlate with lower affinity for antibody binding (150), which was also observed for the activating NK cell receptor 2B4 (CD244) (151). In their recent review, Rosenstock and Kaufmann describe an important contribution of sialic acids to the functions of NK cells, both through the expression of sialic acid-binding receptors and by having sialic acids on their surface (152). Two of these receptors are Siglec-7 and Siglec-9, which have an inhibitory function on NK cells. While Siglec-7 mainly recognizes tumor-expressing gangliosides (153), Siglec-9 has a high affinity for α2,6- and α2,3-linked sialic acids, including the sLex epitope (154). Cytokines such as interleukin 2 (IL-2) and interferon α (IFN-α) have been shown to increase the level of sialylation on the surface of NK cells (155, 156). Although increased sialylation is usually considered to be anti-inflammatory, the functional role of these sialic acids may be to mask Siglec-9 through *cis* interactions, and thus preventing the inhibition of NK cells that would occur through *trans* binding of sialic acids. The importance of Siglec-9 in NK cell immunoregulation has been demonstrated in liver inflammation, where decreased Siglec-9 expression has been associated with disease progression (157). Although glycosylation in NK cells is functionally important, there is little information on the underlying mechanisms that alter N-glycosylation of NK cells during inflammation because of their relatively low abundance. However, the development of methods to enrich human NK cells from a single donor (149) may be a first step toward a more detailed analysis of inflammation induced N-glycosylation changes in NK cells.

ADAPTIVE IMMUNITY

In contrast to innate immunity, adaptive immunity is characterized by high degree of specificity as well as the substantial property of memory. The adaptive immune system can be further divided into cellular immunity mediated by T cells and humoral immunity represented by B cells and secreted antibodies (158). In adaptive immunity, glycans are essential for the majority of signal transduction and cell-cell interactions. N-glycans have been shown to regulate important steps in lymphocyte biology, such as T and B cell activity and cell differentiation and proliferation. Moreover, N-glycans are of great importance for the fate and function of secreted antibodies in chronic inflammation. In this section, we will therefore describe mechanisms by which inflammation can alter N-glycosylation of lymphocytes and antibodies, explain the significance of these changes in chronic inflammatory diseases, and discuss the potential of immunotherapies based on manipulation of the altered N-glycosylation.

T Cells

T cells (T lymphocytes) have a central role in the adaptive immune system. Briefly, after differentiation from thymocytes to naïve T cells, T cells leave the thymus and enter the periphery. There, exposure to antigens by antigen presenting cells (APCs) such as macrophages and/or DCs along with concomitant cytokine stimulation triggers maturation of naïve T cells. In general, mature T cells carry a unique T cell receptor (TCR) and can express either CD4 or CD8 molecules, allowing the identification of $CD4^+$ T helper cells (Th) and $CD8^+$ cytotoxic T lymphocytes (CTLs). While CTLs can exert direct cellular cytotoxicity, Th cells are required for the initiation of humoral

and cell-mediated immune responses. Thus, they can be divided into several subtypes based on functions and the production of specific cytokines - Th1, Th2, Th17, Tfh and Treg cells (159). The involvement of T cells through various mechanisms in the development and progression of chronic inflammation is undisputed (160–163). T cell function in inflammation is highly pleotropic and dependent on intra- and intercellular communication, which is often mediated by N-glycans and their corresponding binding partners (**Figure 4**). In this regard, alterations in the N-glycome of T cells can significantly affect their activation, differentiation, survival, and cytokine production, often leading to autoimmunity, chronic inflammation, or cancer (164). Under homeostatic conditions, galectins are the major immune regulators of T cells, with Gal-1, Gal-3, and Gal-9 consistently showing immunosuppressive effects. The role of galectins in immunomodulation of T cells has been discussed in detail by several authors (111, 128, 129, 165). Therefore, we will specifically discuss galectin functions mediated by N-glycosylation in chronic inflammation, along with the latest findings on the underlying mechanisms affecting N-glycosylation in and by inflammation itself. Gal-1 and Gal-3 preferentially bind to branched N-glycans containing the LacNAc motif found on their T cell counter-receptors such as CD7, CD45, CD43 and TCR. This leads to inhibited transendothelial migration and induced apoptosis of T cells (108). The aforementioned binding is under the direct influence of the activity of glycan-modifying enzymes and the availability of corresponding substrates. One such enzyme is Golgi Beta-1,6-N-acetylglucosaminyltransferase V (MGAT5), which catalyzes the biosynthesis of tetra-antennary N-linked glycans, the preferred intermediates for elongation with (poly) LacNAc and ligands for galectins. MGAT5 expression in T cells is altered in chronic inflammatory diseases at both the genetic and protein levels. At the genetic level, several *MGAT5* single-nucleotide polymorphisms (SNPs), associated with reduced expression of the MGAT5 enzyme, have been found to correlate with pathological changes in T cell glycosylation in chronic diseases such as IBD, COPD, and multiple sclerosis (MS) (47, 166, 167). Deficiency in the N-glycosylation branching pathway increases susceptibility to development of severe forms of disease due to the lack of galectins' binding substrate and consequently their inability to inhibit the exuberant Th1/Th17 immune response (168, 169). In addition, N-glycosylation alterations may occur under the influence of various cytokines; an interesting study showed that in chronic viral infection, IL-10 induced expression of MGAT5 in $CD8^+$ T cells promotes the formation of the Gal-3 lattice and increases the antigen activation threshold. Normally, this would be considered an anti-inflammatory mechanism, but this restriction in viral infection allows rapid replication of the pathogen, and thus leading to the establishment of persistent chronic inflammation (170). In terms of cytokine-mediated T cell N-glycome regulation, IL-2 is one of the most involved. Based on their research in MS, Grigorian and colleagues elegantly explained the paradoxical impacts of IL-2 on N-glycan branching and MGATs in T cells. Interestingly, IL-2 reduces N-glycan branching in

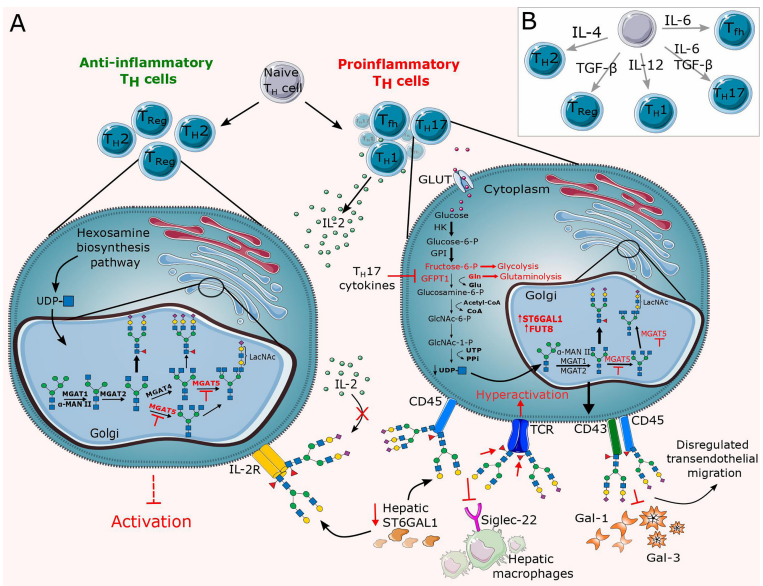

FIGURE 4 | Overview of altered N-glycosylation pathways regarding T cells during chronic inflammation. **(A)** Differentiation of lymphocytes and thus their surface N-glycome is under the direct influence of cytokines and stimulation by antigen presenting cells (APCs). Cytokines control differentiation in favor of proinflammatory T cells (Th1, Th17, Tfh), thereby altering their N-glycome by dysregulating the expression of glycosyltransferases such as MGAT5, ST6GAL1 and FUT8 and abrogating substrate availability for the hexosamine biosynthesis pathway (HBP). The resulting N-glycan changes significantly reduce the binding affinity of inhibitory galectins and Siglecs. **(B)** Schematic representation of the relevant cytokines responsible for the T cell differentiation. GLUT, glucose transporter; TCR, T cell receptor; Tfh, T follicular helper cell; Th, T helper cell; Treg, T regulatory cell.

resting T cells, whereas it has the opposite effect in activated T cells. This is thought to be a consequence of IL-2 induced upregulation of MGAT1, an enzyme that catalyzes the biosynthesis of mono-branched N-glycans, in resting T cells. MGAT1 has a ~250-fold higher affinity for UDP-GlcNAc than MGAT5, thus increased MGAT1 expression inhibits further N-glycan branching by limiting UDP-GlcNAc availability to MGAT5. In contrast, in active T cells, TCR signaling appears to increase levels of MGAT5 and UDP-GlcNAc, thereby exploiting IL-2 induced upregulation of MGAT1 to increase N-glycan branching by providing more substrates for downstream enzymes (47, 171). IL-2 is also involved in T cell differentiation. It suppresses the formation of Th17 and Tfh while promoting the development and activation of Treg cells (172–174). The latter is critical for maintaining immune homeostasis, as Treg cell dysfunction is associated with several inflammatory diseases. Therefore it is no surprise that low-dose IL-2 therapy has shown improvement in various autoimmune and inflammatory conditions (175–177). Also, mature Treg cells on their surface carry IL-2R receptor consisting of three subunits, IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγc (CD132), of which CD25 is heavily N- and O-glycosylated (178). Reduced branching decreases surface expression and retention of CD25, inhibits proper IL-2 binding, and eventually prevents Treg cell activation which consequently promotes inflammation (179). In addition to glycosyltransferase activity, substrate availability is another critical factor for successful N-glycan branching. The hexosamine biosynthetic pathway (HBP) is the main source of UDP-GlcNAc, which is required for N-glycan branching. *De novo* synthesis of UDP-GlcNAc is characterized by the conversion of fructose-6-phosphate to glucosamine-6-phosphate by the rate-limiting enzyme glutamine-fructose-6-phosphate transaminase (GFPT). To complete the conversion, GFPT also requires glutamine. Thus, the synthesis of UDP-GlcNAc by HBP may directly compete with glycolysis and glutaminolysis for fructose-6-phosphate and glutamine, respectively (179, 180). Inflammatory Th1 and Th17 undergo a metabolic switch from oxidative phosphorylation to glycolysis and glutaminolysis during inflammation (181). Therefore, by switching to glycolysis alone during inflammation, Th1/Th17 indirectly starve the hexosamine pathway of fructose-6-phosphate and consequently UDP-GlcNAc. In addition, Th17 cytokines were shown to induce down-regulation of GFPT, UDP-GlcNAc and branching in abundantly present proinflammatory T cells. These data suggest that glycolysis drives Th17 over Treg differentiation, with Th17 cytokines further maintaining reduced N-glycan branching (179). Therefore, a potential treatment for autoimmune diseases could be with metabolites of the hexosamine pathway (180). As can be seen, alteration of N-glycan branching seems to have dual function in promoting inflammation; it abrogates immunosuppression by galectins and shifts fate toward inflammatory T cells. Nevertheless, N-glycan branching is not the only feature that influences immune modulation and polarization of T cells. It has long been known that Gal-1 preferentially kills proinflammatory Th1 cells over anti-inflammatory Th2 and Treg cells. The latter is explained by the fact that Th2 and Treg cells have higher expression of ST6GAL1, which is responsible for the synthesis of terminal α2,6-sialic acids, compared with Th1 cells, and are thus protected from galectin-mediated apoptosis (109, 130, 182). Not surprisingly, the expression of ST6GAL1 is altered in chronic inflammation. In SLE, the expression of ST6GAL1 is increased in autoimmune-activated T cells, which inhibits the binding of Gal-1 and thus contributes to the pathophysiology of SLE (183). Moreover, besides the lymphocyte-specific ST6GAL1, there is a soluble form of ST6GAL1 released from the liver which is also involved in the immunomodulation of T cells. Interestingly, in mice with hepatocyte-specific ablation of ST6GAL1, there was an increase in local inflammation and a decrease in systemic Ag tolerance projected *via* increased T cell activation, and thus greater susceptibility to T cell dependent inflammatory diseases. Paradoxical as this may seem with respect to galectin inhibition, this clearly demonstrates that galectins are not sufficient to carry T cell immunosuppression alone. This is consistent with the recent discovery that liver macrophages expressing the α2,6-sialic acid-specific Siglec, CD22, can inhibit α2,6-sialic acid decorated T cells, which provides an alternative liver-driven mechanism for maintaining systemic immune homeostasis (184). APCs also have a key role in T cell polarization and activation. Sialylation of antigens has been shown to cause a shift in the differentiation of effector T cells toward tolerogenic Treg through the sialic acid-siglec axis on DCs. This could open a new way to treat patients suffering from autoimmune diseases or allergies (142, 185). Finally, another important glycosylation trait on T cells that is altered in chronic inflammation is fucosylation. The TCR receptor requires core fucosylated N-glycans for its proper activation and function. This is mediated by the Alpha-1,6-Fucosyltransferase, FUT8. In SLE and IBD, the expression of FUT8 is strongly upregulated, resulting in a hyperfucosylated TCR and thus hyperactivated T cells that contribute to the pathophysiology of the aforementioned diseases (186, 187). On the other hand, core fucosylation is required for the expression of programmed cell death receptor 1 (PD-1), which is responsible for attenuating TCR signaling, resulting in depleted and unresponsive T cells (188). The hyper-core fucosylation induced upregulation of PD-1 expression could then provide an explanation for the impairment of T cells in chronic viral infections (189, 190). Unfortunately, the underlying mechanism of upregulated core fucosylation in chronic inflammation is still unclear and is a topic for further study. In summary, inflammation has apparently found every loophole in the N-glycosylation life cycle of T cells to turn the tide in its favor. Therefore, it is necessary to consider N-glycosylation during the development of anti-inflammatory therapy, and particularly in case of a therapy specifically targeting critical steps in the transition from homeostasis to inflammation.

B Cells

B cells, also called B lymphocytes, are the major central effector immune cells in the humoral branch of adaptive immunity.

During inflammation, naïve or memory B cells are exposed to antigens by APCs under co-stimulation of Th cells in the germinal center (GC) in secondary lymphoid organs. This induces activation and rapid proliferation of B cells and selection of high-affinity B cell receptors (BCRs) (191, 192). B cells expressing a high-affinity receptor enter the periphery, where they differentiate into plasma cells that secrete large amounts of antibodies (193). Once antibodies encounter their antigen, pathogen, or infected cells, their functions include neutralization, ADCC, phagocytosis, and complement-dependent cytotoxicity (CDC) (194). In addition to their function as precursors of antibody-secreting plasma cells, B cells are involved in suppression of T cells and secretion of relevant cytokines that control adaptive immunity (195, 196). N-glycosylation has a tremendous impact on B cell proliferation, differentiation, and effector functions (**Figure 5**), but research on this topic lags far behind that of T cells. Nevertheless, there are implications that altered N-glycosylation in B cells may contribute to the development of various chronic inflammatory (autoimmune) diseases.

In B cells, among the best understood roles for lectin-glycan interactions are those of sialoglycans and Siglecs in BCR signaling. Sialic acids are often referred to as inhibitory "self-signals" because of their high local concentration on the surface of B cells. Thus, it is not surprising that Siglecs are considered major B cell immunomodulators (197). B cells express siglec-2 (CD22) and siglec-10 (ortholog of mouse siglec-G), both of which preferentially bind α2,6-sialic acid and act as inhibitory co-receptors of the BCR to maintain peripheral tolerance and prevent autoimmunity (198, 199). Thus, varying degrees of autoimmunity have been observed in mice lacking CD22, Siglec G, or both (200–202). Interestingly, ST6GAL1 deficient B cells show suppressed BCR signaling, yet mice deficient in both CD22 and ST6GAL1 showed restored BCR signaling, emphasizing the importance of α2,6-sialic/siglec axis in immunomodulation of BCR signaling (203). In addition to ST6GAL1, sialic acid acetyl esterase (SIAE) is another enzyme involved in regulation of BCR signaling. SIAE deacetylate sialic acid ligands, thereby allowing CD22-mediated inhibition of BCR signaling (204). Several studies showed that congenital mutations in SIAE are associated with an increased risk of autoimmune disease in humans (205–207). Nevertheless, while the contribution of sialic acid/siglec interactions to B cell function has been extensively defined (97, 199, 208, 209), sialoglycans represent only a fraction of the glycans involved in interactions regulating B cell immunity. Recently, Giovannone and colleagues discovered that B cells express significant amounts of tri- and tetra- complex N-glycans decorated with (poly)LacNAcs. Interestingly, the (poly)LacNAc structures were linear on naïve B cells but modified by Glucosaminyl (N-acetyl) Transferase 2 (GCNT2) with I-branches in GC B cells. In addition, I-branches were found to selectively impair B cell binding to Gal-9. Specifically, Gal-9 was found to be predominantly expressed by naïve B cells and to bind mainly the glycoprotein receptor CD45 carrying (poly)LacNAc decorated N-glycans. Functionally, binding of Gal-9 to CD45 induces inhibitory signaling through interaction with CD22, abrogated BCR calcium flux, and attenuated B cell activation. On the contrary, in GC B cells,

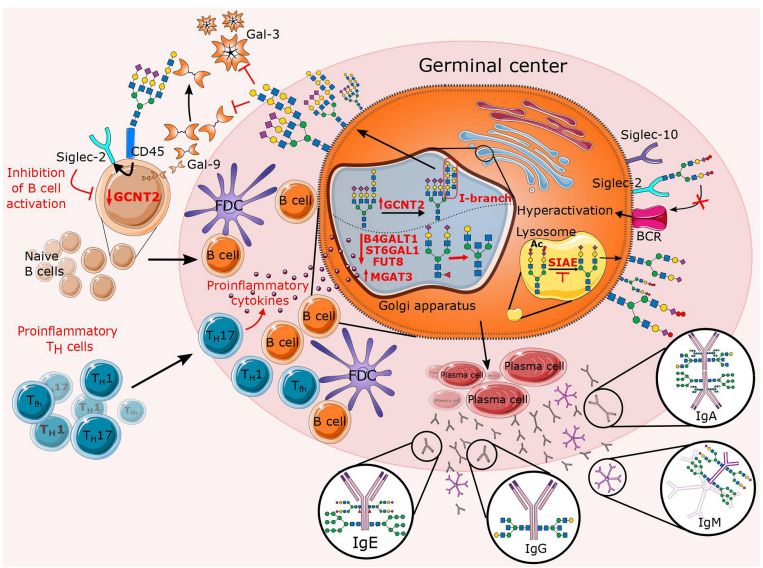

FIGURE 5 | Overview of altered N-glycosylation pathways regarding B cells during chronic inflammation. In the presence of proinflammatory stimuli, inflammatory T cells significantly affect B cell proliferation and their N-glycan profile by deregulating a specific subset of glycosyltransferases (B4GALT1, ST6GAL1, FUT8, MGAT3, and GCNT2). The latter is reflected in an increase in features such as bisecting GlcNAc, agalactosylation, afucosylation, and the presence of I-branches that have been shown to inhibit Gal-3 and Gal-9 binding. In addition to the affected Golgi enzymes, lysosomal sialic acid acetyl esterase (SIAE) is also downregulated so that it is unable to deacetylate sialic acids, which is necessary for immunomodulation of B cell receptor (BCR) signaling. This figure also summarizes the Fc N-glycome of secreted immunoglobulins, which reflects inflammation-related changes that may further contribute to disease progression.

Gal-9 mediated inhibition is down-modulated by the combined downregulation of Gal-9 and upregulation of GCNT2 (210). Although this is a novel BCR regulatory axis involving Gal-9 and GCNT2, further studies are needed to unravel the underlying mechanisms controlling the expression of these proteins. However, increased expression of Gal-9 has been reported in various autoimmune inflammatory diseases (211), therefore the exact involvement of Gal-9 in B-cell (dys)function in chronic diseases remains to be elucidated. In addition to Gal-9, other galectins such as Gal-1 and Gal-3 are also known to regulate BCR signaling, plasma cell differentiation, and survival (212–214). Interestingly, besides Gal-9, I-branches have also been shown to selectively impair B cell binding to Gal-3 but not Gal-1 (210, 215). A proposed explanation includes the fact that Gal-3 and Gal-9 preferentially bind to internal LacNAc residues, while Gal-1 favors binding to (poly)LacNAc termini (128). This suggests that I-branches may inhibit Gal-3 and Gal-9 binding, whereas terminal modifications such as α2,6-sialylation by ST6GAL1 may more selectively inhibit Gal-1. In addition, branched N-glycans are not exclusively a feature of B cell regulation but also of B cell mediated T cell regulation in autoimmunity. Branched N-glycans have been reported to suppress B cell triggered proinflammatory Th1/Th17 differentiation by promoting Toll-like receptor-2 (TLR2) and Toll-like receptor-4 (TLR4) endocytosis and downstream APC activity in B cells, thereby reducing inflammatory demyelination in a murine model of MS. At the same time, it was observed that minimal branching promotes surface retention of BCR and its co-receptor CD19, thereby stimulating adaptive B cell function. Although branching may represent another way to prevent Gal-9 binding and enhance BCR signaling, MGAT1 deficiency has been observed to prevent both branching and poly-LacNAc synthesis, leading to a decrease in BCR/CD19 surface expression and BCR signaling in this MS model (216). Because altered expression of glycosyltransferases may be a mechanism of differential regulation of galectin activity and receptor expression in B cells, future studies are needed to determine potential roles of these enzymes and their corresponding effector molecules in the development of autoimmune diseases. Lastly, it was demonstrated that core fucosylation of IgG-BCR mediates antigen recognition, along with cell signal transduction *via* BCR and antibody production (217).

The final, but not less important, role of B cells is the secretion of immunoglobulins (Igs) - the major executive glycoproteins of the humoral adaptive immune response. In humans, five classes of immunoglobulins exist: IgG, IgA, IgE, IgM, and IgD. All human Ig classes are N-glycosylated, with N-glycans attached to the conserved glycosylation regions on the fragment crystallizable (Fc) and/or on the variable fragment antigen binding (Fab), where new glycosylation sites can be acquired during somatic hypermutation (218). N-glycans can affect the structural stability and conformation of immunoglobulins as well as their effector functions (219). While alterations in N-glycosylation of IgG have been observed in several chronic (inflammatory) diseases and discussed in detail elsewhere (3), not much is known about alterations in the N-glycosylation profile of other immunoglobulins under pathological conditions. In the following paragraphs, the current knowledge about this topic is summarized with the focus on the possible underlying mechanisms mediated by inflammation that could contribute to the alterations in N-glycosylation of Igs.

IgG

IgG represents 75% of all antibodies in human serum, making it the most abundant immunoglobulin class in the bloodstream (220). Its Fab region recognizes and binds antigens, while the Fc fragment interacts with type I and type II Fcγ receptors (FcγRs) on the surface of many immune cells (including macrophages, neutrophils, B cells, NK cells, etc.), triggering various immune responses such as antigen neutralization, macrophage phagocytosis, ADCC, and complement activation (221). Each IgG molecule contains a conserved N-glycosylation site at the Asn297 of the constant heavy 2 (CH2) domain on each of its heavy chains. This site is where most of the contact with the various IgG Fc receptors and ligands occurs, and it is critical for maintaining both the pro- and anti-inflammatory effector functions of IgG (222). Glycosylation traits that are of most importance for IgG effector functions, and so mostly altered in/by inflammation, are galactosylation, sialylation, fucosylation and bisecting GlcNAc.

Galactosylation

Increased abundance of agalactosylated IgG glycans is considered a hallmark of various diseases with an underlying inflammatory component (3). Fc glycans lacking terminal galactoses are thought to be proinflammatory by activating complement through the alternative pathway along with the lectin pathway by binding to mannose-binding lectin (MBL) (223, 224). While agalactosylated glycans are considered strictly proinflammatory, terminal galactosylation seems to be quite controversial in this regard. Glycans decorated with galactoses have been held responsible for attenuating inflammation by binding to the inhibitory FcγRIIB, followed by inhibition of the proinflammatory activity of complement component C5a (225). On the other hand, Fc galactosylation is shown to activate the classical complement pathway by facilitating IgG hexamerization, thereby increasing C1q avidity and enhancing CDC (226). It has also been found to increase the affinity of IgG for activating FcγRs, leading to ADCC (227, 228). Although biological functions of (a)galactosylated IgGs are described, the underlying mechanism of how this is regulated in inflammation remains unclear. In this context, decreased levels of IgG galactosylation have been shown to associate with decreased activity of Beta-1,4-Galactosyltransferase 1 (B4GALT1) in peripheral B cells from RA patients, but no difference in expression of B4GALT1 was observed in RA patients compared with healthy controls. Proposed explanation points toward a stress-induced disruption of Golgi (heat shock and other stress proteins are elevated in RA), which could affect the proper targeting of B4GALT1 and thus impair its catalytic function (229). On the other hand, proinflammatory cytokines are observed to alter glycosylation of IgG indirectly *via* T cell-

dependent (TD) activation of B cells. Accordingly, low levels of IgG galactosylation were dependent on the effects of the Th1 cytokine interferon γ (IFN-γ) *via* IFN-γRI signaling, as decreased agalactosylation was observed in Ifngr1$^{-/-}$ mice (230). To support this, a novel B-cell intrinsic IFN-γR signaling pathway has been defined that is required for Tfh cell development and promotes autoreactive B cell formation and autoimmunity (231). Tfh cells secrete cytokines such as interleukin 6 (IL-6), IFN-γ, and interleukin 17 (IL-17), which maintain the agalactosylated state of IgGs (232). Also, a recent genome-wide association study (GWAS) showed that IL-6 signaling [SNPs in the *IL6ST* (gp130) gene] correlates with low serum IgG galactosylation (233). Interestingly, binding of these cytokines to their receptors leads to activation of JAK/STAT pathway known to target genes that appear to promote inflammation (234), therefore it is plausible that targeted genes include galactosyltransferases.

Sialylation

The addition of sialic acid to the terminal end of IgG N-glycans is essential for the control of inflammatory immune responses. Highly sialylated IgG have a lower affinity for activating FcγRIIIa, resulting in reduced ADCC (235, 236), whereas they stimulate upregulation of inhibitory FcγRIIb and thus inhibition of CDC (237). In autoimmunity, hyposialylation is thought to be responsible for the development of chronic inflammation. The results of more in-depth studies have shown that IL-23 stimulates Th17 cells to secrete IL-21 and IL-22, which are responsible for decreased expression of ST6GAL1, and thus sustaining hyposialylated state of IgG (238). Another explanation for IgG hyposialylation includes Tfh cells, and especially Tfh17 and Tfh1 cells. Tfh17 cells negatively regulate ST6GAL1 from autoantibody-producing B cells *via* the OX40-OX40L (TNF receptor superfamily) interaction. An increased number of OX40-overexpressing Tfh17 cells was observed in RA patients, and their frequency was negatively correlated with ST6GAL1 expression. However, blocking the OX40-OX40L pathway resulted in a decrease of Tfh17 cells and upregulation of IgG sialylation (135). Moreover, IL-27 stimulates Tfh1 to secrete IFN-γ, which can downregulate ST6GAL1 expression in cultured B cells by binding to the B cell intrinsic IFN-γR, and activating the JAK1/2 signaling pathway (232). Consistent with this effect of T cell cytokines on sialylation of IgG, it has been shown that T cell-independent B cell activation leads to the development of immunosuppressive sialylated IgG capable of abrogating B cell activation independent of FcγRIIb (230), possibly promoting an inhibitory feedback mechanism by binding to CD22 expressed on the B cell surface (239). In addition to inflammatory cytokines, increased risk of RA under conditions of low estrogen levels (e.g., menopause) correlate with estrogen induced increase in IgG Fc sialylation through increased expression of ST6GAL1 in splenic plasmablasts (240). Of note, recent evidence suggests that IgG glycans can be extracellularly sialylated by hepatic ST6GAL1 present in the bloodstream (241, 242), although this appears to be an inflammation-dependent process rather than a constitutive one (243).

Core Fucosylation

More than 90% of Fc glycans of IgG in healthy individuals have fucose bound to their core, which acts as a "safety switch" and attenuates potentially harmful ADCC (94). More recently, decreased fucosylation of the IgG core has been found in autoimmune thyroid diseases. The underlying mechanism is thought to be abnormal expression of the *FUT8* and *IKZF1* genes in B cells producing thyroid peroxidase antibody (TPOAb) (244). Both genes have previously been associated with afucosylated IgG N-glycans (233). Although the exact mechanism is still unclear, the *IKZF1* gene encodes the transcription factor Ikaros, a potential indirect regulator of fucosylation in B cells by promoting the addition of bisecting GlcNAc, which then inhibits fucosylation (233). Interestingly, several SNPs surrounding the *IKZF1* gene have been associated with other autoimmune diseases, including SLE (243) and IBD (244). Of note, elevated plasma levels of α-L-fucosidase (FUCA-1) were significantly associated with chronic inflammation and autoimmune diseases (245), raising the question of extracellular IgG defucosylation in inflammation. On the contrary, Plomp et al. found that IgG fucosylation is increased in individuals with a higher degree of inflammation, sometimes even in autoimmune patients (246). This was further investigated by Huang et al. and they found that increased IgG core fucosylation was observed in the serum of RA patients with a concomitant decrease in α2,6-sialylation. Moreover, α2,6-sialylation of IgG was increased in Fut8$^{-/-}$ mice (247). These findings may represent a novel mechanism for disease-specific, inflammation-related changes in IgG glycome that are consistent with distinctive observations regarding fucosylation and sialylation in autoimmune diseases differing in mechanisms of pathophysiology.

Bisecting N-Acetylglucosamine (GlcNAc)

Bisecting GlcNAc has been classified as a proinflammatory trait in many inflammatory diseases (3). Although afucosylated IgG plays the most important role in enhancing ADCC, the addition of bisecting GlcNAc to IgG Fc glycans has also been reported to boost ADCC (248). However, because the presence of bisecting GlcNAc blocks the addition of the core fucose residue (233, 249), it is difficult to distinguish the functional roles of these two glycosylation features (248). Nevertheless, epigenetic modifications and proinflammatory stimuli are shown to be responsible for increased abundance of bisecting GlcNAc on IgG Fc glycans in inflammation. It has been demonstrated that aberrant methylation in the promoter region of the *MGAT3* gene (encoding the MGAT3 enzyme responsible for the production of bisecting GlcNAc structures) results in an increased percentage of bisecting GlcNAc on IgG glycans in CD patients, suggesting a possible involvement of bisecting GlcNAc in the pathogenesis of CD (168). Moreover, Ho et al. demonstrated that the cytokine transforming growth factor β1 (TGF-β1) exerts paradoxical activity, depending on the inflammation state, in relation to the presence of tissue fibrosis and bisected IgG (250). Although further studies are needed to derive specific mechanisms that influence the

formation of bisected IgG, the functional importance of this feature in inflammation is undisputed.

IgA

Immunoglobulin A (IgA) is by far the most abundant antibody in the human body (251). The majority of IgA is secreted as a dimer and is known for its protective role on mucous membranes. In serum, IgA is the second most abundant isotype, usually produced as a monomer (252). For a long time, IgA was considered 'passive' or anti-inflammatory, but recently it has become clear that IgA also actively triggers immune responses. IgA can trigger inflammation *via* FcαRI (CD89) by directing the secretion of cytokines. Therefore, its involvement in the pathogenesis of various chronic inflammatory diseases (253) is not surprising. IgA has two conserved N-linked glycosylation Fc sites (Asn263 and Asn459) (254), but there are limited data on how the Fc N-glycome of IgA modulates binding to FcαRI. The fact that FcαRI has no direct mouse homolog (255, 256) may be a possible explanation for the lack of research on this topic. Nonetheless, N-glycosylation of IgA appears to be associated with inflammation. One of the best studied chronic inflammatory diseases related to IgA N-glycosylation is IgA nephropathy (IgAN). Recently, a study by Dotz et al. showed that a decrease in N-linked sialylation and galactosylation, and increased bisection in IgAN is associated with worsening renal function (257). Interestingly, it has been shown that mice lacking B4GALT1 develop human IgAN-like glomerular lesions and have high serum levels of polymeric IgA with agalactosylated N-glycans (258). The elevated levels of polymeric form of IgA in patients with IgAN is also associated with increased immune complex formation (259). While monomeric IgA induces inhibitory immunoreceptor tyrosine-based activation motif (ITAMi) signaling *via* FcαRI, binding of IgA immune complexes to FcαRI triggers classical ITAM signaling and activates inflammatory responses (260, 261). Furthermore, quantitative analysis revealed significant differences in N-linked glycosylation between monomeric IgA and polymeric IgA, including the presence of oligomannose exclusively on polymeric IgA (262). The differential N-glycosylation of polymeric IgA may contribute to its enhanced binding to mesangial cells and their subsequent activation, as well as to its ability to activate complement *via* binding to MBL. Moreover, the absence of terminal α2,6 linked sialic acid enhances the pro-inflammatory capabilities of IgA (263) and may serve as a predictor of poor prognosis in patients with IgAN (264). On the contrary, elevated plasma ST6GAL1 levels have been shown to be associated with IgAN disease severity (265), possibly representing an anti-inflammatory positive feedback loop. Overall, these findings may suggest a link between N-glycosylation of IgA and the pathogenesis of IgAN *via* increased formation of polymeric IgA. However, further in-depth studies are required for a better understanding of the potential role of IgA N-glycome in the development and progression of inflammatory diseases.

IgE

Immunoglobulin E (IgE) is best known for its role in allergic immune responses. Specifically, IgE binds to high-affinity IgE receptors (FcεRI) expressed on the surface of basophils and mast cells, triggering degranulation and the release of proinflammatory mediators (266). IgE is the most glycosylated immunoglobulin, having seven N-glycosylation sites (267). However, because IgE is the least abundant immunoglobulin in the bloodstream (268), analysis of N-glycosylation of IgE is significantly limited, leaving the biological function of IgE N-glycosylation largely unclear. However, it has been shown that there is a single N-glycosylation site at Asn394 consisting exclusively of oligomannose N-glycans which is critical for IgE-mediated initiation of the allergic cascade. Specific amino acid mutations or complete deglycosylation of Asn394 alter the secondary IgE structure, abolishing FcεRI binding and subsequent IgE-mediated degranulation and anaphylaxis (269, 270). Interestingly, mutation of all other N-linked sites of IgE, which consist of complex N-glycans, had almost no effect on the ability of IgE to elicit an anaphylactic response (270). Although the underlying mechanism is not yet known, the functional significance of oligomannose N-glycans at Asn394 may provide a unique therapeutic target. On the other hand, galectins such as Gal-3 and Gal-9 have also been shown to be involved in the regulation of IgE-mediated functions. Gal-3, previously known as IgE-binding protein, has the ability to cross-link IgE and FcεRI *via* their N-glycans and trigger basophil or mast cell activation (271). Moreover, both Gal-3 (272) and IgE (273) are overexpressed in atopic dermatitis (AD), suggesting that they are important players in mediating chronic inflammation in AD. In contrast, Gal-9 has been shown to reduce mast cell degranulation and anaphylaxis by blocking the formation of the IgE-antigen complex (274). Given the affinity of these galectins for complex N-glycans (128), it is likely that the galectin-IgE interactions mentioned above are mediated by complex N-glycans on IgE. Strikingly, the removal of terminal sialic acid on IgE N-glycans, as well as coexistence of other asialylated glycoproteins, attenuates degranulation of effector cells (275). The exposed terminal galactoses could exert a suppressive function by binding to inhibitory galectins, although the exact mechanism remains to be elucidated.

IgM

Immunoglobulin M (IgM) is the largest antibody in serum and its level is elevated in various inflammatory and autoimmune diseases (276). It is another highly N-glycosylated antibody, as its constant domain contains five N-linked glycosylation sites, three of which belong to the biantennary complex form (Asn171, Asn332, Asn395) and two to the oligomannose type (at Asn402, Asn563) (219). Oligomannose N-glycans have been shown to be important for MBL binding and subsequent elimination of IgM aggregates by opsonization (277). On the other hand, complex N-glycans are involved in immunomodulation of T and B cells. Sialylated N-linked glycans have been demonstrated to induce

internalization of IgM by T cells, which in turn causes inhibition of T cell responses. The authors hypothesized that IgM-mediated immunosuppression occurs through the binding of sialylated IgM to the constitutively expressed IgM Fc receptor (FcμR) on the surface of T cells (278). On the other hand, B cell activation is under the direct influence of Gal-9-mediated negative regulation. It has been proposed that Gal-9 organizes IgM-BCR and the inhibitory molecules CD45 and CD22 into larger clusters by binding to their N-linked glycans, and thus directly inhibiting BCR signaling (279). Considering Gal-9 binding preferences (128), the above N-glycan-mediated interaction could be facilitated by complex N-glycans on IgM molecules. In addition, sialylated N-glycans on soluble IgM are preferential *trans*-binding ligands for CD22, which further contributes to the abrogation of BCR signaling (280). These results support the concept that the presence of α2,6-sialic acid on Igs contributes to immunosuppression, as previously demonstrated for the anti-inflammatory effects of intravenous immunoglobulin therapy (IVIg) (281).

IgD

Even though O-glycans of Immunoglobulin D (IgD) are associated with autoimmune diseases (282), nothing is known about the role of N-glycosylation in IgD effector functions, despite having three N-glycosylation sites in the Fc domain (Asn354, Asn445, Asn496) (283). The oligomannose glycans at Asn354 are inaccessible for potential lectin interactions because the complex N-glycans at Asn445 block binding (284). Nevertheless, oligomannose N-glycans are critical for IgD production, and elimination of the Asn354 site by mutagenesis results in incomplete assembly and failure of secretion (285), proposing that the N-glycans are necessary for maintenance of the correct Fc structure, which is important for IgD secretion.

ACUTE PHASE PROTEINS

APPs are mainly synthesized and secreted by hepatocytes. During inflammation, proinflammatory cytokines such as IL-1, IL-8, IL-6, and TNFα stimulate the acute phase response (286–289), increasing APP serum levels up to 1000-fold (288). Several APPs are glycoproteins and changes in their N-glycans have been observed in chronic inflammation. The most significant N-glycosylation changes observed in APPs are high branching (tri- and tetra-antennary glycans) and increased levels of sLex epitope as detected on haptoglobin (HPT), α1-acid glycoprotein (AGP-1), α1-antitrypsin (A1AT), and α1-antichymotrypsin (ACT) (29, 290–292). The sLex epitope on AGP contributes to its antineutrophil capacity (75) and is critical for binding to endothelium-expressing E-selectin, where AGP competes with sLex-expressing leukocytes, providing a feedback inhibition mechanism (293). Proinflammatory cytokines IL-1β, IL-6, and TNFα, involved in the induction of the acute phase response, may also be involved in the regulation of APP glycan biosynthesis in hepatocytes (294–298). *In vitro* studies have shown that AGP expresses N-linked glycans with increased branching and sLex epitope when hepatocytes are stimulated with IL-1β and IL-6 (294), possibly through cytokine mediated upregulation of enzymes responsible for biosynthesis of sLex epitope, ST3GAL4 and FUT6 (295). Furthermore, TNFα has also been shown to increase sLex synthesis by stimulating the expression of ST3GAL4 and FUT4 *via* NFkB-p65 dependent transcriptional regulation (298, 299). In addition to *in vitro* studies, TNFα induced increase in sLex epitope has also been observed in RA patients (300). Based on the results of their GWAS study, Lauc et al. described another pathway for the regulation of plasma protein sLex formation involving hepatocyte nuclear factor 1α (HNF1α) and its transcriptional cofactor HNF4α. HNF1α/HNF4α induce both *de novo* and salvage synthesis of GDP-fucose, upregulate antennary fucosyltransferases (FUT3/4/6) and downregulate core fucosyltransferase (FUT8), ultimately leading to increased sLex-expressing APPs (301). Interestingly, HNF1α mediated transactivation of hepatic genes is stimulated by IL-6 (302), adding to the molecular mechanism behind the reported association between proinflammatory cytokines and increased levels of sLex-expressing APPs. While sLex epitope formation is highly dependent on cytokine mediated increase in the expression of relevant glycosyltransferases, increased HBP flux and consequently higher levels of UDP-GlcNAc in hepatocytes lead to increases in tri- and tetra-antennary N-glycans on APPs in chronic inflammation. Donor molecules directly involved in modulating UDP-GlcNAc levels and HBP flux are glucose and glutamine (303). During sustained inflammation, increased hepatic uptake of glutamine and increased hepatic glucose production *via* TNFα-activated NF-kB transcriptional regulation have been observed (304, 305). Consequently, increased hepatic HBP flux leads to high levels of UDP-GlcNAc, the crucial substrate for N-glycan multistep branching of APPs and other hepatic glycoproteins. The biosynthesis of tri- and tetra-antennary N-glycan-decorated APPs is ultrasensitive to UDP-GlcNAc content, as the affinity for UDP-GlcNAc decreases from MGAT1 to MGAT5 (306). Furthermore, N-glycan branching of hepatic membrane transporters (for glucose and glutamine) increases galectin binding affinity, protecting them from endocytosis and thus establishing a positive feedback loop by increasing HBP substrate uptake (307). In addition to the aforementioned APPs, hepatic ST6GAL1 is also upregulated and released into the circulation during inflammation (308). Although certain anti-inflammatory effects of hepatic ST6GAL1 have been observed (242, 309), its role still remains elusive. However, Oswald and coworkers have shown that loss of hepatic ST6GAL1 leads to dysregulation of hepatic metabolic pathways and consequent changes in the N-glycan profile of circulating glycoproteins. It has been observed that loss of α2,6-sialic acid, core and/or antennary fucose, and an increase in α2,3-sialylation, branching, and bisection ultimately lead to spontaneous liver inflammation and disease (310). Interestingly, chronic alcohol exposure has previously been shown to downregulate hepatic *ST6GAL1* gene expression, leading to metabolic dysfunctions, including altered glycosylation (311). This highlights the fact that lifestyle may

contribute to the loss of hepatic ST6GAL1, which in turn triggers the development of inflammation and activates the cascade of proinflammatory cytokines responsible for the increased expression of hepatic ST6GAL1 during the peak of inflammation (308, 309, 312, 313), providing a positive feedback loop that may explain hepatic ST6GAL1 paradox.

CONCLUSION

N-glycosylation is one of the key mediators in intercellular interaction and communication, which makes it highly susceptible to changes in inflammation. On the other hand, as discussed above, altered N-glycosylation affects the immune response, which may further enhance the inflammatory reaction. Therefore, N-glycans are essential for normal immune system function, from innate to adaptive immunity. This opens up the possibility for development of new therapeutic approaches for various inflammatory diseases targeting altered N-glycan structures or biosynthetic enzymes associated with glycosylation. Moreover, the potential of N-glycosylation alterations as novel biomarkers or as enhancements of existing ones for disease predisposition and progression, as well as for diagnosis, prognosis, and response to therapy, cannot be ignored. However, further in-depth research is needed to elucidate the precise mechanism underlying some of these alterations so that these discoveries can be translated into clinical practice and diagnostic test development.

AUTHOR CONTRIBUTIONS

BR collected the data and wrote the manuscript. IG provided valuable guidance and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Glucose metabolism and glycosylation link the gut microbiota to autoimmune diseases

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Carbohydrates serve as important energy sources and structural substances for human body as well as for gut microbes. As evidenced by the advances in immunometabolism, glucose metabolism and adenosine triphosphate (ATP) generation are deeply involved in immune cell activation, proliferation, and signaling transduction as well as trafficking and effector functions, thus contributing to immune response programming and assisting in host adaption to microenvironment changes. Increased glucose uptake, aberrant expression of glucose transporter 1 (*e.g.*, GLU1), and abnormal glycosylation patterns have been identified in autoimmunity and are suggested as partially responsible for the dysregulated immune response and the modification of gut microbiome composition in the autoimmune pathogenesis. The interaction between gut microbiota and host carbohydrate metabolism is complex and bidirectional. Their impact on host immune homeostasis and the development of autoimmune diseases remains to be elucidated. This review summarized the current knowledge on the crosstalk of glucose metabolism and glycosylation in the host with intestinal microbiota and discussed their possible role in the development and progression of autoimmune diseases. Potential therapeutic strategies targeting glucose metabolism and glycosylation in modulating gut ecosystem and treating autoimmune diseases were discussed as well.

