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The Contribution of Immunoglobulin G to Inflammation is Regulated by The Site of Production via Glycosylation

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Abstract: Immunoglobulin G (IgG) is one of the main contributors to inflammation due to its function and amount in circulation. This function of IgG can swing from pro- to anti-inflammatory states with small changes in glycan structure at Asparagine-297 of IgG Fc domain. This variation is implicated in pathophysiology of autoimmune diseases such as rheumatoid arthritis. Although our understanding about the structure-function relation of IgG Fc glycosylation has immensely increased for the last four decades, it is still limited about the regulators of this glycan structure. To broaden our understanding, ex-vivo cell cultures derived from lymph node (LN), spleen (SP) and bone marrow (BM) of K/BxN mouse, which is a model for autoantibody-dependent rheumatoid arthritis, were established. The glycan structures of antigen purified anti-glucose-6-phosphate isomerase (anti-GPI) autoantibody heavy chains produced in these cultures were characterized using HPLC. According to the results, while IgG produced in LN is decorated with the highest amount of pro-inflammatory agalactosylated structure, IgG produced in SP has the highest amount of anti-inflammatory sialylated structure. Interestingly, IgG produced in BM stands in between them due to more balanced distribution of glycan structures. In conclusion, the ex-vivo cell cultures of LN, SP and BM were successfully established, and the contribution of IgG to on-going inflammation is regulated by the production site of anti-GPI autoantibody via glycan structures. In addition, N-glycan profile of anti-GPI autoantibody produced in LN closely replicates the N-glycan profile for anti-GPI autoantibody from K/BxN serum indicating that anti-GPI autoantibody in circulation is heavily produced in LN.

İmmünoglobulin G'nin İnflamasyona Katkısı, Glikozilasyon Yoluyla Üretim Yeri Tarafından Düzenlenir

Anahtar Kelimeler
İmmünoglobuin G,
IgG,
Romatoid artrit,
İltihaplanma,
Siyalilasyon,
Galaktosilasyon,
Proinflamatuar,
Antiinflamatuar,
Glikozilasyon

Öz: İmmünoglobulin G (IgG), işlevi ve dolaşımdaki miktarı nedeniyle inflamasyona katkıda bulunan başlıca faktörlerden biridir. IgG'nin bu işlevi, IgG Fc bölgesindeki Asparagin-297 glikan yapısındaki küçük değişikliklerle pro-inflamatuar durumdan anti-inflamatuar duruma geçebilmektedir. IgG Fc glikozilasyonunun yapı-fonksiyon ilişkisi hakkındaki bilgi son kırk yılda büyük ölçüde artmış olsa da, bu glikan yapısının düzenleyicileri hakkında hala sınırlıdır. Anlayışımızı genişletmek için, otoantikor bağımlı romatoid artrit modeli olan K/BxN faresinin lenf nodu (LN), dalak (SP) ve kemik iliğinden (BM) türetilen ex-vivo hücre kültürleri oluşturuldu. Bu kültürlerde üretilen antijen ile saflaştırılmış olan anti-glikoz-6-fosfat izomeraz (anti-GPI) otoantikor ağır zincirlerinin glikan yapıları HPLC kullanılarak karakterize edildi. Sonuçlara göre, LN'de üretilen IgG en yüksek miktarda pro-inflamatuar agalaktosile yapıya sahipken, SP'de üretilen IgG en yüksek miktarda anti-inflamatuar siyalile yapıya sahiptir. İlginç bir şekilde, BM'de üretilen IgG, glikan yapılarının daha dengeli dağılımı nedeniyle bunların arasında yer almaktadır. Sonuç olarak, LN, SP ve BM'nin ex-vivo hücre kültürleri başarıyla oluşturulmuştur ve

IgG'nin devam eden inflamasyona katkısı, glikan yapıları aracılığıyla anti-GPI otoantikorunun üretim bölgesi tarafından düzenlenmektedir. Ayrıca, LN'de üretilen anti-GPI otoantikorunun N-glikan profili, K/BxN serumundan elde edilen anti-GPI otoantikorunun N-glikan profiliyle yakından örtüşmektedir; bu da dolaşımdaki anti-GPI otoantikorunun LN'de yoğun olarak üretildiğini göstermektedir.

1. Introduction

Immunoglobulin G (IgG) glycosylation is implicated in the pathophysiology of many autoimmune diseases, viral and bacterial infections, and normal physiology of aging and gender differences [1-13]. IgG is the major immunoglobulin in circulation [14, 15]. It is composed of two heavy-chains (HC) and two light-chains (LC) with an N-glycan attached to Asparagine-297 of each HC [16-21]. The quaternary structure of these HCs and LCs generates functionally two distinct domains (Fab and Fc) connected via flexible hinge region [22, 23].

The protein backbone of IgG Fc domain is germ-line encoded and therefore constant for each IgG subclasses [14]. Regardless of IgG subclasses, all of them has an N-glycan structure in a conserved sequence of EQYN$_{297}$STYR on their Fc-domains [21]. Unlike most glycan structures, this N-glycan structure is placed on the surface of IgG but is uniquely enclosed within a confined space determined by three-dimensional structure of the protein backbone of the Fc domain (Figure. 1A) [17, 18, 24, 25]. It is a biantennary complex-type oligosaccharide with variable structures (Figure. 1B) [26, 27]. Its core structure is constant as GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asparagine-297 (Figure. 1B). This core structure can be further modified by the addition of fucose (Fuc, core-fucosylation), N-acetylglucosamine (GlcNAc, bisecting GlcNAc), galactose (Gal) and sialic acid (Sialyl) moieties by synthetic enzymes in endoplasmic reticulum and Golgi pathway to form the mature glycoforms before secretion to plasma (Figure. 1B) [28, 29]. As a result of differential processivity by the synthetic enzymes, this glycan can have at least 36 different structures which can be easily detected and characterized by liquid chromatography and other physical techniques [30-32].

Historically, IgG glycan structures mentioned above are grouped based on the total number of galactose residues on this glycan structure. It can contain none, one or two galactose residues and is classified as G0, G1 and G2, respectively. These different N-glycan structures modulate the conformation of IgG Fc domain and therefore its affinity toward Fc gamma receptors (FcgRs) and complement component proteins leading to different physiological outcomes [24, 34-42]. In fact, in *in vitro* and *in vivo* experiments, the total removal of this glycan structure using molecular biology techniques or enzymes such as *Streptococcus pyogenes* endoglycosidase (EndoS) totally abrogates the binding of IgG Fc domain to FcgRs and therefore dismantles the IgG-dependent inflammation [42-50]. More strikingly, even the small changes in the glycan structure such as sialylation and core-fucosylation have immense effect on the inflammatory potential of IgG [51-62]. The fully processed IgG glycan structure capped with sialic acid is considered as an anti-inflammatory due to the higher affinity toward FcgRIIB, an inhibitory FcgR [63-66]. However, its exact mechanism is yet to be discovered [63, 64, 67-71]. The absence of the core-fucosylation translates into higher antibody (ab)-dependent cellular cytotoxicity due to the considerably higher affinity toward FcgRIIIA [36, 58, 72]. In addition, both sialylation and core-fucosylation can modulate the inflammatory milieu of their microenvironments via changing the cytokine secretion such as IL-10 and IL-1b, and TNF, respectively [73, 74]. In return, the modified microenvironment by cytokines such as IFN-g, IL-21 and IL-17A changes the IgG glycan structure in *in vitro* experiments with human B cells [75, 76]. These results clearly show that IgG glycosylation is a dynamic process and regulated by feedback mechanisms.

In patients with RA, the effect of G0 structure is intensely investigated and the results show that the increase in the ratio of agalactosylated to monogalactosylated glycan structures (G0 ratio) predates the RA onset and correlates with the RA disease activity [8, 9]. It is more relevant to the mechanism of RA as an autoimmune disease that this aberrant galactosylation of IgG enriches in autoantibody (auto-ab) pool reactive with the citrullinated protein epitope in patients with RA [9, 77-81]. This phenotype is not limited to RA but rather extends to different autoimmune diseases such as juvenile idiopathic arthritis, psoriatic arthritis, systemic lupus erythematosus and anti-cytoplasmic ab as well as viral and bacterial infections [2, 81, 82]. In addition, the G0 ratio for healthy human shows variation with respect to gender and age [9, 10].