KEYWORDS

autoimmune diseases, glucose metabolism, glycosylation, gut microbiota, systemic lupus erythematosus

Introduction

Accumulated evidence showed an increased glucose uptake in autoimmune diseases as revealed by ^{18}F-FDG PET-CT (1) and implied the impact of distinct glucose metabolic pathways on the immune system. Glucose, serving as an important energy resource and the major substrate for adenosine triphosphate (ATP) generation, is deeply involved in multiple physiological and pathological bioactivities such as immune cell activation, proliferation, signaling transduction, leukocyte trafficking, and cytokine production. Based on the current evidence, immune cells can modulate their cytology to accommodate environmental glucose levels. T cells separated from mice with hyperglycemia showed a more active response to T cell receptor (TCR) stimulation, with accelerated proliferation of $CD4^+$ T cells and a higher expression of Th1, Th2, and Th17 cytokines compared with those separated from healthy controls (2), indicating the proinflammatory effects of hyperglycemia circumstances. Glucose deprivation or restricted energy availability steers the metabolic preference from glycolysis towards fatty acid oxidation and consequently adjusts cell fate and polarization; thus, it can be taken as an immune-metabolic checkpoint (3).

During activation, T cells undergo increased glycolysis and oxidative phosphorylation (OXPHOS) to meet the energy demand for proliferation and effector functions (4). Not surprisingly, the upregulation of glycolysis leads to the restricted but rapid generation of ATP and nucleotides, amino acids, and fatty acids synthesis, which are important for immune cells to fulfill multifold tasks, *e.g.*, cytokine production and proliferation, and switch to inflammatory phenotypes. Increased glucose uptake and accelerated glycolysis are indispensable for immune cells to adapt to microenvironment changes and to cope with external stimuli like infectious pathogens.

Glucose can chemically attach to proteins and nucleic acids without the aid of enzymes, and excessive nonenzymatic glycosylation is involved in multiple biological effects such as inhibiting regulatory molecule binding, decreasing proteolysis susceptibility, influencing nucleic acid function, altering macromolecular endocytosis, and increasing immunogenicity (5). What is more, glycoproteins count on carbohydrates to achieve a proper three-dimensional conformation which is critical for the function and bioactivity of glycoprotein.

Fucosylation is one of the major glycosylation patterns in the human body and is of utmost importance in immune cell maturation, inflammatory cytokine secretion, and gut bacteria adhesion (6). Increased levels of fucosylation had been shown in patients with systemic lupus erythematosus (SLE) and was positively correlated with disease severity (7). Meanwhile, studies have shown that the state of gut microbes is largely regulated by their own carbohydrate modification and host dietary intake as well as host glycosylation (8–16). Both glucose metabolism and microbiota composition as well as the interaction between them are suggested to participate in host immune regulation (17–23). Dysbiosis of intestinal microbiota, such as a lower Shannon diversity index, symbiont translocation, and pathobiont enrichment, has been documented in many types of autoimmune diseases like SLE, rheumatoid arthritis (RA), type 1 diabetes mellitus (T1DM), and multiple sclerosis (MS) with the potential of promoting aberrant immune activation and self-tolerance breakdown (24–29). As a beneficial paradigm, *Akkermansia muciniphila*, a species of human commensal bacterium with mucin-degrading capability that abundantly resides in the mucus layer, can counteract inflammation-associated insulin resistance, display favorable metabolic effects on host glucose and adipose metabolism, reinforce intestinal barrier function, and influence host immune responses. Indeed *A. muciniphila* has shown therapeutic potentials in several inflammation-related diseases, including inflammatory bowel disease (IBD) and diabetes (30–32). In autoimmune diseases characterized by the overactivation of immune cells and aberrant self-attack (33), how glycosylation links glucose metabolism to the multiple physiopathological changes in the immune system and gut ecosystem is of great interest. However, studies on the molecular mechanism linkage of autoimmune diseases with glucose metabolism and glycosylation and their interaction with the gut microbiota were limited.

By analyzing the contribution of distinct glucose metabolic pathways to cell fate determination (34, 35), glycomics provides novel insights into the molecular metabolic pathogenesis of autoimmune diseases. Further research dissecting the complex mutual influence of host glucose metabolism and gut microbiota, together with host immune system programming, might help to exploit new targets or novel intervention strategies to control these diseases. In this review, the interaction of carbohydrate metabolism pathways and glycosylation with the intestinal microbiota and host immune response was discussed based on current knowledge and research. We also discussed their role in the development of autoimmune diseases and the potentials of measures targeting glucose metabolism and microbiota in the treatment of autoimmune diseases.

Glucose metabolism and glycosylation

Glucose metabolism

Glucose metabolism is fine-tuned to accommodate the cell demand for energy and biosynthesis. Glycolysis is a fast way to acquire energy, though not as efficient as OXPHOS in the mitochondria, and it is of preference for cells in rapid growth and proliferation. Interestingly, pro-inflammatory cells (*e.g.*,

effector T cells, type 1 macrophage) seemed to more preferentially rely on glycolysis process to acquire ATP for their fate determination and cytokine production, whereas regulatory cells (*e.g.*, regulatory T cell, type 2 macrophage) might rely more on mitochondrial OXPHOS for their function (36). During glycolysis, glucose is catabolized into pyruvate and lactate which can subsequently be converted into acetyl-CoA to fuel the tricarboxylic acid cycle.

There are three glucose catabolic pathways: aerobic oxidation, anaerobic glycolysis, and pentose phosphate pathway (37). Among all three pathways, hexokinase (HK) is in the first step and the vital enzyme crucial in modulating the intracellular glucose concentration.

As known, mammalian target of rapamycin (mTOR) is also a key regulator in immunometabolism that can regulate glucose uptake and glycolysis in immune cells and, coupled with phosphoinositide 3-kinase/Akt, can modulate diverse cell activities.

Gluconeogenesis typically occurs in the liver but is unveiled recently as a novel function in the gut metabolism. The human intestinal mucosa can express glucose 6-phosphatase and phosphoenolpyruvate carboxykinase gene which is responsible for gluconeogenesis. It is suggested that dietary fiber fermentation products—butyrate and propionate—can induce gluconeogenesis gene expression and benefit body energy homeostasis (38).

Glycoconjugates and glycosylation

The existence of carbohydrates in the human body is presented in several forms: monosaccharides (*e.g.*, fructose, galactose, sucrose, ribose), complex carbohydrates (*e.g.*, oligosaccharides or polysaccharides), and glycoconjugates (*e.g.*, glycoprotein, glycolipid) (39). Sugars from the daily diet are digested to provide energy source through catabolic pathways or produce structural substances (*e.g.*, fatty acids, FAs) through anabolic pathways (39). Some intermediate products formed in carbohydrate catabolism become raw materials and carbon frames for synthesizing biological macromolecules such as lipids, proteins, and nucleic acids. Small non-carbohydrate substances can also be converted into carbohydrate molecules or covalently polymerized with carbohydrate molecules to form glycoconjugates in the form of glycoproteins, proteoglycans, glycolipids, and glycophosphatidylinositol-linked proteins (40) and take part in diverse human physiological and biochemical processes.

A layer of glycocalyx covers almost every cell surface in human organs, which is a highly hydrated gel-like layer composed of a meshwork of proteoglycans, glycoproteins, glycosaminoglycans, and glycolipids and assembled with proteins embedded in the plasma membrane to form the external cell surface (41). Mounting evidence has revealed the function of endothelial glycocalyx in maintaining vasculature homeostasis by acting as vascular permeability barrier, physical force transmitter, antagonist of adhesion, and oxidative stress shield, *etc.* (41, 42). Under vascular pathological circumstances, the endothelial glycocalyx can be damaged by hypervolemia or hyperglycemia (43). The glycocalyx is involved in gut epithelial mucosal barrier formation as well, which is mainly composed of sulfated mucin glycoproteins produced by goblet cells (44–46). The gut barrier prevents the invasion of enteric pathogens, and its disruption can cause a "leaky gut" and subject the host to microbial invasion and translocation, which has been reported in multiple autoimmune diseases. In addition, the mucin barrier of the gastrointestinal epithelium might be disrupted under certain a pathological state like IBD, which brings the collapse of its barrier function and promotes bacterial adherence (47, 48). The disruption was found related with both host genetic susceptibility and environmental etiologies (49, 50).

The surfaces of immune cells are also equipped with a mixture of glycoconjugates with complex glycosylation, which play a vital role in regulating immune cell maturation and differentiation (51, 52).

By appending monosaccharide residues to saccharides, peptides, and lipids with glycosidic bonds, catalyzed by glycosyltransferase, glycoconjugates are formed, and the glycosylation process can transform molecular conformation and even alter the antigenicity. Human glycans are basically composed of 10 monosaccharides, including L-fucose, D-galactose, GalNAc, N-acetylglucosamine (GlcNAc), *etc.* (53). Glycosylation of protein is an important way of post-translational modification and ubiquitously involved in human physical activities. Based on whether adding a monosaccharide residue to the free-NH_2 group or to the oxygen atom of the hydroxyl group in a polypeptide chain, protein glycosylation can be categorized into N-glycosylation and O-glycosylation (47, 53, 54). Though not completely understood, the regulation of the glycosylation process and the glycosyltransferase expression are determined by cell types, respond actively to environmental changes, and participate in cell–cell interactions and distinct protein activity regulation (47, 53). Fucosylation is one of the major forms of glycosylation with in-depth studies and known to participate in the formation of ABO blood group antigen determinants, Lewis antigen determinants, and selectin ligands (6). Fucosylation is accomplished with fucosyltransferases, GDP-fucose synthetic enzymes, and GDP-fucose transporter. Studies found that certain genes in the fucosylation process can upregulate the expression of PD-1 on the cell surface, thus subsequently inactivating the T cells and decreasing the alert threshold towards tumor cells (55). Furthermore, fucosylation of some classifications has been found to have a positive correlation with TNF expression (6), contributing to the inflammation.

Association of glucose metabolism and glycosylation with host immune response

Glucose metabolism in immune cells

Immune cells undergo metabolic reprogramming upon antigen challenge. The development, proliferation, and cytokine production of cytotoxic and effector T cells (Teff) rely greatly on glucose metabolism with increased glucose transportation *via* glucose transporter 1 (56), accelerating glycolysis and the bioactive intermediates of the glycolytic pathway (57). The switch of oxidative phosphorylation to aerobic glycolysis is regarded as a hallmark of Teff cell activation (58, 59). Intriguingly, Treg shows unique energetic metabolism by relying on glycolysis to support its proliferation and migration whereas relying on OXPHOS (36) to maintain its suppressive capacity. Glycolysis suppression may reprogram T cell differentiation toward Treg and mTOR inhibitor by inhibiting glucose uptake and inducing hypoxia-inducible factor-1α, subsequently leading to the upregulation of FOXP3 expression, which may partially account for the immune tolerance restoring effects of rapamycin. Foxp3, the well-known transcription factor of Treg, functions to suppress glycolysis and enhance oxidative phosphorylation, thus reprogramming Treg cell metabolism to accommodate low glucose and high lactate conditions (57).

Immune cells also respond to the change of glucose level in the environment with metabolic adjustment, which, in turn, alters their own immune function. Long-term carbohydrate and caloric restriction (CR) lowers the inflammation risks but maintains the T cell repertoire (3, 60). Data from several studies suggested that CR led to the reduction of circulating inflammation markers, including lymphocytes, monocytes, and dendritic cells (DCs) (17–19), but increased the retention of pro-inflammatory monocytes and T cells in the bone marrow (20, 21). Jordan et al. (18) used HK inhibitors to block glycolysis in mice and found a similar shrink of a pool of circulating monocytes, which is mediated by the activation of 5-AMP-activated protein kinase, a key cellular energy sensor (18, 61).

Based on the current evidence, glucose deprivation or restricted energy availability steers the metabolic preference from glycolysis towards fatty acid oxidation and consequently transforms cell fate and polarization; thus, it can be taken as an immune-metabolic checkpoint (3).

Glycosylation in innate immune response

Innate immune response is the first line to defend against microorganism invasion, which is initiated through the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) from pathogens with pattern recognition receptors (PRRs) (*e.g.*, Toll-like receptors, TLRs) either secreted by or anchored in the membrane of innate immune cells (Table 1). Most PAMPs have glycan components (47, 62), and bacteria also produce extracellular glycocalyx to mediate cell attachment, protect against antibacterial agents, retain humidity, *etc.* (64)—for example, lipopolysaccharide (LPS) from Gram-negative bacteria, known as endotoxin and the most studied PAMP, is a glycolipid that can induce an acute or chronic inflammatory reaction *via* TLR4 recognition and subsequent NF-κB activation and is associated with a poor prognosis of sepsis. LPS is mainly produced by gut microbes, and increased serum LPS may act as a clue of damaged gut mucus barrier which has been reported in some immune-mediated diseases such as SLE and IBD. Recently, it is suggested that LPS exposure in early life may induce endotoxin tolerance, help suppress Th-2 skewing effects, and modify allergic response, which may act as supportive evidence of the hygiene hypothesis (63). Polysaccharide A (PSA) from *Bacteroides fragilis* is another typical PAMPs, recognized by TLR2, and activates downstream MyD88- and TRIF-dependent pathways, allowing the translocation of transcription factors like NF-κB, AP-1, IRF5, and IRF7 into the nucleus, promoting the expression of inflammatory cytokine genes, and increasing the production of NO and secretion of TNFα in macrophages (22). PRR like TLRs are also heavily glycosylated themselves (62). The glycosylation sites of TLR determine its three-dimensional features and affect its function (47). Lectins are a group of carbohydrate-binding proteins with opsonization capability to mediate the attachment and binding of bacteria by aggregation (23). C-type lectins belonging to PRRs have a specific affinity to different glycosyls and can trigger a series of downstream immunoreactions including T cell activity regulation, cell adhesion, and cytokine secretion (72). Their function as information transmitter in regulating immune response and autoimmunity is coded by diverse glycosylation. According to their affinity to distinct monosaccharide, lectins can be classified into five groups: mannose, galactose/GalNAc, GlcNAc, fucose, and N-acetylneuraminic acid (73). Mannose-binding lectins (MBLs) have a high binding specificity with mannose and GlcNAc. The MBL pathway, as one of the three activation pathways in the complement system, is significant in innate, nonspecific immunity, especially in the initial immune response to sugar-decorated pathogen (74). MBLs and ficolins (another type of lectins in humans that can only bind with GlcNAc) can discriminate self and non-self and bind with carbohydrate groups on the surface of bacteria, fungi, or other pathogens through a COOH terminal carbohydrate recognition domain and activate MBL-associated serine protease (MASP) by a configuration change. MASP1cleaves C3 directly; MASP2 catalyzes the production of C4b2a (C3 convertase), thus triggering the complement cascade by the classical pathway (23). Once C3 is cleaved and C3b is generated, C3b (or C4b,

TABLE 1 Summary about pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and pattern recognition receptors (PRRs) with their characteristics.

	Definition and characteristic	Reference
Pathogen-associated molecular patterns	A common non-specific, highly conserved molecular structure on the surface of pathogens Necessary for the survival and pathogenicity of the pathogen The molecular basis for the recognition of "non-self" by innate immune cells (PRRs)	(22, 47, 62–64)
Damage-associated molecular patterns	The immune system does not distinguish between "self and non-self" but senses danger cells respond to distress (stress, injury, necrosis), danger signals (alarmin), and then respond Including certain proteins or peptides, nucleic acid, unsaturated fatty acids, lipoproteins, purine metabolites, uric acid crystal, cholesterol crystal, and cytokines	(65–68)
Pattern recognition receptors	A class of receptors that directly recognize PAMPs or share specific molecular structures (DAMPs) on the surface of host apoptotic and senesis-damaged cells Present on the surface of various immune cell membranes, intracellular organelles, and serum, such as phagocytes and dendritic cells	(65, 69–71)

iC3b) is fixed to the bacterial cell surface and binds with complement receptor 1 (CR1) (or CR3, CR4), which is expressed on the surface of phagocytic cells (macrophages, neutrophils, monocytes, *etc.*), and phagocytosis of these bacterial cells is initiated, which is also called opsonization (74–77). The galectins are a group of soluble members of lectin family, excreted by immune cells, endothelial cells, or epithelial cells, and can lead the microbes to the target cells (47, 78) (Figure 1).

DCs are an important component in innate immunity as the professional antigen-presenting cells. Brigitta et al. verified that the nonenzymatic glycosylation of human β2 glycoprotein I (β2GPI) was capable of activating immature DCs, priming of Th2 cell differentiation, and triggering the signaling pathway of ERK, p38 MAPK, and NF-κB. Glycosylation is known to be capable of influencing the immunogenicity of proteins, so it is rational to postulate that glycation of β2GPI may lead to the exposure of cryptic epitope or the formation of neo-determinants to trigger immunogenic DC (79).

Glycolysis induced by mTOR-HIF1α is also involved in neutrophil migration, neutrophil extracellular trap production, and killing activity (80).

Glycosylation in adaptive immune response

Exploration of the role of carbohydrates and glycosylation on adaptive immune responses revealed that proper MHCII N-glycosylation is critical for the binding and presentation of PSA to CD4+ T cells for their subsequent activation and proliferation (81–83). Mice treated with mannosidase inhibitor to interfere N-glycan complex formation would lose the ability to expand PSA-specific T cells, while the transfer of normally glycosylated APCs to the mice helps restore the ability (84). Other studies found the exposure to PSA results in the upregulation of IFNγ, TNFα, IL-6, CXCL10, and surface and intracellular markers associated with M2 macrophages (85, 86) (Figure 1).

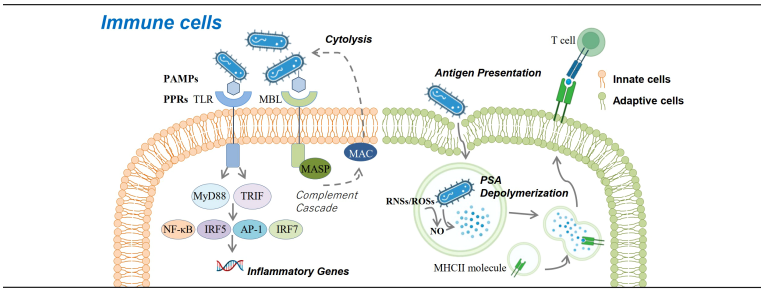

FIGURE 1
Roles of carbohydrates in immune cells. Carbohydrates are involved in immune response against bacteria at the cellular level. Glycans contained in the glycocalyx of bacteria serve as pathogen-associated molecular patterns that bind with pattern recognition receptors (*e.g.*, Toll-like receptor and mannose-binding lectin) and activate downstream immune response pathways, either promoting the expression of inflammatory genes or activating the complement cascade and eliminating pathogens. Bacteria can also be presented by antigen-presenting cells *via* the MHCII pathway, which requires NO to help degrade bacterial polysaccharide A into small molecules and present them to CD4+ T cells.

During inflammatory response, leukocyte adhesion and extravasation, homing to lymphoid organs, and recruitment to peripheral tissues are closely linked with selectins. Selectins are a group of C-type lectins that are found in the Weibel–Palade bodies of endothelial cells and platelet α-granules (P-selectins), on the surface of skin endothelial cells (E-selectins), and on the surface of most leukocytes (L-selectins) and are inducibly expressed during inflammatory responses (87–90). Most selectin ligands contain a glycan epitope named sialyl Lewis x (sLex) with fucose and sialic acid-decorated carbohydrate chains (90, 91). The main selectin ligand expressed by all calculating leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1), supports the capture and rolling of a broad range of leukocytes (monocytes, DCs, mast cells, NK cells, neutrophils, basophils, eosinophils, activated T cells, and Treg cells) (88, 92). Therefore, malfunction of selectin due to the aberrant structure of selectins and their ligands mediated the impaired leukocyte trafficking which participated in the pathogenesis of autoimmune diseases, including MS, autoimmune skin inflammation, IBD, T1DM, RA, SLE, and systemic sclerosis (88) (Figure 2A).

Immune response mediated by antibodies, including phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement activation involves the immunoregulatory role of glycosylation as well (93). IgG is the most abundant immunoglobulin in human serum. Each one of the two heavy chains of an IgG molecule contains an N-linked glycan at Asn-297 in its constant region (Fc), with the ingredients of the glycan including GlcNAc, fucose, mannose, galactose, and sialic acid (94). Distinct glycan modifications affect the shapes of the hinge or the CH_2–CH_3 interface of Fc domains and determine the specificity of receptor binding (94, 95). Fc gamma receptor I (FcγRI) is the only type of high-affinity IgG Fc receptors in promoting immune response against bacterial infection and may contribute to several autoimmune diseases. A significant structure to determine its higher affinity is the bond between the receptor D2 domain FG loop and the proximal carbohydrate units (GlcNAc) of Fc glycan from IgG, in the form of a hydrogen bond (95, 96). The FG loop of FcγRII and FcγRIII instead has a distance too far to form a hydrogen bond with the glycan of the IgG Fc domain, which leads to a lower affinity of these two receptors (95). Furthermore, the fucosylated Asn-162 of FcγRIIIA was found to bind with the nonfucosylated glycan of Fc more stably, and studies revealed that FcγRIIIA reached a significantly higher affinity to a fucosylated IgG (absence of core fucose at Asn-297) than fucosylated IgG, which means that the core fucosylation of the Fc domain inhibits the binding of IgG to FcγRIIIA, thus inhibiting ADCC (95, 97–99). On the contrary, the addition of GlcNAc and sialic acid might have the effect of promoting the affinity (100, 101).

Glucose metabolism and intestinal bacteria

Host glucose metabolism and glycosylation influence the intestinal ecosystem

Various kinds of carbohydrates and glycosyls exert bidirectional effects on the colonization of different bacteria species in the gut and contribute to the establishment of healthy infant gut microbiota (9, 102, 103). Human intestinal epithelial cells (IEC) mainly carry α (1, 2)-fucosylated glycans, which constitute an important part of the interface between host

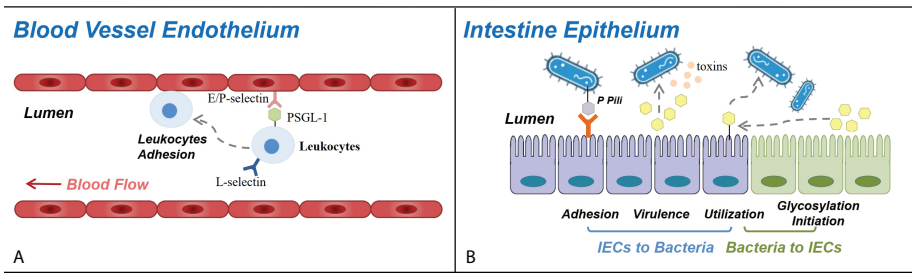

FIGURE 2
Roles of carbohydrates in blood vessel endothelium and intestine epithelium. **(A)** Selectins and selectin ligands participate in leukocyte trafficking in immune response and autoimmune diseases. E-selectins and P-selectins expressed on the blood vessel wall bind with PSGL-1 and other selectin ligands expressed on the surface of circulating leukocytes, controlling their adhesion and recruitment to the endothelium of CNS venules (in multiple sclerosis), dermal vessels (in autoimmune skin inflammation), ileum and colon vessels (in inflammatory bowel disease), joint synovium (in rheumatoid arthritis), *etc.* **(B)** Carbohydrates and glycosylation mediate the interactions between the bacteria and the intestinal epithelial cells in a variety of approaches. The adhesion and the colonization of bacteria can be mediated by their cell surface glycans binding specifically with receptors on intestinal epithelial cells (IECs). The virulence of bacteria can be influenced as glycogroups on IEC function as bacterial toxin targets, and their breakdown releases products that regulate the expression of bacterial virulence genes. Certain species of bacteria can degrade IEC glycosyls and utilize them, thus influencing the condition of both the bacteria and the intestine epithelium, and several bacterial species can induce the fucosylation initiation of IECs.

and microbiota (104). The protective intestinal (also respiratory and genitourinary) mucus layer is built up with large glycoproteins called mucins (MUC) in high density, which is characterized by high O-glycan ratio binding with water (47). MUC2, the major normal mucin in the intestinal tract, forms trimers once secreted and becomes an ideal net-like filter (105). The composition, function, and regulation of mucins are complex and only limitedly understood (45), but mucus glycosylation defection has been implied to be associated with gut flora diversity reduction and composition alteration (8) as exemplified by the finding that Fut2-deficient mice have lower intestinal flora diversity with a shrinking population of an unclassified genus of *Clostridiales* and expanded *Parabacteroides*, *Eubacterium*, *Parasutterella*, *Bacteroides*, and family *Lachnospiraceae* (9, 104), whereas Johansson et al. (106) found that germ-free (GF) mice had increased mucus penetrability and a decreased amount of MUC2 in colon mucus compared to conventional mice but can develop impenetrable mucus and normalized MUC2 after the colonization of conventional cecal flora, demonstrating the contribution of intestinal microbes to the development and glycosylation pattern of mucin.

The carbohydrate modification of mucin domain determines the selection and adhesion of colonized bacteria partially *via* single carbohydrate epitope–protein interactions. As described earlier, bacteria express a layer of extracellular glycocalyx, consisting of adhesive structures named adhesins, whose interaction with host cells are mediated by either protein or glycan (45, 47, 107). The colonization of *Escherichia coli* (*E. coli*) is mediated by its P pili (Figure 2B) containing a terminal PapG unit that specifically binds with a digalactoside receptor determinant on gastrointestinal and urinary tract epithelial cells (10, 11, 47). Another study revealed that *E. coli* and *Salmonella typhimurium* bind to glycoprotein 2 on the epithelial M cells *via* bacterial FimH on their pilis (12, 104), and *Vibrio parahaemolyticus* targets the sulfated and fucosylated glycans on the surface of epithelial cells (13, 104).

The virulence of intestinal bacteria is also affected by the glycocalyx structure (Figure 2B). *Vibrio cholera* produces cholera toxin which binds to GM1 ganglioside, a type of glycolipid molecule expressed on epithelial cells and which conducts downstream signals to cause watery diarrhea. Galactose and sialic acid compose the sugar moieties of GM1 and might be responsible for the binding with cholera toxin (104, 108). Another study reported that L-fucose released from epithelial cells could inhibit the expression of virulence genes of enterohemorrhagic *E. coli* through a chemical sensing system of the bacteria called FusKR (14, 104).

Carbohydrate fermentation and absorption occurs in the gut, and the decomposition of dietary fibers rely on symbionts residing in the gut (Figure 2B). Apart from dietary starch, protein, and fiber (109) in the small intestine, some bacteria species, *e.g.*, *Bacteroides thetaiotaomicron*, are able to express enzymes to sensor, transport, and degrade glycosyls from the host into short fatty acids (SCFAs) and acquire energy and structure material (45, 110). SCFAs serve as not only energy substrates but also immunomodulators *via* binding to G-protein-coupled receptors (GPCRs) that can induce DC tolerogenic phenotype, promote Treg differentiation, facilitate M2 macrophage conversion, and protect gut homeostasis. Upon stimulation of SCFAs, GPCRs would induce the production of peptide YY, modulating intestinal motility and nutrient absorption (111, 112). Different bacteria have different survival status on O-glycan dense mucus.

In addition, maternal milk contains a thousand types of fucosylated components and a large amount of oligosaccharides (human milk oligosaccharides) which can inhibit the binding of *Campylobacter jejuni* (*C. jejuni*) with fucosyl and prevent diseases associated with it. Both *in vitro* experiment of human epithelial cells and a mouse model infected *in vivo* with *C. jejuni* have proved that 2′-fucosyllactose (2′-FL), the dominant glycosyls in milk, inhibited the colonization, resisted the infection of *C. jejuni*, and mitigated the intestinal mucosa immune response towards *C. jejuni* (113, 114). 2′-FL can also be utilized by other species of bacteria like *Bacteroides* as substrates to produce fucose, lactose, and SCFAs (115).

Gut microbiota form the glycosylation environment in the intestine

In turn, some intestinal symbiotic bacteria can affect the glucose metabolism and glycosylation status in the host intestinal tract. A germ-free mouse showed defective epithelial α (1, 2)-fucosylation which recovered after commensal bacteria cultivation, implicating that the initiation of epithelial fucosylation partially relies on commensal bacteria (104, 116, 117) (Figure 2B). The frequency of fucosylated epithelial cells (F-Ecs) in different locations along the length of the small intestine was found to be positively correlated with the population of microorganisms, and the dependence of fucosylation on bacteria was further verified in both antibiotic-treated mice and GF mice such that a dramatic reduction in F-Ecs frequency and *Fut2* expression was exhibited, which can be restored with antibiotic cessation or conventionalized cultivation (118). Additionally, researchers also found that commensal bacteria selectively induce the fucosylation of columnar epithelial cells and goblet cells instead of Paneth cells (118). Commensal bacteria may have unequilibrim potency in inducing fucosylation, such as *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*), *Bacteroides fragilis* (*B. fragilis*), *Citrobacter rodentium*, and segmented filamentous bacteria, while *Lactobacillus murinus*, *Bifidobacterium*, and *Peptostreptococcus* cannot initiate fucosylation (104, 117, 119, 120). However, once *Bacteroides* are deficient in fucose metabolic pathway, they lose the function of inducing epithelial fucosylation (104). Apart from commensal

bacteria, some pathogenetic bacteria including *S. typhimurium* and *Helicobacter bilis* have also been reported to induce epithelial Fut2 expression and fucosylation (104, 121).

On the flip side, *B. thetaiotaomicron* and *B. fragilis* can also express fucosidase and cut off the terminal and subterminal fucosylation on epithelial cells for self-utilization. This may help to explain why *B. fragilis* has advantages in colonization compared with mutation strains with defects in fucosyl-utilizing enzymes (104, 122).

The interaction of glucose metabolism and intestinal bacteria contributes to host immune response and autoimmunity

Intestinal bacteria impact host immune response *via* modifying the glucose metabolism

The human intestinal microbiota is made up of five dominant bacterial phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia (111). Changes of the intestinal ecosystem have been reported in multiple autoimmune diseases, and the shifted gut microbiota architecture is suggested to be involved in skewing host immune responses through various mechanisms, which have been summarized elsewhere previously (26, 123). Table 2 shows a summary of the changes in gut micobiota composition in patients with different autoimmune diseases. Specifically, reduced microbial diversity and decreased *Firmicutes/Bacteroides* ratio were found in T1D patients (143), and the gut microbiota composition and the associated metabolites in adult patients with latent autoimmune diabetes (LADA) closely correlated with circulatory autoantibodies, glucose metabolism, islet function, and inflammatory factors (144). PSA from *B. fragilis* can reduce IL-17 expression and upregulate IL-10-producing inducible Tregs. *B. fragilis* colonization was proved to be negatively associated with LPS-induced inflammatory cytokines and chemokines, thus suppressing inflammatory risks (145, 146). An intraperitoneal injection of the bacterial cell wall from certain bacterial species including *Bifidobacterium*, *Streptococcus*, *Lactobacillus*, *etc.*, induced arthritis in susceptible strains of rats, and researchers found that the peptidoglycan component in the bacteria determined their proinflammatory property (147).

Some pathogenetic bacteria in the intestine might stimulate host autoimmune responses *via* molecular mimicry, which may also associate with the glucosyl in bacterial peptide as glucosyl structure and density affect the configuration and even the antigenicity of glycoconjugates. GM1- and GD1a-like LPS on *C. jejuni* surface might be responsible for the generation of anti-GM1b antibodies in Guillain–Barré syndrome patients (148). *Streptococcus agalactiae* can produce sialic acid-capped structures mimicking host glycosylation patterns and bind with sialic acid recognizing Ig-superfamily lectins on DCs, macrophages, and NK cells (51, 149, 150). Group B *Streptococci* can present sialylated glycans on the cell wall as well and prevent attacks from the complement system (150). Studies also proved that the peptides homologous with human RNA binding autoantigen Ro60 and β2-glycoprotein I from *B. thetaiotaomicron* and *Roseburia intestinalis* could provoke autoimmunity and lupus-like manifestations in GF mice models (26, 151).

Goto et al. (118) pointed out that, besides the microbiota–epithelia crosstalk, the stimulation of $CD90^+ROR\gamma t^+$ILC3 cells is also essential in the initiation of EC fucosylation. By producing IL-22 and regulating epithelial *Fut2* expression, $CD90^+$ILC3 induces epithelial fucosylation and creates a protective barrier against infection in a commensal-bacteria-dependent and commensal-bacteria-independent manner, respectively (118). *Fut2* or α (1, 2)-fucosedeficient mice were found to have an increased risk of *Salmonella typhimurium* infection and vaginal *Candida albicans* (104, 152).

In addition, the mucin barrier of the gastrointestinal epithelium might be disrupted under a certain pathological state like IBD, which brings the collapse of its barrier function and promotes bacterial adherence (35, 36). The disruption was found to be related with both host genetic susceptibility and environmental etiologies (37, 38).

Disturbed glucose metabolism and glycosylation in autoimmune diseases

There is a broad spectrum of autoimmune diseases but with common features of chronic excessive inflammation, necessitating extra energy and biomaterial supply. Typically, upregulation of GLU1 and increased glucose uptake have been recognized in RA, SLE, and psoriasis. The interference of glycolysis with GLU1 knockout or inhibitor displayed a therapeutic potential and improved the autoimmune phenotype in animal models (153).

In RA patients, $CD4^+$ T cells transfer glucose to the pentose phosphate pathway to meet its energy and biosynthesis demand for rapid proliferation (154). In hypoxic synovial cavity, a metabolic shift to glycolysis instead of oxygen-consuming mitochondrial OXPHOS is required for synovium hyperplasia and invasive activity (153). Studies have also found that infiltrating lymphocytes in the synovium of RA mice model expressed increased HK2, the rate-limiting enzyme in glycolysis (155). Using an HK inhibitor, 2-deoxidation-D-glucose (2-DG), can obviously reduce the proportion of Tfh and Th17 cells (156). The galectin-1 expression of RA patients was found to be correlated with the regulation of cell apoptosis, and a galectin-

TABLE 2 Changes in gut microbiota composition in patients with different autoimmune diseases.

Autoimmune diseases	Increased prevalence	Decreased prevalence	Reference
Rheumatoid arthritis	Eggerthella, Faecalibacterium prausnitzii, Firmicutes, Lactobacillaceae, Bacteroides, Clostridium asparagiforme, Lactobacillus, Holdemania filiformis, Bifidobacterium dentium, Coprobacillus, Eggerthella, Gordonibacter pamelaeae, Ruminococcus lactaris	Bacteroidetes, Bifidobacterium, Clostridium coccoides, Eubacterium, Klebsiella, Haemophilus, Veillonella, Coprococcus, Dialister invisus, Sutterella wadsworthensis, Megamonas hypermegale, Lactobacillus sanfranciscensis	(27, 124–126)
Systemic lupus erythematosus	Eggerthella, Rhodococcus, Klebsiella, Eubacterium, Lachnospiraceae, Genera Rhodococcus, Prevotella, Flavonifractor, ATCC BAA-442, Atopobium rimae, Shuttleworthia satelles, Actinomyces massiliensis, Bacteroides fragilis, Clostridium leptum	Christensenellaceae, Dialister, Odoribacteraceae, Firmicutes, Lactobacillaceaeb, Rikenellaceae, genera Eubacterium, Dialister, Pseudobutyrivibrio	(24, 127–131)
Sjogren syndrome	Bacteroidetes, Firmicutes	Bifidobacterium, Leptotrichia, Escherichia, Shigella, Enterobacter, Fusobacterium	(131–134)
Ankylosing spondylitis	Megamonas, Dorea, Blautia, Clostridiales bacterium, Clostridium bolteae, Clostridium hathewayi, Prevotella copri, Dialister invisus	Lachnospira, Ruminococcus, Clostridium_XlVb, Bifidobacterium adolescentis, Coprococcus, Lachnospiraceae, Roseburia inulinivorans	(135, 136)
Multiple sclerosis	Genus Streptococcus, genera Akkermansia, Clostridium, Blautia, Dorea, Adlercreutzia, genera Oscillibacter, Ruminiclostridium, Anaerostipes, Erysipelatoclostridium, Blautia, Collinsella, Anaerofilum, Flavonifractor, Dorea, Akkermansia, Marvinbryantia	Genus Prevotella, Slackia, genera Bacteroides, Parabacteroides, Butyricimonas, Romboutsia	(137–139)
Type 1 diabetes	Clostridium, Veillonella, Bacteriodetes, Firmicutes, Genera Bacteroides, Veillonella, Alistipes	Lactobacillus, Bifidobacterium, Blautia coccoides/ Eubacterium rectale, Prevotella, genera Prevotella, Lactobacillus, Lactococcus, Bifidobacterium, Streptococcus, Akkermansia, Faecalibacterium, Subdoligranulum	(140–142)

1 injection can help relieve the clinical and histopathological manifestations (157). Another study also revealed the increased galectin-3 levels in the sera and synovial fluids of RA patients (72). From the fucosylation perspective, the expression of Futs catalyzing terminal and subterminal fucosylation was found to be significantly increased in synovial tissues, predominantly in M1 macrophages, which indicated that terminal fucosylation might be a novel hallmark of inflammatory macrophages (158). The antibody glycosylation condition was also altered in RA patients. Parekh et al. (159) found a lower rate of IgG containing galactose residues in RA and OA patients compared with healthy controls.

A similar aberrant glycosylation phenomenon was found in SLE, the prototype of autoimmune disease. Vučković et al. found decreased galactosylation and sialylation of IgG in SLE patients, along with a decreased core fucosylation level and an increased GlcNAc level (160). These alternations lead to a decreased immunosuppressive activity of immunoglobulins and are associated with disease severity (160).

Patients with SLE are prone to present insulin resistance, abnormal glucose tolerance, and diabetes (161), which might be attributed to the metabolic abnormalized effect of inflammatory factors and the functional changes of adipose tissue in SLE patients (162, 163). $CD4^+$T cells in lupus patients and lupus-susceptible mice depend on enhanced glycolysis for rapid ATP production to meet their glucose requirements (154). The leukocyte recruitment pathway is mediated by selectin, the level of which is higher in SLE patients' plasma, indicating that it may contribute to endothelial cell activation and the proinflammatory status in SLE patients. Higher levels of PSGL-1 with enhanced fucosylation on Treg cells in SLE downregulate the TGFβ pathway and inhibit the suppressive activity of Treg cells, which may facilitate self-tolerance breakdown (164).

MS patients, perceived to result from the autoimmune effect of T cell in damaging the myelin sheath, were found to have an elevated level of blood pyruvate in both fasting and postprandial times (165). By treating primary cultures of oligodendrocyte progenitor cells from rat cerebrum with MS-diseased cerebrospinal fluid, Mathur et al. revealed a decreased gene expression related with carbohydrate metabolism, glucose metabolism impairment, and reduced ATP availability for cellular damage repair (166, 167).

Potential mechanisms underlying the interaction of glucose metabolism and intestinal microbiota changes in autoimmune diseases

Several potential mechanisms have been raised to explain how the interaction between glucose metabolism and intestinal microbiota affects autoimmune diseases. According to the preceding discussion, prior to microbiota colonization, aberrant glucose metabolism and glucosylation condition might occur in patients with autoimmune diseases. On the one hand, this abnormality can cause potential damage to the intestinal mucosa, thus influencing the interaction between gut microbiota and host. On the other hand, aberrant glucose metabolism and glucosylation would also interfere with the function of the host immune system. Genetic factors that

participate in the expression of glucose metabolism-related enzymes, like Fut, or intrinsic carbohydrate metabolism disturbance, like mucin disruption in IBD, would lead to the defective formation of the gut protective barrier, which, in turn, alters the gut ecosystem through influences on adhesion, virulence, and metabolism. An abnormal glycosylation level of antibodies either downregulates or upregulates immune responses to certain types of microbes and might damage organs and lead to disease development. *Fut2* might be an important target gene in IBD. By constructing *Fut2*△IEC mice (*Fut2* knockout mice), Tang et al. found that the intestinal epithelium-specific *Fut2* deficiency increases the susceptibility to IBD by regulating the intestinal microbiota and the production of lysolecithin (168). After certain types of microbiota have interacted with the host–microbe interface and colonized, the PAMPs like PSA would be recognized by PRRs and mediate the immune responses. The process of glycosylation might be initiated, thus affecting the intestinal mucosa condition. The microbial metabolism in the gut might produce metabolites such as SCFAs, influencing the immune environment and either improving or exacerbating the disease manifestations (169).

A study revealed that the rapid fucosylation of intestinal epithelium sustained host–commensal symbiosis in sickness. Activation of the TLR signaling pathway leads to the rapid α (1, 2)-fucosylation of mouse IEC. The fucosylated proteins enter the intestinal lumen, and the fucose is then utilized by the intestinal microbiota, which affects bacterial metabolic pathways and decreases the expression of virulence genes (120).

Prospects of interventions targeting glucose metabolism, glycosylation, and microbiota in autoimmune diseases

Up to now, there are several studies about the treatment of autoimmune diseases targeting different underlying mechanisms, including glucose metabolism, glycosylation, microbiota, *etc.* They might have already shown potential therapeutic effects or indicated future research interests (Table 3).

Anti-adhesion drugs

As previously mentioned, the interaction between bacteria (*C. jejuni*) and fucosylation prompts a bacterial infection, and 2′-FL inhibits this adhesion. In a mouse infection model, feeding 2′-FL orally has been proven to lead to a strong protection of the intestinal mucosa against infections (113). This result might indicate the possibility of a novel kind of 2′-FL-derived drug which can be used orally and might be effective in IBD patients.

Fucosylation inhibitors

Studies have revealed the positive therapeutic effects of small molecules of fucosylation inhibitors. Fucosylation inhibitors can be categorized as competitive inhibitors and metabolic inhibitors; the former blocks the interaction between fucosyl and selectin, while the latter reduces the process of fucosylation (6). Focustatin II, designed as an analog of fucose-1-phosphonate, competitively reduces the fucosylation of therapeutic monoclonal antibodies and improves the efficacy without combination with antibodies (183). The treatment of 2-fluorofocus on a sickle cell disease mice model showed a significant decrease in the adhesion of leukocytes, neutrophils, and sickle red blood cells with the endothelium (170). Other competitive inhibitors include hyperacetylated 6-alkynyl-fucose, hyperacetylated 5-thiofucose (5T-Fuc), *etc.* 5T-Fuc can also play an important role in metabolic inhibition (6).

Glycoregulation therapy with 2-DG and metformin changes the T cell surface glycosylation patterns and enables immune tolerance induction as evidenced by the reduced anti-dsDNA antibody titers and the decreased renal deposition of immune

TABLE 3 Potential glucose metabolism, glycosylation, and microbiota targeting therapeutic strategies.

Interventions	Theory	Indication	Reference
Anti-adhesion drugs	Inhibit the interaction between bacteria (*C. jejuni*) and fucosylation Protection of intestinal mucosa against infection	Inflammatory bowel disease	(113)
Fucosylation inhibitors	Competitive inhibitors: block the interaction between fucosyl and selectin Metabolic inhibitors: reduce the process of fucosylation	Sickle cell disease mice model	(6, 170)
Glycoengineering	Glycan modification of IgG Fc domains modulates divergent functions	Arthritis and tumor	(150, 171, 172)
Probiotics, prebiotics, and diet regulation	Probiotics: a group of bacteria that can be beneficial for human immune function, nutrition condition, intestinal microbiota constitution, *etc.*	Rheumatoid arthritis	(173–182)
	Prebiotics: indigestible food ingredients that improve gut microbiota structure by providing carbon sources and metabolic substrates and by adjusting the gut microenvironment	Resisting bacterial infection	
	Diet regulation: a helpful and safe intervention to guide the establishment of healthy gut microbiome	Lupus-prone mice, multiple sclerosis	

complex as well as the restored tolerance to allografts in lupus-prone mice, probably *via* targeting glycolysis and oxidative phosphorylation (184).

Glycoengineering

Inspired by the mechanism that glycan modification of IgG Fc domains modulates divergent functions, scientists have come up with the idea of glycoengineering therapy. Pagan et al. increased the sialylation level of IgG *in vivo* in mice models through the administration of engineered glycosyltransferases and found that it successfully exerted an anti-inflammatory effect and was effective at relieving arthritis (171, 172). Furthermore, activated platelets were found to be significant ingredient donors of *in vivo* sialylation, and coordinately giving clopidogrel or other platelet inhibitor drugs would inhibit the anti-inflammatory effect of this glycoengineering therapy though the platelets themselves have pro-inflammatory effects (171, 185, 186). Glycoengineering has also attracted interest in the field of tumor as the mechanism of immune evasion of tumor cells also involves the sialylation of related proteins (150).

Probiotics, prebiotics, and diet regulation

Probiotics are a group of bacteria that can be beneficial for human immune function, nutritional condition, intestinal microbiota constitution, *etc.* Prebiotics are indigestible food ingredients that improve the gut microbiota structure by providing carbon sources, making metabolic substrates available for the gut microbiota to utilize, and adjusting the gut microenvironment, such as in terms of pH value, gastrointestinal absorption, and enzyme hydrolysis (173, 174). Fructo-oligosaccharide (FOS) is a well-studied effective prebiotic consisting of one glucose and one fructose, and it has shown multiple beneficial effects in several animal models including the following: (a) resisting bacterial infection such as that of enterotoxigenic *E. coli* (176), (b) promoting the colonization of probiotics like *Bifidobacterium* and *Lactobacillus* (177–180), (c) increasing the production of SCFAs by the use of these probiotics (180), and (d) downregulating the pro-inflammatory cytokine expression and protecting the mucosal barrier (181). Besides FOS, inulin, galacto-oligosaccharides, and some foods containing indigestible β-glucans such as termites, a kind of Chinese traditional medicine, also have a similar effect (174).