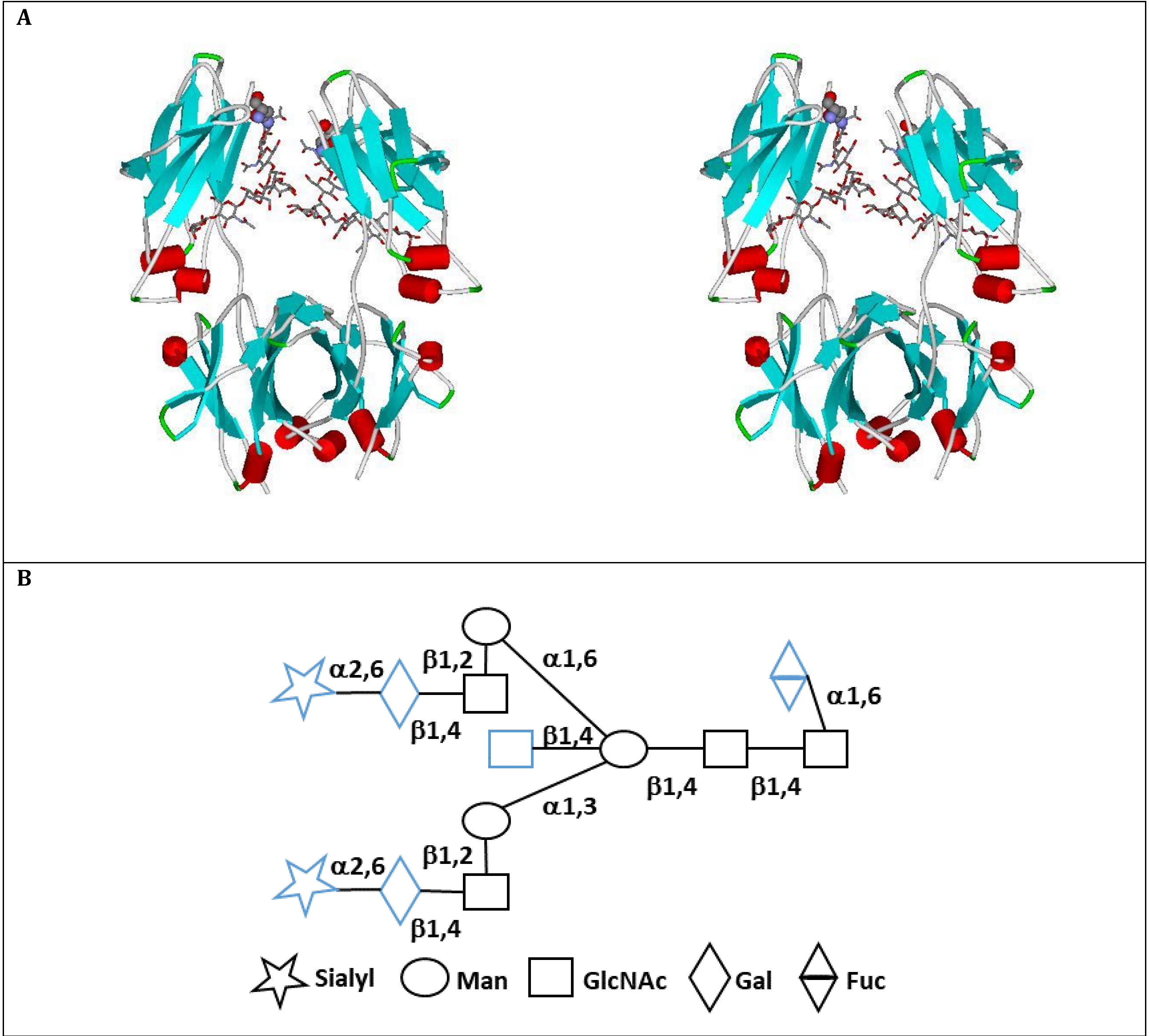

Figure 1. The variable N-glycan structure is sequestered within the IgG Fc domain. (**A**) The stereo view of IgG Fc domain with Galβ1,4GlcNAcβ1-2Manα1–6(Galβ1,4GlcNAcβ1-2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1 attached to Asparagine-297 is shown. While glycan structure is presented as stick according to the identity of the atoms, the protein backbone of the Fc domain is presented as schematic colored according to the secondary structures and Asparagine-297 which glycan structures attached to, is presented as ball and stick. This figure is generated using crystal structure from the PDB entry 5GSQ [33]. (**B**) The fully processed glycan structure is shown where the constant core and the variable glycan structures are colored in black and blue, respectively.

IgG has a critical function for the defense of the host as well as autoimmune diseases in bridging the adaptive and the innate immune arms by the interaction of Fab with the antigen and Fc domain with FcgRs on the effector cells [83]. This brings out the antigen-dependent destructive nature of innate arm which heavily depends on the glycan structure on IgG Fc domain. Therefore, it is crucial to understand the regulators of IgG Fc glycan structure. Currently, it is shown that Estradiol and the nature of the antigen regulate IgG glycosylation by *in vivo* experiments using human samples [6, 58]. In *in vitro* experiments, the treatment of B cell by all-trans retinoic acid, IL-21, interferon-gamma and CpG oligodeoxynucleotide can also change the IgG Fc glycan structure [76]. In addition, the regulation of IgG glycan structure also exists at cellular level. For example, in *in vivo* mouse experiments, T-cell dependent IgG production leads to more sialylated IgG Fc glycan than T cell-independent production of IgG and the depletion of neutrophils also shows changes in IgG glycosylation pattern [84, 85]. In combination with these observations, the proven presence of LN-like structures in the synovium of RA patients and IgG isolated from synovium of the active RA patients with more G0 structure than the matching serum samples suggest that IgG Fc glycosylation is regulated by the microenvironment with soluble and cellular factors [78, 86, 87]. However, the effect of microenvironment such as LN, spleen (SP) and bone marrow (BM) on the IgG glycan structure hasn't been investigated yet.

IgG is solely produced by B cell [88, 89]. Shortly, the development of B cell starts in BM from pluripotent hematopoietic stem cells [88, 89]. After developing to an antigen specific naïve B cell with surface IgM, it leaves BM and circulates among different secondary lymphoid organs such as LN and SP to find the cognate antigen presented by T follicular helper (Tfh) cells in the B cell follicles compartment of the germ centers [90-92]. Once B cell successfully recognizes the cognate antigen and acquires T cell help, it undergoes clonal expansion and somatic hypermutation to increase the affinity of B cell receptor (BCR) toward the antigen [88]. After the T-cell dependent affinity maturation of the BCR, B cell undergoes class-switching to produce IgG rather than IgM. At this point, B cell simply can develop into memory B cell, long-lived plasma cell or short-lived plasma cell [93]. Once this decision is made, long-lived plasma and memory B cells move to a special niche in BM where they can stay for years and provide memory for the next infection [94, 95]. For the short-lived B cell, it stays in the B cell follicular compartments of the germ centers in LN, produces lots of IgG and dies off [96-98].

The detailed mechanistic investigation of human disease requires an animal model replicating the features of related human disease. Development of arthritis in human and K/BxN mouse model depends on the production of auto-abs against autoantigens; the anti-citrullinated protein (ACPA) and glucose-6-phosphate isomerase (anti-GPI) abs, respectively [99-109]. As in human, anti-GPI auto-ab in K/BxN mouse is aberrantly galactosylated as G0 being the dominant glycan structure. This aberrant galactosylation of anti-GPI ab correlates with disease activity in K/BxN mouse as ACPA ab in human with RA [9, 26, 110]. Therefore, K/BxN mouse model is a good model to study IgG glycosylation [99-102, 111, 112]. In this model, the effect of microenvironment such as LN, SP and BM on the glycosylation of anti-GPI auto-ab is investigated to determine whether the glycan structure varies according to the microenvironment where it is synthesized. Although the number of mice utilized in the experiments is small, the results clearly show that IgG HC N-glycan profile of anti-GPI auto-ab from LN closely replicates the *in vivo* glycan profile with the dominant presence of proinflammatory G0 structure while glycan profile from SP has the highest anti-inflammatory sialylated structure. Interestingly, IgG HC N-glycan structure from BM is between LN and SP. These results show that the contribution of anti-GPI auto-ab to ongoing inflammation is regulated by the site of IgG production.