As has been proved that chemically synthesized oligosaccharides with core fucosylation have the function of promoting *Bifidobacterium* and *Lactobacillus* growth, it is put forward that the core fucosylation in maternal milk is a promising prebiotic for infants (102). Gestational diabetes mellitus during maternity results in higher fucosylation and sialylation levels in milk that affects the immune function of infants, which indicates the importance of guidance in beneficial nutrition and healthcare of gestating women (187).

Furthermore, diet regulation has been raised as a helpful and safe intervention to guide the establishment of a healthy gut microbiome and restore beneficial metabolism. Resistant starch or rich-fiber diet has shown many benefits in lessening the inflammation status and improving metabolic aberrance by retarding glucose absorption, strengthening the gut barrier, flourishing beneficial commensals, and reducing cytokine release. Kearney et al. (188) reported an increase of relative abundance of *Bacteroides plebeius* by more than two orders of magnitude in mice fed with a diet containing 1% raw seaweed. Dietary resistant starch is proven to be beneficial to lupus-prone hosts by suppressing the abundance and translocation of *Lactobacillus reuteri* which drives autoimmunity (182). Studies also report that the Mediterranean-style diet, characterized by rich unsaturated fatty acids and fibers, has anti-inflammatory effects and produces a beneficial effect to relieve the symptoms of MS patients (189, 190).

Other therapy strategies

Though carbohydrates play a vital role in physiological and pathological events, they can be easily eliminated by the digestive and urinary systems; therefore, the development of drugs directly derived from carbohydrates was limited. Some carbohydrate-derived drugs, like sodium hyaluronate (decreasing the risks of infection and pain in arthroscopic debridement) (191) and sulfated glycosaminoglycans (binding to antithrombin and promoting anticoagulation in thrombosis) (40, 192), have been indicated as beneficial for osteoarthritis. Researchers also attempted to deliver carbohydrates orally or by injection and observe their pharmacokinetics and effects. Pozharitskaya et al. intragastrically administrated fucoidan to rats and found that it accumulated firstly in the kidney, spleen, and liver and had a relatively long elimination time from circulation, thus revealing the possibility of fucoidan-derived drugs (193). Wu et al. (194) treated induced obese mice with fucose by intragastric administration and found decreased body weight gain, fat accumulation, and hepatic triglyceride elevation in mice.

In addition, several novel oral heptadecaglycoside antibiotics have been studied and developed. Saccharomicins A and B, isolated from *Saccharothrix espanaensis*, showed inhibition effects to pathogenetic bacteria growth both *in vivo* and *in vitro* (195). The microscopic observations showed that the

treatment of these drugs could inhibit the synthesis of DNA, RNA, and proteins in bacteria (195, 196).

In conclusion, the mutual influence of glucose metabolism, glycosylation, and intestinal microbes is complicated, and their contributions to autoimmune diseases are largely unclarified. A well-defined integrated network can improve our substantial understanding about the dynamic change of immunity and metabolism under the era of microbiota. From the perspective of the synergistic effect of glucose metabolism, glycosylation, and intestinal microbiota, new inspirations and intervention strategy may be developed for autoimmune diseases.

Author contributions

All authors were involved in drafting this paper or revising it critically for key intellectual content. JZ and LZ had full access to all the data in the article. Conception: LZ and JZ; methodology: LZ, HX, and LW; interpretation and writing: LW, HX, HY, and LZ; and supervision: LZ and JZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Alterations of m6A RNA methylation regulators contribute to autophagy and immune infiltration in primary Sjögren's syndrome

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N6-methyladenosine (m6A) RNA modification is a new epigenetic regulation mechanism on eukaryotic mRNA. Few autoimmune diseases focused on the role of m6A in their pathogenies, and m6A modulation in the pathological process of primary Sjögren's syndrome (pSS) is still unknown. In this work, three microarray datasets of pSS patients were downloaded from the GEO database: datasets #1 and #2 from the whole peripheral blood (PB) samples, dataset #3 from the labial salivary gland tissue samples, as well as a PB cohort collected from our hospital. Six differentially expressed m6A regulators were identified by comparing the PB dataset #1 of pSS and healthy controls using the Wilcox test and logistic regression analysis. Among them, four (ALKBH5, RBMX, RBM15B, and YTHDF1) were confirmed as down-regulated in PB dataset #2 and in our PB cohort by RT-PCR, and four (ALKBH5, METTL3, RBM15B, and YTHDF1) were confirmed as down-regulated in the dataset #3 of the labial gland tissue. In addition, discrepantly expressed m6A regulators accompanied by diverse immunocytes, including dendritic cells (DCs), T cells, and CD56dim natural killer cells, and among the regulators, ALKBH5 and METTL3 were comprehensively linked with the infiltrated immune cells. Notably, the most enriched autophagy mechanism mediated by m6A was observed in pSS using functional annotation analysis. Ten hub genes were identified using a protein-protein interaction network, and their expression in PB dataset #2 and the expression of three genes (PIK3CA, STAT1, and MAPK3) in the labial gland tissue dataset #3 were confirmed. Our study provides evidence that m6A methylation is widely involved in the immune infiltration and autophagy of pSS, thus contributing to the pathogenesis of this disease and potentially representing a novel therapeutic target.

KEYWORDS

primary Sjögren's syndrome, m6A, autophagy, immune microenvironment, GEO

Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease that mainly affects the exocrine glands, and it is characterized by dryness of the eyes and mouth (1). Genetic and environmental factors may contribute to its occurrence and development (2). Activated epithelial cells of the salivary gland represent the initiating factor that leads to the immune and inflammatory disorders in pSS, immunocytes infiltration, as well as amplified production of autoantibodies and interferon alpha, consequently leading to tissue damage (2). Thus, pSS is also defined as "autoimmune epithelitis" (3). Nonetheless, the pathological mechanisms regulating pSS remain unclear.

N6-methyladenosine (m6A) RNA modification has emerged in recent years as a new epigenetic regulatory mechanism influencing mRNA stability, translation, translocation, and control of gene expression in eukaryotes. It is a reversible process driven by three groups of enzymes called "writers" (methyltransferases including METTL3, METTL14, METTL16, and WTAP), "erasers" (demethylases including FTO and ALKBH5), and "readers" (m6A-binding proteins, including YTHDC1, YTHDC2, YTHDF1, YTHDF2, FMR1, and RBMX) (4). m6A plays important roles in almost all vital biological processes, including the inflammatory and antitumor immune response, antiviral immunity, and T-cell homeostasis, therefore affecting both health and diseases (5, 6). Some studies demonstrated the key role of m6A regulators in tumor initiation, progression, and metastasis by disturbing the balance between apoptosis, differentiation, and proliferation (7), or by regulating glycolytic metabolism to evade immune surveillance (8), or controlling the interaction between cancer cells and microenvironment to promote metastasis (9).

In recent years, m6A modification has attracted more and more attention in a wide range of non-cancer areas. Nie et al. found that m6A regulators interact with risk genes of inflammatory bowel diseases (10). Zhang et al. reported that m6A modification plays a crucial role in the abundance of monocytes and multiple immune reactions involving TNF family member receptors and cytokines in patients with periodontitis (11). Li et al. demonstrated that m6A regulators are related to the degree of immune infiltration of central memory T cells, macrophages, mast cells, gamma delta T cells, and NK CD56 bright cells in patients with abdominal aortic aneurysms. To be specific, m6A-modified genes are involved in body metabolism and autophagy (12). All this weight of evidence implies that aberrant m6A modification may underlie the autoimmunity and dysfunction of other important pathological processes through various mechanisms (13, 14). However, few autoimmune diseases focused on the role of m6A in their pathogenesis (15–20), and the mechanism of action of m6A in the pathological process of pSS is still unknown.

Nowadays, the public database has become a promising resource for extracting valuable data. Therefore, this study evaluated several datasets from the GEO database and our collected cohort covering the peripheral blood (PB) and salivary gland samples to analyze the expression pattern of m6A regulators in pSS. The aim of this study was to uncover the key m6A regulators by bioinformatic tools, acting as diagnostic biomarkers and their involvement in vital biological mechanisms for an epigenetic-based therapy in pSS.

Methods

Data collection

Three datasets (dataset #1: GSE51092; dataset #2: GSE84844; dataset #3: GSE40568) were downloaded from gene expression omnibus (GEO) using the search words "((Sjögren's syndrome) OR (Sjogren syndrome)) AND microarray expression data AND Homo sapiens". Dataset #1 was the discovery set composed of 222 whole PB samples from 190 pSS and 32 healthy controls (HCs) samples; the sequencing for the expression profile was performed on the Human WG-6 BeadChip microarray (Illumina, San Diego). Dataset #2 was composed of 60 PB samples from 30 pSS and 30 HCs; the sequencing for the expression profile was performed on the Affymetrix Human Genome U133 plus 2.0 Array (Affymetrix, Santa Clara, California, USA). Dataset #3 was composed of 8 samples from labial salivary glands biopsy of 5 pSS and 3 HCs; the sequencing for the expression profile was performed on the Affymetrix GeneChip Human Genome U133 plus 2.0 Array (Affymetrix, Santa Clara, California, USA). The gene matrix of each dataset was normalized by the "normalizeBetweenArrays" function in the "limma" package. The gene list of m6A regulators was obtained from previous studies (4, 11, 21).

m6A signature in pSS

Protein-protein interaction (PPI) network of 26 m6A regulators was constructed using STRING (https://cn.string-db.org/). The correlation matrix analysis was performed to visualize the association between m6A regulators, and rectangles of clustered m6A regulators on the matrix were drawn based on the hierarchical cluster (hclust). The correlation analysis of m6A "writers" and "erasers" with the significant spearman correlation $r > 0.3$ ($p < 0.05$) was presented.

Differential expression of m6A regulators

The expression of m6A regulators between pSS and HCs samples from dataset #1, and between groups of pSS patients

with high and low anti-Ro/SSA antibody levels (high: higher than the median level, low: lower than the median level) in dataset #2 was compared using the Wilcox test. A volcano plot was drawn to visualize the different profiles in the expression in m6A regulators (DEMRs). The heatmap was constructed to show the expression of DEMRs in pSS and HCs. The univariate logistic regression was performed to identify the m6A regulators related to pSS, and multivariate logistic regression analysis was performed to construct the classifier for pSS from HCs by DEMRs. The receiver operating characteristic (ROC) curve analysis was used to evaluate the distinguishing performance of the m6A regulator panel. The identified DEMRs from the dataset #1 were validated in the dataset #2 (PB samples), dataset #3 (samples of labial salivary glands biopsy) and our PB cohort.

Construction of the nomogram

A nomogram was constructed using the "rms" R package to quantify the predicted risk for pSS based on the results of the logistic regression analysis through a simple visualization figure (22). The calibration and decision curve analyses (DCA) were used to determine the performance of the nomogram. The calibration curves were graphically assessed with a bootstrap of 1000 samples by mapping the nomogram-predicted probabilities against the observed rates, and the 45° line represented the best predictive values. The "rmda" package was used for a net benefit-dependent DCA, which is a novel method for assessing clinical predictive models by examining the range of threshold probabilities and the overall therapeutic advantage (23).

Single-sample gene set enrichment analysis of immune characteristics

Single-sample gene-set enrichment analysis (ssgsea) was performed using the "GSVA" R package to assess the enrichment score of 23 specific immunocytes (24) and immune reactions for each sample within a given data set. The gene-set containing 17 types of immune reactions was downloaded from the ImmPort database (http://www.immport.org). The diverse immunocyte abundance and the immune reaction between groups were identified according to the enrichment scores using the Wilcox test. The correlation matrix between DEMRs and immune scores was constructed by Spearman correlation analysis.

Functional annotation analysis of genes mediated by DEMRs

The target genes of DEMRs were predicted by searching them in the m6A2Target database (http://m6a2target.canceromics.org/#/). Spearman correlation analysis was performed to search the co-expressed genes of DEMRs using the criteria $r > 0.3$ and $p < 0.01$. The genes mediated by DEMRs in pSS were defined as the intersection of the predicted target genes and the co-expressed genes. The functional annotation analysis (FAA), including gene ontology (GO) annotation incorporated by biological process (BP), cellular components (CC), and molecular functions (MF), as well as the Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis were performed using the "clusterProfiler" R package to obtain the biological functions and signaling pathways of genes mediated by DEMRs. The criterion to evaluate the significant terms was set as a q value less than 0.05.

Identification of the differentially expressed genes between groups

The differentially expressed genes (DEGs) between pSS and HCs samples from dataset #1, and between groups of pSS patients with high and low anti-Ro/SSA antibody levels in dataset #2 were identified using the lmFit and contrasts.fit functions of the limma package. The empirical eBayes command in the limma package was used to calculate the consensus pooled variance of genes and adjust the associated p-value. Genes with logFC absolute values ≥ log2 (1.2) and p-value <0.05 were defined as DEGs.

Protein-protein interaction network and hub gene of autophagy

Taken together, 1027 unique genes involved in autophagy were obtained by integrating the gene sets from GO_Autophagy, KEGG_ Autophagy, and HAMdb database (http://hamdb.scbdd.com/). The HAMdb database contains 796 genes including 545 genes from the literature and 251 collected from online databases.

The confidence of 0.15 was set as the minimum required interaction score, and the protein-protein interaction (PPI) network of DEMRs and autophagy-related genes was developed using STRING to unveil the m6A modified autophagy genes. In addition, the PPI network of autophagy-related genes and differentially expressed genes in pSS setting confidence > 0.4 was constructed. PPI maps were then visualized using Cytoscape (Version 3.9.1). CytoHubba plugin was applied to extract the top 10 key nodes/hubs ranked by maximum clique centrality (MCC). Molecular Complex Detection (MCODE) plugin was used to detect densely connected regions in large PPI networks (25) by parameters keeping a degree cut-off = 0.2, node score cut-off = 0.2, k-score = 2, and max.Depth = 100, as previously described (26), and the top 3 MCODE clusters were retrieved. The association between the hub genes of autophagy

and DEMRs was analyzed using the spearman correlation analysis and visualized using the chord diagram.

Validation of DEMRs and autophagy hub genes

Dataset #2 and dataset #3 were used to validate the 6 DEMRs and 10 hub genes of autophagy identified in dataset #1. Their expression between pSS and HC PB/tissue samples was compared using the Wilcox test.

Patients and samples

pSS predominantly affects women and has peak incidence at 50 years old (1). Therefore, the whole blood samples were obtained from 17 women with pSS and 17 age-matched female HCs at the Peking Union Medical College Hospital (PUMCH) from March 2022 to April 2022. This study was approved by the Medical Ethics Committee of PUMCH and informed consent was obtained from the enrolled subjects (JS-2049). The clinical characteristics of the subjects are listed in Supplementary Table 1.

RNA isolation and RT-PCR

Total RNA was isolated from the whole blood samples of the enrolled subject at the day of blood collection according to the manufacturer's instructions (Mei5 Biotechnology, Co., Ltd). Then, the RNA samples were preprocessed using gDNA plus remover mix to remove genomic DNA contamination, and then, the RNA was reverse transcribed to cDNA using M5 RT Super plus Mix. HiPerSYBR Premix Estaq (Mei5 Biotechnology, Co., Ltd) was used to perform the real-time PCR on a Roche LightCycler 480 system, and the relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method after the normalization with the endogenous GAPDH mRNA expression. The primer sequences of the 6 DEMRs are listed in Table 1.

Association of m6A regulators and autophagy hub genes with clinical features

The association of DEMRs and autophagy hub genes with clinical features of pSS was found by Spearman analysis based on dataset #2, including age, gender, rheumatoid factor, antinuclear antibodies (ANA), IgG, IgA, IgM, anti-Sjögren's-syndrome-related antigen A autoantibodies (anti-SSA), ant-Sjögren's-syndrome-related antigen B autoantibodies (anti-SSB), and EULAR Sjögren's syndrome disease activity index (ESSDAI), to investigate the clinical value of these genes in the disease activity, immune response, and diagnostics.

Results

Overview of m6A regulator signature in pSS

The gene list of 26 m6A regulators was obtained from previous studies (4, 11, 21) (Figure 1A). The PPI network of the m6A regulators demonstrated their compact interactions and synergistic effect (Figure 1B). Altogether, 17 m6A regulators were detected in dataset #1, including 5 "writers" (METTL3, WTAP, RBM15, RBM15B, and CBLL1), 1 "eraser" (ALKBH5), and 11 "readers" (YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, LRPPRC, IGFBP2, IGFBP3, RBMX, ELAVL1, and IGF2BP1). Furthermore, the correlation matrix of the 17 m6A regulators in pSS revealed the universal correlation and their function as a complex (Figure 1C). The "writer" RBM15B showed a close association with the "eraser" ALKBH5" with a coefficient r = 0.34 ($p < 0.0001$) (Figure 1D).

Identification of pSS-related m6A regulators

The comparison of 17 m6A regulators from PB samples of pSS and HCs was performed. The volcano plot shows that the expression of 6 m6A regulators was different; 4 were up-

TABLE 1 Primer sequences for real-time PCR.

Gene	Forward primer	Reverse primer
METTL3	AAGCTGCACTTCAGACGAAT	GGAATCACCTCCGACACTC
RBM15B	GGGAGCATTCGGACCATTGA	CTCATTTTAGCACAGGCGGC
YTHDC2	GGTATCCCCTGCCGTATATTTTG	CTTTCCCGTCTCTCTGCGG
YTHDF1	ATACCTCACCACCTACGGACA	GTGCTGATAGATGTTGTTCCCC
RBMX	TGGAAGCAGTCGCTATGATG	GAGGGTACCCCCTTTCCATA
ALKBH5	CCCGAGGGCTTCGTCAACA	CGACACCCGAATAGGCTTGA
GAPDH	CCTCAAGATCATCAGCAAT	CCATCCACAGTCTTCTGGGT

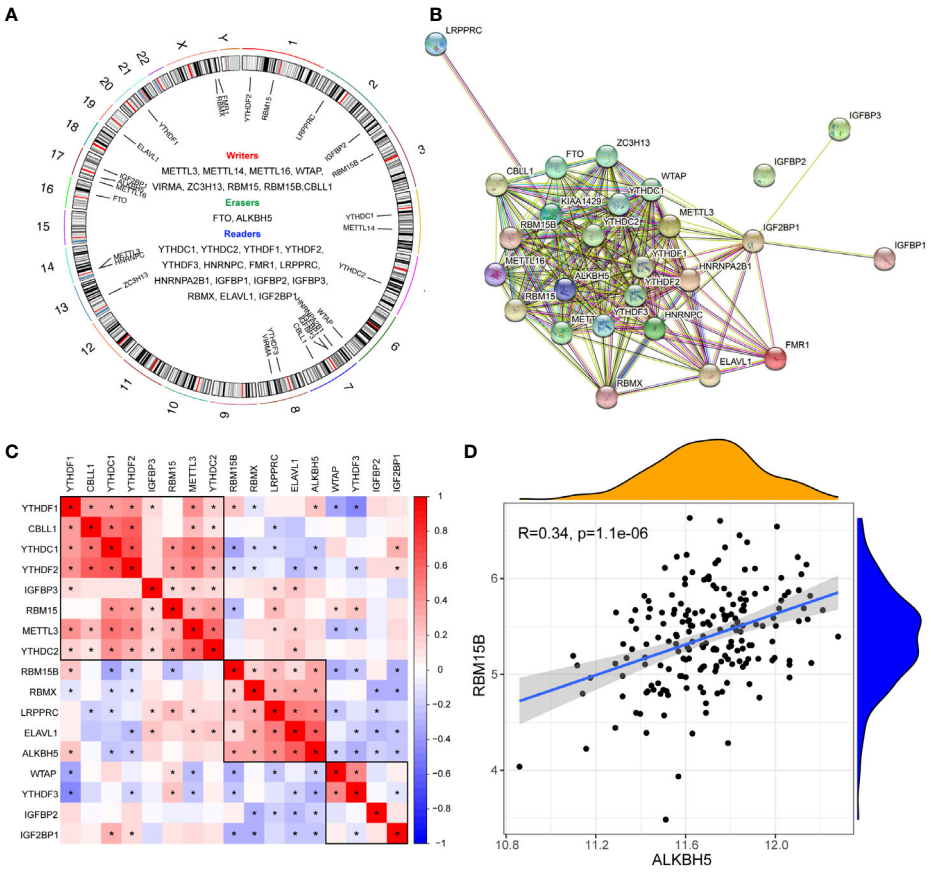

FIGURE 1
Expression landscape of m6A regulators in primary Sjögren's syndrome. **(A)** Circus diagram indicating the location of m6A regulators in the chromosome. **(B)** The protein-protein interaction network of m6A regulators. **(C)** The correlations among the expression of 27 m6A regulators in pSS PB samples. **(D)** The scatter plot demonstrated the correlations between dysregulated m6A "writers" and m6A "erasers" with r>0.3 and p<0.01; *, p<0.05.

regulated (RBM15B, RBMX, ALKBH5, and YTHDF1) and 2 were down-regulated (YTHDC2 and METTL3) in pSS compared to HCs (Figure 2A). The expression pattern of the 6 DEMRs is shown in Figures 2B, C. All DEMRs in validation dataset #2 were validated except RBMX, which was not detected by the Affymetrix Human Genome U133 plus 2.0 Array (Figure 2D). However, METTL3 showed a significantly opposite trend compared with that in the dataset #1.

Univariate logistic regression analysis was performed to investigate the contribution of the 17 detected m6A regulators to the pathogenesis of pSS (Figure 3A). Six m6A regulators were essential for pSS in the univariate logistic regression consistently with the differentially expressed analysis. The ROC analysis indicated that each DEMR discriminated pSS from HC with an AUC > 0.6 (Figure 3B). Multivariate logistic regression analysis was performed to develop a quaternary classifier to distinguish pSS and HCs (Figure 3C). The ROC curve verified that the quaternary panels composed of METTL3, YTHDC2, YTHDF1, and RMBX presented the best performance in distinguishing pSS from HCs (AUC = 0.812), revealing the involvement of m6A regulators in the pathogenesis of pSS (Figure 3D).

Construction of a nomogram based on DEMRs

A nomogram was constructed using 6 DEMRs to provide a quantitative approach for clinicians to predict the risk of pSS (Supplementary Figure 1A). In this nomogram based on the logistic regression model, a scale was used to assign points to the 6 DEMRs. A straight line was drawn upward to determine the points for each DEMR. The points of all the 6 DEMRs were accumulated as the "Total Points". The probability of pSS was determined by drawing a vertical line from the "Total Points" axis straight downwards to the axis of the "Risk of Disease". RBMX and YTHDF1 contributed to the most risk points compared with the other DEMRs, and this result was

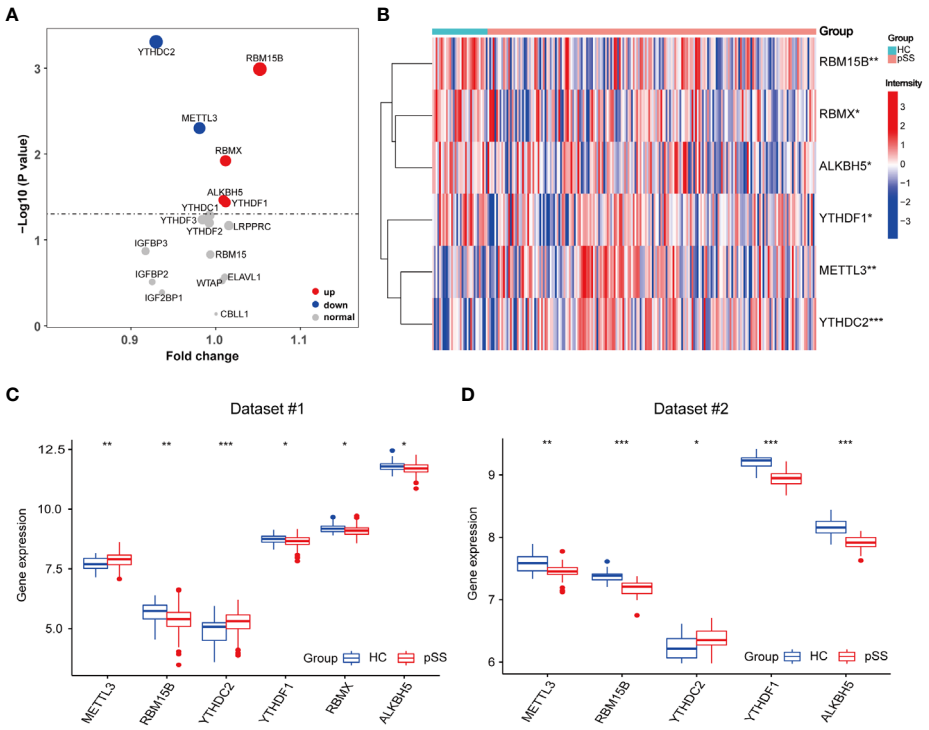

FIGURE 2
Identification of pSS-related m6A regulators. **(A)** The volcano plot illustrates the m6A regulators that are dysregulated between the HCs and pSS groups. Upregulated and downregulated significant genes ($p < 0.05$) are indicated in red and blue dots, respectively. B-D. The heatmap **(B)** and the box plots **(C, D)** demonstrated the transcriptome expression status of the 6 DEMRs between healthy and pSS PB samples in dataset #1 **(B, C)** and dataset #2 **(D)**. *, p<0.05; **, p<0.01; ***, p<0.001.

consistent with our logistic regression results. The bias-corrected line in the calibration chart was close to the ideal curve (the 45-degree line), suggesting a good consistency between the observation and prediction (Supplementary Figure 1B). The DCA showed that the nomogram had a high clinical application value (Supplementary Figure 1C). These findings suggested that the nomogram represented a better tool for predicting pSS in clinically suspected patients.

m6A regulators were associated with immune dysfunction in pSS

Immune dysfunction plays a key role in the pathophysiology of pSS (27–30). The ssgsea analysis and the correlation analysis for DEMRs and immune characteristics were performed, to explore the involvement of m6A regulators in the immune microenvironment. Patients with pSS showed dysregulation of immune cell infiltrate, including dendritic cells (DCs), T cells, and CD56dim natural killer cells (Figure 4A). Decreased fractions of activated DCs and plasmacytoid DCs were found in pSS ($p < 0.01$), in accordance with the findings of Vogelsang et al. (28), which could be due to their migration from the circulatory system to the inflammatory tissues, including salivary glands and secondary lymphoid organs (29). Conversely, type 1, type 2, and type 17 T helper cells increased in pSS (Figure 4A). The correlation analysis revealed that m6A regulators were related to immunocytes, and among them, ALKBH5 and METTL3 showed a comprehensive linkage with the infiltrating immune cells (Figures 4B-D and Supplementary Table 2). METTL3 displayed the strongest negative correlation with the abundance of activated DCs and plasmacytoid DCs (r = -0.6, $p < 0.001$, Figure 4D), consistent with the findings that mRNA methylation mediated by METTL3 promoted DCs activation and T cell stimulation (5). These results indicated the key role of m6A regulation in mediating the immune dysfunction in pSS.

Additionally, the immune reaction score enriched by ssgsea revealed that natural killer cytotoxicity was decreased in pSS (Supplementary Figure 2A), which was consistent with our results of immune infiltration enrichment that showed a decrease in CD56dim natural killer cells in PB of pSS patients (Figure 4A). TCR signaling pathway and TNF family members receptors were also decreased in pSS (Supplementary Figure 2A). It is worth noting that METTL3 still showed the most significant

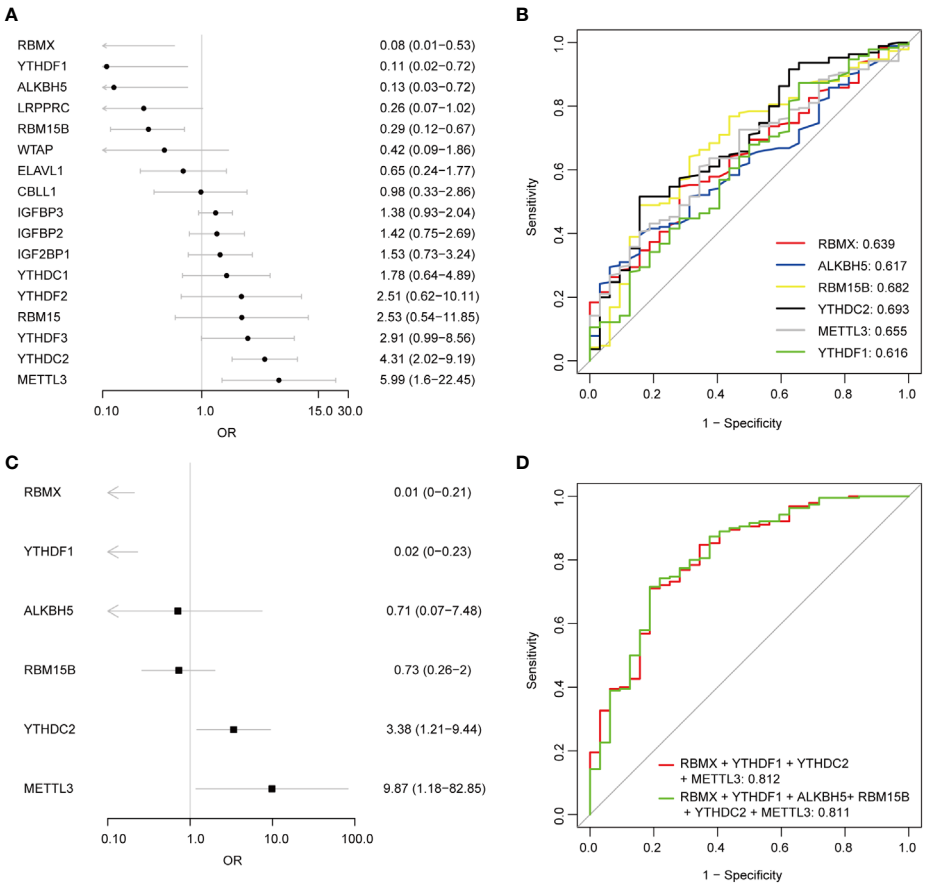

FIGURE 3
Logistic regression models investigating the relationship between m6A regulators and pSS. **(A)** Univariate logistic regression investigated the relationship between m6A regulators and pSS. **(B)** The discrimination ability of the 6 DEMRs for HCs and pSS samples was analyzed by ROC curve and AUC value. **(C)** Distinguishing signature was developed by multivariate logistic regression. **(D)** The discrimination ability of the m6A regulator panels for HCs and pSS samples was analyzed by ROC curve and AUC value.

correlation with the immune reaction gene-sets (Supplementary Figure 2B).

FAA indicated the involvement of autophagy in pSS

The list of the target genes of DEMRs was obtained referring to the m6A2Target dataset. The co-expression analysis was also performed and the intersection of the two gene sets was defined as the genes mediated by DEMRs in our study, including 2071 genes regulated by METTL3, 1130 genes regulated by YTHDF1, 1090 genes regulated by ALKBH5, 293 genes regulated by RBM15B, 107 genes regulated by YTHDC2 and 1 gene regulated by RBMX. FAA was then performed on these genes mediated by DEMRs, and a list of significantly enriched pathways was identified (Figure 5A-C). Overall, the emapplot of GO : BP revealed three main biological mechanisms that included the most enriched terms emerging from the FAA analysis in pSS: RNA splicing, nuclear transport, and autophagy (Figure 5C).

Notably, the FAA results of KEGG and GO converged particularly to autophagy (Figures 5A, B), covering the most enriched KEGG terms including “protein processing in endoplasmic reticulum”, “ubiquitin mediated proteolysis”, “autophagy” and “mitophagy”, (Figure 5A) and GO : BP terms including “proteosome-mediated ubiquitin-dependent protein catabolic process”, “proteasomal protein catabolic process”, “regulation of protein modification by small protein conjugation or removal”, and “macroautophagy” (Figure 5B). The KEGG signaling pathway showed the comprehensive involvement of DEMR-mediated genes and their coaction in autophagy (Figure 5D, Supplementary Figure 3A). The cnetplot shows the gene names involved in terms related to KEGG_Autophagy and GO_Autophagy (Supplementary Figures 3B, C).

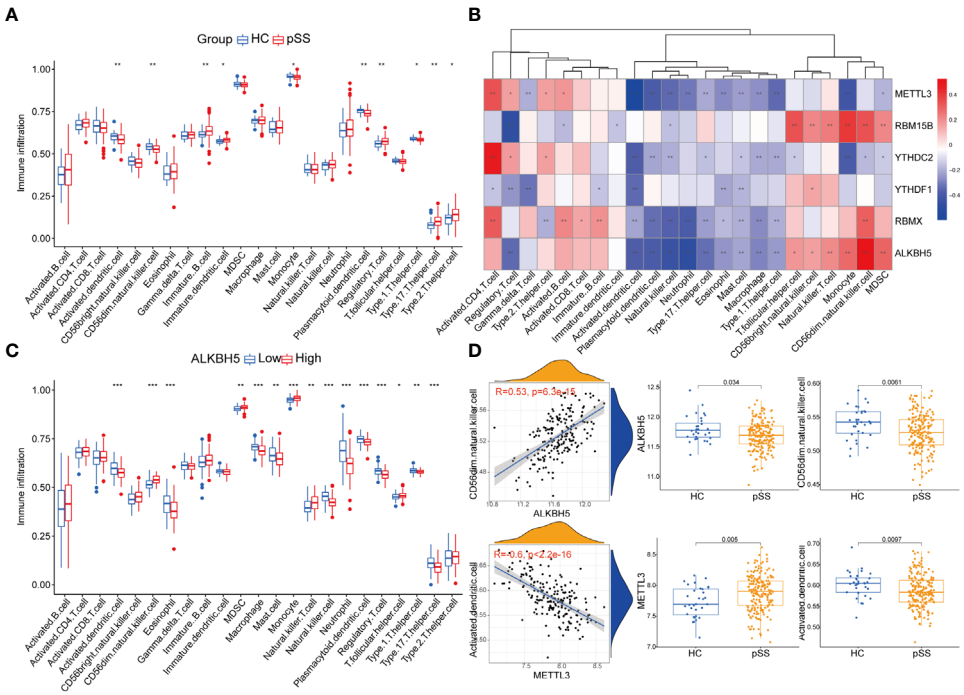

FIGURE 4
Immune microenvironment characteristics in pSS. **(A)** The immune score differences of each immune microenvironment infiltrating immunocyte in HCs and pSS. **(B)** The correlation matrix between the immune score and the 6 DEMRs. **(C)** The correlation between immune score and ALKBH5. **(D)** ALKBH5 and METTL3 showed a comprehensive linkage with the infiltrating immune cells. As for ALKBH5, the most correlated pair is ALKBH5 - CD56dim natural killer cells, the expression differences of ALKBH5 and activated dendritic cells in HCs and pSS is presented by dot plots at the top. As for METTL3, the most correlated pair is METTL3 – activated dendritic cells, the expression differences of METTL3 and activated dendritic cells in HCs and pSS is presented by dot plots at the bottom. *, p<0.05; **, p<0.01; ***, p<0.001.

Construction of the DEMR regulatory network for autophagy in pSS

The Venn plot revealed the intersection of m6A-mediated genes, autophagy-related genes, and the differentially expressed genes (DEGs) in pSS (Figure 6A). Among the 3626 target genes of the 6 DEMRs, 286 were involved in autophagy. Further PPI network analysis showed the interaction among DEMRs and these autophagy-related genes (Figure 6B), revealing the regulatory role of m6A in autophagy. In addition, 211 autophagy-related genes that were dysregulated between pSS and HCs (Figure 6A) were uploaded into the PPI network analysis (Figure S4). The top 10 hub genes filtered by CytoHubba based on the MCC rank were identified (Figure 6C, Supplementary Table 3). The top 3 subnetwork gene clusters (cluster #1 with a score of 8.2; cluster #2 with a score of 2; cluster #3 with a score of 4.118) were exported using MCODE (Figure 6D). All hub genes fell in the three clusters, where MAPK1, MAP2K7, KRAS, PIK3CA, and TSC2 were located in cluster #1 (Figure 6D), MTOR, IL1B, and STAT1 2 were located in cluster #2 (Figure 6E) and GAPDH and MAPK3 2 were located in the cluster #3 (Figure 6F). PIK3CA, STAT1, and MAPK3 were located at the center of cluster #1, #2, and #3, respectively.

The expression of 10 autophagy hub genes in dataset #1 was compared between pSS and HCs (Figure 6G). The expression of MTOR, STAT1, and PIK3CA was up-regulated, while the other hub genes were downregulated. The chord diagram demonstrated the correlation between DEMRs and autophagy hub genes (Figure 6H), and the correlation coefficient r > 0.3 is shown in Supplementary Table 4. These results revealed the key regulation of the autophagy process by m6A in pSS.

Validation of autophagy hub genes and the association with clinical features

Among the top 10 hub genes related to autophagy in pSS, the mRNA expression of STAT1 and PIK3CA in PB was significantly higher in pSS samples than in HC samples, whereas the mRNA expression of MAP2K7, MTOR, GAPDH, MAPK3, MAPK1, and TSC2 was higher in the HCs samples than in the pSS samples (Figure 7A).

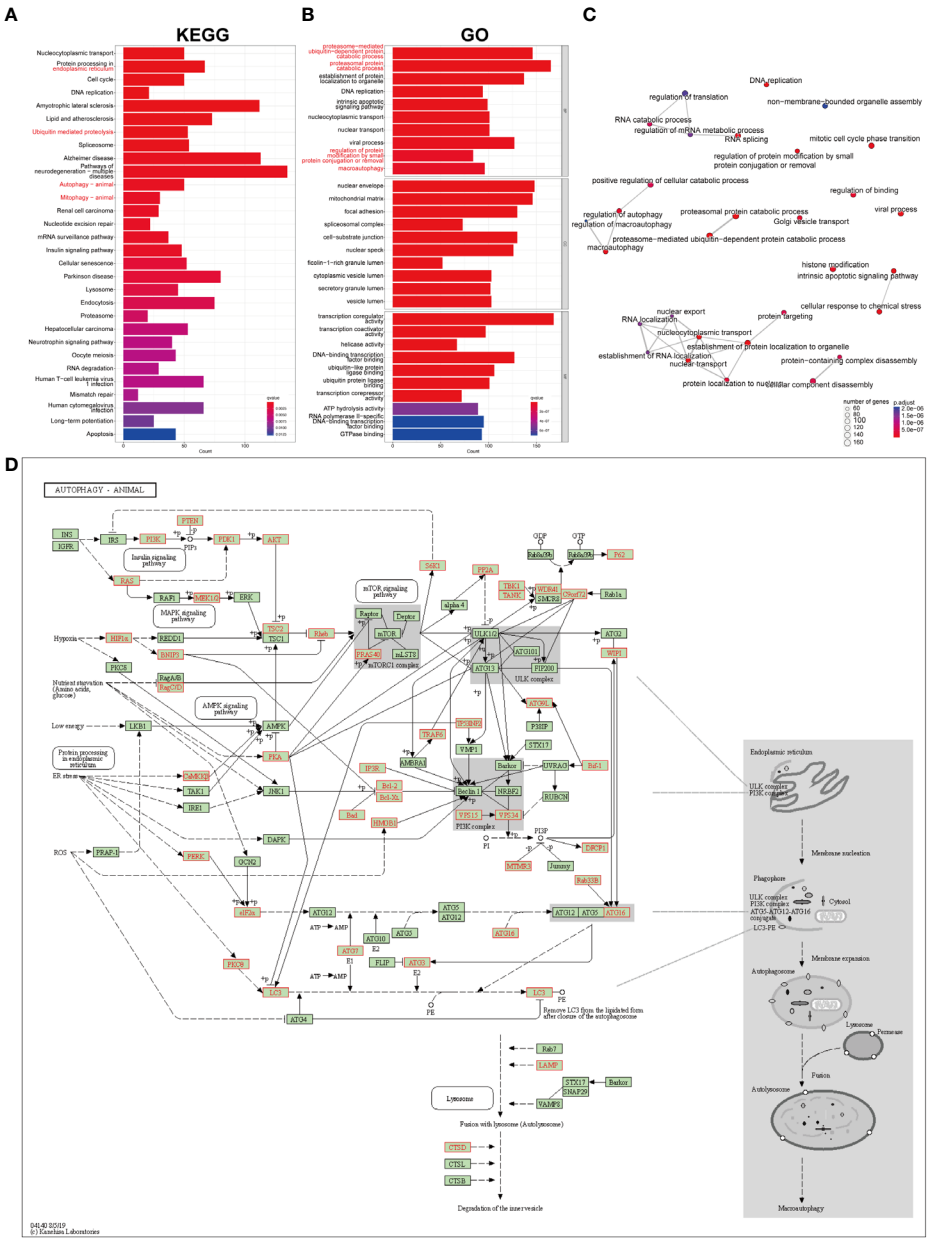

FIGURE 5
GO and KEGG pathway enrichment analysis on the genes mediated by key pSS-related m6A regulators. **(A)** The significantly enriched top 30 KEGG pathways. **(B)** The significantly enriched top 10 terms for GO: are BP, CC, and MF. **(C)** The emapplot visualizes gene sets of biological processes as a network, mutually overlapping gene sets tend to cluster together, making it easier for interpretation. Top 30 GO: BP terms were presented. **(D)** The "Autophagy-animal" pathway identified with KEGG enrichment.

The clinical features of 30 pSS patients in dataset #2 were downloaded. The diagnosis of pSS requires the presence of immunologic abnormalities, including ANA immunofluorescence, positive anti-SSA, and anti-SSB. The Spearman correlation analysis showed that the expression of STAT1 in PB showed a moderate to strong positive correlation with the immune response of pSS patients, including anti-SSA ($r = 0.75$, $p < 0.001$), anti-SSB ($r = 0.47$, $p = 0.011$), ANA ($r = 0.52$, $p = 0.0031$), and IgG ($r = 0.58$, $p < 0.001$), and associated with the disease activity based on the ESSDAI index (Figure 7B), in accordance to the results of Barrera et al (31). Conversely, PIK3CA showed a negative association with IgA, anti-SSA and age (Figure 7C).

Heterogeneity related to anti-Ro/SSA antibody

Four m6A regulators in dataset #2 were identified as related to anti-Ro/SSA level or seroreactivity, including two writers (RBM15B and ZC3H13) and two readers (YTHDC1 and YTHDF1) (Figure S5). Compared with the group with low anti-Ro/SSA antibody levels, the B cells (activated B cells and immature B cells) of pSS patients in the group with high anti-Ro/SSA antibody levels showed higher infiltration ($p<0.01$) (Supplementary Figure 6A). However, this immune dysfunction was less correlated with m6A regulators (r=-0.38) (Supplementary Figure 6B). In addition, 122 genes were identified as differentially expressed between the pSS patients with high and low anti-SSA antibody levels, and they were dominantly involved in the defense response to viruses (Supplementary Figure 6C). Among them, 38.6% of genes (34/122) were mediated by the four m6A regulators related with

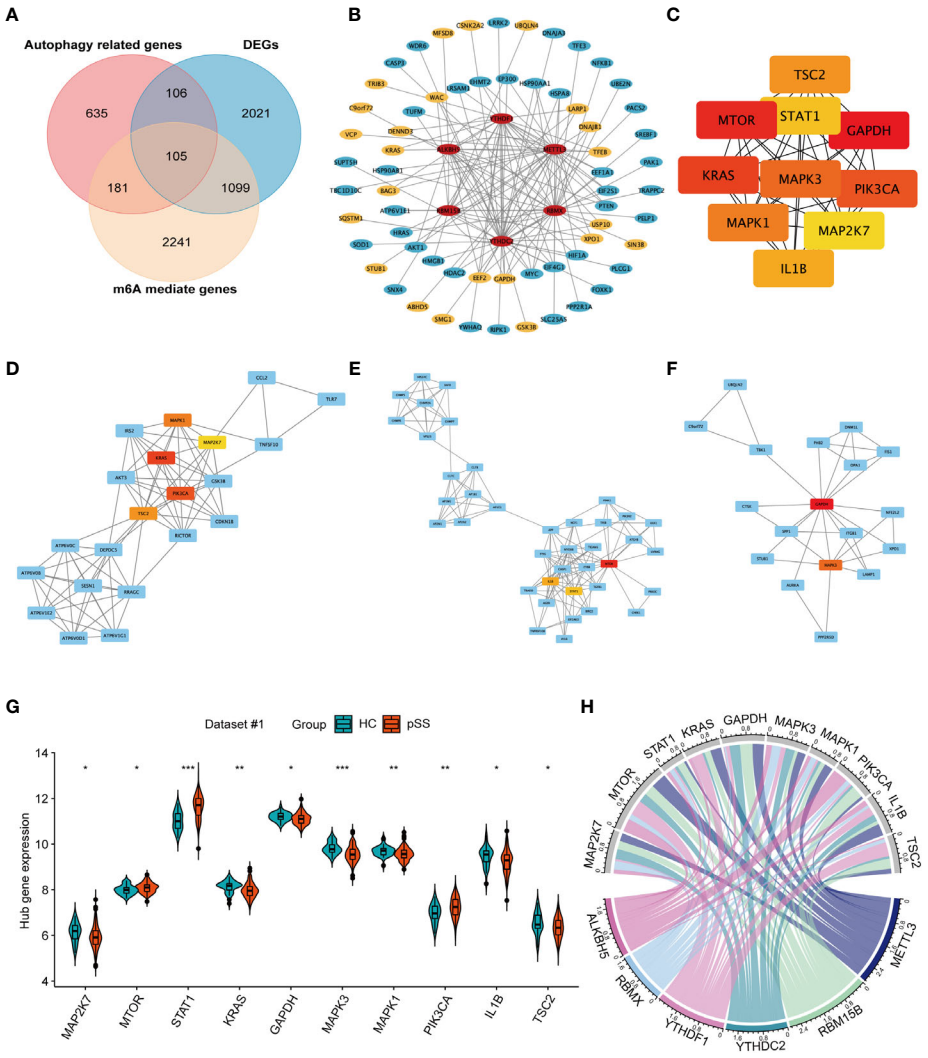

FIGURE 6
The network for autophagy-related genes modified by key pSS-related m6A regulators. **(A)** The venn plot illustrates the intersection of m6A mediated genes, autophagy-related genes, and the differentially expressed genes in pSS. **(B)** Protein-protein interaction (PPI) network among the pSS-related DEMRs and autophagy-related genes mediated by m6A regulators (n=286). Red represents m6A regulators, blue represents autophagy-related genes, among which the significantly expressed genes in pSS compared with HCs are highlighted in yellow. **(C)** The top 10 candidate hub genes of the differentially expressed autophagy-related genes (n=211) ranked by the maximum clique centrality (MCC) method. **(D-F)**. The top 3 subnetwork gene clusters were exported using the MCODE program. The hub genes were highlighted in red or yellow color as in Figure 6C. **(G)** The violin plot illustrates the autophagy hub genes that are dysregulated between the HCs and pSS groups in dataset #1. **(H)** The chord diagram demonstrated the correlation between DEMRs and autophagy hub genes. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

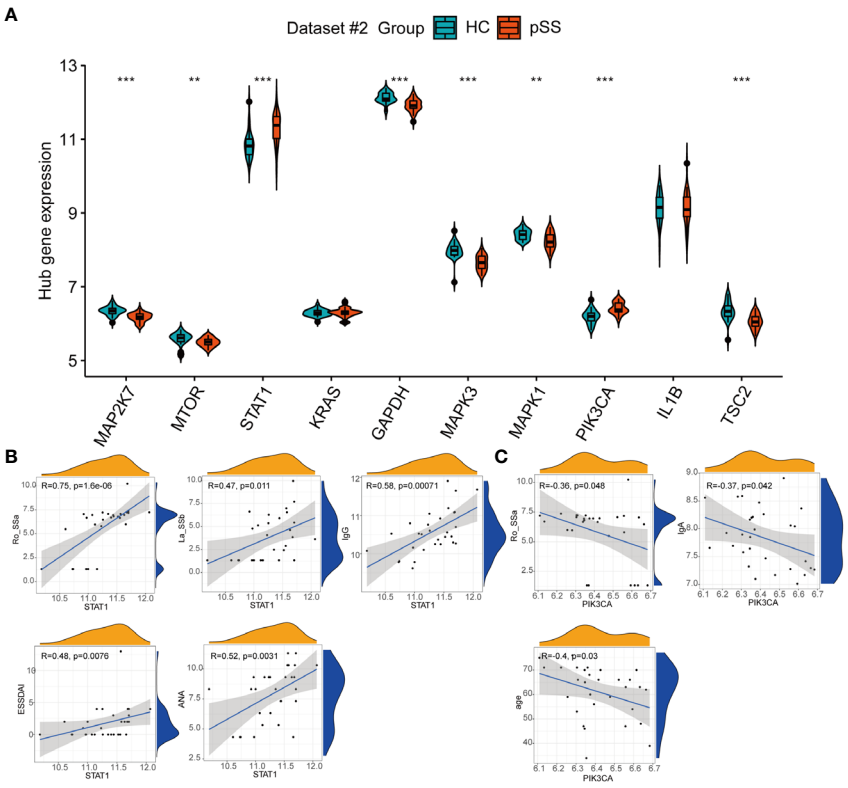

FIGURE 7
The expression of autophagy hub genes in pSS. **(A)** The violin plot illustrates the autophagy hub genes that are dysregulated between the HCs and pSS groups in dataset #2. **(B)** Correlations between STAT1 and laboratory tests in pSS patients in dataset #2. **(C)** Correlations between STAT1 and laboratory tests in pSS patients in dataset #2. **, p<0.01; ***, p<0.001.

anti-Ro/SSA antibodies and also mainly involved in the defense response to viruses and response to type I interferon (Supplementary Figures 6D, E).

Verification of gene expression in our PB samples and labial salivary glands

The six DEMRs in the PUMCH PB cohort were validated. The mRNA expression of ALKBH5, RBMX, RBM15B, and YTHDF1 was significantly lower in pSS PB samples than that in HCs (Figure 8A). These results were consistent with those from dataset #1 and dataset #2. METTL3 mRNA expression was higher although not significant and YTHDC2 decreased significantly in pSS PB samples compared with that in HCs, results that were the opposite of those from the two GEO datasets of PB samples.

Considering the histopathology and the classification role of labial salivary glands in pSS, these findings, together with DEMRs, were further verified in samples from labial salivary gland biopsy of pSS and HCs in the dataset #3 (Figure 8B). RBMX was also not targeted in dataset #3, in which the expression profile was detected by Affymetrix GeneChip Human Genome U133 plus 2.0 Array as in dataset #2. Consistently, ALKBH5, METTL3, RBM15B, and YTHDF1 were downregulated in the tissue sample of pSS compared with those of HCs, whereas the upregulation of YTHDC2 was not verified in dataset #3. Notably, the hub genes PIK3CA, STAT1, and MAPK3 located at the center of the subnetwork cluster #1, #2, and #3 by MCODE respectively (Figure 6) were confirmed as significantly changed in the tissue samples of pSS than those in HCs (Figure 8C). Notably, the expression of 8,124 genes detected in dataset #1 and #3 showed a moderate correlation (r=0.455, p<0.0001, Figure S7), indicating that most genes maintained consistent changes between PB and labial salivary glands and their transcriptomes in pSS were overall moderately comparable, although inter-individual heterogeneity existed.

Discussion

Recently, the epigenetic mechanism has been considered an emerging concern in human health and disease. It can alter gene

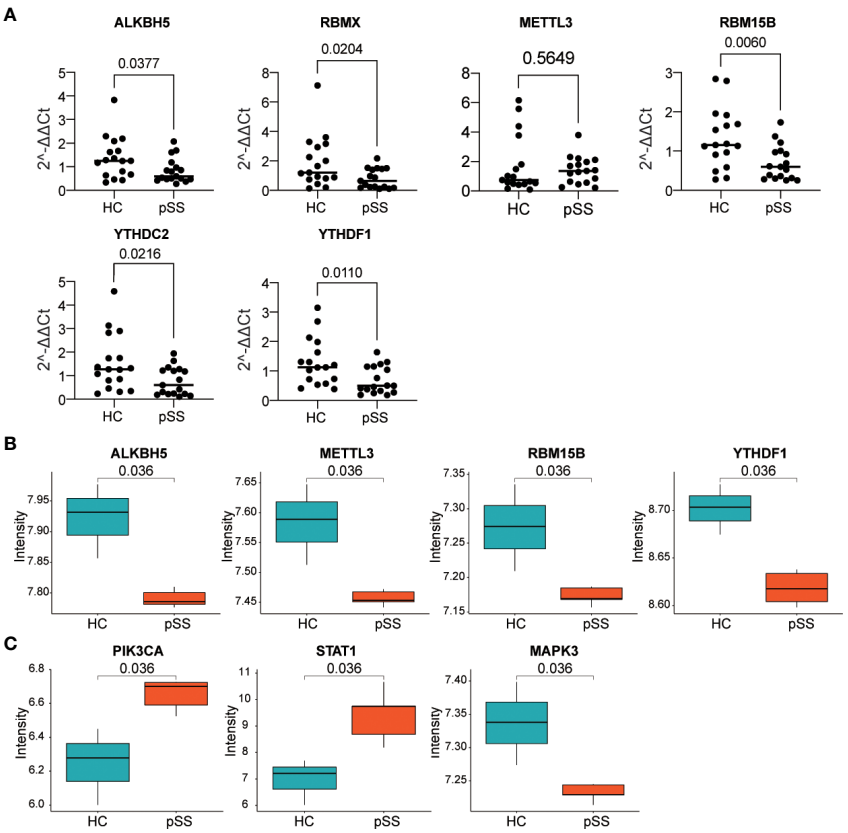

FIGURE 8
Verification of gene expression in our PB samples and labial salivary glands. **(A)** Verification of DEMRs in PB samples of our cohort. **(B, C)** Verification of DEMRs **(B)** and autophagy hub genes **(C)** in labial salivary glands of dataset #3.

expression without changing the DNA nucleotide sequence. Post-transcriptional modifications of mRNA, including m6A, 5-methylcytosine (m5C), and pseudouridine (ψ), are involved in the epigenetic regulation of multiple cellular processes, with broad roles in influencing mRNA stability, translation, and translocation (5, 6). The present study provided evidence that a peculiar profile of m6A regulators could distinguish pSS patients from HCs. The DEMRs identified from the array sequencing data were eventually validated in the other two GEO datasets of PB and in the samples of salivary gland tissue, as well as our collected fresh PB samples. Multiple immune reactions were disturbed in pSS, which could be modulated by m6A. Notably, the functional analysis of m6A mediated genes highlighted their relevance in the autophagy in pSS, hence suggesting the epigenetic regulation of m6A in the autophagy mechanisms in this disease.

m6A modification plays important roles in various cellular responses (5). The immune infiltration score for each subject was evaluated using ssgsea. The results revealed a significantly changed infiltration of the immunocyte profile including decreased activated DCs, plasmacytoid DCs, and CD56dim natural killer cells in pSS, in accordance with previous findings (27, 28). Since the cell infiltrates mainly consist of T cells and B cells, little attention has so far been directed to DCs. However, studies have uncovered the significance of DCs in pSS (28, 32). Plasmacytoid DCs circulate with PB and migrate to the inflamed tissues. Vogelsang et al. discovered that plasmacytoid DCs are decreased in PB, but their infiltration is detected in the salivary gland, implying their pathological role in the exocrinopathy of pSS patients (28). Besides the altered population and enumeration of DCs in PB (28), the activation of plasmacytoid DCs converges with the increased type-I IFN production and enhanced survival (32). The current study first proposes that m6A may modulate DCs in pSS, which has been previously reported in cancer, since m6A marked mRNAs encoding lysosomal proteases recognized by m6A "readers" can suppress the antigen presentation and promote immune evasion (33). Our results on METTL3 with the strongest negative correlation with the immune infiltration score of DCs, are remarkably consistent with those of Wang et al. who revealed that the specific depletion of METTL3 in DCs results in the impaired activation of DCs, with a decreased expression of the co-

stimulatory molecules CD40, CD80 and IL-12, and reduced T cell responses (5). The immune score of other immunocytes was also identified, including CD56dim natural killer cells, T helper cells and monocytes, and their close association with m6A regulators further provided insight to the m6A epigenetic regulation on the immune responses in pSS. Interestingly, heterogeneity within pSS patients existed, and the abunt B cell infiltration (activated B cells and immature B cells) was identified related to the higher levels anti-Ro/SSA antibody.

The FAA on the m6A mediated genes derived from the database and co-expression results was performed to further illustrate the downstream molecular mechanisms and functions of DEMRs related to pSS. In particular, the most innovative finding emerging from the FAA results confirmed the undebated role of m6A methylation on autophagy in pSS, consistent with previous study on abdominal aortic aneurysm (12) and epithelial ovarian cancer (34). Autophagy is an evolutionarily conserved mechanism, which widely exists in eukaryotes, and includes at least three types: macroautophagy, microautophagy, and chaperone-mediated autophagy (35). It promotes cell survival and the homeostatic state in harsh conditions by breaking down dysfunctional or unnecessary organelles and proteins, or microorganisms such as viruses and bacteria (35, 36). It is accepted that autophagy or the unique functions of autophagic proteins may serve as a central fulcrum to balance the beneficial and harmful effects of the host's response to infections and other immunological stimuli (36). Altered autophagy or autophagic protein function results in maladaptive inflammatory and metabolic responses, therefore causing the development of more severe diseases (36). Various pathophysiological conditions have identified different m6A modifications regulating aberrant autophagy (34, 37–39).

The pathologic role of autophagy in the lymphocyte infiltration of exocrine glands, as well as in the survival and activation of epithelial cells in the salivary gland has previously been described in pSS (3). Colafrancesco et al (3, 40, 41) demonstrated that up-regulated autophagy pathways induced by inflammation activate salivary gland epithelial cells in pSS, causing the high expression of adhesion molecules, and it is associated with histologic severity. This study discovered hub genes of autophagy in pSS, including PIK3CA, STAT1, MAPK3, and MTOR. Since the activated epithelial cells of the salivary glands could be the initiating factor causing immune and inflammatory disorders and the dryness symptoms in pSS, their expression in salivary gland tissues was further verified, which was closely interlinked to the altered expression of m6A regulators. Consistently, the modulation of m6A on autophagy has been previously reported. Liang et al. unveiled that the m6A reader YTHDC1 modulates autophagy in diabetic keratinocytes through the regulation of the stability of the nuclear sequestosome 1 (SQSTM1) mRNA (an autophagy receptor) (37). The depletion of METTL3, a primary m6A methyltransferase, under hypoxia activates autophagy-associated pathways in cultured hepatocellular carcinoma cells (38). As regards metabolic diseases, METLL3 and the partner YTHDF1 promote the hepatic autophagic flux and the clearance of lipid droplets in nonalcoholic fatty liver disease (39). PI3K-AKT and MAPK-ERK are upstream signals of mTOR, which activate TOR and consequently inhibit autophagy. ALKBH5 regulates autophagy through the mTOR pathway. Silencing the ALKBH5 inhibits p-AKT, p-MAPK, and p-ERK, while the ectopic expression of ALKBH5 promotes its expression (34).

Discrepancies were observed between different datasets and several potential factors should be considered. First, the clinical background, including the disease severity and activity of patients, was different among the cohorts of these datasets, which greatly affects the dysregulation of m6A regulators (19). Second, the subjects in different datasets had different treatment histories and the treatment received may affect the expression of m6A regulators (19, 42). For example, pSS patients in dataset #1 who were treated with moderate to high doses of corticosteroids, immunosuppressants, or biological agents were excluded, and HCs were those who did not receive any drug. However, all patients and HCs in dataset #3 were naive to immunosuppressive therapy. Third, different detection platforms were applied to perform the high-throughput sequencing.

This study presents some limitations. First, the molecular mechanisms underlying the influence of m6A modification on pSS should be explored by *in vivo* and *in vitro* experiments for epigenetic-based therapy. Second, the further exploration of the interaction between m6A and autophagy in the gland tissues and the difference in the expression pattern with PB was hampered due to the limited cohorts in dataset #3. Third, the expression of DEMRs and autophagy hub genes in dataset #3 should be further verified considering the key role of the epithelial cells of the salivary gland in the pathogenesis of pSS. However, the samples from dataset #3 were entirely salivary gland tissues rather than epithelial cells isolated from the primary salivary gland and were contaminated with lymphocytes, potentially compromising the observation (31). Further studies recruiting a larger cohort with PB samples and exocrine gland epithelial tissue samples should be performed to discover the pathological mechanisms of DEMRs in the autophagy involved in pSS.

Conclusion

To date, this study represents the first prospective study on PB and exocrine glands from pSS patients in the investigation of the m6A epigenetic mechanism. The crosstalk between m6A epigenetic modification and the remarkable up-regulation of autophagy pathways in pSS was revealed, which acted as a contributor to the pathogenesis of pSS and represents a novel therapeutic target. Despite the available biological agents and disease-modifying antirheumatic drugs for nearly all rheumatic diseases, firm evidence should be acquired to recommend drugs

for pSS (1). The present findings together with previous studies on autophagy in pSS strongly suggest the therapeutic potential of innovative alternative strategies disrupting this mechanistic connection through m6A modification.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Peking Union Medical College Hospital (JS-2049). The patients/participants provided their written informed consent to participate in this study.

Author contributions

YZL conceived and designed the research. LLC extracted data, performed software analysis and visualized graphs and tables. LLC, HLL, and HTZ went on validation experiments. LLC wrote the paper and HTZ proofread the paper. YZL, XML, and YH categorized and supervised graphs and tables. LW and FCZ provided the samples and the clinical information. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.949206/full#supplementary-material

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Effects of low-calorie and different weight-maintenance diets on IgG glycome composition

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Obesity-induced inflammation activates the adaptive immune system by altering immunoglobulin G (IgG) glycosylation in a way to produce more proinflammatory antibodies. The IgG glycome has already been well studied, and its alterations are correlated with a high body mass index (BMI) and central adiposity. Still, the IgG N-glycome susceptibility to different dietary regimes for weight control after the initial weight loss has not been studied. To explore changes in IgG glycosylation induced by weight loss and subsequent weight-maintenance diets, we analyzed 1,850 IgG glycomes from subjects in a dietary intervention Diogenes study. In this study, participants followed a low-calorie diet (LCD) providing 800 kcal/d for 8 weeks, followed by one of five weight-maintenance diets over a 6-month period. The most significant alteration of the IgG N-glycome was present 8 weeks after the subjects underwent an LCD, a statistically significant decrease of agalactosylated and the increase of sialylated N glycans. In the follow-up period, the increase in glycans with bisecting GlcNAc and the decrease in sialylated glycans were observed. Those changes were present regardless of the diet type, and we did not observe significant changes between different diets. However, it should be noted that in all five diet groups, there were individuals who prominently altered their IgG glycome composition in either proinflammatory or anti-inflammatory directions.

KEYWORDS

IgG, glycosylation, calorie reduction, weight maintenance, healthy diet

Abbreviations: LCD, low-calorie diet; LP, low protein; HP, high protein; LGI, low glycemic index; HGI, high glycemic index; U(H)PLC, ultra-(high-)performance liquid chromatography.

Introduction

Being overweight or obese is associated with numerous non-communicable and infectious (including Coronavirus disease 2019 (COVID-19) (1)) diseases, yet overweight/obesity is on its, seemingly, unstoppable global rise; over 2 billion people have excess body weight, which is over a quarter of the world population (2). Moreover, the global study on over 10.6 million participants showed that both overweight [body mass index (BMI) ≥ 25 kg/m^2] and obesity (BMI ≥ 30 kg/m^2) are associated with increased all-cause mortality (3). Given these overweight/obesity-related associations, weight loss is often a target that attenuates inflammation and reduces the risk of developing further health complications. Yet, losing weight is a more achievable goal than the maintenance of the reduced weight, which is crucial to achieving long-term health improvements (4).

Obesity-induced inflammation is often the cause of these comorbidities and eventually mortality; the inflammation usually starts with the activation of the innate immune system and consequently activates the adaptive immune system affecting multiple organs, from adipose tissue, pancreas, liver, and skeletal muscle to heart and brain (5). During this adaptive immune system activation, there is an accumulation of B cells in the adipose tissue, the production of a more inflammatory repertoire of cytokines and pathogenic immunoglobulin G (IgG) antibodies (6). Each IgG molecule has an N-glycosylation site on the Asn–297 of the constant heavy 2 (CH2) domain on each of its heavy chains, which influences its structural stability, conformation, and half-life, as well as effector functions (7). As with most cell surface and secreted proteins, IgG glycosylation varies widely depending on the inflammatory state. Unlike other proteins, these changes directly contribute to altered effector function and add an extra dimension to the functional diversity of antibodies (8). Under homeostatic conditions, the IgG glycome composition within an individual is stable over time. However, it changes gradually with age and can change rapidly in many homeostatic disorders. Significant changes in the IgG glycome have also been reported in several diseases such as cancer, autoimmune diseases, infectious diseases, and inflammatory diseases and conditions (7). By modulating its affinity for different FcγR receptors, the different terminal monosaccharides of N-glycans direct IgG binding to a preferential receptor and thereby effect its function. For example, a higher proportion of sialic acid and galactose residues is associated with anti-inflammatory activity, while the lack of sialic acid and galactose and presence of bisecting N-acetylglucosamine (GlcNAc) residues are linked with proinflammatory activity (7). Indeed, these proinflammatory IgG glycome traits have already been associated with a higher BMI and the measures of central adiposity (9, 10). It is also important to emphasize that not only IgG glycosylation is associated with chronic inflammation and associated diseases but also the glycosylation of other proteins (e.g., other plasma proteins), which indicates that glycan processing may be, *per se*, altered under these conditions (11).

A wide range of high-throughput analytical platforms can be used for the profiling, characterization, and analysis of IgG N-glycans. Liquid chromatography coupled with fluorescence detection (LC-FLR) is the most widespread (12). High-throughput sample preparation for the separation and detection of LC-FLR glycans usually begins with the enzymatic release of glycan moieties from the protein backbone, followed by the labeling of free glycans with tags containing fluorophores (13). The most used tags are 2-aminobenzamide and procainamide. Both labels use the same reductive amination mechanism to bind stoichiometrically to a glycan molecule, allowing for relative quantification based on Fluorescence (FLR) intensity. Recently, a new labeling compound was introduced, RapiFluor MS (RFMS). RFMS is an instant labeling agent containing a quinoline fluorophore for FLR detection and an n-hydroxysuccinimide group that binds to N-glycan-glycosylamine creating a stable urea linkage. Compared to traditional tags, RFMS enables fast N-glycan labeling and allows for better throughput, which comes in handy when larger numbers of samples are analyzed (12, 13).

To explore IgG glycosylation alterations due to weight loss and subsequent weight-maintenance diets, we analyzed 1,850 IgG glycomes in plasma from subjects of the Diogenes study (14, 15), one of the largest dietary intervention studies in which subjects underwent a low-calorie diet (LCD) followed by one of five weight-maintenance diets [low protein (LP)/low glycemic index (LGI), LP/high glycemic index (HGI), high protein (HP)/LGI, HP/HGI, and control] in a period of 6 months when subjects are vulnerable to regain the weight.

Material methods

Subjects

The glycome composition of IgG isolated from 1,850 plasma samples was analyzed from eight different centers of the Diogenes study, described elsewhere in detail (14, 15), at three different time points: at the beginning of the diet intervention (time point 1), after 8 weeks on an LCD diet providing 800 kcal/d (time point 2), and after 6 months on a weight-control diet (time point 3). Subjects on a diet according to current national dietary guidelines ("healthy diet") in each of the countries served as the control group. After the initial screening, 938 subjects, 620 women and 312 men, with a mean age of 40 years for women and 42 years for men and a mean BMI of 33.8 kg m^{-2} for both sexes entered the LCD phase of the study. The mean waist circumference of the women who participated in the LCD intervention was 103.0 cm and that of men was 112.4 cm (14).

A total of 773 participants who completed this phase with an achieved target weight loss of 8% of baseline body weight were randomly assigned to one of the five maintenance diets, of which only 548 participants completed the intervention (71%). Fewer participants in the HP and LGI groups than in the LP/HGI group discontinued from the study, 26.4% and 25.6% *vs*. 37.4%, respectively (16). In-depth information on the number of subjects and types of diets per each center is presented in Supplementary Table 1.

Sample preparation, rapid rapifluor-MS N-glycan labeling and hydrophilic interaction chromatography–solid-phase extraction clean-up

Samples were randomly positioned into 26 96-well plates. Each 96-well plate contained approximately 70 samples, as well as five randomly chosen sample replicates from the same plate and five from other plates. IgG was isolated using 96-well protein G monolithic plates (BIA Separations, Ajdovscina, Slovenia) following a protocol first described by Pučić et al. (17) and Trbojević-Akmačić et al. (18). Briefly, plasma samples were first diluted and filtered through a 0.45 µm GHP filter plate (Pall Corporation, Port Washington, New York ,USA) and transferred to a protein G monolithic plate. Samples were then repeatedly washed with 1× phosphate-buffered saline (1× PBS) and eluted with 0.1 mol/L formic acid (Merck, Darmstadt, Germany) followed by an immediate neutralization with ammonium bicarbonate (Acros Organics, Waltham, Massachusetts, USA). An appropriate volume of isolated IgG (average mass of 15 µg) was dried in a vacuum centrifuge.

Further sample analysis (deglycosylation, released N-glycan labeling, and clean-up) was performed using the Waters GlycoWorks RapiFluor-MS N-Glycan Kit (USA) according to the Producer's instructions (Waters Corporation, 2017), with some adaptations of the protocol to suit high-throughput analysis in the 96-well PCR plate format (19). In short, isolated and dried IgG was first resuspended in ultrapure water, swiftly denatured with a RapiGest SF surfactant solution, and enzymatically deglycosylated with GlycoWorks Rapid PNGase F. Released N-glycans were then labeled with the RapiFluor-MS fluorescent dye. Labeled IgG N-glycans were purified by hydrophilic interaction chromatography–solid-phase extraction (HILIC-SPE) cleanup by repeated washing steps with formic acid/ultrapure water/acetonitrile (ACN) (1:9:90, v/v/v) and eluted in three steps with an SPE elution buffer, 200 mmol/L ammonium acetate/ACN (95:5, v/v), pH 7. To dilute the samples, a sample diluent, dimethylformamide/ACN (32:68, v/v) was added to each sample and mixed. Diluted samples were either immediately used in further chromatographic analysis or were stored at -20°C until further use.

Hydrophilic interaction chromatography–ultra-high-performance liquid chromatography with fluorescence detection N-glycan analysis

The analysis of RapiFluor-MS-labeled IgG N-glycans was performed on Waters Acquity UPLC H-class instruments monitored by Waters Empower 3 software and using Waters UPLC Glycan bridged ethylene hybrid (BEH) amide chromatographic columns (130 Å, 1.7 µm BEH particles, 2.1 × 100 mm) with 50 mmol/L ammonium formate, pH 4.4 as solvent A, and 100% LC-MS grade ACN as solvent B, as previously described by Keser et al. (20). The adjustments of the separation method made by Deriš et al. (19) included a linear gradient of 75%–61.5% acetonitrile (v/v) at a flow rate of 0.4 ml/min over 30 min in a 42-min analytical run and an injection volume of 30 µl. Chromatograms acquired in the analysis were automatically integrated, separated into 22 glycan peaks (Supplementary Figure 1), and total area-normalized (%Area) values were obtained for each peak to enable a relative quantification of IgG N-glycans.

Data analysis

Normalization and batch correction were performed on UHPLC glycan data to remove the experimental variation of measurements. To make the measurements across samples comparable, normalization by the total area was performed where the peak area of each of 22 glycan structures was divided by the total area of the corresponding chromatogram. Prior to batch correction, normalized glycan measurements were log-transformed due to the right-skewness of their distributions and the multiplicative nature of batch effects. The batch correction was performed on log-transformed measurements using the "ComBat" method (R package sva) where the technical source of variation was modeled as a batch covariate. Estimated batch effects were subtracted from log-transformed measurements to obtain measurement correction for experimental noise.

Six derived traits were calculated from 22 glycan structures directly obtained by UHPLC analysis. Derived glycan traits represent a portion of structurally similar glycan species with common biosynthetic pathways. The total IgG-derived glycan traits were calculated as the ratios of glycan peaks (GP1–GP22) with the same structural characteristics in a total IgG glycome: total agalactosylated glycans, G0 = (GP1 + GP2 + GP3 + GP4)/SUM (GP1-GP22)*100; total monogalactosylated glycans, G1 = (GP5 + GP6 + GP7 + GP8 + GP9 + GP10)/SUM (GP1-GP22)*100; total digalactosylated glycans G2 = (GP11 + GP12 + GP13)/SUM (GP1-GP22)*100; total sialylated glycans,

S = (GP14 + GP15 + GP16 + GP17 + GP18 + GP19 + GP20 + GP21 + GP22)/SUM (GP1-GP22)*100; total fucosylated glycans F = (GP1 + GP3 + GP4 + GP7 + GP8 + GP9 + GP10 + GP12 + GP13 + GP14 + GP16 + GP17 + GP21 + GP22)/SUM (GP1-GP22)*100; total glycans with bisecting GlcNAc, B = GP4 + GP9 + GP10 + GP13 + GP17 + GP20 + GP22)/SUM (GP1-GP22)*100.

The longitudinal analysis of patient samples through their observation period was performed by implementing a linear mixed-effects model where glycan measurement was the dependent variable and time was modeled as a fixed effect, while individual ID was included in the model as a random intercept, with age, gender, and BMI included as additional covariates. The analyses were firstly performed for each center separately and then combined using a random effects meta-analysis approach (R package meta, metagen (method = "ML")). Prior to the analyses, glycan variables were all transformed to standard normal distribution (mean=0, sd=1) by the inverse transformation of ranks to Normality (R package "GenABEL", function rntransform). The usage of rank-transformed variables in analyses makes the estimated effects of different glycans in different centers comparable as transformed glycan variables have the same standardized variance. The false discovery rate was controlled using the Benjamini–Hochberg procedure (function p.adjust(method = "BH")). Data were analyzed and visualized using R programming language (version 3.0.1).

Results

The N-glycome composition of IgG isolated from subjects' plasma samples was determined by the UHPLC analysis of glycans labeled with RapiFluor-MS as described in the Materials and Methods section. Statistical analysis was performed on six derived traits calculated from 22 directly measured glycan structures, corresponding to 22 glycan peaks obtained by UHPLC analysis.

The glycan analysis was firstly performed for each center of the Diogenes study separately on rank-transformed glycan variables (Supplementary Table 2; Supplementary Figure 2). After performing meta-analysis for all the centers, four derived traits showed statistically significant alterations in their levels, either in the first (T1–T2) or second (T2–T3) time period (Figure 1). The most significant change after meta-analysis in the first time period, representing an 8-week long period on LCD with a weight loss of ~11 kg, is a decrease of agalactosylated structures (adjusted p-value < 0.001) accompanied by an increase of sialylated structures (adjusted p-value < 0.03) (Supplementary Table 3; Figure 2). On the other hand, in the second time period with weight maintenance, there was a statistically significant increase in the abundance of digalactosylated structures and structures with bisecting GlcNAc (adjusted p-value < 0.02), while sialylated glycans went to the opposite direction when compared to the first time period (adjusted p-value < 0.02) (Supplementary Table 3; Figure 2).

Additionally, we did not observe any significant changes in derived traits between different weight-control diets (Supplementary Figure 3), nor did we find any correlation between IgG N-glycan traits and age, sex, and BMI after the LCD period or after the second, the weight maintenance period (Supplementary Table 4).

Discussion

The Diogenes dietary intervention study deepened our understanding of the impact of the weight-loss and weight-maintenance diets on the adipose tissue (AT) transcriptome (21), different clinical parameters [e.g., blood pressure (BP) (22) and fasting insulin and glucose (23)], blood proteome, and steroid hormones (4), and this is now further extended by IgG glycome analysis. The IgG glycome has already been well studied, and its alterations are associated with different

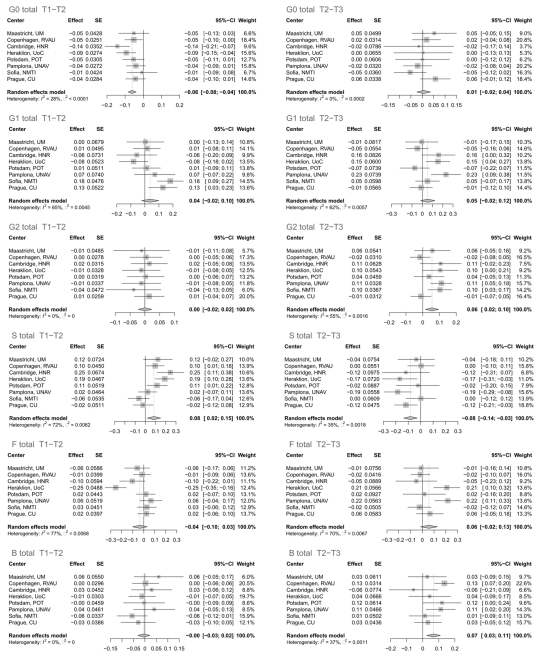

FIGURE 1
Effect of calorie loss on the immunoglobulin G (IgG) glycome in the first time period (T1–T2) and effect of different weight maintenance diets on the IgG glycome in the second time period (T2–T3). Changes in IgG glycome composition after performing meta-analysis for all the centers are shown. SE, standard error; 95% CI, 95% confidence interval; T1, time point 1; T2, time point 2; T3, time point 3; G0, agalactosylated glycans; G1, monogalactosylated glycans; G2, digalactosylated glycans; S, glycans containing sialic acid; F, glycans with core fucose; B, glycans with bisecting GlcNAc.

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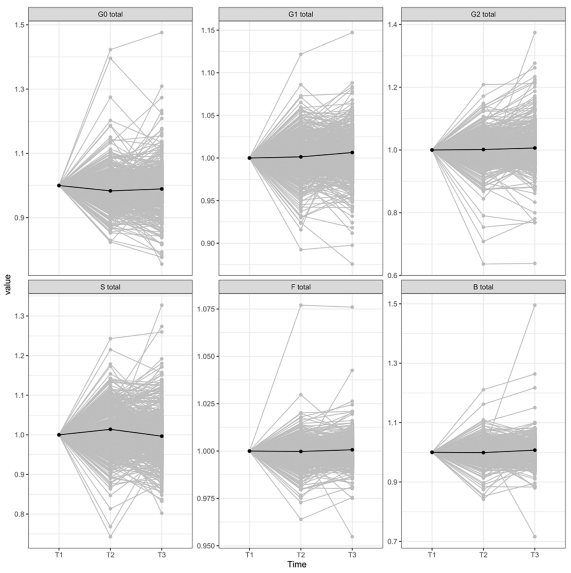

FIGURE 2
IgG glycome composition changes between different time-periods normalized to the first point. T1, time point 1; T2, time point 2; T3, time point 3; G0, agalactosylated glycans; G1, monogalactosylated glycan structures; G2, glycans with two galactoses; S, glycans containing sialic acid; F, glycans with core fucose; B, glycans with bisecting GlcNAc.

diseases and conditions, including high BMI and central adiposity (7, 9, 10), yet this is the first study that shed light on its susceptibility to different dietary regimes for weight control after weight loss.

The most prominent alteration of the glycome was observed 8 weeks after the subjects underwent LCD with a weight loss of approximately 11 kg; a statistically significant decrease of agalactosylated N-glycans and an accompanying increase of sialylated N-glycans in the IgG glycome present shifting from pro- to the anti-inflammatory activity of IgG (7). These observations are expected knowing that adipose tissue, apart from being an energy storage depot, is also an endocrine organ that produces adipokines such as interleukin (IL-6) and tumor necrosis factor-α (TNF-α), which are cytokines already well linked with inflammation (24, 25). Since their adipose tissue production is affected by the degree of adiposity, the attenuation of systematic inflammation is an obvious outcome after a weight-reducing energy-restricted diet. Indeed, both of these cytokines and the attenuation of inflammation have been associated with the alteration of the IgG glycome (7). Thus, gene and protein association studies have found a correlation between IL-6 and proinflammatory IgG glycosylation patterns in humans (26, 27), while TNF-α serum levels are correlated with a proinflammatory IgG glycome pattern that is shown to be susceptible to anti-TNF-α therapy (28–30). Unfortunately, aside from these association studies, there is no study that illuminated the underlying mechanism in which inflammation modulates IgG glycosylation. However, our study is not the first one that observed an increase of anti-inflammatory IgG glycan repertoire after LCD (31). The IgG glycome changes toward the anti-inflammatory direction were reported just after a week of fasting, yet they did not correlate significantly with the clinical improvement after the vegetarian diet period (31).

The mean weight gain in 548 participants who completed the 6-month intervention was 0.565 ± 44 kg. Only the participants assigned to the LP/HGI diet had a significant weight gain of approximately 1.7 kg. Body weight changes differed between the diet groups, and each group did not differ significantly in terms of diet-related adverse events (16). Similarly, we did not observe significant changes between different diets during the weight-maintenance period, and an increased abundance of glycans with bisecting GlcNAc and a decrease of sialylated ones, which were present regardless of the diet type, suggest the proinflammatory direction of IgG glycome alteration in the period of weight maintaining or regaining.

Therefore, our study together with the study published by Kjeldsen-Kragh et al. suggests that calorie intake and weight loss rather than a diet type is the main driver of IgG glycome changes (31). Berry et al. also published a study where they challenged the logic of the standardized, universal diet and recommended personalized nutrition for disease prevention (32), and the IgG glycome alterations we observed seem to support this claim. Interestingly, this distinguishes IgG glycosylation and its susceptibility to change from clinical and biochemical parameters observed/measured after a vegetarian diet or Diogenes diets (31, 33). Specifically, in rheumatoid arthritis patients' IgG glycome, significant changes were observed already after 7–10 days after fasting and there were no significant changes after following a vegetarian diet even though clinical improvement was reported also after the vegetarian diet period (31). Similarly, of the Diogenes study diets, the HP/LGI diet was associated with reduced body fatness and beneficial effects on blood pressure, blood lipids, and inflammation (33), yet this diet's beneficial influence had no impact on the IgG glycome despite its association with all of these clinical/biochemical parameters (7).

In addition, a comprehensive evaluation of several antiaging diets, published by Lee et al. (34), revealed how calorie reduction and similar diets (including fasting, time-restricted feeding, and ketogenic diet), may not always be uniformly beneficial in extending a life span and their outcome is highly dependent on the genotype. Moreover, they concluded that a ubiquitous endorsement of a particular dietary intervention for healthy longevity would be impractical, which strongly coincides with our findings regarding the influence of dietary composition on IgG glycosylation.

In conclusion, this study is limited by the types of included diets and the number of measured biochemical parameters and consequently unable to illuminate the link between the IgG

glycome and adipose tissue inflammation. However, by analyzing 1,850 IgG glycomes from subjects following one of five different diets and finding no association between glycome alterations and diet types, this study reinforces transition from a universal, standardized to a personalized nutrition approach for the achievement of overall health benefits and the need for further research of relations between obesity, inflammation, and glycosylation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the local ethics committees in the respective countries. The protocol was in accordance with the Declaration of Helsinki; all study participants signed an informed consent document after they received verbal and written instructions and according to local legislation. The ethics committee waived the requirement of written informed consent for participation.

Author contributions

HD, NB and PT carried out the experiments. FV was involved in data analysis and interpretation, and visualization. AA, EB and GL were involved in funding acquisition, study design, and reviewing and editing the manuscript. HD and IG were involved in original draft preparation, reviewing, and editing, and IG supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

GL is the founder and owner and HD, PT, FV and IG are employees of Genos Ltd, a company that specializes in high-throughput glycomics and has several patents in this field. Author AA was employed by The Novo Nordisk Foundation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.995186/full#supplementary-material

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Blood DNA methylation marks discriminate Chagas cardiomyopathy disease clinical forms

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Chagas disease is a parasitic disease from South America, affecting around 7 million people worldwide. Decades after the infection, 30% of people develop chronic forms, including Chronic Chagas Cardiomyopathy (CCC), for which no treatment exists. Two stages characterized this form: the moderate form, characterized by a heart ejection fraction (EF) ≥ 0.4, and the severe form, associated to an EF < 0.4. We propose two sets of DNA methylation biomarkers which can predict in blood CCC occurrence, and CCC stage. This analysis, based on machine learning algorithms, makes predictions with more than 95% accuracy in a test cohort. Beyond their predictive capacity, these CpGs are located near genes involved in the immune response, the nervous system, ion transport or ATP synthesis, pathways known to be deregulated in CCCs. Among these genes, some are also differentially expressed in heart tissues.

Interestingly, the CpGs of interest are tagged to genes mainly involved in nervous and ionic processes. Given the close link between methylation and gene expression, these lists of CpGs promise to be not only good biomarkers, but also good indicators of key elements in the development of this pathology.

KEYWORDS

chagas disease, cardiomyopathy, blood, biomarkers, methylation

Introduction

Chagas disease is an endemic disease from South America, caused by a parasite, *Trypanosoma cruzi*, and affecting around 7 million people. With migration flow, this disease can now be found in non-endemic country, notably in North America (1) (n > 300,000), Europe (2) (n > 100,000), Japan (3) (n > 4,000) or Australia (4) (n > 1,000). After the infection, patients present an acute stage which is mostly asymptomatic (ASY). Then comes the chronic forms, where 70% of them remains asymptomatic, with no end organ damage (the so-called indeterminate stage). However, 30% develop Chagas disease Cardiomyopathy (CCC) (5). CCC had been divided in two stages based on heart ejection fraction: moderate CCC (EF ≥ 0.4) and severe CCC (EF < 0.4) (6–8). Some drugs are effective on *T. cruzi*, but does not cure the CCC, reducing the parasitemia, without having any effect on heart damage (9). The only way out for CCC patients is the placement of a pacemaker, or a heart transplant. The early diagnosis of Chagas disease is therefore essential.

During the acute stage, disease diagnosis is commonly made by microscopy, considering the limited sensitivity of the direct test (10). However, in chronic stage, parasitemia is very low, or even null. The Pan American Health Organization (PAHO) recommends using two serological tests (two techniques based on different antigens) in parallel and, in case of discordant results, to perform these tests again on a new sample (11). If the results remain unclear, a confirmation test should be achieved (12). For CCC especially, an ECG and/or an echocardiogram is made to confirm cardiac involvement (13). BNP and NT-proBNP, well-known markers be associated with cardiac dysfunction (14), have been associated to Chagas cardiomyopathy (15, 16), but are not specific to this pathology. Others markers, including miRNAs (17, 18), cytokines (19) or metalloproteinases (20) have been proposed as biomarkers for CCC, but no confirmation has been made in a test cohort at this time. The only diagnosis of CCC currently in place is a clinical diagnosis, which is difficult to access for the most remote populations.

A previous analysis (21) has highlighted differences of DNA methylation in blood of asymptomatic and CCC patients. Moreover, some differences have also been demonstrated between moderate and severe CCC. Blood DNA methylation has already been proposed as biomarker for several diseases (22–24). Here, we used machine learning methods on both asymptomatic and CCC blood DNA methylation data to predict Chagas disease, as well as Chagas disease stage.

Methods

Ethical considerations

The protocol was approved by the institutional review boards of the University of São Paulo School of Medicine and INSERM (French National Institute of Health and Medical Research). Written informed consent was obtained from all patients. All experimental methods comply with the Helsinki Declaration.

Blood DNA collection and DNA methylation analysis

Blood samples (5 to 15 ml of blood) from CCC patients were collected in EDTA tubes. Genomic DNA was isolated using standard salted methods and the methylation analysis was done using the same protocol as tissue DNAs.

Blood DNA methylation data

138 patients were selected randomly from our Chagas bank. It included 48 asymptomatic subjects, 46 moderate CCC patients and 44 severe CCC patients (Supplementary Table 1). The age and sex ratio were not significantly different between the 3 groups (age mean and ratio female/male for all phenotypes: asymptomatic: age: 57.63, ratio =1; moderate: age: 56.89, ratio=1.14; severe: age: 59.59, ratio=0.95). In a second time, these 138 samples were randomly distributed between the training (70%) and validation (30%) cohorts. This random distribution was done in such a way that the age and sex ratio was still not so different between the groups in the two sub-cohorts (Training cohort (age mean and ratio female/male for all

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phenotypes: asymptomatic: age: 62.45, ratio=1; moderate: age: 60.13, ratio=1.14; severe: age: 57.18, ratio=0.86), validation cohort (age and ratio female/male for all phenotypes: asymptomatic: age: 52.82, ratio=1; moderate: age: 53.67, ratio=1.13; severe: age: 62, ratio=1)). The methylation data are available under the reference: (GEO accession: GSE191082).