2. Materials and Method

2.1 Production, partial characterization and immobilization of GST-GPI protein on Glutathione-Sepharose beads

The expression plasmid pGEX-GPI carrying mouse GPI gene was constructed. Then, pGEX-GPI was used to transform the T7 express competent *E. coli* cells to express GST-GPI protein. Shortly, *E. coli* cells carrying pGEX-GPI plasmid and growing on a LB/AMP agarose plate were transferred into 1L of LB/AMP media placed on a shaker at 250 RPM at 37°C. When the OD_{590} of growth media reached 0.5, the expression was induced by the addition of isopropyl beta-d-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 5 h induction to produce GST-GPI, *E. coli* cells were spun down and washed with cold phosphate-buffered saline (PBS) (x 2). Then, the cells were lysed using freeze/thaw cycles (4 x), nonylphenoxy polyethoxy ethanol (NP40) and sonication. This was followed by spinning samples at 30k x g for 30 min. The soluble cell fractions were removed and passed through 1 mL of the Glutathione-Sepharose beads in a column equilibrated with PBS at the benchtop. After washing the column with PBS (5 x column volume (CV)), the bound protein was released from the column by applying 5 mM reduced glutathione in PBS and collected in 100 µL fractions. Then, 10 µL of the samples from each fraction was used to determine protein concentrations with Bradford assay and loaded onto 12.5% SDS-PAGE gel which showed relatively clean bands with expected protein size around 59 kDa (Figures 2A and 2B).

The functional interaction between GST-GPI protein and anti-GPI ab was investigated using Enzyme-Linked Immunosorbent Assay (ELISA) and affinity column purification approaches. In ELISA experiment, GST-GPI protein was immobilized on 96-well plate and was titrated with ab pool purified from K/BxN mouse serum using Protein G (GE Healthcare) as described in the manual. It was visualized using Peroxidase AffiniPure® $F(ab')_2$ Fragment Donkey Anti-Mouse IgG (H+L) (Jacson Immunoresearch) as secondary ab.

In column characterization, the GST-GPI immobilized Sepharose beads (1 mL) was synthesized as described above. Serum (100 µL) from K/BxN mouse with active arthritis was loaded on to this column. After washing with PBS (10 x CV), anti-GPI ab bound to the column was released with 10 mM phosphate buffer pH 2.5 and collected in 100 µL fractions. Then, 10 µL from load, flow-through and each fraction were loaded on 12.5% SDS-PAGE gel (Figure. 2D). Anti-GPI ab ELISA performed as follows. GST-GPI was produced in *E. Coli* cell and purified using affinity column with GST-Tag. Each well of 96-well plate was coated by applying 100 µl of 5µg/ml GST-GPI in 0.01M Sodium Bicarbonate buffer at pH 7.0 and incubating overnight at 4°C. After removing the unbound material from the plate, it was washed twice with 100 µl of PBS containing 0.01% Tween-20 following 5 min incubation at RT. The wells on the plate were coated with 100 µl of Super Block containing 40 gr Whey, 150 ml normal goat serum, 5 ml Tween-

20 and 0.5 gr NaN_3 in 1 L of PBS for 1 h at RT. Then, the plate was washed twice with 100 µl of PBS- 0.01%Tween-20 for 5 min per wash at RT. To each well, 50 µl of anti-GPI ab, which was purified from K/BxN mouse serum and diluted with Super Block solution to different concentrations, was applied. Then, the plate was incubated for 1h at RT and washed four times with 100 µl of PBS containing 0.01% Tween-20 (x4), 5 min per wash at RT. For detection, 100 µl of 1/400 diluted Horseradish Peroxidase AffiniPure® $F(ab')_2$ Fragment Donkey Anti-Mouse IgG (H+L) (Jacson Immunoresearch) was applied to each well as secondary ab in 0.5% BSA-PBS. Then, the plate was incubated for an hour at RT and washed with 100 µl of PBS with 0.01% Tween-20 (x 4). After adding 100 µl of substrate TMB (3,3',5,5'-tetramethylbenzidine) per well and incubating for 5 min at RT, the readings were measured at 650 nm.

2.2 Extraction of LN, SP and BM from K/BxN mice and establishing their cell cultures

Inguinal and popliteal LNs, SP and BM were extracted from 8-9 weeks old arthritic K/BxN mouse (n = 5) with active RA in two different experiments and single cell suspensions for LN and SP were generated by gentle rubbing them in between microscope slights in 10 mLs of 10% Ultra-Low IgG Fetal Bovine Serum (Invitrogen) in RPMI 1640 medium (Thermo Fisher Scientific). Single cell suspension for BM was prepared by flushing out BM from femur and tibia after cutting off the epiphyses from both ends. BM was pushed through a needle to generate single cell suspension. The single cell suspensions from LN, SP and BM were then passed through 100 µm screen. The red blood cells were lysed with ACK treatment according to the manual provided by the producer (Invitrogen). Then, the cells were spun down. Pelleted cells were resuspended in 1 mL of Serum Free Media (SFM) and incubated at 37°C, 5% CO_2 and 95% humidity. After incubating for an hour, cells in suspension were collected, washed and placed in new petri dishes. This was repeated three times. Then, cells in suspension were placed in new petri dish and incubated for IgG production for 5 days.

2.3 N-glycan analysis of anti-GPI ab heavy chain

Anti-GPI ab was produced in the ex-vivo cell culture of LN, SP and BM as described above and purified with GST-GPI immobilized Glutathione-Sepharose beads on the benchtop. Shortly, the growth media from each microenvironment (1 mL) was mixed with 10 µl of the GST-GPI immobilized beads from 20% slurry. After incubation for an hour at RT, samples were spun down at 500 x g for 5 min and unbound material was removed. After washing the beads with PBS (5 x), the beads with the bound materials were mixed with the SDS-PAGE gel sample buffer. Following boiling and cooling down the samples to RT, they were loaded onto the gel to separate HC from LC. The bands for HC were cut off from the gels and placed in 1.5 mL Eppendorf tubes. The rest of the procedure was followed according to the procedure described for in-gel N-glycan analysis for the SDS-PAGE gel bands using High Performance Liquid Chromatography (HPLC) by Royle et al. 2008 (119). Shortly, the piece of the gel containing HC in the Eppendorf tube was washed with acetonitrile followed by 20 mM NaHCO3 pH 7.2. After repeating this step, gels were dried and treated with N-glycosidase F (PNGaseF) in 50 ul of PNGase buffer (Prozyme). N-glycans released from IgG were collected, labeled with 2-aminobenzamide (ProZyme) and purified using the Whatman 3MM chromatography paper. These labeled glycans were separated on a normal-phase TSK gel amide-80 column (4.6 mm internal diameter and 25.0 cm long) attached to a Waters 1525 binary HPLC and areas under the peaks were quantified by Waters Empower software.