Biomarker identification for disease forms

Since data contains a lot of features (736,661), feature selection was performed in two steps, on the training group only. The scripts used for the following steps are available on Github (https://github.com/TAGC-ComplexDisease/biomarkersChagas). First, the delta beta (difference of beta means) was computed between the two phenotypes of interest. Only the CpGs having at least 10% methylation differences were retained. Then, a machine learning (ML) analysis was done in Python with Scikit-learn library. Four supervised ML methods were considered: decision tree, random forest, logistic regression and linear SVM (Support-Vector Machine, a linear classificator). For each method, recursive feature elimination (RFE) was performed, and the best model (best accuracy) with the minimal set of feature was selected using 10-time cross-validation. Finally, model parameters were optimized with a grid search to obtain the final prediction on the validation group.

Results

Symptomatic cardiac form prediction

After feature selection based on delta beta values, 86 CpGs were selected. Among all the tested models, linear SVM seems to have the better prediction on training dataset with the minimal number of features (Figure 2A). According to this analysis, the SVM was trained with 35 features (Supplementary Table 2). The model parameters optimization was performed using a grid search where the L2 penalty varies between 0.01 and 10. Finally, with a L2 penalty of 1, 42 of 44 patients phenotype of the validation dataset were correctly predicted (accuracy = 0.95), with a sensitivity of 0.96 and a specificity of 0.94 (area under the curve: 0.996) (Figure 1A). Those 35 features are mainly located in the body of genes (n=20), or in intergenic regions (n=11). Particularly, 3 CpGs are located in LHX6, and 3 in POU6F2. All those genes are involved in biological process associated to Chagas disease: nervous system (LHX6, POU6F2, MDGA1, DISC1, PCSK9), immune system (ZMIZ, HLA-DRB1), Wnt pathway (DISC1), ion transport (KCNK15, PCSK9), striated muscles (SMYD3) or ATP metabolic process (ATP5S).

Chagas cardiomyopathy stage prediction

After feature selection based on delta beta, 108 CpGs were selected. Among all the tested models, random forest seems to have the better prediction on training dataset with the minimal number of features (Figure 2B). According to this analysis, the SVM was trained with 33 features (Supplementary Table 3). The model parameters optimization was performed using a grid search where the number of estimators varies between 50 and 200. Finally, 150 estimators, 27 of 28 patients phenotype (accuracy = 0.96) of the validation dataset were correctly predicted, with a sensitivity of 1 and a specificity of 0.93 (area under the curve: 1) (Figure 1B). Those 33 features are mainly located in 18 intergenic regions. Other CpGs are located in 15 genes, and more precisely in 6 gene body and 7 promoter regions. Here, genes are involved in various biological processes, from ion transport (KCNC1, MFI2), actin filament (PACSIN1), generation of neurons (TNN, PACSIN1) or MAPK cascade (DUSP22). 2 CpGs are in common with those used as biomarker between ASY and CCC: cg24000535 (LOC101928909) and cg21873524 (intergenic).

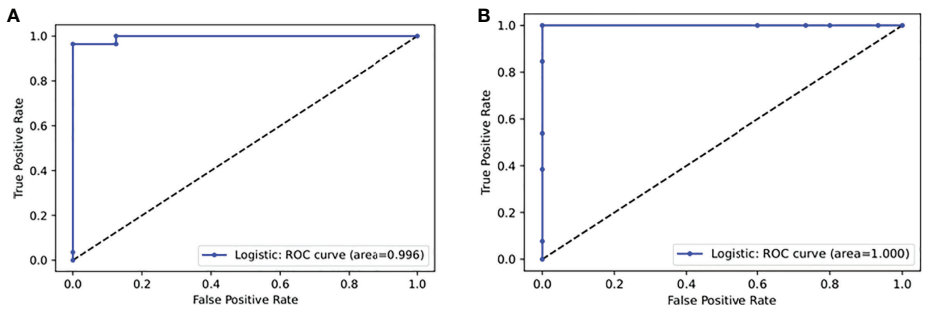

FIGURE 1
Receiver Operating Characteristic (ROC) curves produced by **(A)** linear SVM to predict CCC on 44 patients and **(B)** random forest to predict CCC stage on 28 patients.

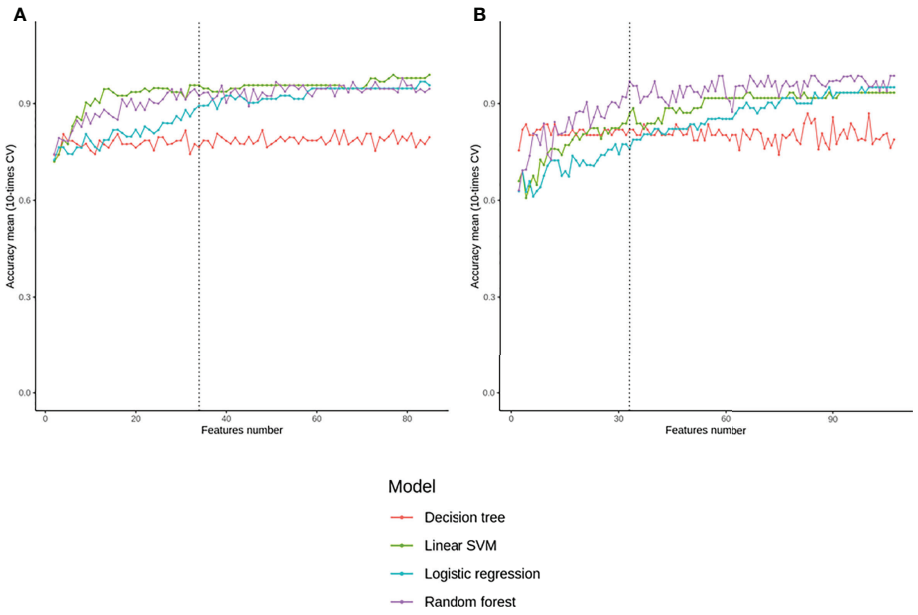

FIGURE 2
Evolution of the accuracy obtained with different machine learning models according to the number of top-explicative features chosen for **(A)** predict CCC or **(B)** predict CCC stage.

Discussion

Alterations of heart tissue DNA methylation profiles have been associated to the development of dilated cardiomyopathies (25, 26) and chagas diseases (21, 27). Recently, we have studied the DNA methylation in the blood of asymptomatic, moderate and severe CCC by hypothesizing that the blood data reflect the phenotype. We had found 12624 DMPs (Differentially Methylated Position) between asymptomatic and severe CCC blood samples and 6735 CpGs were found as DMPs between moderate and severe CCC.

In our study, based on machine learning approaches, we have identified 35 CCC-specific methylation markers. Those CpGs could distinguish controls (asymptomatic) to CCC from blood samples with 96% of sensitivity and 94% of specificity in independent validation sets. 3 of them (cg02872767, cg24540763, cg25134647) are also differentially methylated between asymptomatic and severe CCC in heart tissue (21). Similarly, 33 CpGs have been identified, allowing to predict the progression of this pathology (from moderate to severe CCC), with a sensitivity of 100% and a specificity of 93%. In conclusion, we identified two set of methylation markers potentially useful for Chagas disease diagnostic. The first one permit to discriminate patients with Chagas cardiomyopathy from asymptomatic patients, with 95% of precision. The second allows to predict Chagas cardiomyopathy severity stage, according to the heart ejection fraction rate, with 96% of precision.

Interestingly, most of these markers were not differentially in heart tissue of patients. The main message of this report is the finding that peripheral blood epigenetic marks are good markers of clinical form, implying that epigenetic events are closely related to CCC progression. These markers are highlighting the same biological processes that have been associated to the disease development such as ion transport, ATP metabolic process, immune system, Wnt system, nervous systems, striated muscles and actin filament. These findings are important as these are under-lighting biological pathways that will have to be targeted in drug design.

To propose large-scale reproducible biomarkers, a consensus Target Product Profile (TPP) has been developed for Chagas disease (28) stipulating that marker should be able to detect the effects of drug treatments, be detectable with limited resources and not vary according to the strain of the parasite. Given the high specificity of these assays, these methylation sites appear to be good candidates to decipher the pathogenic process, or to be used as blood biomarkers, and further studies will be necessary to potentially validate their possible use in the clinic, in accordance with the TPP consensus.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by INSERM IRB University of Sao Paulo. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study design: PBr, JK, LS, ECN, CC. Phenotype characterization: BI, CM, SS, CWP, BS, FD, MS, JAMN, AF, GDLP, PBu, HTL-W, AS, MM, MHH, ED, ACP, VRJ Experimental analysis: PBr, AFF, JPSN, PCT, LRPF, AK, DDSC, RCFZ, VOCR, RRA. Statistical analysis: PBr, LS, ECN, CC. Manuscript preparation: PBr, LS, ECN, CC.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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The IgG glycome of SARS-CoV-2 infected individuals reflects disease course and severity

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Immunoglobulin G (IgG) antibodies play an important role in the immune response against viruses such as SARS-CoV-2. As the effector functions of IgG are modulated by *N*-glycosylation of the Fc region, the structure and possible function of the IgG *N*-glycome has been under investigation in relation to divergent COVID-19 disease courses. Through LC-MS analysis we studied both total IgG1 and spike protein-specific IgG1 Fc glycosylation of 129 German and 163 Brazilian COVID-19 patients representing diverse patient populations. We found that hospitalized COVID-19 patients displayed decreased levels of total IgG1 bisection and galactosylation and lowered anti-S IgG1 fucosylation and bisection as compared to mild outpatients. Anti-S IgG1 glycosylation was dynamic over the disease course and both anti-S and total IgG1 glycosylation were correlated to inflammatory markers. Further research is needed to dissect the possible role of altered IgG glycosylation profiles in (dys)regulating the immune response in COVID-19.

KEYWORDS

IgG glycosylation, SARS-CoV-2, COVID-19, anti-spike IgG, total IgG

Introduction

The rapid spread of the infectious agent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the first global pandemic in the 21st century that gave rise to extensive hospitalizations and a significant economic burden. While the majority of people infected with SARS-CoV-2 remain asymptomatic, a minority of symptomatic infections require hospitalization due to the development of severe coronavirus disease 2019 (COVID-19). Severe COVID-19 is characterized by a dysregulated immune response, of which the nature remains incompletely understood (1). However, certain demographic risk factors for severe COVID-19 have been identified including higher age, male sex and ethnicity, as well as pre-existing comorbidities such as type II diabetes, COPD and hypertension (2–4). Moreover, both immune cell exhaustion (5) and unusual phenotypes thereof (6), a pro-inflammatory cytokine and chemokine milieu in various body fluids (7), together with proteomics and metabolomics signatures (8) have been shown to characterize severe COVID-19 (9).

The observed inflammatory environment is inherent to severe respiratory viral infections (10), but the peculiar, and often unexpected exacerbation of COVID-19 appears to coincide with immunoglobulin G (IgG) seroconversion (11–13). IgGs play an important role both in the neutralization of viral antigens *via* the fragment antigen binding (Fab) portion, and in mediating effector functions *via* its fragment crystallizable (Fc) moiety (14). The effector functions of IgG are modulated by its *N*-glycosylation. IgGs contain two conserved *N*-glycosylation sites in the Fc region at Asn-297 in each of the constant heavy chain 2 domains. At these sites oligosaccharides (glycans) are attached that are made up of a pentasaccharide core of two *N*-acetylglucosamine (GlcNAc) moieties and three mannose residues. This core can be modified by the addition of a fucose residue (fucosylation), a bisecting GlcNAc residue (bisection) and elongated with up to two antennae, each consisting of a GlcNAc and optionally a galactose (galactosylation), of which the latter may be terminated by a sialic acid (sialylation). These Fc region-associated *N*-glycans affect the affinity of the antibody to its cognate receptors on immune cells and consequently modulate the immune response (14, 15). For example, IgG1 with an afucosylated *N*-glycan attached to its Fc domain has, compared to fucosylated IgG, a greatly increased affinity to the Fcγ-receptor IIIa (FcγRIIIa), which regulates antibody-dependent cell-mediated cytotoxicity (ADCC) (16, 17). IgG glycosylation has thus been under investigation for its potential role in COVID-19 and its potential as an early severity marker (17).

Several studies have found that severe COVID-19 is associated with SARS-CoV-2 spike protein-specific (anti-S) IgG afucosylation (18, 19). For example, it has been shown that afucosylated anti-S IgG enriched from sera of severe COVID-19 patients stimulates pro-inflammatory cytokine production by alveolar-like macrophages *in vitro* (11). Furthermore, in an *in vivo* model, afucosylated IgG immune complexes from COVID-19 patients have been shown to induce inflammation and infiltration of the lungs by immune cells (20). A similar afucosylated pathogen-specific antibody response has likewise been observed in Dengue virus infection, malaria and HIV, as well as in alloimmune settings (21–24). A hypothesis has been proposed that the expression of foreign antigens on host or viral membranes triggers such afucosylated IgG responses (18). Interestingly, SARS-CoV-2 mRNA vaccination of SARS-CoV-2 naïve individuals also induced a transient afucosylated IgG response, yet to a lesser extent than in severely ill COVID-19 patients (25). Another study found anti-S IgG1 to be highly fucosylated and enriched in sialylation following mRNA vaccination against SARS-CoV-2, albeit this group has neither investigated early timepoints nor longitudinal changes (20).

Besides these patterns of anti-S IgG glycosylation, characteristic total IgG glycosylation signatures have been observed as well. For example, severe COVID-19 patients have been shown to display decreased bisection of the total IgG *N*-glycome, compared to mild inpatients (26). Additionally, a case-control study has shown that COVID-19 patients had decreased levels of total IgG fucosylation, sialylation and galactosylation compared to controls (27). Moreover, low levels of total IgG galactosylation and sialylation at diagnosis of SARS-CoV-2 infection have been shown to be predictive of poor prognosis (28).

In a previous longitudinal, prospective observational cohort study of hospitalized COVID-19 patients (29), we found skewed glycosylation patterns characterized by increased bisection and decreased galactosylation and sialylation of anti-S IgG1 (normalized to total IgG1) to be associated with increased COVID-19 severity as well as with markers of inflammation, both at hospitalization and at highest disease severity.

Here, we explored IgG1 glycosylation in two geographically distinct cohorts including outpatients and negative controls through affinity purification of IgG from plasma samples and tryptic digestion followed by liquid chromatography mass spectrometry (LC-MS) analysis. We were able to confirm and expand on previous findings regarding both total and anti-S IgG1 glycosylation in COVID-19 patients in two cohorts that were diverse with regards to geographical origin (Brazil and Germany), days since symptom onset and disease severity. Both total and anti-S IgG1 Fc glycosylation were correlated to inflammatory markers. After correction for known confounders of IgG1 glycosylation using logistic regression analysis, we found that decreased total IgG1 bisection and galactosylation as well as decreased anti-S IgG1 fucosylation and bisection were associated with hospitalization.

Materials and methods

Study cohorts

Samples from COVID-19 patients with varying disease severity as well as controls included in this study originated from Tübingen (Germany) and São Paulo (Brazil). The Tübingen cohort consisted of 12 inpatients (COV-HCQ, ClinicalTrials ID: NCT04342221), 10 outpatients (COMIHY, ClinicalTrials ID: NCT04340544) and 107 outpatients that were sampled at a late timepoint (TüCoV) (Table 1). In the COV-HCQ and COMIHY cohorts SARS-CoV-2 infection was confirmed using reverse transcription polymerase chain reaction (RT-PCR) tests, while in the TüCoV patients infection was confirmed with either an RT-PCR test or an enzyme-linked immunosorbent assay (ELISA) against anti-S antibodies. The São Paulo cohort was made up of 73 inpatients (of which 68 were treated at Hospital Santa Casa de Misericórdia and 5 at Hospital São Paulo), 20 outpatients, 70 convalescent patients (post-hospitalization) from the AEROBICOVID project (30) and 87 SARS-CoV-2 negative control subjects (Table 1). In most (>85%) of the participants from the São Paulo cohort SARS-CoV-2 infection was confirmed using RT-PCR tests (Biomol OneStep Kit/COVID-19-Instituto de Molecular Biology of Paraná-IBMP Curitiba/PR, Brazil). In the remaining participants serology-specific IgM and IgG antibodies tests (SARS-CoV-2 antibody test®, Guangzhou Wondfo Biotech Co., Ltd., Guangzhou, China) or immunochromatographic tests were used. All clinical trials were performed according to the principles of the Declaration of Helsinki. Ethical clearance was obtained from the Ethical Committee of the University of Tübingen and the units of the University of São Paulo involved in this study. Written informed consent was obtained for trial participation.

Chemicals, reagents and enzymes

Disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, sodium chloride and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany). From Sigma-Aldrich (Steinheim, Germany) ammonium bicarbonate, formic acid, potassium chloride and tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin from bovine pancreas were purchased. The Visucon-F pooled healthy human plasma standard was obtained from Affinity Biologicals (Ancaster, Canada). HPLC-supra-gradient acetonitrile originated from Biosolve (Valkenswaard, The Netherlands). From GE Healthcare (Uppsala, Sweden) protein G Sepharose 4 Fast Flow beads were purchased. Recombinant trimerized S protein was prepared as described previously (31). An ELGA Purelab Ultra system (Elga LabWater, High Wycombe, United Kingdom) was used to produce type I Ultrapure Water that was used in solutions throughout.

TABLE 1 Patient characteristics. The patient characteristics can be viewed separately for each cohort in supplementary materials (Tables S2, S3).

	Negative controls (n = 87)	Convalescent patients* (n = 70)	Outpatients (n = 30)	Inpatients (n = 85)	Outpatients (late timepoints) (n = 107)
Age *median (1st quartile - 3rd quartile)*	35 (28 - 46)	50 (41 - 55)	44 (33 - 52)	62 (55 - 76)	31 (24 - 53)
BMI *median (1st quartile - 3rd quartile)*	26 (23 - 28)	30 (27 - 33)	27 (25 - 30)	28 (25 - 32)	24 (22 - 27)
Days since onset of symptoms *median (1st quartile - 3rd quartile)*	–	–	15 (10 - 19)	11 (8 - 15)	121 (106 - 141)
Date of sample collection *min - max*	04/05/2020 - 07/08/2020	–	29/04/2020 - 25/06/2020	08/04/2020 - 21/01/2021	28/05/2020 - 04/09/2020
Male *n (%)*	35 (40%)	26 (37%)	15 (50%)	52 (61%)	41 (38%)
Female *n (%)*	52 (60%)	44 (63%)	15 (50%)	33 (39%)	66 (62%)
São Paulo *n (%)*	87 (100%)	70 (100%)	20 (67%)	73 (86%)	0 (0%)
Tübingen *n (%)*	0 (0%)	0 (0%)	10 (33%)	12 (14%)	107 (100%)

*Convalescent patients are patients that had been hospitalized, but at the time of sample collection were recovering at home.

Sample preparation

Anti-S IgG was captured through affinity purification with recombinant trimerized S protein-coated Maxisorp NUNC-Immuno plates (Thermo Fisher Scientific, Roskilde, Denmark) (18), while total IgG was enriched using protein G Sepharose Fast Flow 4 beads (32). A 100 mM formic acid solution was used for antibody elution, followed by sample drying through vacuum centrifugation and reconstitution in 25 mM ammonium bicarbonate. The purified antibodies were subjected to tryptic digestion to obtain glycopeptides, as described previously (29, 32). For the samples from Brazil, a minimum of 2 Visucon-F standards, 4 pooled anti-S IgG samples and 2 blanks were included per plate. For the German samples at least 1 Visucon-F standard and 1 blank was included on each plate.

IgG Fc glycosylation analysis

The obtained glycopeptides were detected with an Impact HD quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA) following separation using an Ultimate 3000 high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA), as described (29, 32). IgG1 glycoforms were assigned on the basis of accurate mass and specific migration positions in liquid chromatography. Other glycoforms were excluded from analysis to circumvent interference of IgG3- with IgG2- and IgG4-glycopeptides due to the potential overlap in the amino acid sequences of allotypic variants (14).

Cytokine quantification

All cytokines (IL-6, IL-8, IL-1b, IL-10, and TNF) were quantified in heparinized plasma using the BD Cytometric Bead Array Human Inflammatory Kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, after sample processing, the cytokine beads were counted using a flow cytometer (FACSCanto II; BD Biosciences, San Jose, CA), and analyses were performed using FCAP Array (3.0) software (BD Biosciences). The concentrations of cytokines were expressed as pg/ml. Cytokines were measured in samples collected at the same timepoint as the samples used in this study for determining IgG1 Fc *N*-glycosylation profiles and infection status for SARS-CoV-2.

Data processing

Liquid chromatography-mass spectrometry (LC-MS) data were converted into mzXML files. Alignment and targeted extraction of the raw data was performed using the in-house developed software LacyTools (33). LacyTools was first used to align the runs based on the average retention time of a minimum of three abundant IgG1 glycoforms, and second, to perform targeted data extraction. The extraction list consisted of analytes in 2^+ and 3^+ charge states (Table S1) (29). Repeatability was assessed by measuring a pre-COVID-19 plasma pool (Visucon-F) and, in the case of the Brazilian samples, pooled anti-S IgG samples from patients present in replicates on each plate. Spectra were excluded from further analysis if their sum intensity was below the average sum intensity plus three times the standard deviation of the anti-S IgG1 signal of negative controls. Signals were integrated by covering a minimum of 95% of the area of the isotopic envelope of glycopeptide peaks. Isotopic peaks of a glycopeptide that may have overlapped with contaminants were excluded from integration (Table S1).

Statistical analysis

Total area normalization of IgG1 Fc glycopeptides was applied to calculate the relative abundance of each glycoform. The relative abundances of related glycopeptide species were summarized for calculating the glycosylation traits fucosylation, bisection, galactosylation and sialylation, as described previously (29). To compare the anti-S and total IgG1 Fc glycosylation profiles Wilcoxon signed-rank tests were performed (Figure 1). Spearman's correlations were computed to assess the effects of the days since onset of symptoms and of body mass index (BMI) on IgG1 glycosylation and to explore correlations with inflammatory markers (Figures 3, S3, 6, S6). The comparisons between different biological groups for both total and anti-S IgG1 glycosylation were performed using logistic regression (Figures 4, 5, S4, S5). For each glycosylation trait a model was built to predict for example whether a patient was hospitalized or not. Age, sex, BMI, the cohort and the interaction between age and sex were included as covariates in all the logistic regression models to adjust for potential confounding effects, whereas the days since symptom onset was included when applicable (Figures 5, S4, S5). The *p*-values of the coefficients corresponding to the glycosylation traits were used to determine whether that particular glycosylation trait was a significant predictor of for example hospitalization and were shown as significance levels in Figures 4, 5, S4 and S5. Odd ratios with 95% confidence intervals can be found in Tables S6, S7. To explore the confounding effect of the cohort on IgG1 glycosylation Wilcoxon rank sum tests were performed (Figure S1).

Results

In this study, anti-S and total IgG1 Fc glycosylation profiles were characterized of 163 COVID-19 infected patients from a Brazilian and 129 COVID-19 patients from a German cohort. Both cohorts comprised hospitalized (inpatients), convalescent

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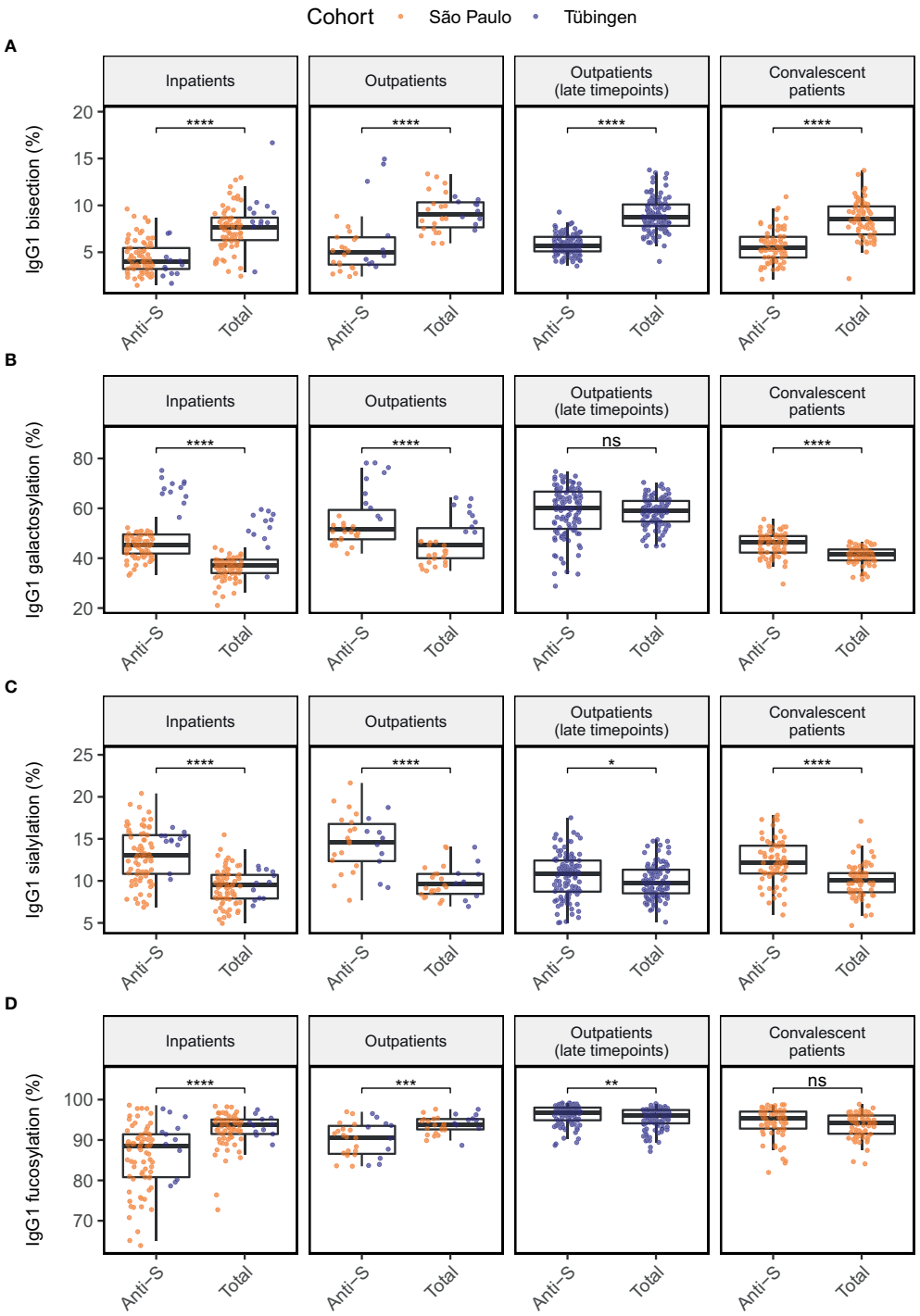

FIGURE 1
Comparison of anti-S to total IgG1 glycosylation in four patients groups for glycosylation traits bisection **(A)**, galactosylation **(B)**, sialylation **(C)** and fucosylation **(D)**. Significance levels shown are based on the *p*-values from Wilcoxon signed-rank tests. *, **, ***, ****: *p*-value < 0.05, 0.01, 0.001, 0.0001, respectively and ns, not significant (*p*-value ≥ 0.05).

as well as non-hospitalized patients (outpatients). In addition, 87 negative control subjects were included in the Brazilian cohort. The characteristics of the study groups are summarized in Table 1.

Anti-S IgG1 glycosylation differs from total IgG1 glycosylation

To explore how the obtained results align with previous studies, we first compared anti-S IgG1 glycosylation of the patient groups to their total IgG1 glycosylation in a paired manner (Figure 1). In all patient groups, glycosylation of anti-S IgG1 was skewed towards low bisection (Figure 1A), high galactosylation (Figure 1B) and high sialylation (Figure 1C) when compared to total IgG1. Anti-S IgG1 was skewed towards low fucosylation in outpatients and in inpatients. In contrast, slightly increased fucosylation characterized the late timepoints of outpatients (Figure 1D). In addition, anti-S IgG1 galactosylation at the late timepoints of outpatients (days since onset of symptoms >= 41, median = 121) was similar to total IgG1 galactosylation (Figure 1B). No significant difference was found between the anti-S and total IgG1 fucosylation profiles of convalescent patients.

Interestingly, the level of IgG1 galactosylation seemed to be higher overall in patients from the German cohort compared to those from the Brazilian cohort (Figure 1B). Upon further examination, we found that there was a significant difference in both anti-S and total IgG1 galactosylation, as well as in total IgG1 bisection and anti-S IgG1 fucosylation between the Brazilian and German patients (Figure S1). Because of these observations, we decided to adjust our later analyses not only for the confounding effects of age, sex and BMI, but also for the cohort.

Anti-S IgG1 glycosylation is dynamic

Various studies have described IgG glycosylation to be dynamic over the course of COVID-19 (11, 18, 29), suggesting that the days since symptom onset could be an important factor in the association of IgG glycosylation with disease severity. Therefore, we studied longitudinal samples of 35 patients to assess the dynamics of IgG1 glycosylation over the COVID-19 disease course. Anti-S IgG1 fucosylation appeared to increase with time (Figure 2A). In contrast, total IgG1 glycosylation did not appear to be dynamic over the disease course in these cohorts (Figures 2A–D).

To further explore the potential confounding effect of the sampling day we assessed the Spearman's correlations between the days since onset of symptoms and the levels of glycosylation traits in baseline samples (Figure 3). In the inpatients and outpatients combined, anti-S IgG1 fucosylation was positively correlated to the days since onset of symptoms (Figure 3A). In the outpatients a positive correlation was found between the days since onset of symptoms and total IgG1 fucosylation (Figure 3A). Therefore, we concluded that the days since onset of symptoms is an additional confounder of IgG1 fucosylation and therefore decided to include it as an additional covariate for later analyses.

IgG1 glycosylation profiles differ between patient groups and negative controls

Total IgG1 Fc glycosylation profiles of both inpatients and outpatients were compared to those of negative controls through logistic regression analysis. For each glycosylation trait three logistic regression models were built. The first two models predicted the probability of a patient being an outpatient or a hospitalized patient, respectively, rather than a negative control. The third model served to predict the probability of a patient being a hospitalized patient rather than an outpatient. In each model the patients' age, sex, BMI and the cohort were included as covariates to account for possible confounding effects. Increased BMI is known to be associated with decreased IgG galactosylation (34), which was reflected in these cohorts (Figure S3). The differences in the levels of the various glycosylation traits between the patient groups and negative controls are visualized in Figure 4. The significance levels shown are based on the *p*-values of the glycosylation traits' coefficients in the regression models (Table S6). These *p*-values give an indication of whether the glycosylation trait has predictive value of the patient group, while taking confounding factors of glycosylation into account. Inpatients were characterized by decreased total IgG1 bisection (Figure 4A) and galactosylation (Figure 4B) as compared to outpatients and negative controls. In contrast, outpatients were not significantly different from negative controls with regards to their total IgG1 glycosylation (Figures 4A–D).

Next, anti-S IgG1 glycosylation of inpatients was compared to that of outpatients (Figure 5 and Table S7). The levels of anti-S IgG1 fucosylation (Figure 5A) and bisection (Figure 5B) were slightly decreased in inpatients compared to outpatients. Additionally, anti-S IgG1 glycosylation was compared between patients that had been admitted to an intensive care unit (ICU) and patients that had not been admitted to an ICU (non-ICU) (Figure S5). However, no difference was found between the anti-S IgG1 glycosylation of ICU and non-ICU patients in this study.

Associations between IgG1 glycosylation and cytokine levels

The concentrations of various cytokines including several interleukins (ILs) and tumor necrosis factor alpha (TNFα) at baseline were available for 57 inpatients and 20 outpatients from the Brazilian cohort. We studied the associations between IgG1 Fc glycosylation and cytokine levels using Spearman's

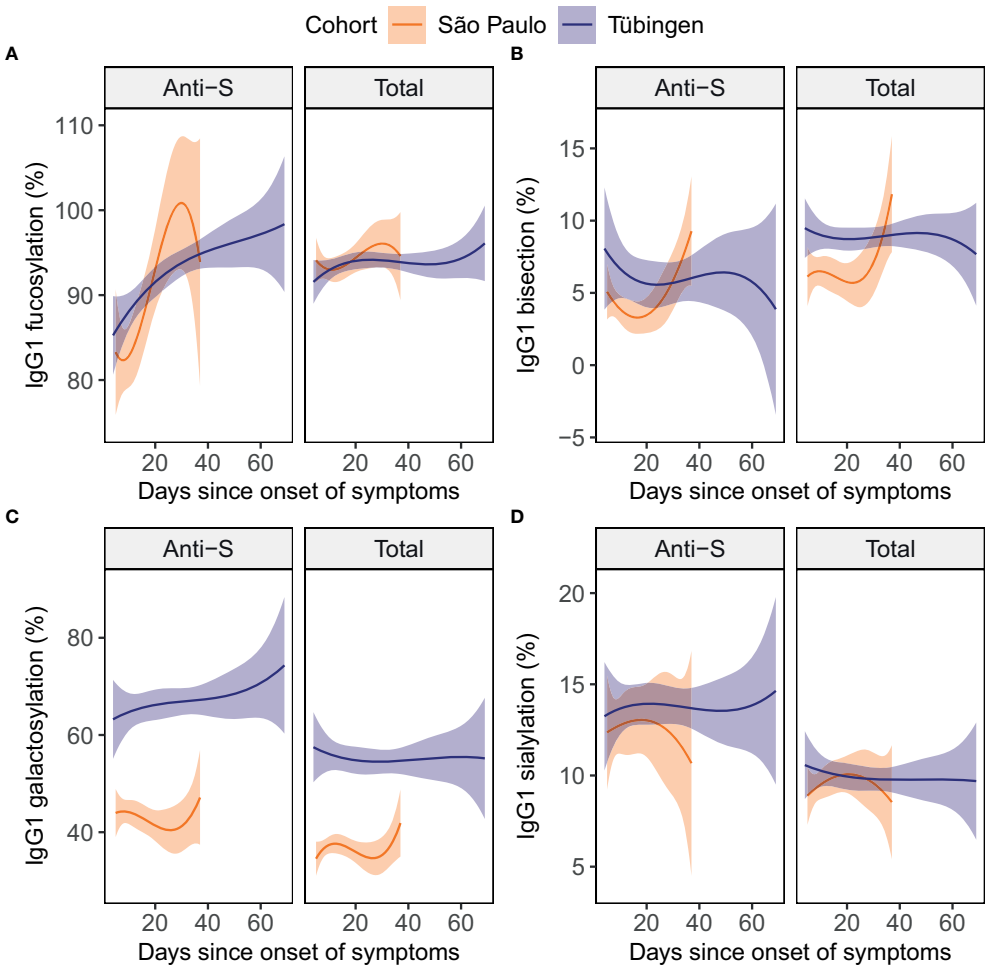

FIGURE 2
Total and anti-S IgG1 glycosylation in patients over time. For the glycosylation traits fucosylation **(A)**, bisection **(B)**, galactosylation **(C)** and sialylation **(D)** both anti-S (left) and total (right) IgG1 glycosylation are plotted against the days since onset of symptoms. To illustrate the dynamics in each cohort, cubic polynomial curves fitted to the data are shown as lines with 95% confidence intervals shown in orange and purple for the São Paulo (15 inpatients) and the Tübingen (10 inpatients and 10 outpatients) cohort, respectively. Datapoints per individual patient can be viewed in supplementary Figure S2.

correlations (Figures 6, S6; Table S8). The concentration of the proinflammatory cytokine IL-6 was negatively correlated to anti-S and total IgG1 galactosylation and sialylation as well as to total IgG1 bisection. The cytokine IL-8 showed a similar pattern of correlations to that of IL-6, except for a lack of significant correlation with anti-S IgG1 sialylation. In addition, the concentration of the proinflammatory cytokine IL-1β correlated negatively with anti-S IgG1 galactosylation.

Discussion

In this study we determined both the total and anti-S IgG1 Fc glycosylation profiles of 163 Brazilian and 129 German COVID-19 patients. This cohort represents the full spectrum of COVID-19 disease severities, comprising inpatients with varying disease severity, large groups of outpatients at different times during and after their illness as well as convalescent patients. The reliability of our results was bolstered by adjusting for known confounders of glycosylation including age, sex (and their interaction) and BMI, as well as for the cohort (country of residence) and time since symptom onset, of which the latter was found to be a major confounder of anti-S IgG1 glycosylation in line with a previous study (29). Of note, adjustment for these confounding factors has been lacking in most preceding studies (18–20, 26, 27), despite their strong association with glycosylation. We observed that anti-S IgG1 glycosylation profiles diverged from those of total IgG1 in all

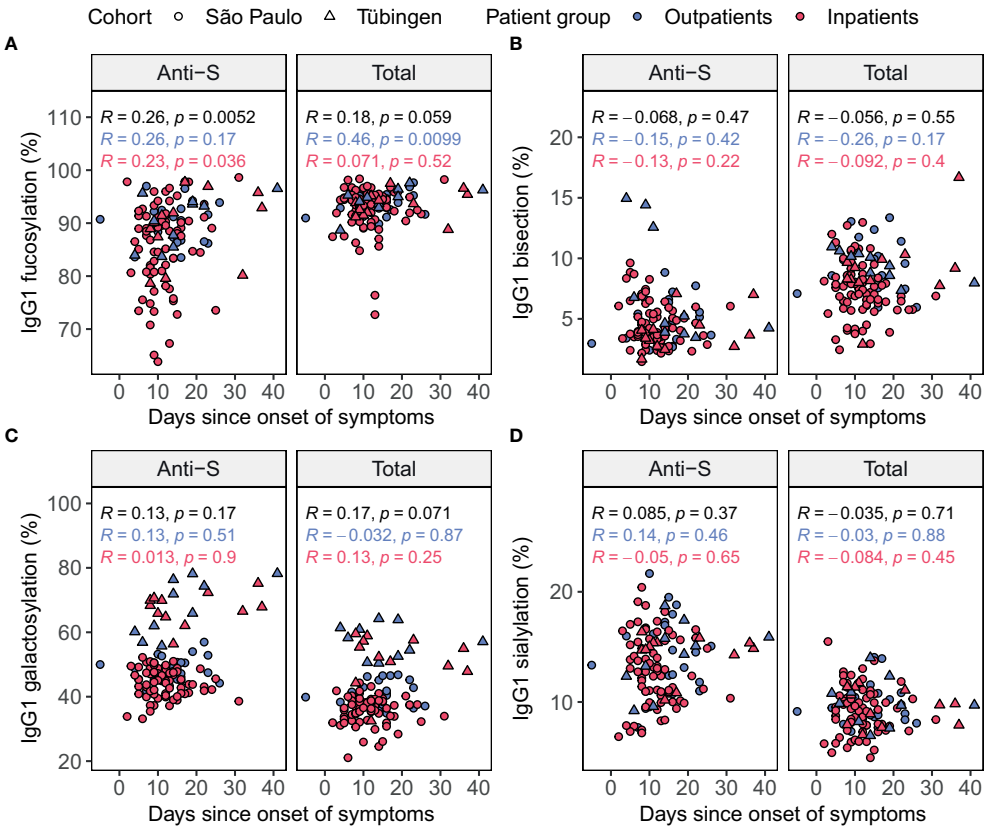

FIGURE 3
Spearman's correlation of days since symptom onset and IgG1 glycosylation in patients at home (blue) and inpatients (red) in each cohort. Glycosylation traits fucosylation **(A)**, bisection **(B)**, galactosylation **(C)** and sialylation **(D)** were plotted both for anti-S (left) and total (right) IgG1 Fc glycosylation against the days since onset of symptoms. Brazilian patients are shown as circles, while German patients are shown as triangles. Spearman's correlation coefficients (*R*) are shown with corresponding *p*-values both separately for the patients at home (blue) and inpatients (red) and for all patients combined (black).

patient groups, largely in line with previous reports (18, 29). We found that both the anti-S and total IgG1 glycome of COVID-19 infected individuals reflected disease course and severity.

The observed differences in anti-S IgG1 glycosylation compared to total IgG1 glycosylation were similar to what has previously been reported for inpatients (18, 29) and for a small cohort of outpatients (18). Moreover, we found comparable differences in convalescent patients, except regarding fucosylation, which remained unchanged. Similar to previous reports, we found anti-S IgG1 skewing towards low fucosylation in inpatients to be transient (18, 29). This temporary nature is underlined by the absence of skewing of anti-S IgG1 towards afucosylation in convalescent patients, suggesting that the low levels of anti-S IgG1 fucosylation act as an early inflammatory signal in the transition of mild-to-severe COVID-19 (18, 20). Indeed, we found that anti-S IgG1 afucosylation was significantly increased in inpatients relative to outpatients. Anti-S IgG1 afucosylation has been associated with COVID-19 severity in a number of studies (11, 18–20, 29) and reports using both *in vitro* (11) and *in vivo* (20) experiments have shown that afucosylated anti-S IgG can stimulate pro-inflammatory cytokine production. However, in line with our previous report on anti-S IgG1 Fc glycosylation (29), we observed no negative correlations between anti-S IgG1 fucosylation and inflammatory markers in patients' plasma, which may merely be due to the fact that fucosylation of the studied circulatory anti-S IgG1 does not represent the fucosylation of IgG in lung tissues in the form of immune complexes with the S protein, that potentially evoke inflammation at an earlier stage of the disease. In contrast to Hou *et al.* (27), we observed no increase in total IgG1 fucosylation in COVID-19 patients compared to controls, nor did we observe an association between total IgG1 fucosylation and disease severity. Alterations of total IgG glycosylation profiles are largely influenced by anti-S levels, a potential additional source of biological variation that may contribute to this contrasting observation in our study.

When compared to outpatients, inpatients were characterized by decreased anti-S and total IgG1 bisection,

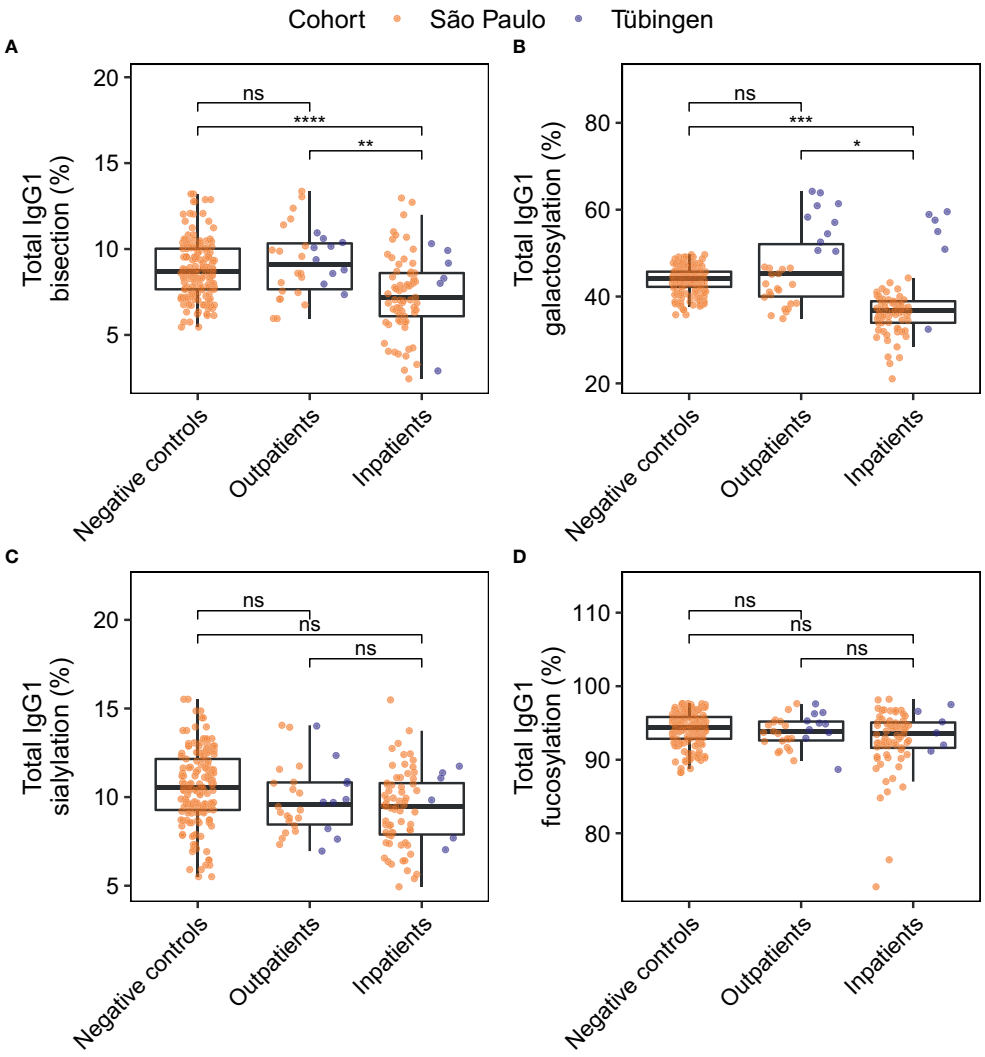

FIGURE 4
Comparison of total IgG1 glycosylation between patient groups. For the derived glycosylation traits bisection **(A)**, galactosylation **(B)** and sialylation **(C)** and fucosylation **(D)** total IgG1 glycosylation was compared between negative controls (n = 81) and patients at home (n = 30), and between negative controls and inpatients (n = 73). Study participants of whom the BMI was unknown were not included in this analysis. The significance levels shown are based on the *p*-values of the coefficients for the glycosylation traits in the logistic regression models with adjustment for the effects of age, sex, BMI, the cohort and the interaction between age and sex. *, **, ***, ****: *p*-value < 0.05, 0.01, 0.001, 0.0001 and ns: not significant (*p*-value ≥ 0.05). Differences in bisection and galactosylation between outpatients and inpatients remained significant after correction for days since disease onset (Figure S4).

in agreement with our previous study (29) and with Petrovíc et al., who reported that total IgG bisection was associated with COVID-19 severity (26). Additionally, in a longitudinal observational study total IgG bisection decreased over the COVID-19 disease course in severe, but not in mild and asymptomatic patients (35). A recent study has indicated that bisection may increase the affinity of monomeric IgG to FcγRIIIa (36), albeit little is known about the functional implications of bisection of IgG antibodies *in vivo*.

Contrasting findings surround the functional effect of IgG galactosylation. Decreased IgG galactosylation has been associated with many autoimmune and infectious diseases and has been linked to inflammation *via* several mechanisms (37). In addition, agalactosylated IgG has been suggested to play a role in the activation of complement *via* the alternative pathway and the mannose-binding lectin pathway (37). On the other hand, galactosylation of IgG1 immune complexes has been described to stimulate dectin-1-mediated signaling that leads to phosphorylation of FcγRIIb which inhibits the pro-

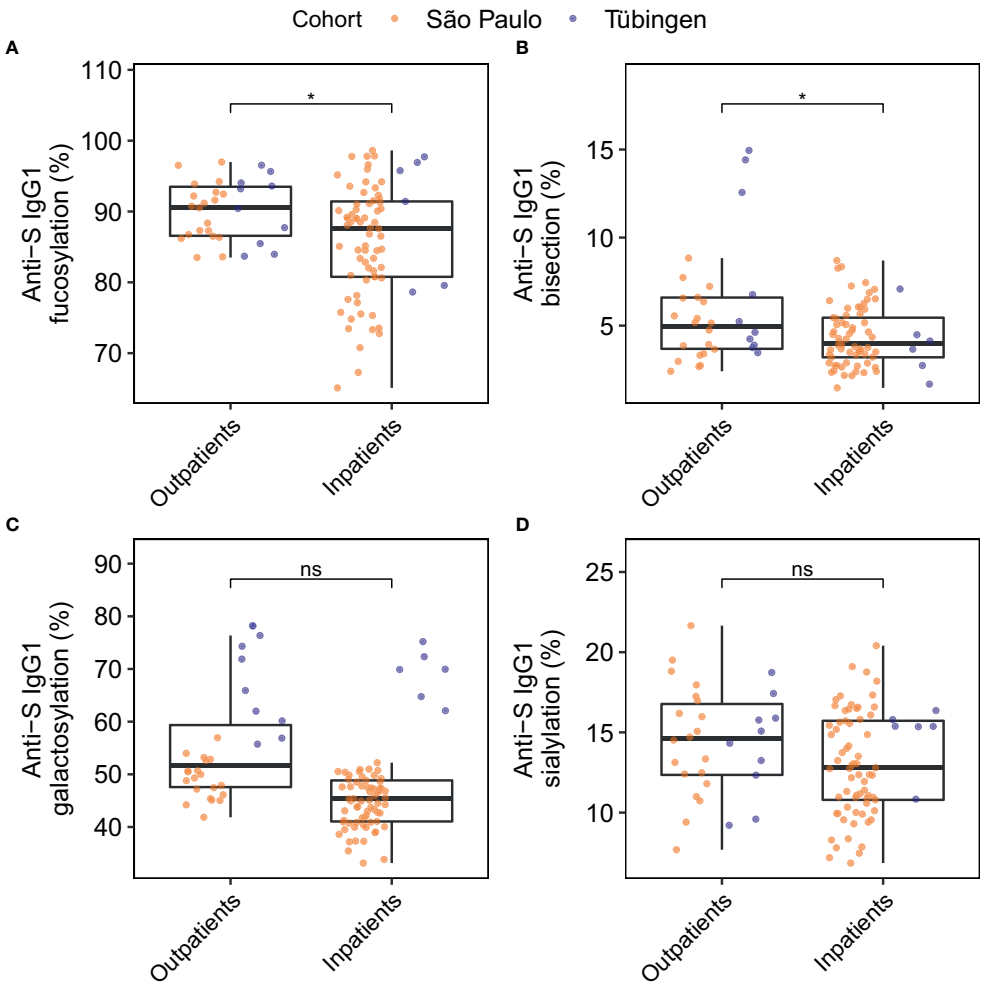

FIGURE 5
Comparison of anti-S IgG1 glycosylation of inpatients and outpatients for the glycosylation traits fucosylation **(A)**, bisection **(B)**, galactosylation **(C)** and sialylation **(D)**. The significance levels shown are based on the *p*-values of the coefficients for the glycosylation traits in the logistic regression models with adjustment for the effects of age, sex, BMI, the days since onset of symptoms, the cohort and the interaction between age and sex. Study participants of whom the BMI was unknown were not included in this analysis (number of inpatients = 73, number of outpatients = 30). **p*-value < 0.05 and ns, not-significant (*p*-value ≥ 0.05).

inflammatory activation of complement (38). Contrary to this, it has recently been shown that galactosylation of IgG promotes hexamerization and thereby enhances complement activation (39). In the context of COVID-19, and in line with previous findings (27, 28), we found that hospitalized COVID-19 patients had decreased levels of total IgG1 galactosylation compared to control subjects and outpatients. Hou *et al.* reported decreased total IgG galactosylation in COVID-19 cases compared to controls (27). Moreover, Vicente *et al.* reported that decreased total IgG galactosylation at diagnosis indicates poor prognosis, and is accompanied by higher NK cell activation (28). These observations and the link between IgG galactosylation and inflammation suggest that decreased galactosylation of IgG may play a role in the inflammation observed in severe COVID-19. In contrast to previous studies (18, 29), we found no association between anti-S IgG1 galactosylation and COVID-19 severity. This discrepancy might partly be explained by our more thorough adjustment for confounding factors. Moreover, in contrast to Vicente *et al.*, we did not find a sialylation signature associated with COVID-19 severity or hospitalization of COVID-19 patients.

Cytokines and chemokines play an important role in inflammatory settings such as COVID-19. For example, IL-6 has been shown as an important marker of COVID-19 severity (7, 40). In addition, IL-8 is a chemoattractant for neutrophils and can induce the generation of neutrophil extracellular traps (NETs), which have been suggested to contribute to organ damage in severely ill COVID-19 patients (41). We observed

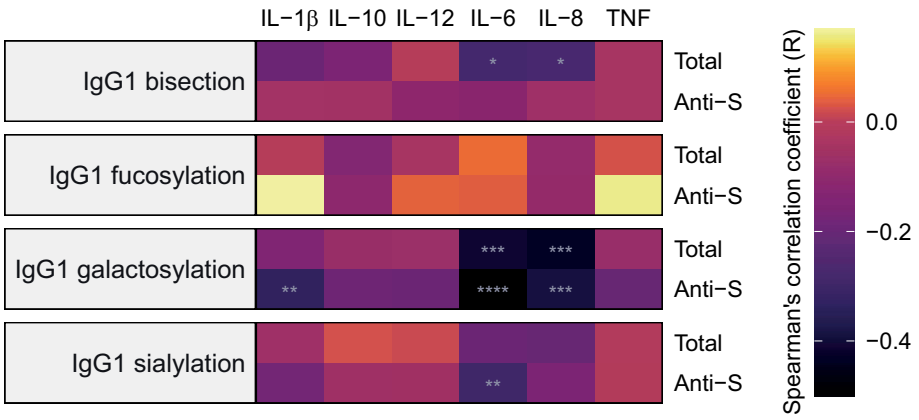

FIGURE 6
Heatmap of Spearman's correlations between inflammatory markers and IgG1 glycosylation traits at baseline. Anti-S IgG1 glycosylation is shown on the bottom part of each panel and total IgG1 glycosylation on the top for 57 inpatients and 20 outpatients from the São Paulo cohort. Cytokines were measured in samples collected at the same timepoint as the samples used for determining IgG1 Fc *N*-glycosylation profiles and infection status for SARS-CoV-2. The significance levels indicated with grey asterisks are based on the *p*-value of the Spearman's correlation coefficient (*R*). *, **, ****: *p*-value < 0.05, 0.01, 0.0001, respectively.

negative correlations between the pro-inflammatory markers IL-6, IL-8 and IL-1β and IgG1 glycosylation traits galactosylation, bisection and sialylation, in line with our previous study (29).

Glycosylation has been described to be influenced by both genetic and environmental factors (37). Accordingly, glycosylation of IgG varies between different populations, with the largest variations being in the level of galactosylation (42). For example, individuals living in developing countries were shown to have decreased IgG1 galactosylation, which has been associated with immune activation (43), indicating that environmental factors and immune activation may be another plausible cause for the differences in glycosylation, besides genetics (37, 42). Likewise, we observed differences between the Brazilian and German patients with regard to galactosylation, justifying the addition of the cohort as a covariate in our logistic regression model.

In conclusion, this study explored both total and anti-S IgG1 glycosylation profiles of inpatients and outpatients at various times during and after their COVID-19 disease course. Inpatients when compared to outpatients and SARS-CoV-2-negative control subjects were characterized by low total IgG1 galactosylation and bisection as well as low anti-S IgG1 fucosylation and bisection. Anti-S IgG1 glycosylation was dynamic over the disease course, in contrast to total IgG1 glycosylation, but both were correlated with markers of inflammation. This study included large cohorts from two continents, supporting the general validity of our results. Furthermore, we were able to replicate some of the previously reported IgG glycosylation patterns in COVID-19, which we believe to be a valuable step towards possible clinical translation, with the added value of thoroughly accounting for known confounders of both anti-S and total IgG1 glycosylation, while furthering our understanding of the potential role of IgG1 glycosylation in COVID-19 progression.

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Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

This study was reviewed and approved by the Ethical Committee of the University of Tübingen and the units of the University of São Paulo involved in this study. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SS: Data (pre-)processing, data curation, formal analysis, writing – original draft preparation, visualization. TP: Data (pre-) processing, data curation, formal analysis, writing – original draft preparation, supervision. WW and JN: Sample preparation and data generation, data quality control. PK, RF, IS: Study design, development of data analysis plan. AT: Study design, acquisition and development of data. PS-N: Acquisition of data and development of data analysis plan. ME, AK, JH: Study design. MW: Funding acquisition, coordination, supervision, data interpretation, writing - review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.993354/full#supplementary-material

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Breakthrough of glycobiology in the 21st century

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As modern medicine began to emerge at the turn of the 20th century, glycan-based therapies advanced. DNA- and protein-centered therapies became widely available. The research and development of structurally defined carbohydrates have led to new tools and methods that have sparked interest in the therapeutic applications of glycans. One of the latest omics disciplines to emerge in the contemporary post-genomics age is glycomics. In addition, to providing hope for patients and people with different health conditions through a deeper understanding of the mechanisms of common complex diseases, this new specialty in system sciences has much to offer to communities involved in the development of diagnostics and therapeutics in medicine and life sciences.This review focuses on recent developments that have pushed glycan-based therapies into the spotlight in medicine and the technologies powering these initiatives, which we can take as the most significant success of the 21st century.

KEYWORDS

glycobiology, glycan, carbohydrate, nanotechnology, drug development, vaccine

Glycobiology and glycomics

Over the past few decades, the use of computational modeling in the field of glycobiology has grown due to the rising popularity of glycobiology and glycomics (1). One of the newest omics disciplines to emerge in the contemporary post-genomics age is glycomics. In addition, to providing hope for patients and people with different health conditions through a deeper understanding of the mechanisms of common complex diseases, this new specialty in system sciences has much to offer to communities involved in the development of diagnostics and therapeutics in medicine and life sciences (2). Glycomics is a branch of glycobiology that focuses on defining the structure and function of glycans in living organisms. Glycobiology is the study that focuses on the structure, function, and biology of carbohydrates (1, 3). An emerging discipline is known as

"systems glycobiology (the impact of systems biology on the study of glycome)," which is hopeful given the current availability of advancing Wet-Lab techniques in the fields of glycobiology and glycomics (4).

A long chain of carbohydrate-based polymers called glycans is made up of repeating monosaccharide monomer units joined by glycosidic connections. All cells in nature appear to contain complex and varied glycans, which are crucial to all biological systems. In living things, glycans play physical, structural, and metabolic roles (4). The last century appeared to be a significant expansion in our understanding of the biochemistry and biology of proteins and nucleic acids (5, 6).

Genomics revolution and biotechnology

Scientific interest in understanding the characterization, function, and interaction of other essential biomolecules such as DNA transcripts, proteins, lipids, and glycans for the cell has grown due to the genomics revolution and the advent of high-throughput technologies (7). High-throughput technology enables the production of massive amounts of data for omic analysis, for example, genomics, transcriptomics, proteomics, phenomics, and metabolomics (8). At present, the growth of these technologies and their application go hand-in-hand with the growth of bioinformatics (9). Glycopeptide-based antibiotics (GBA) such as Vancomycin, Teicoplanin, Telavancin, Ramoplanin, and Decaplanin, including Corbomycin, Complestatin, and antitumor antibiotic Bleomycin, are another breakthrough invention of this century (10). Two blockbuster drugs, Acarbose (Bayer) and Heparin, including influenza treatment drugs Tamiflu (Oseltamivir, Roche) stand out as monosaccharide-based drugs that have been used therapeutically for a long time and saving the lives of people (11, 12).

Studies on glycobiology have grown as high-throughput technologies have advanced, allowing for fast cell screening. Additionally, more sophisticated analytical methods and data processing tools offer the chance to enhance high-throughput approaches for glycan screening as a disease marker and for categorizing glycan structure in therapeutic proteins (13). In addition, nanotechnology is a process of modifying matter at a size close to the atomic level to create a novel structure, materials, and devices. This technique offers advances in science across a wide range of industries, including manufacturing, consumer goods, energy, transportation, food safety, environmental science, and medicine, among many others (14).

Glyco-nanoparticles and nanotechnology

In addition, nanoparticles (NPs) are currently gaining a lot of attention due to their use in biology and medicine. The primary biological applications include the identification, estimation separation, purification, and characterization of biological molecules and cells, as well as the use of fluorescent biological labels, MRI contrast enhancement agents, pathogen and protein detection, DNA probing, tissue engineering applications, tumor targeting, targeted delivery of drugs, genes, and small molecules (15).

The development of powerful tools with diagnostic, therapeutic, and analytical applications through the use of nanotechnology has changed the approach of biomedical sciences and fight against human diseases (16). Millions of lives have been saved annually from vaccination, which is a success story in global health and development. More than 20 deadly diseases (such as Polio, Tetanus, Flu (influenza), Hepatitis B and Hepatitis A, Rubella, Hib, Measles, Whooping Cough, Pneumococcal, Rotavirus, Mumps, Chickenpox, Diphtheria, and off course end of 2021, COVID-19 and so on) can now be saved by vaccines, allowing individuals of all ages to live longer (17, 18). The milestone intervention has been created in medical history by developing the vaccine for cancer, such as the HPV (human papillomavirus) vaccine, to prevent cervical, vaginal, and vulvar cancer, anal cancer, and genital warts used for oral cancer. Likewise, the Hepatitis B vaccine treats existing liver cancer (also called therapeutic vaccine-immunotherapy) (12, 19).

Nanotechnology focuses on hybrid materials made of inorganic nanostructures and biomolecules (20–22). Synthetic scaffolds made of iron oxide, noble metal, and semiconductor nanoparticles have been used to multimerize glycans and increase their affinity for receptors. Hybrid material's physical features, such as magnetic and fluorescence, have led to applications in sensing, delivery, and imaging, e.g., ultraviolet-visible (UV-Vis) spectroscopy, infrared (IR) spectroscopy, elemental analysis, nuclear magnetic resonance (NMR), transmission electron microscopy (TEM), and X-ray photoelectron spectroscopy (XPS) (23). Likewise, as contrast agents for magnetic resonance imaging (MRI), magnetic nanoparticles (MNPs), such as iron oxide and manganese oxide nanoparticles (MONPs), are of particular interest. MRI uses a radio frequency (RF)-induced electromagnetic field to generate internal tomographic tissue pictures; modification of that field's signal by particles (called "contrast") allows their location to be noticed. Targeted magnetic, photodynamic, and gene therapy have all been used to battle cancer using nanocarriers based on heparin and heparin derivatives (24). Multifunctional gold NPs (nanoparticles) containing polysaccharide-functionalized gold NPs have been developed in various applications, including imaging, photodynamic treatment, and apoptosis activation of metastatic cells (24, 25). Likewise, heparin's capacity to inhibit blood clot formation has enabled significant medical advancements since world war-II, such as heart transplants, renal dialysis, and coronary artery dilations (angioplasties) (26).

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Glyco-science and therapeutics uses

In recent decades, various functions of glycans in biological systems have been discovered due to the growing glycoscience study. Numerous scientific fields, including immunology, development and differentiation, biopharmaceuticals, cancer, fertility, blood types, infectious illnesses, etc., have identified significant roles of glycans" (9). Glycan receptors are being targeted to treat viral diseases. The antiviral drugs Zanamivir (Relenza) and Oseltamivir (Tamiflu) are perhaps the most successful sugar-based medications these days. Likewise, carbohydrate-based antivirus medicines are Remedesivir, Molnupiravir, Azvudine, Entecavir, Telbivudine, Clevudine, Sofosbuvir, and Maribavir; those drugs are competitive neuraminidase ligands that bind to the enzyme and prevent the virus particle from being released from host cells (16, 27).

Due to their diversity, glycans have a wide range of biological functions and play essential roles in many physiological and pathological processes, including cell division, differentiation, and tumour formation (28, 29). Glycans are essential biomarker candidates for many diseases, including cardiovascular diseases, immune system deficits, genetically inherited disorders, various cancer types, and neurological diseases, carry information in biological system (30–33). During the onset and progression of these disorders, altered glycan expression is seen, brought on by improperly controlled enzymes, including glycosyltransferases and glycosidases. As a result, altered glycan structures may be helpful in the early detection of certain disorders. Glycans play an important role in illness diagnosis and management. Still, they can also be employed therapeutically as markers to identify and isolate particular cell types and as targets for developing new medications (3, 34).

Geoinformatics databases and glycosylation

Numerous biological processes, such as cell growth and development, tumour growth and metastasis, immunological detection and response, cell-to-cell communication, and microbial pathogenesis, are significantly influenced by glycosylation. Due to course, one of the most prevalent and critical posttranslational modifications of proteins is glycosylation (35, 36). Several factors can influence and modify glycosylation, including genetic determinants, monosaccharide nucleotide levels, cytokines, metabolites, hormones, and ecological factors (35–39). To get a large picture of the entire biological system, it is crucial to integrate omics methods such as proteomics, genomics, transcriptomics, and metabolomics into the field of glycobiology (35, 36, 40). Additionally, a wide variety of geoinformatics resources and databases are now available to investigate glycans and glycosylation pathways, which is also one breakthrough invention of the century (13).

Chromatography, diagnostic and therapeutic

Several methods have been developed and used in recent years to determine the structure of glycans to various degrees of detail (41). A conventional approach involves radioactively labelling the glycoconjugates, followed by enzymatic or chemical treatments, anionic exchange, gel filtration, or paper chromatographic analysis. Studies using nuclear magnetic resonance (NMR), gas chromatography with mass spectrometry (GC-MS), and other methods were carried out extensively. In recent years, simple chromatography methods have been replaced by High-performance anion-exchange chromatography (HPLC) and Ultra-performance liquid chromatography (UPLC), and fluorescence labelling has taken the role of radioactive labelling. Chromatography columns can be utilized in conjunction with the proper enzymatic/chemical treatments, for example, graphitized carbon, reversed-phase (RP), anion exchange, normal phase, or hydrophilic interaction resins (9, 42). The most precise and widely used separation and purification method is column chromatography. Column chromatography can be used to separate and purify both solid and liquid materials. The extraction of pesticides of animals origin (made up of lipids, waxes, and pigments) has been aided by column chromatography. The chromatography process is used in medicine to create the peptide hormone pramlintide (an analog of amylin), which is used to treat diabetes and many more (43).Various glycan detection technologies have shed light on the nature of several diseases, including COVID-19, diabetes, cancer, and congenital abnormalities (44–46).

Numerous diseases that afflict humans are treated, cured, or even prevented using information from DNA. Researchers have already worked on gene sequencing to find specific genes that cause diseases, allowing them to develop remedies. The development of biomedicine has been greatly aided by gene therapy. The health community and the general public believe the human genome draft sequencing will enable researchers to provide cures or at least effective therapies for all ailments (47).

A more detailed and exact understanding of skin ageing is made possible by the recent improvement in glycobiology, which has been made possible by cutting-edge technological advancements. The field of longevity and anti-aging has been revolutionizing by the anti-ageing healthcare technology (48, 49). Cutting-edge technology is the use of the latest and most advanced version of technology or applications that make function easy, cost-effective, reliable, and fast. Cutting-edge technology can be software that is also regarded as a "game

changer". The cloud, containers, AI (artificial intelligence), and machine learning are all considered cutting-edge technologies (50). Patient care services, chronic disease management, and patient health initiatives, including rapid virus testing during COVID-19, digital diagnostics, telehealth, drug delivery, vaccine development, skin grafting, cancer and diabetics treatment and modern, etc., are examples of applications (51, 52). Glycans are now undeniably proven to be essential skin elements and play a critical function in skin homeostasis. Glycans, which are essential for skin health, also change qualitatively and quantitatively as we age (53).

Conclusion

Since the beginning of modern medicine in the last century, the genomics revolution, geoinformatics database, biotechnology, and development in chromatology including the progress of glycan-based therapies have advanced rapidly. Research and development of structurally defined carbohydrates have led to the use of new tools and methods that have fueled interest in the therapeutic applications of glycans. DNA- and protein-centered therapies became widely used and progressed toward success. However, more precise targets for glycomimetics need to be found. Studying complex glycosylated structures, particularly glycoproteins, has advanced significant developments in synthetic procedures, analytical tools, and high-resolution biophysical approaches in glycobiology. Nanoparticles and other polyvalent structures have been developed to improve specially formulated glycopeptides' avidity and therapeutic potential, which can be considered the biggest success of the 21st century.

Author contributions

GM was responsible for the concept, design and draft of the manuscript. CT, XX contributed to the collect the information and reviewed this manuscript. JZ reviewed the final version of the manuscript and approved by all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Identification and validation of IgG N-glycosylation biomarkers of esophageal carcinoma

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Introduction: Altered Immunoglobulin G (IgG) N-glycosylation is associated with aging, inflammation, and diseases status, while its effect on esophageal squamous cell carcinoma (ESCC) remains unknown. As far as we know, this is the first study to explore and validate the association of IgG N-glycosylation and the carcinogenesis progression of ESCC, providing innovative biomarkers for the predictive identification and targeted prevention of ESCC.

Methods: In total, 496 individuals of ESCC (n=114), precancerosis (n=187) and controls (n=195) from the discovery population (n=348) and validation population (n=148) were recruited in the study. IgG N-glycosylation profile was analyzed and an ESCC-related glycan score was composed by a stepwise ordinal logistic model in the discovery population. The receiver operating characteristic (ROC) curve with the bootstrapping procedure was used to assess the performance of the glycan score.

Results: In the discovery population, the adjusted OR of GP20 (digalactosylated monosialylated biantennary with core and antennary fucose), IGP33 (the ratio of all fucosylated monosyalilated and disialylated structures), IGP44 (the proportion of high mannose glycan structures in total neutral IgG glycans), IGP58 (the percentage of all fucosylated structures in total neutral IgG glycans), IGP75 (the incidence of bisecting GlcNAc in all fucosylated digalactosylated structures in total neutral IgG glycans), and the glycan score are 4.03 (95% CI: 3.03-5.36, P<0.001), 0.69 (95% CI: 0.55-0.87, P<0.001), 0.56 (95% CI: 0.45-0.69, P<0.001), 0.52 (95% CI: 0.41-0.65, P<0.001), 7.17 (95% CI: 4.77-10.79, P<0.001), and 2.86 (95% CI: 2.33-3.53, P<0.001), respectively. Individuals in the highest tertile of the glycan score own an increased risk (OR: 11.41), compared with those in the lowest. The average multi-class AUC are 0.822 (95% CI: 0.786-0.849). Findings

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are verified in the validation population, with an average AUC of 0.807 (95% CI: 0.758-0.864).

Discussion: Our study demonstrated that IgG N-glycans and the proposed glycan score appear to be promising predictive markers for ESCC, contributing to the early prevention of esophageal cancer. From the perspective of biological mechanism, IgG fucosylation and mannosylation might involve in the carcinogenesis progression of ESCC, and provide potential therapeutic targets for personalized interventions of cancer progression.

KEYWORDS

glycomics, esophageal squamous cell carcinoma, immunoglobulin G, glycosylation, biomarkers

Introduction

Esophageal cancer (EC) is the seventh most common cancer type worldwide and ranks sixth in the cause of cancer-related death (1). In China, there have been an amount of estimated 0.25 million new cases of esophageal cancer and 0.19 million related deaths as of 2018, accounting for 43% and 37% of the global morbidity and mortality (2). The 5-year relative survival rate of the localized esophageal cancer at the point of confirmed diagnosis is 47%, while the rate declines to only 20% for all esophageal cancer patients (3). In addition, esophageal squamous cell carcinoma (ESCC) predominates subtype of esophageal cancer and is among the most aggressive forms of squamous cell carcinoma. ESCC belongs to the most deadly malignancy with late stage diagnosis, metastasis, therapy resistance and frequent recurrence (4).

Most patients of ESCC lack obvious symptoms at the early stage and progress insidiously to a relatively advanced stage when detected (5). Therefore, exploring the reliable biomarkers associated with early stage of ESCC is critical for improving the prognosis and life quality of patients, which fits with in the paradigm of predictive medicine. Esophagogastroduodenoscopy (EGD) is the main method for screening EC in the clinical practice, and it is of high cost, discomfortable and invasive. In addition, there are some serum biomarkers recommended for the assistant screening of EC, such as carcinoembryonic antigen (CEA), P53-Ab, Cytokeration fragment antigen21-1 (CYFRA21-1), squamous cell carcinoma antigen (SCC), protein kinase D1 (PRKD1), matrix metalloproteinase 2 (MMP-2), tissue inhibitor of metalloproteinases-2 (TIMP-2) and serum macrophage colony-stimulating factor (M-CSF). However, these tumor markers could alter in various tumor types, and even relate with the acute infection (6–8). Therefore, it is of great significance to identify novel biomarkers of high specificity and sensitivity for the early detection of ESCC, contributing to the early diagnosis and prevention of ESCC.

The glycomics analysis is a promising 'omics' technology (9), providing novel biomarkers for diseases diagnosis and prognosis, which could advance the personalized medicine and intervention strategy (10). Immunoglobulin G (IgG), as the most abundant immunoglobulin in blood, constitutes approximately 75% of the serum immunoglobulin proteins (11). IgG activates a series of effector pathways, such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) (12, 13), which are regulated by the N-linked glycosylation process at the Fc segment of IgG. N-glycosylation is one of the most common post-translational modifications of membrane and secretory proteins, with an important role in the biological processes, such as intercellular recognition, adhesion, communication and mutual interactions (14, 15). It plays an important role in the antibody functions and almost all the tumor markers approved by FDA are modified through glycosylation (16). The attached N-glycans on IgG are essential for the proper functional activity of the immune system. IgG N-glycosylation has been reported to be affected by the pathophysiological conditions, and thus associated with various diseases, such as the metabolic diseases (17–22), aging (23, 24), inflammatory and autoimmune diseases (25, 26) The profile of IgG N-glycans could alter its effector functions on tumor cells, and the variability of IgG N-glycosylation has also been identified in some tumor types (27–30).

Our previous study found that the IgG N-glycosylation profiles were independently associated with the esophageal precancerosis for squamous cell carcinoma beyond inflammation (31). However, the association of IgG N-glycosylation pattern with ESCC remains

Abbreviations: ESCC, esophageal squamous cell carcinoma; EGD, Esophagogastroduodenoscopy; CEA, carcinoembryonic antigen; CYFRA21-1, Cytokeration fragment antigen21-1; SCC, squamous cell carcinoma antigen; PRKD1, protein kinase D1; MMP-2, matrix metalloproteinase 2; IgG, Immunoglobulin G; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PBS, phosphatebuffered saline; UPLC, ultra-performance liquid chromatography; GP, glycan peaks; GlcNAc, *N*-acetylglucosamine; SD, standard deviation; FDR, false discovery rate; AIC, Akaike information criterion; ROC, receiver operating characteristic; AUC, area under-the curve.

unknown to date. In this study, we investigated the variation of IgG N-glycans in the stages of normal, precancerosis and early ESCC. We aimed to develop a predictive score using IgG N-glycans data to improve the risk stratification and management of ESCC.

Materials and methods

Study design and population

In total, 516 subjects voluntarily participated in this study and 496 individuals were finally recruited in the analysis according to the inclusion and exclusion criteria as shown in Figure 1. In 2018, 80 cases of early ESCC, 125 cases of precancerosis and 143 controls were enrolled from Feicheng People's Hospital (Feicheng City, Shandong Province). Meanwhile, data of 34 early ESCC patients, 62 precancerosis patients and 52 controls were collected as validation group from Gansu Wuwei Tumor Hospital (Wuwei City, Gansu Province). This two-center respective case control study umbrellaed under a national screening project, aiming at the early screening and diagnosis of ESCC and other gastrointestinal cancers as described previously (31). Before the endoscopic screening, the demographic information, dietary habit, lifestyle, history of gastrointestinal disease and family history of gastrointestinal cancer were surveyed through a standardized questionnaire (Supplementary Table S1). The blood samples were collected and stored at -80°C for the subsequent experiment.

The following were the inclusion criteria: (1) providing informed consent prior to enrollment; (2) initial confirmed diagnosis of precancerosis or ESCC, or neither; (3) the required information and data of IgG glycosylation profile eligible. The exclusion criteria were as follows: (1) diagnosis of other gastrointestinal cancer (gastric cancer or intestinal cancer) before or at the screening; (2) history of mental illness, infectious disease, autoimmune diseases or and other malignant cancers; (3) women in pregnancy or lactation; (4) post-operation or post-radiochemotherapy.

The study was approved by the independent ethics committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (grant number: 17–124/1380) and ethics committee of Capital Medical University (grant number: Z2019SY012). All participants provided their written informed consents before taking part in this study.

Outcome definition

The diagnosis of precancerous esophageal lesions and early ESCC was according to the endoscopic screening and biopsy examination, while the judgment of the controls was only based on the endoscopic diagnosis. In a previous article we described the procedures of routine endoscopy examination (31). The controls in this study were defined as oesophagitis or normal, while esophageal precancerosis were defined as mild or moderate atypical hyperplasia, and the early ESCC included severe atypical hyperplasia, mucosal and submucosal carcinomas.

Covariates

The body mass index (BMI) was defined as weight (in kilograms)/height^2 (in meters squared) and the participants were

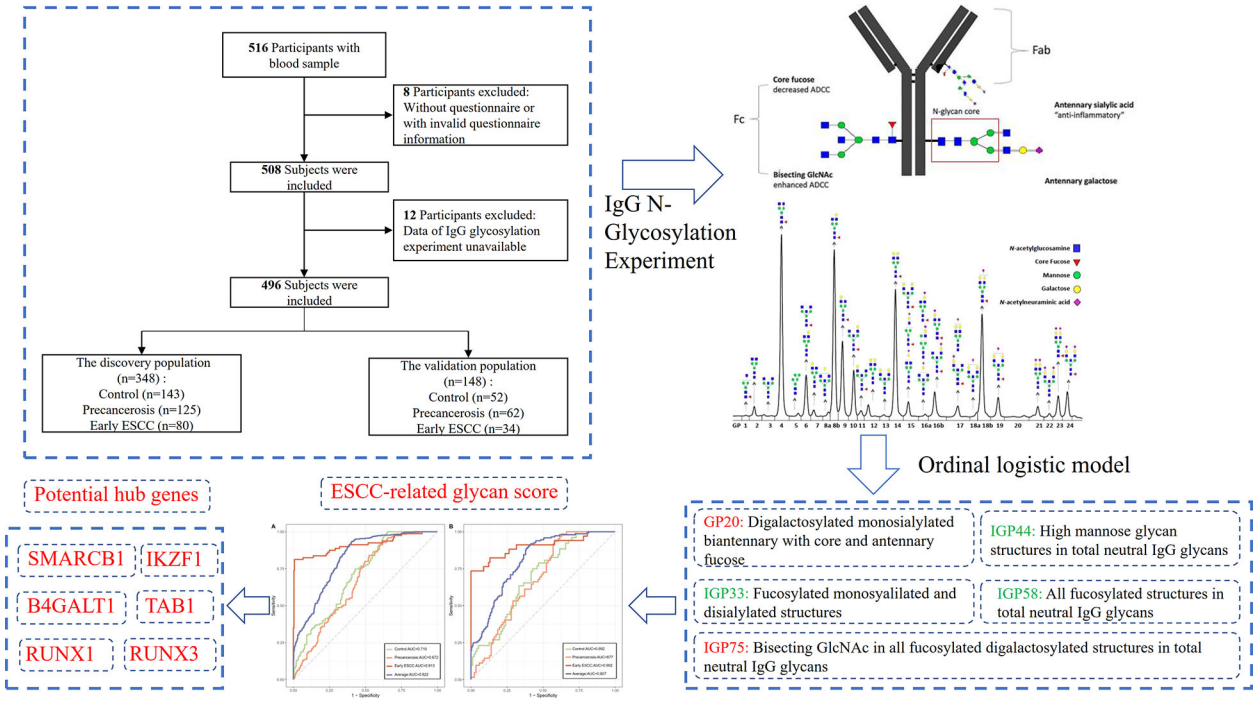

FIGURE 1
A schematic diagram of the study participants.

grouped into <24 kg/m^2 and ≥24 kg/m^2. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice on the right arm using a standard mercury sphygmomanometer in a sitting position after the subjects had rested at least 10 minutes, and the mean value was used for the present analysis. Hypertension was defined as a self-reported history of hypertension, a mean SBP ≥140 mmHg or DBP ≥90 mmHg or taking antihypertensive medications. Education level was classified into illiteracy, primary school, middle or high school, bachelor degree or above. Marriage status was defined as married status or others. Family income was divided into less than and more than 50,000 yuan per capita per year. Smoke was defined as at least one cigarette per day in the past year, while drink was defined as at least 100 ml consumption of alcohol (content ≥50%) per day in the past year. Dietary frequency of pickled food, fried food, hot food and mildew food were grouped into never, seldom and often. History of gastrointestinal disease involved gastroenteritis and peptic ulcer. Family history of gastrointestinal cancer included esophageal cancer, gastric cancer and intestinal cancer.

IgG N-glycosylation experiment

The glycosylation experiment and analysis involved four key processes: IgG isolation and purification from plasma, glycans enzyme digestion and release, fluorescence labeling and quantitative detection, as described previously (32, 33). In brief, IgG was isolated in a high-throughput manner, using 96-well protein G monolithic plates (BIA Separations, Slovenia), starting from 100 µl of plasma. Plasma was diluted 7× with phosphate buffered saline (PBS), applied to the protein G plate and washed. IgG was eluted with 1 ml of 0.1 M formic acid and immediately neutralized with 1 M ammonium bicarbonate. Then, the N-linked glycans were released by incubating at 37°C for 18-20 hours with 1.5 units of PNGase F. The released glycans were fluorescent labeled using 2-aminobenzamide at 65°C for 3 hours. After incubation samples were brought to 96% of acetonitrile (ACN) by adding 700 µl of 100% ACN and applied to each well of a 0.2 µm GHP filter plate. Solvent was removed by application of vacuum using a vacuum manifold. Loaded samples were subsequently washed 5× with 96% ACN. Fluorescently labelled N-glycans were separated by hydrophilic interaction chromatography on Acquity UPLC H-Class instrument (Waters, USA). Labelled N-glycans were separated on a Waters BEH Glycan chromatography column at 60°C, with 100 mM ammonium formate, pH 4.4, as solvent A and ACN as solvent B. Separation method used linear gradient of 75–62% acetonitrile at flow rate of 0.4 ml/min in a 27-min analytical run. Detect N-glycan fluorescence at excitation and emission wave lengths of 330 nm and 420 nm, respectively.

Finally, 24 direct glycan peaks (GPs) were quantitatively expressed with the percentage of the total integrated peak area, as presented in Supplementary Figure S1. In addition, 54 derived traits (IGPs) were derived to reflect the relative abundance of the specific structure, such as galactosylation, sialylation, bisecting *N*-acetylglucosamine (GlcNAc), core fucosylation and mannose. The amounts of GP and IGP were normalized followed by log transformation and batch-effect was considered and corrected. The detailed structural and biological information of each GP and IGP was shown in Supplementary Table S2.

Statistical analysis

Continuous variables adhering to the normal distribution were represented as mean and standard deviation (SD), and the differences between groups were tested by the independent ANOVA tests; otherwise, the median and interquartile range (P25, P75) were used, and the differences were explored by Kruskal-Wallis H tests. Categorical variables were presented as n (%), and the differences were tested by the chi-square tests. The box plots were used to show the differences of IgG GPs and IGPs among the controls, precancerosis and early ESCC.

The false discovery rate (FDR) correction was used to primarily identify the substantially increased or decreased IgG glycans and traits associated with ESCC. Then, the candidate glycans and traits selected above were finally confirmed using the stepwise ordinal logistics regression according to Akaike information criterion (AIC), which composed of an ESCC-related glycan score by the regression coefficients. The glycan score and its components were tested both in the discovery and validation population after the confounding covariates adjusted in three models: model 1 was unadjusted; model 2 was adjusted for age and sex; model 3 was further adjusted for BMI, hypertension, smoke, drink, education, income, marriage status and dietary habits. Formula of the ESCC-related glycan score was listed below:

Score = $\Sigma(\beta_n \times$ amounts of each IgG GP and IGP n), where β is the ordinal logistics coefficient.

The discriminative capacity of the proposed ESCC-related glycan score was illustrated using multi-class receiver operating characteristic (ROC) curve, and the average area under-the curve (AUC) value was provided. Significant differences in the proposed ESCC-related glycan score between different groups in the discovery and validation populations were subsequently assessed using DeLong's test. The robustness of the ESCC-related glycan score was assessed using a bootstrap procedure (k=100). The bootstrap method was used to resample distinct data sets 100 times from the original data set, and the number of subjects in each resampled data set was set to be the same number as the sample size of the original data set. SNPs associated with the proposed ESCC-related glycan score were found out by Meta-analysis of the IgG N-glycosylation GWAS and were annotated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway enrichment analysis were carried as well as protein–protein interaction (PPI) network analysis to find potential hub genes. Finally, we validated the potential hub genes on The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) based on RNA sequence data. Detailed statistical methods are provided in the Supplementary material online. All statistical tests were two-sided at a significant level of 0.05, and the Benjamini-Hochberg method was applied to control the FDR for multiple hypothesis tests (34). All the analyses presented above were performed using the packages of 'MASS', 'forestplot', 'multiROC' in R software (version 4.0.0).

Results

Characteristics

In the discovery population, the median (P25, P75) age was 58.50 (54.00, 63.00), and 163 (46.84%) were males. In the validation population, the median (P25, P75) age was 60.00 (56.00, 64.00), and 65 (43.92%) were males. The characteristics were similar between the discovery and validation populations, except age as shown in Supplementary Table S3. There were no significant differences in sex, education level, marriage status, household income, BMI, hypertension, history, family history, dietary habits among the controls, precancerosis and early ESCC groups both in the discovery and validation populations, apart from age, smoking and drinking. The detailed distributions of the characteristics were shown in Table 1. In addition, the dietary habits, including the frequency of having pickled food, fried food, hot food, and mildew food, were similar among the controls, precancerosis and ESCC groups both discovery and validation populations (Table 2).

Different IgG N-glycosylation patterns in ESCC, esophageal precancerosis, and controls

The detailed distribution of IgG glycans and traits among the controls, precancerous and early ESCC groups were shown in Supplementary Table S4. A total of 7 GPs (GP3, GP6, GP12, GP13, GP17, GP20, GP23) and 11 IGPs (IGP30, IGP36, IGP37, IGP38, IGP46, IGP51, IGP52, IGP57, IGP73, IGP75, IGP77) substantially increased in the carcinogenesis progression of ESCC (Supplementary Figure S2A), while GP5 and 14 IGPs (IGP31, IGP33, IGP34, IGP43, IGP44, IGP47, IGP55, IGP56, IGP58, IGP60, IGP61, IGP62, IGP63, IGP76) showed negative association (Supplementary Figure S2B). After stepwise ordinal logistics regression, GP20 and 4 IGPs (IGP33, IGP44, IGP58, IGP75) retained in the final model and the AIC declined from 749.56 to 531.73. The distribution of these GP and IGPs were presented in Figure 2. In both the discovery and validation populations, compared with the control group, GP20 and IGP75 were elevated ($P < 0.05$); whereas IGP33, IGP44, and IGP58 were

TABLE 1 Social-demographic characteristics in the discovery and validation populations.

	Discovery population (n=348)				Validation population (n=148)			
	Control (n=143)	Precancerosis (n=125)	Early ESCC (n=80)	P	Control (n=52)	Precancerosis (n=62)	Early ESCC (n=34)	P
Age (years)	57.00 (54.00,61.00)	57.00(53.00,63.00)	64.00(57.75,66.25)	<0.001	59.50 (56.75,62.00)	61.50 (56.25,64.00)	59.50(55.25,65.75)	0.456
Male, n (%)	62(43.36)	62(49.60)	39(48.75)	0.550	25(48.08)	28(45.16)	12(35.29)	0.489
Education level, n (%)				0.117				0.574
Illiteracy	28(19.58)	26(20.80)	20(25.00)		16(30.77)	13(20.97)	5(14.71)	
Primary school	41(28.67)	38(30.40)	22(27.50)		19(36.54)	22(35.48)	11(32.35)	
Middle or high school	55(38.46)	46(36.80)	37(46.25)		14(26.92)	21(33.87)	14(41.18)	
Bachelor degree or above	19(13.29)	15(12.00)	1(1.25)		3(5.77)	6(9.68)	4(11.76)	
Married, n (%)	135(94.41)	113(90.40)	75(93.75)	0.419	49(94.23)	53(85.48)	32(94.12)	0.278
Income ≥ ¥50,000, n (%)	41(28.67)	35(28.00)	16(20.00)	0.328	20(38.46)	20(32.26)	7(20.59)	0.218
BMI ≥ 24.0 kg/m^2, n (%)	74(51.75)	57(45.60)	35(43.75)	0.436	22(42.31)	24(38.71)	12(35.29)	0.805
Hypertension, n (%)	86(60.14)	86(68.80)	60(75.00)	0.064	32(61.54)	39(62.90)	28(82.35)	0.091
History *, n (%)	23(16.08)	17(13.60)	16(20.00)	0.477	7(13.46)	12(19.35)	6(17.65)	0.699
Family history *, n (%)	36(25.17)	34(27.20)	25(31.25)	0.620	11(21.15)	18(29.03)	9(26.47)	0.627
Smoke status, n (%)	60(41.96)	39(31.20)	25(31.25)	0.120	18(34.62)	19(30.65)	19(55.88)	0.043
Drink status, n (%)	44(30.77)	34(27.20)	24(30.00)	0.805	13(25.00)	12(19.35)	16(47.06)	0.013

Continuous variable is presented as the median (P_{25}, P_{75}) and examined by using Kruskal-Wallis H test; and categorical variables are presented as the number (percentage) and examined by using chi-square test.

* History refers to the gastroenteritis and peptic ulcer; Family history refers to esophageal cancer, gastric cancer and intestinal cancer.