2.4 Mice

K/BxN mice were obtained from The Jackson Laboratory and housed at the AAALAC-accredited, specific pathogen free Dana-Farber Cancer Institute mouse facility. Mouse experiments started after housing mice for a week.

2.5 Statistical analyses

The statistical differences between groups were performed using Student's T test in GraphPad Prizm 4.

3. Results

Contribution of IgG to inflammation is regulated by N-glycan structure at the IgG Fc domain which changes with age, gender and the health status of individual as in RA. Here, the regulation of this glycan structure is investigated with respect to different microenvironments such as LN, SP and BM from K/BxN mouse using their ex-vivo cell cultures.

3.1 Establishing an affinity column for the purification of anti-GPI abs

N-glycosylation of IgG HC Fc domain in auto-abs such as ACPA in human with RA and anti-GPI in its related mouse model K/BxN bears heavily G0 structures [9]. To assess the effect of the different microenvironments on IgG Fc glycosylation in K/BxN mouse with active RA, an autoantigen Glutathione S-transferase (GST)-GPI protein in this mouse model was produced in *E coli* cells (Figures 2A and 2B). Shortly, the soluble fractions of *E. coli* cell were passed through the column containing glutathione-Sepharose beads. After washing the column thoroughly, the materials captured by the column were eluted with reduced glutathione (Figure 2A). The elution profile is bell-shape as expected (Figure 2A). To confirm the identity of this protein, the collected fractions were run on the SDS-PAGE gel (Figure 2B). On the gel, the dominant protein band has a molecular weight around 59 kD which is very close to the expected molecular weight of GST-GPI protein (Figure 2B). To further investigate the identity of the protein produced in *E coli* cells as being the GST-GPI protein, ELISA approach was utilized. Each well on the 96-well plate was coated with the protein produced in *E. coli* cells and purified on glutathione Sepharose column. Then, this immobilized protein on the plate was titrated with Protein G purified IgG pool from K/BxN mouse serum with active RA. The titration result shows a dose-dependent response supporting the above result as the *E. coli* produced protein being the GST-GPI protein (Figure 2C). In addition, the capacity of this protein immobilized on the Sepharose beads was investigated whether it captures IgG from K/BxN mouse serum or not. Serum sample of K/BxN mouse was loaded onto GST-GPI immobilized glutathione-Sepharose column. After collecting the unbound material, the column was washed with PBS. Then, the bound material was eluted with 10 mM phosphate buffer at pH 2.5 and collected in 100 uL of fractions. These samples were characterized on the SDS-PAGE gel. According to the result, there are three protein bands (Figure 2D). The two bands with lower molecular weight show a similar bell-shape distribution with respect to the fraction number. In addition, the molecular weights of these lower two bands correlate well with the molecular weights of HC and LC of mouse IgG as being 50 and 25 kDa, respectively. This clearly shows that they are IgG HC and LC. The third band is impurity and most likely albumin protein. In

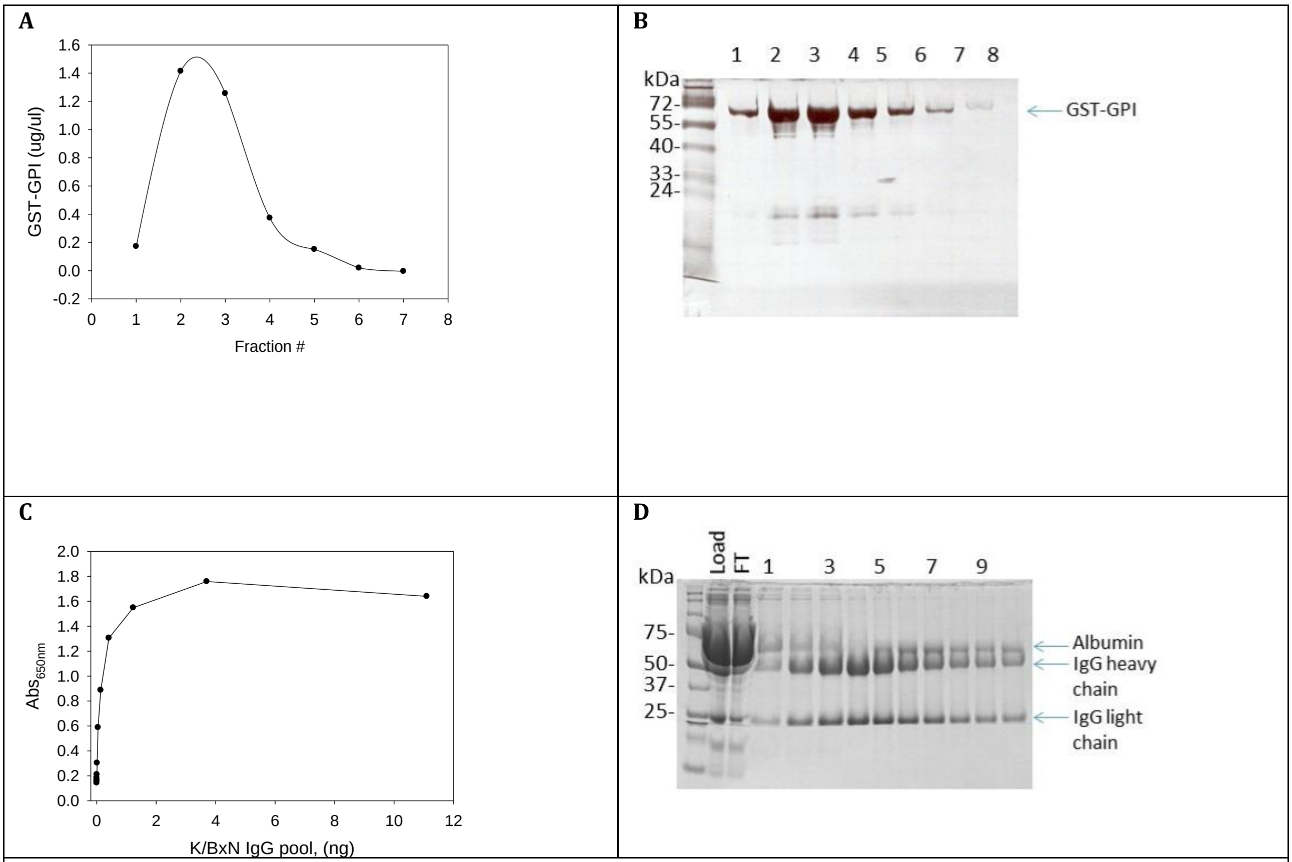

Figure 2. GST-tagged GPI overexpressed in *E. coli* is functional and recognized by anti-GPI ab from K/BxN mouse serum. (**A**) GST-GPI protein produced by *E. coli* cells was purified on Glutathione-Sepharose column and quantified using Bradford protein assay where X- and Y-axis represent the fraction number and absorbance at 595 nm using Bradford protein assay, respectively. (**B**) Purity of GST-GPI was assessed by running 10 uL of each fraction on 12.5% SDS-PAGE gel. (**C**) Functional interaction between *E. coli* expressed GST-GPI protein and *in vivo* produced anti-GPI ab was presented using ELISA. (**D**) Purification of anti-GPI ab was performed using GST-GPI immobilized glutathione-Sepharose column. The success of the purification is assessed using 12.5% SDS-PAGE gel where 10 uL from the K/BxN mouse serum (Load), material which didn't bind the column (FT) and elution fractions were loaded.

conclusion, the above results show that the GST-GPI protein was successfully produced in *E. coli* cells and GST-GPI protein, which was immobilized on Sepharose beads, can capture IgG in serum from K/BxN mouse.

3.2 Effect of microenvironment on anti-GPI auto-ab glycosylation

The functional importance of the IgG glycosylation is well appreciated but the regulators of this glycan structure are not well-understood. Especially, the effect of microenvironment such as LN, SP and BM on IgG glycosylation hasn't been systematically investigated yet. The N-glycan profiles for anti-GPI ab produced in *in vivo*, LN, SP and BM show clearly similarities and difference (Figures 3A and 3B). The comparison of these N-glycan profiles from anatomically and functionally different sites shows that the N-glycan profile of anti-GPI ab produced in LN closely replicates the N-glycan profile of anti-GPI ab in circulation (Figure 3A). In detail, N-glycosylation profiles for anti-GPI ab produced in *in vivo* and ex-vivo cell culture of LN have strong signals for G0 and G1 and a minor peak for G2. N-glycan profiles for anti-GPI ab produced in ex-vivo culture of SP and BM have strong signals for G0, G1 and G2. In addition, N-glycan profile for anti-GPI ab produced in SP has also relatively strong S1 signal compared to the other sites.

According to the results, G0 ratio for anti-GPI ab produced in *in vivo* (mean ± SD, 1.9936 ± 0.3010) is not statistically different from G0 ratio for anti-GPI ab produced in LN (1.5719 ± 0.5632) with the p-value of 0.0754 (Table 1). However, it is statistically significant for SP (0.7765 ± 0.1992) and BM (1.4068 ± 0.5756) with the p-value of <0.0001 for both (Table 1). When G0 ratio is compared among LN, SP and BM, G0 ratio for anti-GPI ab produced in LN is statistically different from G0 ratio for anti-GPI ab produced in SP and BM with the p-values of 0.0088 and 0.0352, respectively (Table 1). In addition, G0 ratio for anti-GPI ab produced in BM is statistically different from G0 ratio for anti-GPI ab produced in SP with the p-value of 0.0303 (Table 1). In terms of overall IgG glycan profiles, IgG produced in *in vivo* and LN are very similar while they differentiate from the anti-GPI ab glycan profiles produced in ex vivo cell cultures of SP and BM (Figure 3A).