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TABLE 2 The dietary habits among the controls, precancerosis and early ESCC groups in the discovery and validation populations.

	Discovery population (n=348)			Validation population (n=148)		
	Control (n=143)	Precancerosis (n=125)	Early ESCC (n=80)	Control (n=52)	Precancerosis (n=62)	Early ESCC (n=34)
Pickled, n (%)	P = 0.878			P = 0.382		
Never	67(46.85)	59(47.20)	34(42.50)	25(48.08)	26(41.94)	15(44.12)
Seldom	27(18.88)	25(20.00)	14(17.50)	14(26.92)	10(16.13)	7(20.59)
Often	49(34.27)	41(32.80)	32(40.00)	13(25.00)	26(41.94)	12(35.29)
Fried, n (%)	P = 0.237			P = 0.493		
Never	62(43.36)	47(37.60)	43(53.75)	23(44.23)	23(37.10)	13(38.24)
Seldom	72(50.35)	67(53.60)	32(40.00)	23(44.23)	35(56.45)	20(58.82)
Often	9(6.29)	11(8.80)	5(6.25)	6(11.54)	4(6.45)	1(2.94)
Hot *, n (%)	P = 0.491			P = 0.689		
Never	80(55.94)	82(65.60)	51(63.75)	33(63.46)	33(53.23)	21(61.76)
Seldom	20(13.99)	11(8.80)	8(10.00)	7(13.46)	12(19.35)	7(20.59)
Often	43(30.07)	32(25.60)	21(26.25)	12(23.08)	17(27.42)	6(17.65)
Mildew, n (%)	P = 0.615			P = 0.246		
Never	141(98.60)	124(99.20)	78(97.50)	50(96.15)	61(98.39)	33(97.06)
Seldom	2(1.40)	1(0.80)	1(1.25)	2(3.85)	0(0.00)	0(0.00)
Often	0(0.00)	0(0.00)	1(1.25)	0(0.00)	1(1.61)	1(2.94)

Categorical variables are presented as the number (percentage) and analyzed using chi-square test.
* Hot refers to beverage or food with temperature above 65°C.

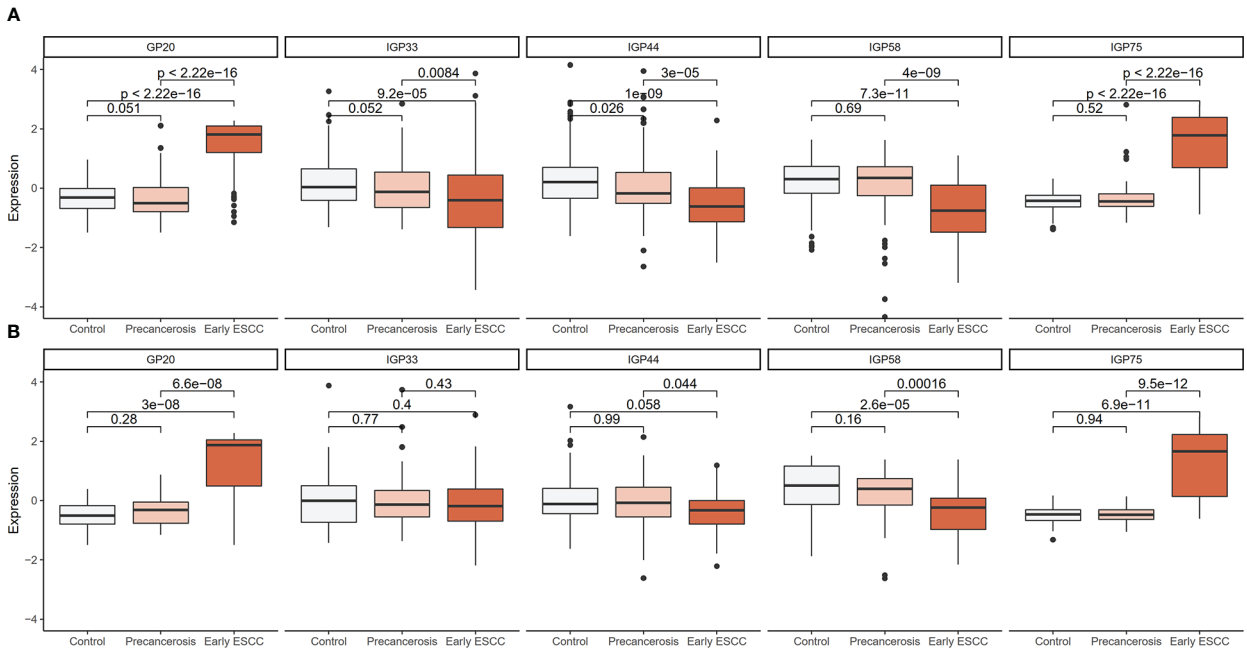

FIGURE 2
Distribution boxplot of differential GP and IGPs among the controls, precancerosis, and early ESCC groups. **(A)** The distribution boxplot of the IgG GP and IGPs in the discovery population; **(B)** The distribution boxplot of the IgG GP and IGPs in the validation population. The vertical position of each histogram represents the relative amount level of GP and IGPs.

decreased in the early ESCC group. Similarly, GP20, IGP33, IGP44, IGP58 and IGP75 differed statistically between the early ESCC group and the precancerosis group. Table 3 summarized the association of IgG glycans and traits with ESCC. In the discovery population, the adjusted ORs of GP20, IGP33, IGP44, IGP58, IGP75 were 4.03 (95% CI: 3.03-5.36, P<0.001), 0.69 (95% CI: 0.55-0.87, P<0.001), 0.56 (95% CI: 0.45-0.69, P<0.001), 0.52 (95% CI: 0.41-0.65, P<0.001), and 7.17 (95% CI: 4.77-10.79, P<0.001) respectively, while in the validation population, the adjusted OR were 7.41 (95% CI: 4.17-13.17, P<0.001), 0.66 (95% CI: 0.45-0.99, P<0.045), 0.60 (95% CI: 0.39-0.92, P<0.020), 0.48 (95% CI: 0.32-0.71, P<0.001), and 14.88 (95% CI: 5.75-38.47, P<0.001).

Construction and assessment of a glycan score for differentiating ESCC from esophageal precancerosis and controls

We screened ESCC-related N-glycan alterations based on ordinal logistic regression analysis. Regression coefficients were used to estimate odds ratios for each of the independent variables. The mathematic formula named ESCC-related glycan score was constructed to differentiate ESCC from esophageal precancerosis and controls (ESCC-related glycan score = 0.612×GP20 - 0.357×IGP33 - 0.623×IGP44 - 0.439×IGP58 + 1.333×IGP75). The distinct distribution of the ESCC-related glycan score was shown in Figure 3. In both the discovery and validation populations, compared with precancerosis and controls, ESCC-related glycan score was elevated (P < 0.001) in the early ESCC group. In the discovery population, compared with the controls, ESCC-related glycan score was slightly increased (P < 0.05) while there was no difference in the validation population. After adjusting confounders including age, sex, BMI, hypertension, smoke, drink, education, income, marriage status and dietary habits (model 3), the ESCC-related glycan score showed significant association with the carcinogenesis progression of ESCC, and the adjusted ORs were 2.86 (95% CI: 2.33-3.53, P<0.001) in the discovery population, and 3.43 (95% CI: 2.32-5.05, P<0.001) in the validation population. Individuals in the highest tertile of the glycan score owned a higher risk compared with those in the lowest, and the adjusted ORs were 11.41 (95% CI: 6.30-20.69, P<0.001) and 14.79 (95% CI: 5.40-40.51, P<0.001), respectively, (Figure 4). Figure 5 illustrated the multi-class ROC curves were of the ESCC-related glycan score for discriminating the controls, esophageal precancerosis and ESCC patients. Accordingly, the AUC value in the discrimination of the controls, esophageal precancerosis and early ESCC patients were 0.710 (95% CI: 0.656-0.775), 0.672 (95% CI: 0.625-0.735) and 0.913 (95% CI: 0.868-0.969) in the discovery population, and 0.692 (95% CI:

TABLE 3 Associations of the IgG GP and IGPs with carcinogenesis progression of ESCC by ordinal logistic models.

	Discovery population			Validation population		
	OR	95%CI	P	OR	95%CI	P
Model 1						
GP20	3.83	2.96-4.96	<0.001	5.20	3.27-8.27	<0.001
IGP33	0.66	0.53-0.81	<0.001	0.68	0.48-0.96	0.027
IGP44	0.57	0.46-0.70	<0.001	0.69	0.48-0.98	0.039
IGP58	0.53	0.42-0.65	<0.001	0.55	0.39-0.78	<0.001
IGP75	6.97	4.77-10.19	<0.001	9.83	4.66-20.75	<0.001
Model 2						
GP20	3.67	2.83-4.77	<0.001	5.20	3.26-8.29	<0.001
IGP33	0.67	0.54-0.83	<0.001	0.64	0.45-0.92	0.016
IGP44	0.57	0.46-0.70	<0.001	0.67	0.47-0.97	0.032
IGP58	0.53	0.43-0.66	<0.001	0.55	0.38-0.78	0.001
IGP75	6.85	4.64-10.11	<0.001	9.93	4.61-21.39	<0.001
Model 3						
GP20	4.03	3.03-5.36	<0.001	7.41	4.17-13.17	<0.001
IGP33	0.69	0.55-0.87	<0.001	0.66	0.45-0.99	0.045
IGP44	0.56	0.45-0.69	<0.001	0.60	0.39-0.92	0.020
IGP58	0.52	0.41-0.65	<0.001	0.48	0.32-0.71	<0.001
IGP75	7.17	4.77-10.79	<0.001	14.88	5.75-38.47	<0.001

Model 1: unadjusted; Model 2: adjusted for age, sex; Model 3: adjusted for age, sex, BMI, hypertension, smoke, drink, education level, income, marriage status, dietary habits.

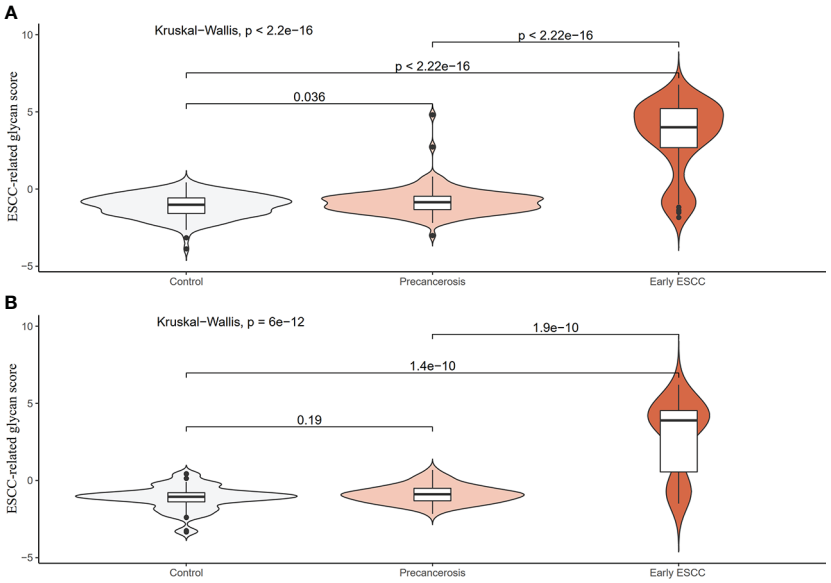

FIGURE 3
Distribution violin plot of the ESCC-related glycan score among the controls, precancerosis, and early ESCC groups. **(A)** The violin plot of the glycan score in the discovery population; **(B)** The violin plot of the glycan score in the validation population.

0.589-0.788), 0.677 (95% CI: 0.597-0.781) and 0.902 (95% CI: 0.824-0.982) in the validation population. The AUC value of early ESCC patients was significantly different from the controls (<0.01) and esophageal precancerosis (<0.001) in both discovery and validation populations. However, no statistically significant difference was found between the ROC curves of the controls and esophageal precancerosis in the validation populations (p>0.05) (Supplementary Table S5). The ESCC-related glycan score achieved an average AUC of 0.822 (95% CI: 0.786-0.849) and 0.807 (95% CI: 0.758-0.864), respectively. The results after combining the two populations were similar to each single population.

After linkage disequilibrium, we found 27 SNPs were associated with the proposed ESCC-related glycan score and 15 of them could be annotated to functional genes (Supplementary Table S6). In total, the genes were significantly enriched in 12 different GO gene sets and 2 different KEGG gene sets (Supplementary Figure S3), and construct a PPI network topology includes 595 nodes and 723 edges (Supplementary Figure S4). Based on the node degree score, the top 6 genes, including SMARCB1, IKZF1, RUNX1, TAB1, RUNX3 and B4GALT1 were considered as potential hub genes. After validation on RNA sequence data in the database online, these 6 genes were differently expressed in ESCC and normal tissues (Supplementary Figure S5), which may be the corroborative evidence of our study.

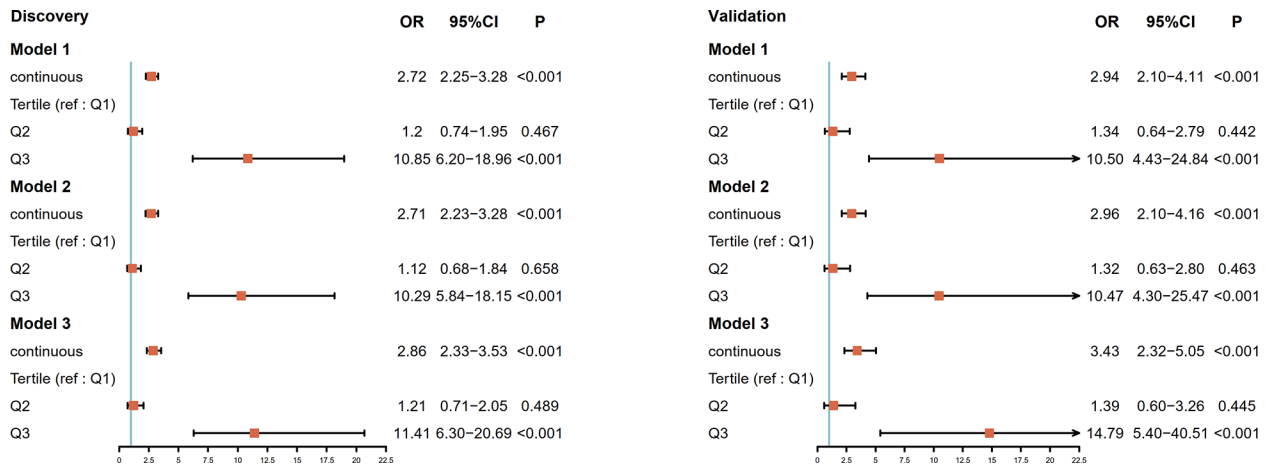

FIGURE 4
Forest plot for the association of the ESCC-related glycan score and progression of ESCC in the discovery population and the validation population. The ESCC-related glycan score estimates the magnitude of the effect as a continuous variable and tertile. The vertical line indicates no effect (odds ratio 1.0); horizontal lines indicate 95% confidence interval. Model 1: unadjusted; Model 2: adjusted for age, sex; Model 3: adjusted for age, sex, BMI, hypertension, smoke, drink, education level, income, marriage status, dietary habits; OR, odds ratio; CI, confidence interval; ref, reference.

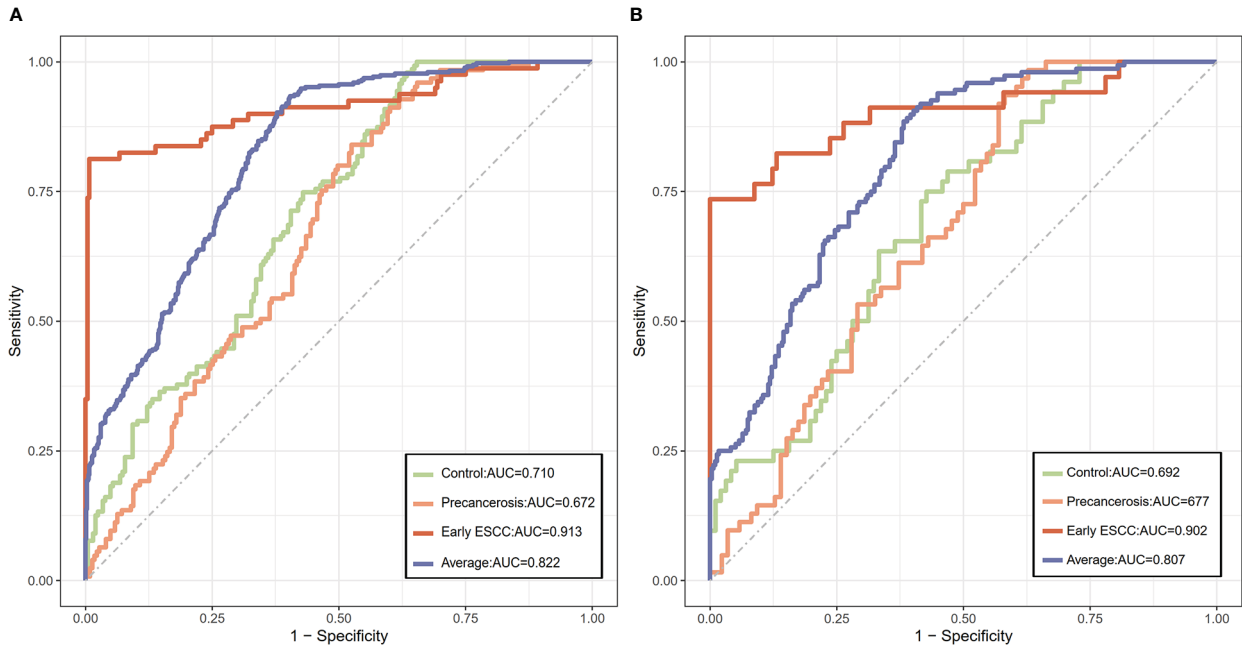

FIGURE 5
The discriminative capacity of the ESCC-related glycan score among the controls, precancerous, and early ESCC populations. **(A)** The ROC plot of the ESCC-related glycan score in the discovery population; **(B)** The ROC plot of the ESCC-related glycan score in the validation population.

Discussion

In this current study, we investigated the association of IgG N-glycosylation profiles and the carcinogenesis progression of ESCC. IgG N-glycans (GP20) and the derived traits (IGP33, IGP44, IGP58, IGP75) were primarily selected and validated to be associated with different stages of ESCC. Specific IgG N-glycosylation pattern participates in the carcinogenesis progression of ESCC, and the proposed ESCC-related glycan score could be a novel indicator. Variation in the fucosylated glycans and the suppressed mannose level, reflected by the altered glycans and traits, could be potential intervention target for ESCC. In addition, an ESCC-related glycan score was composed in this study, which achieved a high AUC value to discriminate different stages of ESCC. Besides, SMARCB1, IKZF1, RUNX1, TAB1, RUNX3, B4GALT1 were considered as potential hub genes of the proposed ESCC-related glycan score.

In our study, we found that GP20 and IGP75 was positively associated with ESCC progression, while IGP33, IGP44 and IGP58 was negatively associated (GP20: digalactosylated monosialylated biantennary with core and antennary fucose; IGP33: the ratio of all fucosylated monosyalilated and disialylated structures; IGP44: the proportion of high mannose glycan structures in total neutral IgG glycans; IGP58: the percentage of all fucosylated structures in total neutral IgG glycans; IGP75: the incidence of bisecting GlcNAc in all fucosylated digalactosylated structures in total neutral IgG glycans). These results above revealed a glycosylation pattern of increased digalactosylated biantennary glycan, the incidence of bisecting GlcNAc in all fucosylated digalactosylated glycans, and decreased high mannose glycan, fucosylated glycan, the ratio of all fucosylated monosyalilated and disialylated glycan among ESCC.

These finding were largely in consistent with previous studies. Liu et al. reported a significantly decreased of mannose glycan in patients with colorectal cancer (35) and we found a decrease of glycans with mannosylation in precancerous lesions and early esophageal cancer. It was also observed that, mannose glycan was distinctively decreased in breast cancer relative to control in total mouse serum proteins, demonstrating that mannosylation may play an important role in cancer progression not only in human but also in other animals (36). Removal of mannose sugar residues resulting in conformational changes in Cgamma2 domain affected the structure and function of IgG-Fc fragments (37), showing the importance of mannosylation. Gornik et al. found that IgG would activate complement and ADCC, and promote anti-inflammatory activity according to the extent of galactosylation and fucosylation of its glycans (38). Sialylation plays a crucial role in the inflammatory potential of IgG. Addition of sialic acid to IgG would decrease its binding to Fcγ receptors, and converts the function from pro- to anti-inflammatory (39). Sethi et al. reported that the expression levels of disialylation was higher in mid-and late-stage colorectal tumors than in early tumors (29) and we found the ratio of all fucosylated monosyalilated and disialylated structures was negatively associated with ESCC progression. Similar to the critical role of sialylated glycan in the regulation of inflammatory action, the fucosylated glycan can also enhance or inhibit IgG-mediated ADCC (40). Liu et al. reported that fucosylation and sialylation were associated with lung tumor cell growth and malignancy (41). Some previous studies pointed out that the decrease of fucosylated glycan was probably associated with

colorectal cancer progression (35, 42), and we found a decrease of glycans with fucosylation in ESCC progression. Therefore, it is of significance to reveal the changes of IgG N-glycans abundance, and to explore the profiling of IgG N-glycans as potential biomarker for early detection of ESCC.

Our study found SMARCB1, IKZF1, RUNX1, TAB1, RUNX3 and B4GALT1 as potential hub genes for the proposed ESCC-related glycan score, which were in agreement with previous studies. B4GALT1, IKZF1, TAB1 and SMARCB1 are reported to associate with IgG N-glycosylation show pleiotropy with autoimmune diseases and haematological cancers (43), while Shen et al. used multivariate methods in a genome-wide association study certified B4GALT1 and SMARCB1 are related to IgG N-glycosylation (44). TAB1 has also been reported associated with the progression and prognosis of esophageal cancer (45). RUNX3 encodes for a transcription factor of the runt domain-containing family. Methylation of RUNX3 promoters has an impact on cancers (46–49) and B-cell maturation (50). By influencing T-cell differentiation, RUNX3 is likely to indirectly affect the glycosylation of antibodies produced by B-cells. IKZF1, attributed to the enzymes of the Ikaros family, can also alter the differentiation process of T-cells (51, 52). Klarić et al. confirmed *in vitro* that knockdown of IKZF1 decreases the expression of fucosyltransferase FUT8, resulting in increased levels of fucosylated glycans, and suggest that RUNX1 and RUNX3, together with SMARCB1, regulate expression of glycosyltransferase MGAT3 (53).

In this study we explored the significant differences in IgG N-glycosylation profile among early ESCC, esophageal precancerosis, and the controls. To our knowledge, this is the first attempt aiming at the association of IgG N-glycans biomarkers with the carcinogenesis progression of ESCC. The identified glycans and proposed glycan score were validated in another population. However, the limitations should be addressed. First, the sample size was relatively small causing an inadequate statistical power. Second, this was a population-based cross-sectional study, hence, no causal relationships or pathophysiological inferences were available, basic experiments *in vivo* or *in vitro* will be conducted to confirm the association of IgG N-glycans biomarkers with the carcinogenesis progression of ESCC. Third, our study was based on two Chinese populations, more collaborations are needed to validate the generalizability of the observed results for other ethnic groups. Fourth, the identification and quantification of glycans were by HPLC in our study, although glycan standards were used, additional cross validation with other techniques, e.g. mass spectrometry, lectin array will be performed in our further research.

Conclusions and perspectives

In summary, we have performed the first analysis so far to identify the association of IgG N-glycans biomarkers with the carcinogenesis progression of ESCC. In this study, GP20, IGP33, IGP44, IGP58, IGP75 are significantly associated with the carcinogenesis progression of ESCC, and the proposed glycan score is a novel indicator for different progressive stages. In addition, the variation of fucosylation level and the suppressed mannose level could provide potential therapeutic intervention targets. These findings support the potential utility of glycomics in the ESCC related personalized therapy. The mechanism studies about the biological or pathological function of the fucosylated protein and mannosed protein in the carcinogenesis of ESCC and other cancers are of paramount importance. The experiment on mice after knocking out the corresponding genes of glycosyltransferase and glycosylhydrolase regulating the fucosylation and mannose levels are the next step for our study to validate the effect of IgG N-glycan patterns in the carcinogenesis of ESCC. Future studies on larger cohorts from diverse populations are expected for the validation of these observed associations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Independent Ethics Committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College and the Ethics Committees of Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XG and GW contributed to conception and design of the study. DZ, ZYZ, YQL, ZZ, ZW, JZ, YuL, YoL, and HZ collected the data. HP, ZL, WF, XTL, and YXL performed the statistical analysis. HP and ZW wrote the first draft of the manuscript. LT, XW, XY, and FZ wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.981861/full#supplementary-material

SUPPLEMENTARY FIGURE 1
UPLC analysis of the IgG glycome. IgG glycome was separated into 24 chromatographic peaks by hydrophilic interaction chromatography.

SUPPLEMENTARY FIGURE 2
Distribution boxplot of substantially changed GPs and IGPs among the controls, precancerosis, and early ESCC groups. A: The distribution boxplot of the substantially increased IgG GPs and IGPs among the controls, precancerosis, and early ESCC groups; B: The distribution boxplot of the substantially decreased IgG GPs and IGPs among the controls, precancerosis, and early ESCC groups. The vertical position of each histogram represents the relative amount level of GPs and IGPs.

SUPPLEMENTARY FIGURE 3
GO and KEGG gene-set enrichment analysis. Adjusted p-value: *P*-value was adjusted by Benjamini-Hochberg method.

SUPPLEMENTARY FIGURE 4
PPI network. The edges between 2 nodes represent the gene-gene interactions. The size of the nodes corresponding to each gene were determined according to the degree of interaction.

SUPPLEMENTARY FIGURE 5
Distribution boxplot of gene expression between the normal and ESCC groups. The vertical position of each histogram represents the relative expression level of genes.

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Synergistic regulation of Notch signaling by different O-glycans promotes hematopoiesis

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Glycosylation of Notch receptors by O-fucose glycans regulates Notch ligand binding and Notch signaling during hematopoiesis. However, roles in hematopoiesis for other O-glycans that modify Notch receptors have not been determined. Here we show that the EGF domain specific GlcNAc transferase EOGT is required in mice for the optimal production of lymphoid and myeloid cells. The phenotype of *Eogt* null mice was largely cell-autonomous, and Notch target gene expression was reduced in T cell progenitors. Moreover, EOGT supported residual Notch signaling following conditional deletion of *Pofut1* in hematopoietic stem cells (HSC). *Eogt : Pofut1* double mutant HSC had more severe defects in bone marrow and in T and B cell development in thymus and spleen, compared to deletion of *Pofut1* alone. The combined results show that EOGT and O-GlcNAc glycans are required for optimal hematopoiesis and T and B cell development, and that they act synergistically with POFUT1 and O-fucose glycans to promote Notch signaling in lymphoid and myeloid differentiation.

KEYWORDS

Notch signaling, O-glycans, HSC, lymphoid, myeloid

Highlights

- O-GlcNAc glycans and EOGT promote lymphopoiesis and myelopoiesis.
- EOGT supports Notch signaling in the absence of POFUT1 and O-fucose glycans.

Introduction

Notch signaling is highly conserved and plays crucial roles in cell fate determination and tissue development (1). There are four Notch receptors (NOTCH1 to NOTCH4) that can be activated by canonical Notch ligands (DLL1, DLL3, DLL4, JAG1 and JAG2) to induce Notch signaling in mammals. Notch ligand binding and Notch signaling are regulated by glycosylation of the extracellular domain (ECD) of Notch receptors (2, 3).

Structural studies reveal direct interactions between Notch ligands and O-fucose in specific epidermal growth factor-like (EGF) repeats of NOTCH1 (4, 5). Following Notch ligand engagement, an ADAM metalloprotease cleaves NECD, followed by a second cleavage by the γ-secretase complex. Notch intracellular domain (NICD) complexes in the nucleus with the transcriptional repressor RBP-Jk, and co-activators including Mastermind-like-1 (MAML1), to induce the expression of Notch target genes, including Hairy enhancer-of-split (*Hes*) and Hairy-related gene families, which regulate the expression of many other genes (6, 7).

Within the hematopoietic system, Notch signaling plays important roles in regulating different stages of lymphoid and myeloid development (8, 9). DLL4-induced NOTCH1 signaling is indispensable for T cell development in the thymus. Thus, conditional inactivation of *Notch1* or *Dll4* using *Mx1-Cre* is sufficient to block T cell development (10, 11). However, *Notch2* is also required for optimal development of early T cell progenitors (12). DLL1-induced NOTCH2 signaling is essential for the generation of marginal zone B cells (MZ-B) in the spleen (13, 14). O-fucose glycans extended by LFNG and MFNG promote the formation of MZ-B cells (15) and all three FNG genes contribute to T and B cell development (16).

Consensus sites within EGF repeats in the NECD of Notch receptors carry O-fucose, O-glucose and O-GlcNAc glycans (17) (Figure 1). EGF repeats with appropriate consensus sites occur in ~50 proteins of the proteome, including Notch receptors and Notch ligands (19, 20). O-fucose is transferred by protein O-fucosyltransferase 1 (POFUT1), which is further extended by the Fringe family of glycosyltransferases (LFNG, MFNG and RFNG). Misexpression of *Lfng* in thymus disrupts T cell development (21–23). Conditional deletion of *Pofut1* in the bone marrow leads to the disruption of hematopoiesis with an increase in granulocyte-monocyte progenitors (GMP), and a reduction in common myeloid progenitors (CMP) (24). This causes a block in T cell production in thymus and MZ-B cell production in spleen, accompanied by an increase in granulocytes in spleen. Notch

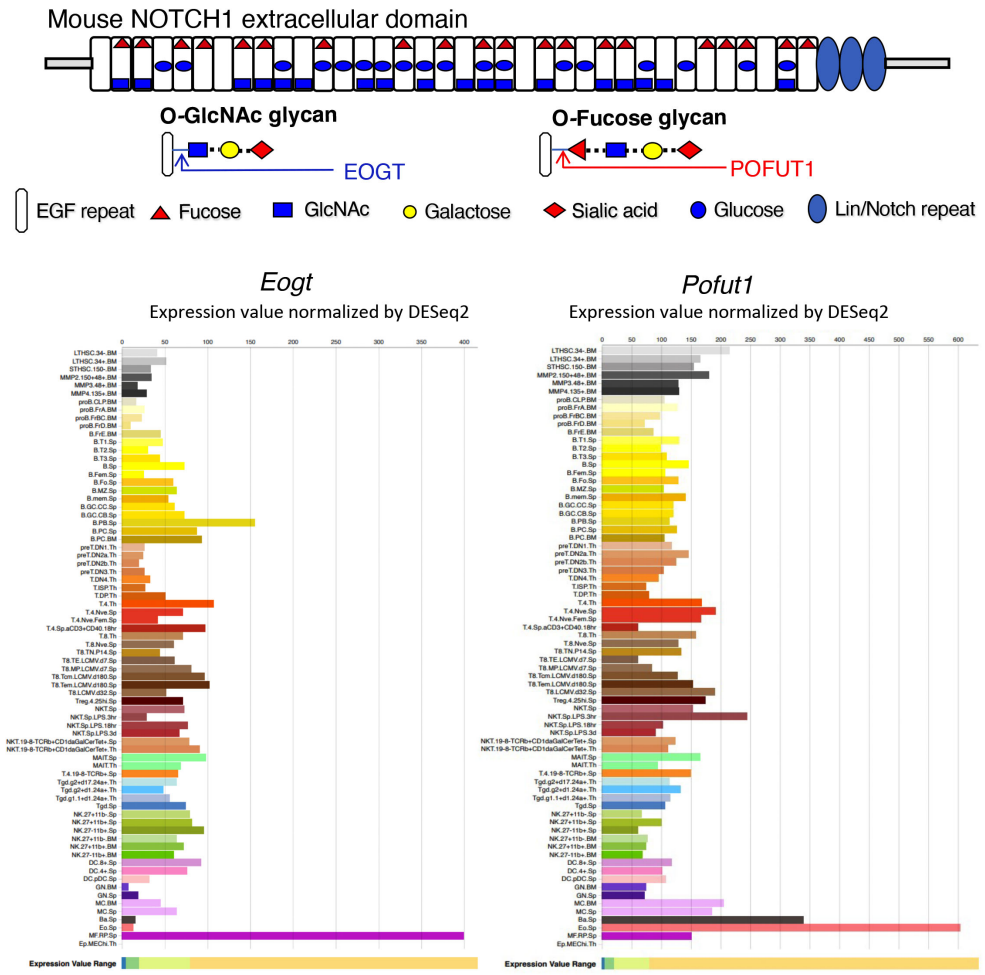

FIGURE 1
Expression of *Eogt* and *Pofut1* in immune cell subsets. The diagram depicts the ECD of mouse NOTCH1 showing the O-glycans attached at respective consensus sites in the 36 EGF repeats. The sugar that initiates each O-glycan is shown in the key. The potential sugar extensions from O-fucose and O-GlcNAc glycans are also shown. The expression of *Eogt* and *Pofut1* in a number of immune cells is taken from ImmGen Gene Skyline ULI RNASeq data group for *Eogt* and *Pofut1* gene expression (18) (http://rstats.immgen.org/Skyline/skyline.html). The definition of each immune cell type is given at the link. Expression of *Eogt* and *Pofut1* vary inversely in certain immune cell subsets.

ligand binding and Notch signaling are markedly reduced in hematopoietic stem cells (HSC) following conditional deletion of *Pofut1*. However, *Pofut1* deletion by *Mx1-Cre* gives a milder reduction in thymic T cells than deletion of RBP-Jk (25), whereas global deletion of *Pofut1* (26, 27) or RBP-Jk (28) causes similarly severe Notch signaling defects, and embryonic lethality. Thus, loss of O-fucose glycans in HSC does not fully abrogate Notch signaling, suggesting that other O-glycans may support Notch signaling in the absence of POFUT1. POGLUT1 adds O-glucose glycans to Notch receptors. Initially cloned from CD34+ human stem cells (29), POGLUT1 promotes Notch signaling via ligand-dependent and -independent mechanisms (30, 31). Synergism between O-fucose and O-glucose glycans on Notch observed in *Drosophila* (32) and in mammalian cells (33) appears to be due to reduced expression of Notch receptors at the cell surface. By contrast, the absence of O-fucose glycans from Notch receptors in CHO and mouse embryonic stem cells only slightly reduces NOTCH1 cell surface levels (34, 35).

The addition of O-GlcNAc to EGF repeats was first identified in *Drosophila* (36), and the EGF domain specific O-GlcNAc transferase EOGT was subsequently revealed (37, 38). *Eogt* null mice exhibit defective perinatal retinal angiogenesis, similar to that observed in mice with disrupted Notch signaling (39). Cell-based experiments showed that EOGT promotes the binding of DLL Notch ligands and Notch signaling (39). *Eogt* is expressed with *Pofut1* in many cells of the immune system (Figure 1). However, their expression levels vary greatly and inversely in some immune cell types, indicating potentially different functional roles (Figure 1). In this paper, we identify roles for *Eogt* in the regulation of Notch signaling during hematopoiesis, lymphopoiesis and myelopoiesis. In addition, we show that *Eogt* supports Notch signaling in the absence of *Pofut1* and O-fucose glycans.

Methods

Mice

Mice with an inactivating mutation in the *Eogt* gene were generated at Nagoya University and previously described (39). *Pofut1*[F/F] mice were also previously described (26). Transgenic mice expressing *Vav1-iCre* (B6.Cg-*Commd10*$^{Tg(Vav1\text{-}icre)A2Kio}$/J Strain) were a kind gift from Britta Will and Paul Frenette at the Albert Einstein College of Medicine, NY, USA. Compound mutant mice termed Pof cKO and EPof dKO, with and without *Vav1-iCre*, were generated by intercrossing. C57Bl/6J male congenic mice expressing CD45.1 (*B6.SJL-Ptprc*^{a} *Pepc*^{b}/BoyJ *#002014*) were obtained from the Jackson Laboratory (Bar Harbor). Genotyping was performed by PCR of genomic DNA using primers that distinguish wild-type and mutant alleles (Supplementary Table 1). Mice were housed in a barrier facility, allowed to eat and drink ad libitum, and used in experiments at 7–8 weeks of age. All experiments were performed with permission from the Albert Einstein Institutional Animal Care and Use Committee under approved protocol numbers 20170709 and 00001311. Euthanized mice were weighed, bone marrow from femurs, tibias, fibulae, & patullae was isolated, thymus and spleen were weighed before making single-cell suspensions.

Antibodies

Supplementary Table 2 has the full description and commercial source of each antibody used in this work.

Flow cytometry of immune cell subsets

Single-cell suspensions were prepared from bone marrow (BM) by crushing femurs, tibias, fibulae, and patullae in a mortar and rinsing vigorously with 20 ml cold FACS binding buffer (FBB: Hank's balanced salt solution (HBSS) with glucose (Corning), 1 mM $CaCl_2$, 2% bovine serum albumin (BSA, fraction V, Sigma) and 0.05% sodium azide, (pH 7.2–7.4). The BM cell suspension was passed through a 70-µm strainer. Thymus or spleen was weighed and squeezed through a 70 µm strainer in 1 ml FBB. Thymocytes were washed in 10 ml cold FBB twice. Bone marrow and splenocytes were centrifuged and incubated in 1 ml RBC lysis buffer (eBiosciences) for 3-5 min before adding 10 ml cold FBB. After centrifugation and resuspension in 5 ml cold FBB, single cell suspensions were counted in a Coulter counter. Cells were centrifuged at 4°C, resuspended in 2 ml cold PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.2, centrifuged and resuspended in 100 µl Zombie NIR dye for live/dead assessment, according to the manufacturer's protocol (Zombie NIR Fixable Viability Kit, BioLegend). After 30 min at 4°C in the dark, 2 ml cold FBB was added. Cells were centrifuged at 4°C, resuspended in 4% paraformaldehyde (PFA, Emsdiasum) in PBS pH 7.2, and incubated 15 min at 4°C in the dark. Cells were washed twice with 2 ml cold FBB, resuspended at 10^6 cells/ml in FBB and stored at 4°C for up to 3 months. For analysis by flow cytometry, ~10^6 cells were washed with 1 ml FBB, resuspended in 90 µl FBB containing 1 µl Fc block (rat-anti-mouse CD16/CD32), and incubated for 15 min on ice. Abs diluted in FBB (10 µl) were added and the reaction mix was incubated for 30 min at 4°C. Cells were washed twice in 1 ml FBB and resuspended in ~500 µl FBB. For all samples, immunofluorescence was analyzed using a Cytek™ Aurora or BD LSRII flow cytometer and data FCS files were analyzed using FlowJo software (Tree Star). Gating strategies shown in Supplementary Figures 1–3 were based on previous work (16, 40, 41) and include the Abs used in the analysis of BM, thymus or spleen immune cell subsets. Data on live cells were collected as frequency percent based on gating strategies. Absolute cell numbers in the different immune cell subsets were calculated from the starting number of live cells and the frequency percent of each subset. Significant differences between genotypes in either frequency percent or absolute cell number are highlighted in the main text while complementary data are presented in supplementary figures as described in the text.

Bone marrow transplantation

Cell suspensions from bone marrow of 7–8 week *Eogt*[+/-] and *Eogt*[-/-] males were prepared as described above. Approximately 3x10^6 cells were resuspended in 50 µl sterile HBSS (Gibco) and injected using a 28-gauge insulin needle via the retro-orbital plexus

into CD45.1+ C57BL/6 lethally irradiated recipients. A split dose of 550 rads γ-irradiation per recipient male was given twice, with a 16 h interval. After 7 weeks, recipients were euthanized, bone marrow, thymus, and spleen were analyzed for lymphoid and myeloid cell subsets by flow cytometry after gating on donor-derived cells positive for anti-CD45.2-Pacific Blue.

Isolation of CD4/CD8 double negative T cells

Fresh thymocytes were resuspended in isolation buffer (PBS lacking cations, pH 7.2-7.4, containing 0.1% BSA, 2 mM EDTA and 1 mg/ml glucose) on ice. For T cell depletion, ~3-5x10^7 thymocytes were incubated with 20 µg anti-CD4 (rat IgG2b clone GK1.5, BioXCell) and 37.5 µg anti-CD8a (rat IgG2a clone 53-6.72; BioXCell) in 5 ml buffer for 20 min at 4°C with tilted rotation. After centrifugation, cells were resuspended in 5 ml buffer, and incubated with 250 µl sheep anti-rat IgG Dynabeads (Thermo Fisher Scientific) for 30 min at 4°C with tilted rotation. The tube was placed in a magnet for 2 min, unbound cells were centrifuged and resuspended in 250 µl Dynabeads for a second 30 min incubation at 4°C. After Dynabeads removal, unbound DN T cells were centrifuged, counted and RNA was extracted from the cell pellet with 1 ml TRIZOL (Ambion) as described below.

Quantitative RT-PCR

DN T cells from ~3-5x10^7 thymocytes were pipetted vigorously in 1 ml TRIZOL and incubated for 5 min at RT before adding 200 µl chloroform. Tubes were vortexed for 15 sec, incubated at RT for 2–3 min, and centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred to a new tube, and 500 µl isopropanol was added. Samples were incubated for 10 min on ice and centrifuged at 12,000 × g for 10 min at 4°C and the supernatant discarded. The RNA pellet was washed once with 1 ml 70% ethanol. Samples were vortexed for 10 sec and centrifuged at 7,500 × g for 5 min at 4°C and the supernatant discarded. The RNA pellet was air-dried for 5–10 min and dissolved in 50 µl RNase-free water. Samples were placed in a 55-60°C water bath for 15-20 min and the RNA concentration determined by Nanodrop. cDNA was prepared from ~250 ng RNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Dc. DiagnoCine) following the manufacturer's protocol. Each sample was assayed in triplicate using a 384 well plate. Relative gene expression was calculated in relation to *Hprt* and *Gapdh* by the log2 dCCT method. Primer sequences used for qRT-PCR are in Supplementary Table 1.

Histopathology

Spleen was collected, weighed, washed and stored in 10% natural buffered formalin (NBF) at 4°C. The samples were processed for paraffin embedding and longitudinal tissue sections (5 µm) were stained with hematoxylin and eosin (H&E), scanned by the 3D Histech P250 High-Capacity Slide Scanner and analyzed using Case Viewer 2.4 software.

Notch ligand binding assay

Soluble Notch ligands DLL1-Fc (#10184-DL-050), JAG1-Fc (#1277-JG-050), and JAG2-Fc (#1726-JG-050) were purchased from R&D Systems, and DLL4-Fc (#DL4-H5259) was purchased from Acro-biosystems. Single-cell suspensions from thymus were washed in FBB at 4°C and fixed in PBS-buffered 4% PFA for 15 min at 4°C, washed twice with FBB and stored in FBB at 4°C. For analysis, ~0.5-1x10^6 fixed thymocytes were washed with FBB, and incubated with 40 µl FBB and 1 µl FcR blocking solution (rat-anti-mouse CD16/CD32) on ice for 15 min. Thereafter, the cells were incubated in 60 µl FBB containing anti-CD4-FITC (1:400), anti-CD8a-PerCp-Cy5.5 (1:400), and 0.75 µg DLL4-Fc, or 1.5 µg of DLL1-Fc, JAG1-Fc, or JAG2-Fc. After incubation at 4°C for ~30 min, cells were washed with 1 ml FBB and incubated with anti-IgG-APC and anti-IgG-DyLight 405 (Fc-specific) Ab (1:100) at 4°C for 30 min. The cells were then washed with 1 ml FBB, resuspended in 500 µl FBB and analyzed in a flow cytometer (Cytek™, Aurora). For detection of NOTCH1 at the cell surface, fixed thymocytes cells were incubated with FcR-block rat-anti-mouse CD16/CD32 (1:100) followed by anti-CD4-FITC mAb (1:400), anti-CD8a-PerCp-Cy5.5 (1:400), sheep anti-mouse NOTCH1 Ab (1:50) at 4°C for 30 min, washed, and incubated with rhodamine Red-X-conjugated donkey anti-sheep IgG (1:100) at 4°C for 30 min. Cells were washed with 1 ml FBB, resuspended in 500 µl FBB and analyzed in a flow cytometer (Cytek™, Aurora).

Statistics

Comparisons are presented as mean ± SEM. Significance was determined by both two-tailed and one-tailed (denoted by parentheses) unpaired, parametric, Student t-test analysis (unless otherwise noted) using Prism software version 9.1.