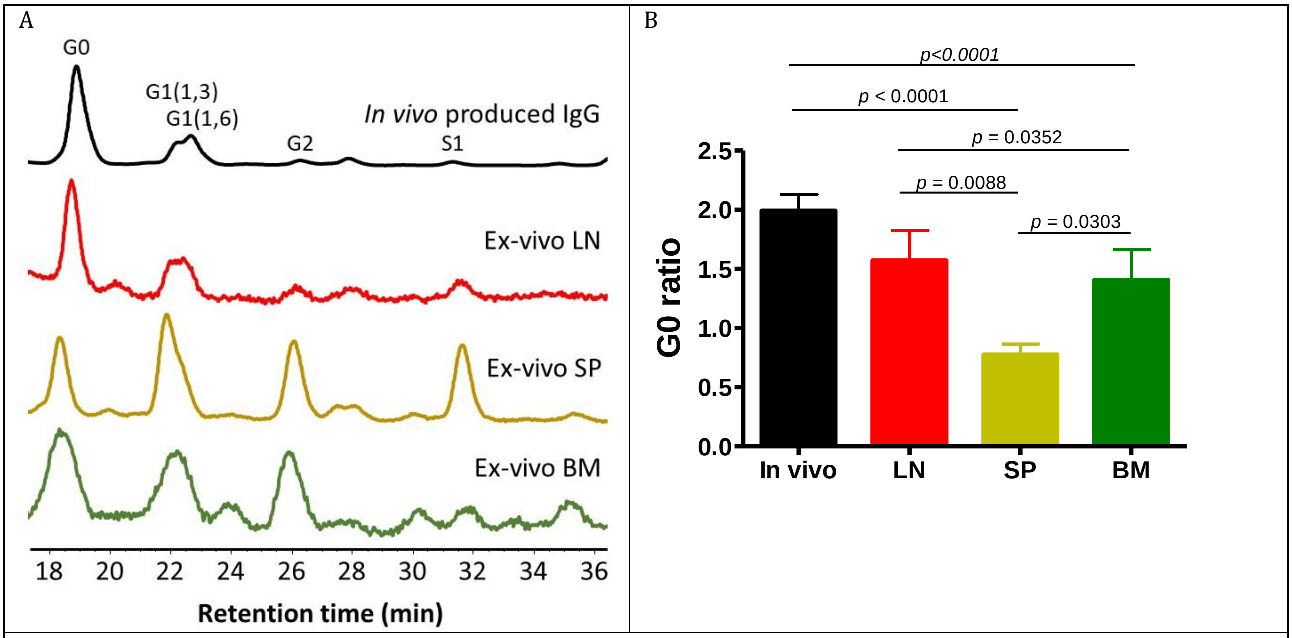

Figure 3. The effect of microenvironment on IgG glycosylation. (**A**) The NP-HPLC traces of N-glycan structures are shown for anti-GPI auto-ab produced *in vivo* (black), and ex vivo culture of LN (red), SP (yellow) and BM (green). The peaks for the G0, G1s, G2 and S1 glycan structures are labeled on HPLC traces. (**B**) The G0 ratios for each of the microenvironments are presented. Blank lines and numbers above represent statistical differences between samples using unpaired Student's t-test.

Table 1. Statistical comparison of G0 contents of anti-GPI auto-ab HC from different microenvironments†

	G0 ratio (mean ± SD)	*p*-values			
		In-vivo	LN	SP	BM
In-vivo	1.9936 ± 0.3010	-	0.0754	<0.0001	<0.0001
LN	1.5719 ± 0.5632	0.0754	-	0.0088	0.0352
SP	0.7765 ± 0.1992	<0.0001	0.0088	-	0.0303
BM	1.4068 ± 0.5756	<0.0001	0.0352	0.0303	-

†Statically significant values (<0.05) are presented in red color.

3.3 Galactosylation and sialylation of IgG depends on microenvironment

To compare the level of galactosylation and sialylation of IgG HC with respect to the microenvironments where anti-GPI ab is produced, they are normalized with G1 as in the case of G1 ratio. According to the results, galactosylation level of IgG produced in LN (0.2898 ± 0.0580) is less than SP (0.4531 ± 0.1109) and BP (0.8889 ± 0.5631) with the p-values of 0.0097 and 0.0087, respectively (Figure 4A and Table 2). However, the difference in G2 ratio between SP and BM is not statically significant with the p-value of 0.0662.

The sialylation level of anti-GPI ab produced in SP (0.5212 ± 0.1037) is different from LN (0.3701 ± 0.1199) and BM (0.2999 ± 0.1878) with the p-value of 0.0329 and 0.0198, respectively (Figure 4B and Table 3). However, sialylation level of anti-GPI ab produced in LN is not different from BM with the p-value of 0.1565. These results show that IgG produced in SP has more anti-inflammatory character than anti-GPI produced in LN and BM.

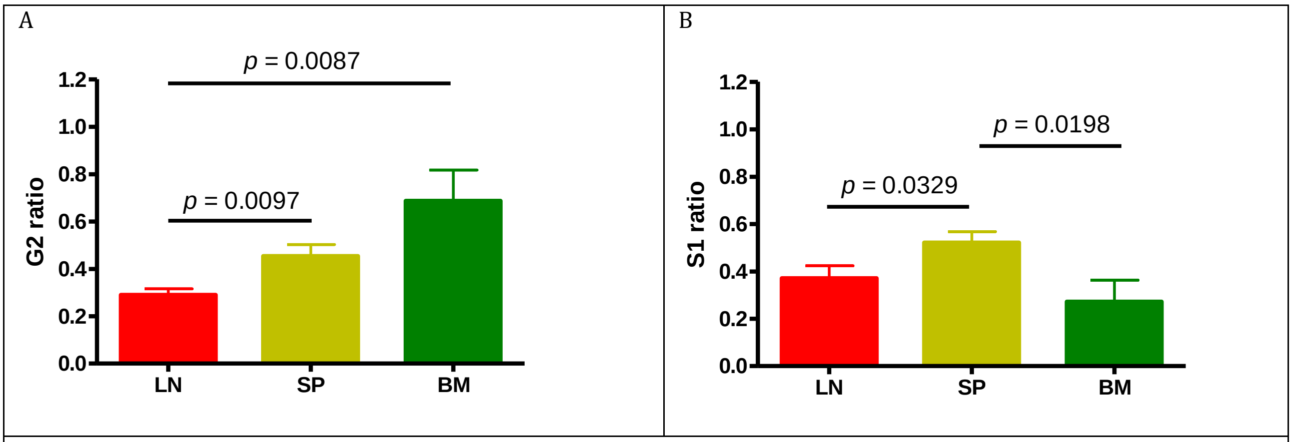

Figure 4. Galactosylation and sialylation of IgG in LN, SP and BM. (**A**) The galactosylation and (**B**) sialylation levels of anti-GPI auto-ab produced in ex-vivo ell culture of LN, SP and BM is presented (**A** and **B**, respectively). The color codes are as described in Figure 3.