Results

Loss of *Eogt* affects myelopoiesis and lymphopoiesis

Initial comparisons of *Eogt*[+/+] and *Eogt*[+/-] heterozygotes revealed no significant differences in T, B and myeloid subset populations in either absolute cell numbers or in the frequency % of different cell subsets (Supplementary Figure 4 and not shown). Therefore, data from *Eogt*[+/+] and *Eogt*[+/-] mice were combined as Control. Thymus and spleen weights, as well as bone marrow (BM) cellularity, were similar in *Eogt* null and Control mice (Supplementary Figure 5A). In bone marrow, the absolute numbers of CD19+/B220+ B cells, and CD11b+/Gr1+ granulocytes were significantly increased in the *Eogt*[-/-] population (Figure 2A). In *Eogt*[-/-] thymus, the frequency % of CD4/CD8 double negative 1 (DN1) T cell progenitors was reduced, although the absolute number

of DN1 T cells was unchanged (Supplementary Figure 5B). The absolute number of DN2 T cell progenitors was strikingly reduced in *Eogt* null cells, while the absolute number of DN4 T cells was significantly increased (Figure 2B). By contrast, the numbers of early T cell progenitors (ETP) and DN3 T cells were unchanged (Supplementary Figure 5B). The proportion of double positive (DP) T cells was slightly decreased, while CD4+ and CD8+ single positive (SP) T cells were increased in *Eogt*[-/-] thymocytes (Figure 2B). These effects were also observed in mice lacking the three Fringe genes (*Fng* tKO) (16, 42). Consistent with inhibition of Notch signaling in the *Eogt* null thymus, there was a significant increase in thymic B cells (CD19+/B220+) and the frequency of myeloid cells (CD11b+) (Figure 2B), but no change in natural killer (NK) T cells (Supplementary Figure 5B). In spleen, no histopathological changes were observed in Control versus *Eogt*[-/-] sections (n=3 for each, not shown), and the absolute numbers of T cells, B cells, marginal zone progenitors (MZ-P) and myeloid cell subsets were similar in Control versus *Eogt*[-/-] mice (Supplementary Figure 5C). However, significant increases were observed in the absolute number of follicular B (Fo-B), MZ-B, CD19+ and B220+ B cells in *Eogt*[-/-] mice (Figure 2C). By contrast, a decrease in the frequency % of natural killer T cells (NK1.1+) and dendritic cells (CD11b/c+) was observed (Figure 2C). Thus, EOGT and O-GlcNAc glycans are required for the optimal generation of lymphoid and myeloid cells from HSC.

The *Eogt* null phenotype is largely cell autonomous

To determine whether the *Eogt* null phenotype was cell intrinsic, bone marrow (BM) transplantation was performed from CD45.2+ *Eogt*[+/-] and *Eogt*[-/-] donor males into CD45.1+ male hosts. *Eogt*[-/-] CD45.2+ donor BM cells reconstituted CD45.1+ hosts to ~57% in bone marrow (Figure 3A), ~80% in thymus and ~64% in spleen (not shown). Thus, host-derived *Eogt*[+/+] cells contributed to each of these populations in recipients. In recipient bone marrow, no changes were seen in B cells (CD19+/B220+) or granulocytes (CD11b+/Gr-1+) from *Eogt*[-/-] donor BM compared to *Eogt*[+/-] BM (not shown); in recipient thymus, *Eogt*[-/-] donor BM generated significantly fewer DN2 and DN3 T cell progenitors compared to *Eogt*[+/-] donor BM (Figure 3B); and in spleen, *Eogt*[-/-] donor BM generated CD19+ cells and Gr-1+ granulocytes in significantly increased numbers compared to *Eogt*[+/-] donor BM (Figure 3C). Thus, the phenotype of *Eogt*[-/-] BM recipients was somewhat milder, than the *Eogt* null phenotype. This could reflect the presence of *Eogt*[+/+] host cells, and/or rescuing effects of the wild type host stroma. Nevertheless, key aspects of the *Eogt* null phenotype were transferred by *Eogt*[-/-] BM.

Notch signaling is reduced in *Eogt*[-/-] DN T cell progenitors

Notch ligand binding was examined using thymic DN T cell progenitors from 7-8 week *Eogt*[+/-] and *Eogt*[-/-] mice. No significant changes were observed in either NOTCH1 cell surface expression, or the binding of soluble ligands DLL1, DLL4, JAG1 or JAG2 between Eogt[+/-] and *Eogt*[-/-] DN T cell progenitors (Figure 4A). However, there were significant reductions in the expression of two Notch target genes, *Hes1* and *Il2ra*, consistent with reduced Notch signaling (Figure 4B). The expression of *Il2ra* was also reduced in DN T cell progenitors from mice lacking the three Fringe genes, along with *Dtx1*, although not *Hes1* (16). The reduction in Notch signaling target gene expression in *Eogt* null T cell progenitors, the changes in T cell subset numbers and frequencies, and the increased numbers of B and myeloid cells observed in *Eogt*[-/-] thymus, indicate that EOGT and O-GlcNAc glycans are necessary for optimal Notch signaling and T cell development. The altered B cell and myeloid subsets in spleen of *Eogt*[-/-] mice are also consistent with reduced Notch signaling. Finally, the B cell and myeloid hyperplasia in *Eogt*[-/-] BM indicate that Notch signaling is required for regulating the differentiation of certain progenitors during hematopoiesis.

Eogt supports lymphoid and myeloid development in HSC lacking *Pofut1*

Previous work showed that inactivation of *Pofut1* using *Mx1-Cre* causes a reduction in T lymphopoiesis in thymus and myeloid hyperplasia in bone marrow (24). In another study, conditional deletion of *Pofut1* in bone marrow was shown to cause a milder T cell phenotype than deletion of RBP-Jk (25). To determine whether *Eogt* and O-GlcNAc glycans support Notch signaling and hematopoiesis in the absence of *Pofut1*, we used *Vav1-iCre* transgenic mice to generate conditional deletion (cKO) of *Pofut1* (henceforth referred to as Pof cKO), and deletion of both *Eogt* and *Pofut1* (henceforth referred to as EPof dKO), in hematopoietic stem cells. The absolute number of BM cells was significantly increased in both Pof cKO and EPof dKO mice (Figure 5). Gating strategies used to define HSC, HSPC, myeloid and lymphoid cell subsets in BM are shown in Supplementary Figure 6. Short-term (ST)-HSC, and LSK (Lineage-Sca1+cKit+) cell numbers were increased in both Pof cKO and EPof dKO mutants, but the frequency % of HSPCs was increased only in EPof dKO BM (Figure 5). By contrast, the frequency % of MPP subsets was reduced except for a small increase in the MPP3 population in EPof dKO BM (Figure 5). The frequency % of lymphoid primed multipotent progenitor cells (MPP4/LMPP) and common myeloid precursors (CMP) were reduced in Pof cKO and further reduced in EPof dKO BM, and megakaryocyte erythrocyte progenitors (MEP) were decreased in both single and double mutants (Figure 5). By contrast, the frequency % of granulocyte-monocyte progenitors (GMP) was increased in Pof cKO (as observed previously (24)), and further increased in EPof dKO BM (Figure 5 and Supplementary Figure 7). The proportion of CD11b+/Gr-1+ granulocytes was increased, but the proportion of common lymphoid progenitors (CLP) was reduced in EPof dKO BM (Figure 5). The frequency % of T- and B-cells in BM was also decreased in EPof dKO mice (Figure 5). NOTCH1 was expressed at the surface of Lin-Sca1+ cells which were increased in frequency in Pof cKO and EPof dKO BM

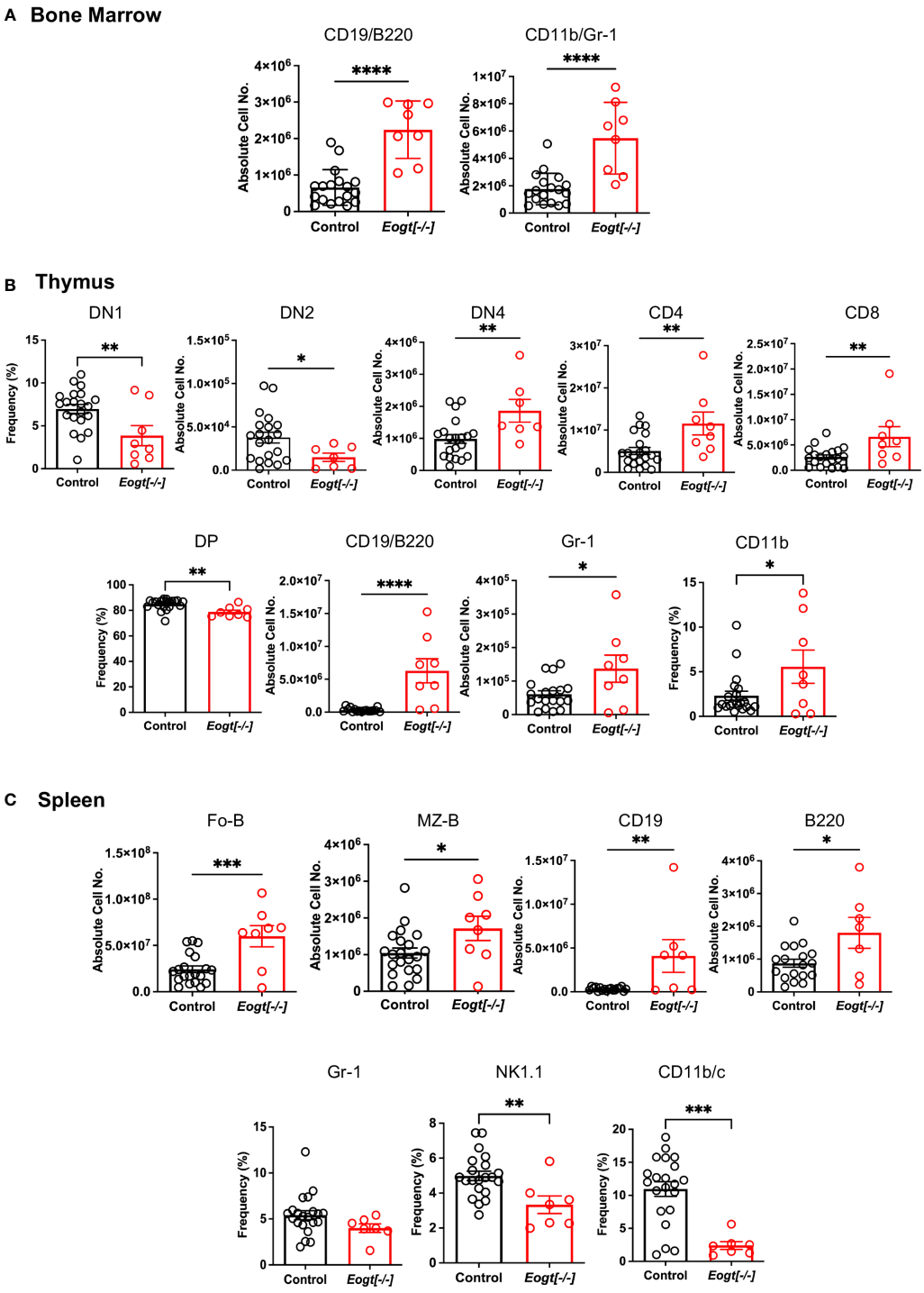

FIGURE 2
Altered lymphoid and myeloid subsets in mice lacking *Eogt* and O-GlcNAc glycans. **(A)** Absolute cell numbers or frequency % for lymphoid and myeloid cell subsets in bone marrow that differed between Control (*Eogt*[+/+] and *Eogt*[+/-]) and *Eogt*[-/-] mice. See Supplementary Figure 1 for gating, Supplementary Figure 4 for *Eogt*[+/+] versus *Eogt*[+/-] data and Supplementary Figure 5 for BM cellularity. **(B)** Absolute cell numbers or frequency % for lymphoid and myeloid cell subsets in thymus that differed between Control and *Eogt*[-/-] mice. See Supplementary Figure 2 for gating, Supplementary Figure 4 for *Eogt*[+/+] versus *Eogt*[+/-] data and Supplementary Figure 5 for subsets that did not differ significantly. **(C)** Absolute cell numbers or frequency % for lymphoid and myeloid cell subsets in spleen that differed between Control and *Eogt*[-/-] mice. See Supplementary Figure 3 for gating, Supplementary Figure 4 for *Eogt*[+/+] versus *Eogt*[+/-] data and Supplementary Figure 5 for subsets that did not differ significantly. Each symbol represents a mouse of 7-8 weeks. Data are presented as mean ± SEM. *p <0.05, **p<0.01,***p<0.001, ****p<0.0001 based on two-tailed Student's t test.

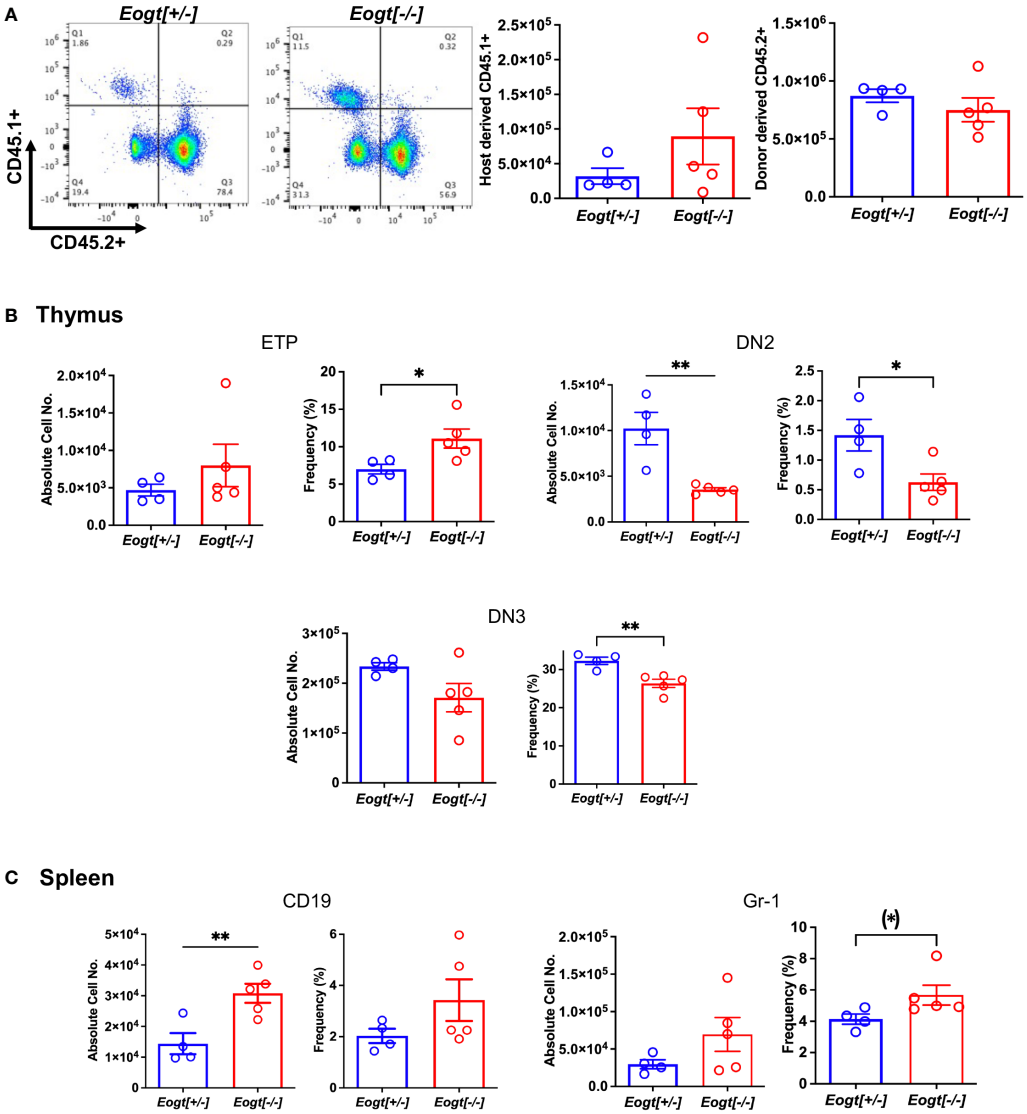

FIGURE 3
The *Eogt* null phenotype is largely cell autonomous. Bone marrow cells (3X10^6) from *Eogt*[+/-] or *Eogt*[-/-] mice expressing CD45.2 were injected via the retro-orbital plexus into lethally-irradiated, wild-type recipient males expressing CD45.1. Recipient BM, thymus and spleen were analyzed 7 weeks after transplantation. **(A)** Flow cytometry profile and histograms of bone marrow from recipient mice (CD45.1+) that received BM from *Eogt*[+/-] or *Eogt*[-/-] mice. The approximate donor contribution was 78% for *Eogt*[+/-] and 57% for *Eogt*[-/-] of recipient BM. **(B)** Absolute cell number and frequency % of T cell progenitors in thymus that differed when derived from *Eogt*[+/-] versus *Eogt*[-/-] donor bone marrow. **(C)** Absolute cell number and frequency % of B cells and granulocyte subsets in spleen that differed when derived from *Eogt*[+/-] versus *Eogt*[-/-] donor bone marrow. Each symbol represents a mouse of 7-8 weeks. Data are presented as mean ± SEM. *p <0.05, **p<0.01 based on two-tailed Student's t test. (*) p <0.05 based on one-tailed Student's t test.

(Supplementary Figure 8). Binding of DLL1 and DLL4 Notch ligands was low and similar in control and mutant Sca1+ cells (Supplementary Figure 8).

Highly disrupted development of T cells in EPof dKO thymus

Deletion of *Pofut1* in HSC via *Vav1-iCre* led to a marked decrease in thymus weight and a similar reduction was observed in EPof dKO thymus (Figure 6A). The reduced size was accompanied by a dramatic change in T cell maturation (Figure 6B). Early thymic progenitors (ETP) were greatly decreased in absolute number, and each DN T cell progenitor population (DN1 to DN4) was also reduced in absolute cell number in Pof cKO, and even further reduced in EPof dKO thymocytes (Figure 6C and Supplementary Figure 9). Interestingly, however, the frequency % of DN4 T cells in both single and double mutant thymocytes was increased relative to control (Supplementary Figure 9), as observed in thymus lacking all Fringe activities (16). The DN T cell population, which includes few DN T cell progenitors and all the non-T cell populations, was greatly

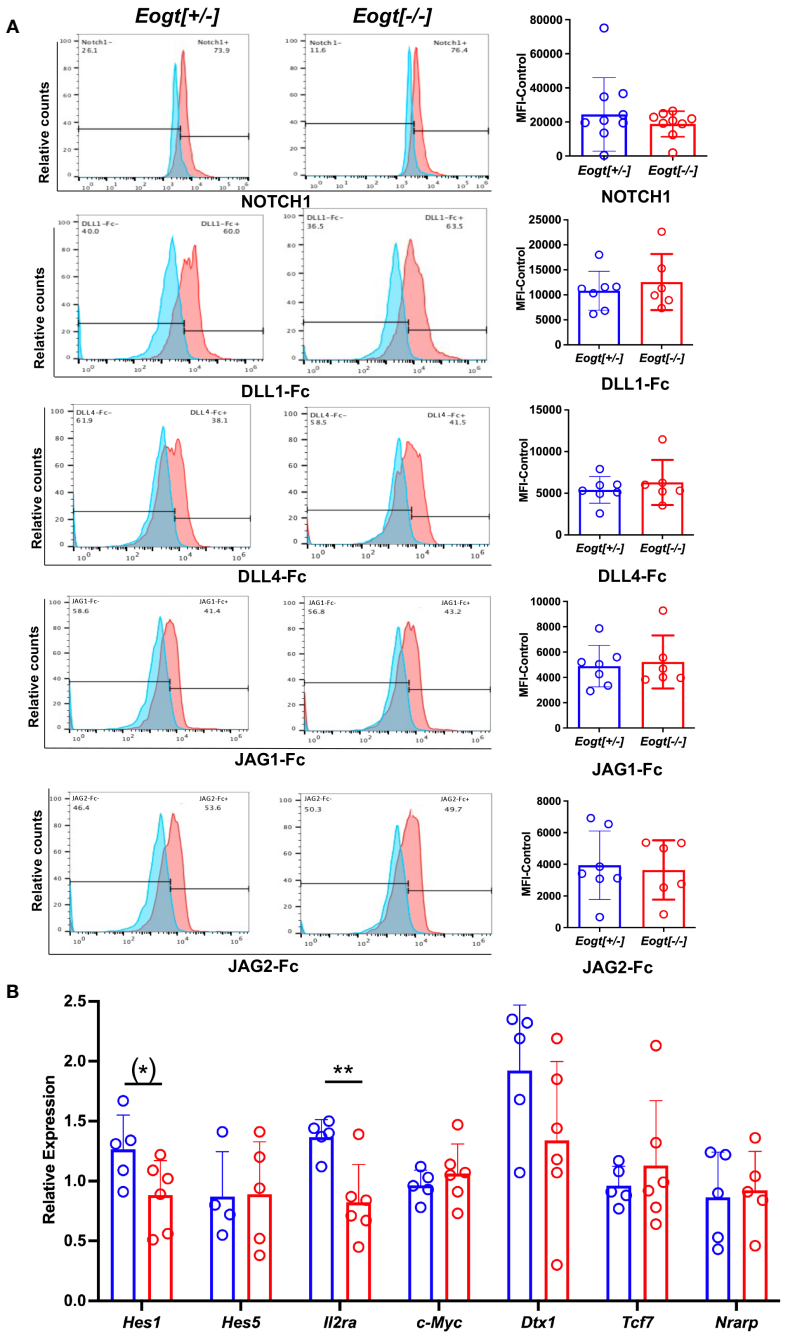

FIGURE 4
Notch signalling in *Eogt* null T cell progenitors. Representative flow cytometry profiles and histogram quantification of **(A)** Cell surface NOTCH1, or binding of DLL1-Fc, DLL4-Fc, JAG1-Fc and JAG2-FC to fixed CD4/CD8 DN T cell progenitors from *Eogt*[+/-] or *Eogt*[-/-] mice. Mean fluorescence index (MFI) for anti-Fc Ab was subtracted from MFI for Notch ligand or NOTCH1 Ab (MFI-control). Symbols represent *Eogt*[+/-] (blue circles) and *Eogt*[-/-] (red circles) DN T cells. Fixed cells had been stored for up to 3 months at 4°C. **(B)** Transcripts from DN T cell progenitors of *Eogt*[+/-] or *Eogt*[-/-] mice were subjected to qRT-PCR as described in Materials and Methods. Relative expression was determined based on the average delta Ct obtained for *Gapdh* and *Hprt* combined. Each symbol represents a mouse of 7-8 weeks. Data are presented as mean ± SEM. *p <0.05, **p<0.01 based on two-tailed Student's t test or (*) p <0.05 based on one-tailed Student's t test.

increased in Pof cKO and EPof dKO, as expected. This reflected loss of Notch signaling leading to the generation of B cell, myeloid and NK cell subsets in thymus (Figure 6D and Supplementary Figure 9). Notably, effects were more severe in EPof dKO than Pof cKO thymus, suggesting that the loss of Notch signaling was greater in EPof dKO thymus.

Defective B, T and myeloid cell development in EPof dKO spleen

Both Pof cKO and EPof dKO mice had an enlarged spleen (Figure 7A). Absolute numbers of splenocytes and spleen weight were increased in Pof cKO, and further increased in EPof dKO mice

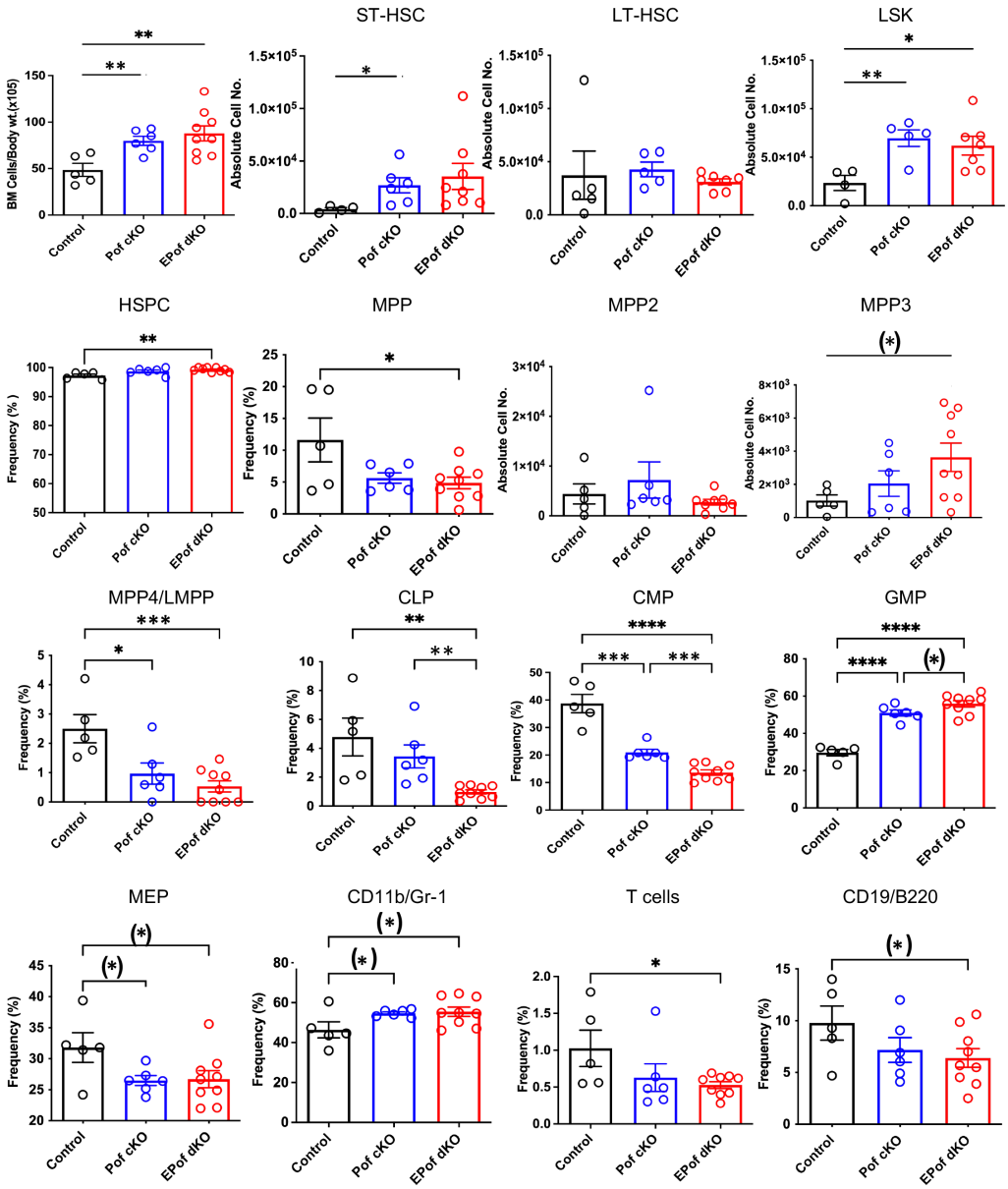

FIGURE 5
Hematopoiesis in EPof dKO bone marrow. Hematopoiesis was analyzed in bone marrow of *Eogt*[+/-]*Pofut1*[F/F] (Control), *Pofut1*[F/F] : *Vav1-iCre* (Pof cKO) and *Eogt*[-/-]*Pofut1*[F/F] : *Vav1-iCre* (EPof dKO) mice. Absolute numbers or frequency % of hemopoietic cell subsets in bone marrow are shown. Gating strategies are shown in Supplementary Figure 6 and additional data are shown in Supplementary Figure 7. Short term-hemopoietic stem cells (ST-HSC), long-term hemopoietic stem cells (LT-HSC), Lin-Sca1+c-Kit+ (LSK) cells, hematopoietic stem progenitor cells (HSPCs), multipotent progenitor-2, -3, -4 (MPP2, -3, -4), lymphoid primed multipotent progenitor cell (LMPP), common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte monocyte progenitor (GMP), megakaryocyte erythrocyte progenitor (MEP), T cells, B cells and myeloid cells. Each symbol represents a mouse of 7-8 weeks. Data are presented as mean ± SEM. *p <0.05, **p<0.01, ***p<0.001, ****p<0.001 based on two-tailed Student's t test or (*) p <0.05 based on one-tailed Student's t test.

(Figure 7B). Histological analysis revealed extramedullary hematopoiesis in both Pof cKO and EPof dKO spleens, with larger areas of extramedullary hematopoiesis observed in EPof dKO spleen (Figure 7A).

The absolute numbers of single positive CD4+ and CD8+ T cells were significantly reduced in Pof cKO, and further reduced in EPof dKO spleen (Figure 7C and Supplementary Figure 10). While CD19+ and B220+ B cells were proportionally increased in both Pof cKO and EPof dKO spleen, the frequency % of the CD19+/B220+ B cell population was reduced (Figure 7D). The frequency of Fo-B and the number of MZ-B cells were also reduced (Figure 7D and Supplementary Figure 10). While the absolute number of MZ-P cells did not change in the mutants, the frequency % of MZ-P precursors was increased in EPof dKO spleen (Figure 7 and Supplementary Figure 10). Myeloid cell subsets such as dendritic cells (CD11b/c+) and Gr-1+

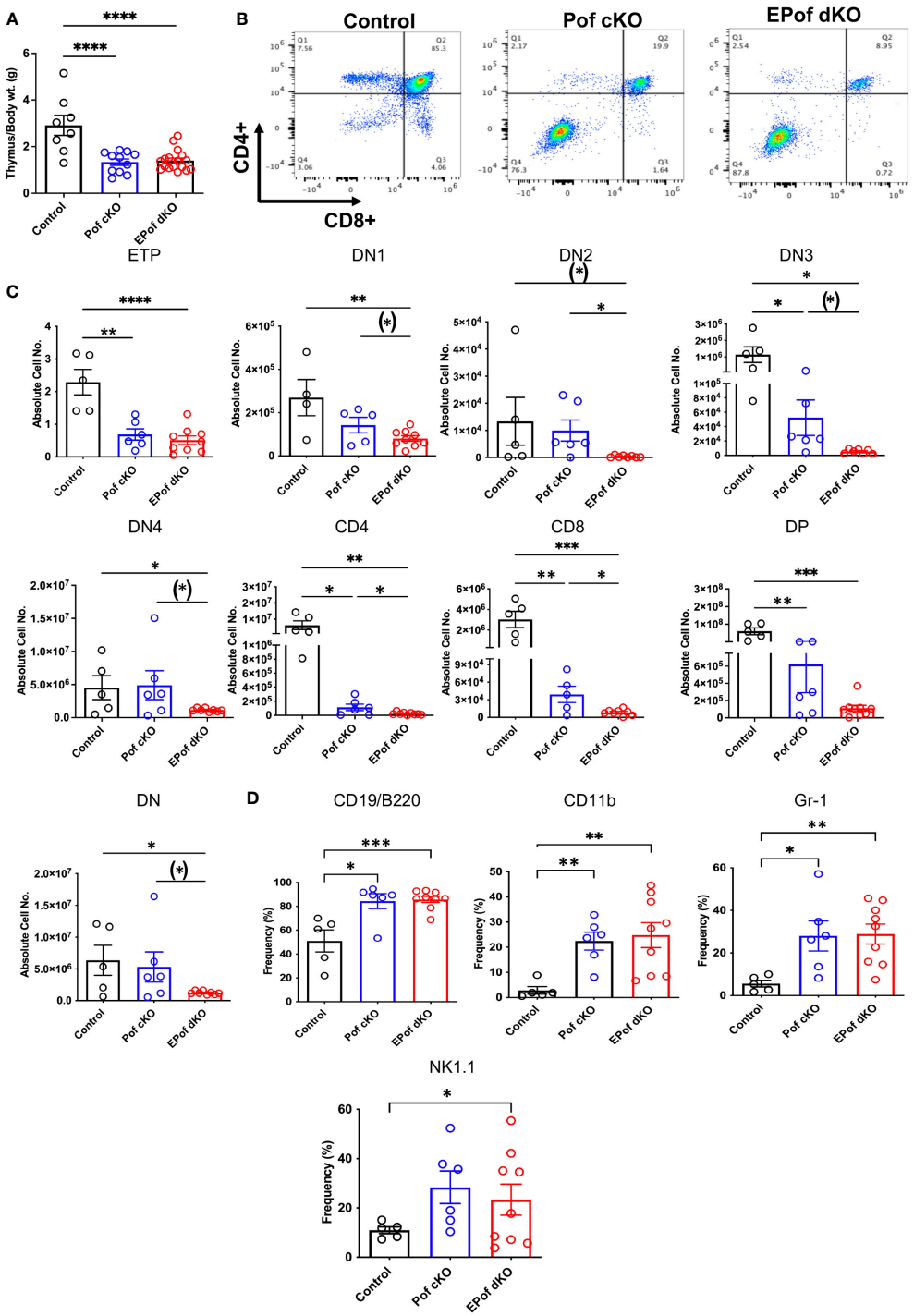

FIGURE 6
Impaired T cell development in Pof cKO thymus is worse in EPof dKO thymus. **(A)** Thymus weight compared to body weight. **(B)** Representative flow cytometry profiles of thymocytes using Abs to CD4 and CD8 cell surface markers after gating on live cells. **(C)** Absolute cell numbers or frequency % of T cell subsets that differed between Control and either mutant. ETP (DN1 cells that were cKit/CD117+), DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD44-CD25+), and DN4 (CD44-CD25-) T cell progenitors, DN (CD4 and CD8 double negative), DP (CD4 and CD8 double positive). **(D)** Absolute cell numbers or frequency % of B cell and myeloid cell subsets that differed from control are shown. Additional data are shown in Supplementary Figure 9. Each symbol represents a mouse of 7-8 weeks. Data are presented as mean ± SEM. *p <0.05, **p<0.01, ***p<0.001, ****p<0.001 based on two-tailed Student 'st test or (*) p <0.05 based on one-tailed Student's t test.

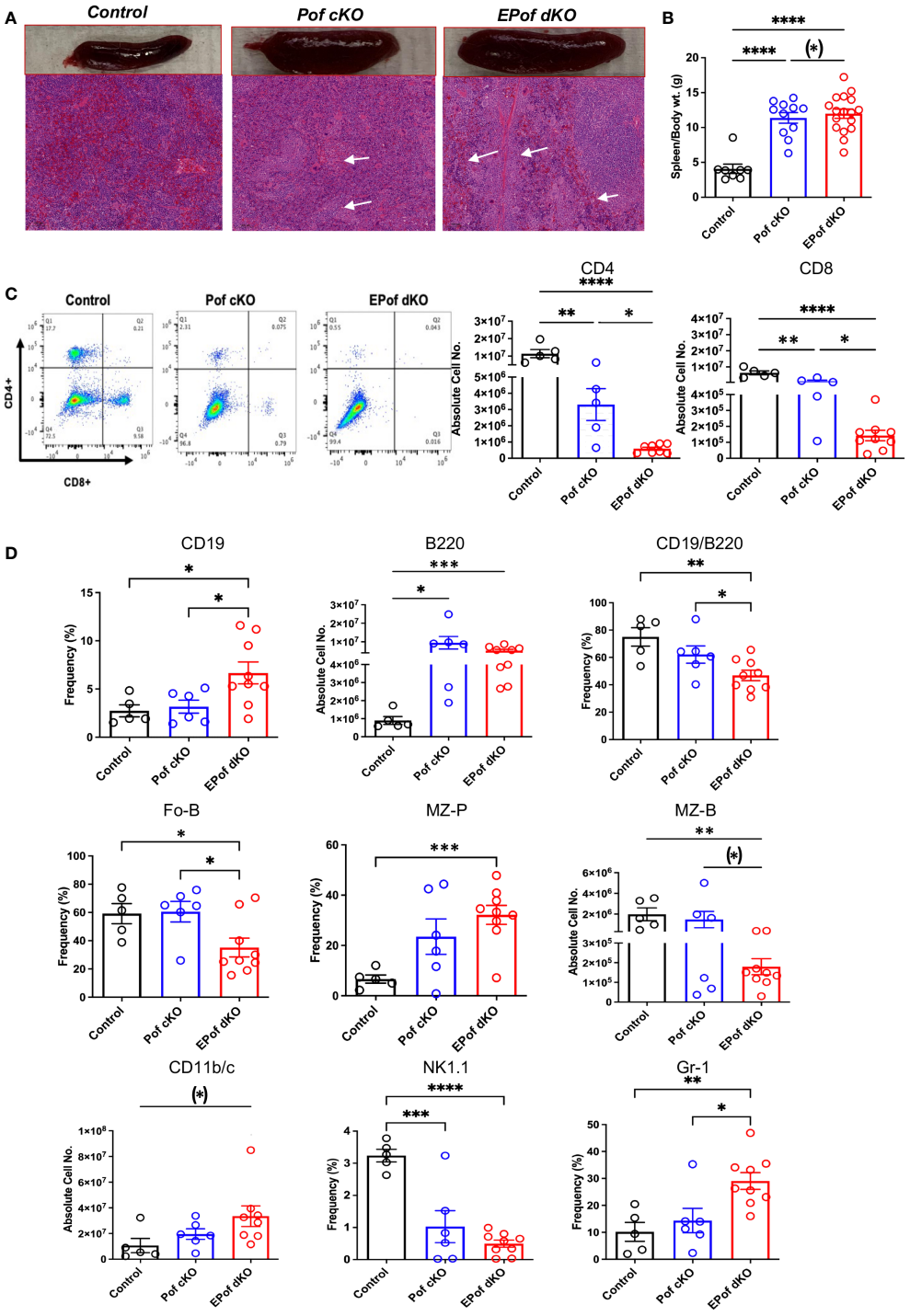

FIGURE 7
Impaired B and T cell development in Pof cKO spleen is worse in EPof dKO spleen. **(A)** Images of spleen sections stained by H&E revealing splenomegaly and extramedullary hematopoiesis (Bars represent 100 μm). **(B)** Spleen weight compared to body weight. **(C)** Flow cytometric profiles and histogram quantification for CD4 versus CD8 cell surface expression after gating on live cells. **(D)** Absolute cell numbers or frequency % of splenic lymphoid and myeloid cell subsets that differed from control are shown. CD19/B220 (B cells), Fo-B (follicular B cells), MZ-B (marginal zone-B cells), MZ-P (marginal zone precursors), CD11b/c (dendritic cells), NK1.1 (natural killer T cells) and Gr-1 (granulocytes). Additional data are shown in Supplementary Figure 10. Each symbol represents a mouse of 7-8 weeks. Data are presented as mean ± SEM. *p <0.05, **p<0.01, ***p<0.001, ****p<0.001 based on two-tailed Student's t test or (*) p <0.05, based on one-tailed Student's t test.

granulocytes were increased in greater proportion in EPof dKO compared to Pof cKO spleens. Natural killer T cells were reduced in frequency in both Pof cKO and EPof dKO spleens (Figure 7D and Supplementary Figure 10).

Discussion

Defining specific roles for the glycans that regulate Notch signaling in lymphoid and myeloid development facilitates our understanding of cell fate decisions controlled by Notch signaling, and of potential consequences for people with congenital diseases that perturb Notch signaling (2, 43). Several congenital diseases inhibit the synthesis or extension of O-glycans that regulate Notch signaling and may induce immune cell defects. Mutations in *EOGT* cause Adams Oliver syndrome (44) and autosomal dominant mutations in *POFUT1* cause Dowling Degos Disease 2 (DDD2) (45). Here we show that the generation of certain lymphoid and myeloid subsets in bone marrow, thymus and spleen was perturbed in mice lacking *Eogt*. In bone marrow, loss of EOGT caused increased numbers of B cells and granulocytes. In thymus, the phenotype of *Eogt* null mice was similar, but not identical to, mice which lack all three Fringe genes (*Fng* tKO) (16). T cell progenitors DN1 and DN2 were reduced in frequency or absolute number, whereas DN4 T cell progenitors were increased, DP T cells were slightly reduced in frequency, and CD4+ and CD8+ SP T cells were increased in absolute numbers. Inhibition of Notch signaling in thymus is well known to lead to the generation of B cells and myeloid cells in thymus (9, 11, 46, 47). *Eogt* null thymus contained significantly increased numbers of B cells and granulocytes, and an increased frequency of myeloid cells. In spleen, *Eogt* null splenocytes included increased numbers of several B cell subsets, although there were no effects on T cells, unlike in *Fng* tKO mice which had a reduced frequency of T cells in spleen (16). Deletion of RBP-Jk by *Vav1-iCre* results in an increased frequency of CD19+ B cells (48), also observed in *Eogt* null spleen. *Eogt* null mice showed increased absolute numbers of CD19+ B cells, increased Fo-B cells, and a decrease in natural killer T-cells and dendritic cells in the spleen. The overall *Eogt* null phenotype was largely cell autonomous following bone marrow transplantation. In addition, expression of Notch target genes *Hes1* and *Il2ra* was reduced, similar to *Fng* tKO DN T cell progenitors that had reduced *Il2ra* and *Dtx1* expression (16). The combined data provide strong evidence that EOGT and O-GlcNAc glycans are required for optimal Notch signaling in the development of lymphoid and myeloid cells from HSC.

Further evidence that EOGT and O-GlcNAc glycans support Notch signaling in lymphoid and myeloid development was obtained in compound mutant mice lacking *Eogt* and conditionally lacking *Pofut1* in HSC. EPof dKO lymphoid and myeloid populations in BM, thymus and spleen were more affected compared to Pof cKO. We conclude that the loss of *Eogt* and O-GlcNAc glycans in EPof cKO HSC exacerbated the deficits in T, B and myeloid differentiation evident in Pof cKO mice. This result, and our findings that *Eogt* is required for optimal Notch signaling and the differentiation of HSC, provide an explanation for the observation that *Pofut1*:*Mx1-Cre* T cell deficiencies were not as severe as those obtained in *RBP-Jk* : *Mx1-Cre* thymus (25). In the absence of POFUT1 and O-fucose glycans, the O-GlcNAc glycans transferred by EOGT to Notch receptors can support a low but significant level of Notch signaling. Thus, as shown here and discussed in a recent review (49), the O-fucose and O-GlcNAc glycans on Notch act synergistically to provide optimal Notch signaling in lymphopoiesis and myelopoiesis.

The conclusions obtained from these experiments are necessarily limited by the difficulty of demonstrating structural changes in the O-glycans on Notch receptors predicted to change in the absence of relevant glycosyltransferase(s). For example, to obtain sufficient NOTCH1 from splenic T cells for analysis by mass spectrometry, it was necessary to activate the T cells in culture thereby increasing cell surface NOTCH1 by ~ 10-fold (50). Furthermore, the effects of conditional deletion of *Eogt* in HSC would allow us to define the contribution, if any, of *Eogt* null stroma to the *Eogt* null phenotype. It would also be important to determine Notch ligand binding and Notch target gene expression in cells from different HSPC lineages in Pof cKO and EPof dKO mice. Single cell RNA-seq of different mutant HSC and HSPCs would greatly expand our understanding of the pathways affected by altered Notch signaling due to loss of regulation by O-glycans in HSC. Moreover, it would be important to investigate roles for the O-glycans on Notch ligands by deleting *Pofut1*, *Eogt* and related glycosyltransferase genes in stromal cells. Another important question for future work is whether changes in immune subset cell numbers reflect apoptosis or proliferation of one or more populations. This question and changes in Notch target gene expression are difficult to determine because changes in Notch signaling lead to changes in cell fates. For example, thymus lacking POFUT1 or EOGT + POFUT1 has few, if any, T cell progenitors and largely comprises B cells, myeloid and NK cells making comparisons with wild type or *Eogt* null thymocytes meaningless in terms of underlying mechanisms. Thus, the major benefit of the experiments reported here is to pinpoint cell fate decisions regulated differently by the strength of Notch signaling which in turn is regulated by the O-glycans of Notch receptors. In addition, our findings contribute to the growing body of evidence that glycans are integral to the functions of all glycoproteins, glycolipids, glycophosphatidylinositol (GPI)-anchored proteins and proteoglycans. Glycans must be included as essential contributors to life, along with proteins, nucleic acids and lipids, to facilitate progress in the personalized medicines of the future (51).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was not obtained from the owners for the

participation of their animals in this study because the mice were bred in my colony at Albert Einstein following approval of the IACUC. I am therefore the owner.

Author contributions

Conceptualization, PS; Methodology, PS and AT; Investigation, AT and PS; Visualization AT and PS; Funding acquisition, PS; Project administration, PS; Supervision, PS; Writing – original draft, AT; review and editing, PS and AT. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1097332/full#supplementary-material

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