Table 2. Statistical comparison of G2 contents of anti-GPI auto-ab HC from different microenvironments†

	G2 ratio (mean ± SD)	*p*-values	
		LN	SP
LN	0.2898 ± 0.0580	-	0.0097
SP	0.4531 ± 0.1109	0.0097	-
BM	0.8889 ± 0.5631	0.0087	0.0662

†Statically significant values (<0.05) are presented in red color.

Table 3. Statistical comparison of S1 contents of IgG HC from different microenvironments†

	S1 ratio (mean ± SD)	*p*-values	
		LN	SP
LN	0.3701 ± 0.1199	-	0.0329
SP	0.5212 ± 0.1037	0.0329	-
BM	0.2999 ± 0.1878	0.1565	0.0198

4. Discussion and Conclusion

Immunoglobulin G is the link between adaptive and innate arms of the immune system for the defense of the host. This link brings out the distractive power of innate immune system in the form of inflammation by two different interactions: IgG Fc domain with FcgRs on the immune cells and IgG Fab domain with the antigen on the surface of the pathogen during the infection or host organ in the case of autoimmune diseases such as RA (135, 136). The interaction of IgG Fc domain with FcgRs is regulated by the variable biantennary complex-type oligosaccharide attached to Asparagine-297 of the IgG Fc domain. This glycan has been recently well-appreciated and become the center of intense research due to its critical function during the infection or autoimmune diseases as well as therapeutic applications (6, 65, 98, 137-142). For example, the removal of this glycan structure from the Fc domain of IgG leads to the inactivation of IgG-dependent immune response by abrogating the interaction with its cognate FcgRs and therefore renders IgG inactive (59, 62, 65, 143, 144). It is shown here that the identity of this critical IgG glycan structure depends on the microenvironment where IgG is produced. According to the N-glycan profiles of IgG HCs, the draining LNs are the center to produce IgG with proinflammatory glycan structures while SP is the place for the production of IgG with more anti-inflammatory glycan structure than other sites, and interestingly, BM is between them (Figures 2, 3 and 4).

According to the results presented above for the glycosylation of anti-GPI auto-ab from K/BxN mouse with active RA, the differences at the glycosylation of IgG Fc domain exist with respect to the production site of IgG (Figures. 2, 3 and 4). While the IgG HC N-glycan profile for IgG produced in the ex-vivo culture of lymphoid cells closely replicates the N-glycan profile of IgG HC isolated from serum of K/BxN mouse, these two N-glycan profiles are very different from the profiles of IgG N-glycan produced in the ex-vivo culture of SP and BM cells (Figure. 3A). For example, IgG HC N-glycan profiles for IgG produced in *in vivo* and LN are dominated by G0 structure and are almost absence of G2 and S1 structures while the profiles for IgG produced in SP and BM have strong presence of S1 and G2 structures, respectively. Therefore, the contribution of IgG to on-going inflammation is regulated by the different microenvironments where IgG is produced. In addition, by considering IgG HC N-glycan profile as a biomarker, the comparison of these profiles shows that anti-GPI ab in serum is heavily produced in LN rather than SP and BM.

These results are very relevant to RA, because the G0 structure correlates with the disease activity in RA pointing to draining LN as the major contributor to IgG in circulation and the on-going inflammation. This aligns well with the literature as peripheral lymphatic organs such as the secondary and tertiary draining LNs close to the site of inflammation in mouse and man are the place where the antibodies are being produced (129, 148-154). For example, ACPA glycan structures from synovium has more G0 and less S1 structures than matching serum samples and therefore ACPA in the synovial fluid has more proinflammatory character than ACPA in the serum (95, 103, 104). Although the tertiary lymphatic structures is proven to be present and active in synovium of RA patients, it isn't clear whether that ACPA auto-ab with the higher content of G0 is due to the capturing auto-ab from circulation by autoantigen in the synovium or locally produced auto-ab in tertiary and secondary LNs. The combination of the data presented here with the above literature strongly suggests that auto-abs with proinflammatory G0 structure are locally produced in draining lymph nodes. As the draining LNs in the center of the IgG-dependent immune response, its response is more vigorous than SP and BM.

Organs such as LN, SP and BM can house B cells at different stages of development. LN and SP are the place where the naïve B cells encounter their cognate antigens the first time and then develop into IgG secreting short-lived and long-lived plasma as well as memory B cells while BM is the place where the early development of B cells from hematopoietic stem cells happen, and the long-lived plasma and memory B cells migrate to (37, 39, 145-147). These organs are functional as well as anatomically different sites, giving rise to unique microenvironments. According to the results presented here, these differences in microenvironments differentially contribute to the inflammation via the production of different glycosylation profiles on the IgG Fc domain. For example, IgG produced in LN, which is anatomically very close to the inflammation site, is to promote the IgG-dependent inflammation with all its capacity to clear pathogen or a cell presenting the autoantigen due to the dominant G0 structure on IgG. On the other hand IgG produced in SP, it has the highest anti-inflammatory character compared to LN and BM. Therefore, it is more to suppress the IgG-dependent inflammation than promote it but being alert at the same time. While these sites promote or suppress the IgG-dependent inflammation, IgG produced in BM can be considered as neither pro- nor anti-inflammatory due to the highest amount of G2 structure among the sites with relatively well-balanced distribution of G0, G1 and G2 glycan structures. This fits well to very well-defined function of BM as a memory compartment. As a conclusion, the contribution of IgG to inflammation is regulated by the microenvironment where it is produced and the method described here as ex-vivo cell culture system provides an important tool to investigate the regulators of IgG glycosylation on a molecular as well as cellular level.

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Conflicts of interest

Authors have no any financial or personal relationships with other individuals or organizations that might inappropriately influence this work during the submission process.

Statement of ethics

All animal experiments were approved by The Dana-Farber Animal Care and Use Committee with the protocol number of 04-009.

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