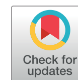

Adaptive antibody diversification through *N*-linked glycosylation of the immunoglobulin variable region

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A hallmark of B-cell immunity is the generation of a diverse repertoire of antibodies from a limited set of germline V(D)J genes. This repertoire is usually defined in terms of amino acid composition. However, variable domains may also acquire *N*-linked glycans, a process conditional on the introduction of consensus amino acid motifs (*N*-glycosylation sites) during somatic hypermutation. High levels of variable domain glycans have been associated with autoantibodies in rheumatoid arthritis, as well as certain follicular lymphomas. However, the role of these glycans in the humoral immune response remains poorly understood. Interestingly, studies have reported both positive and negative effects on antibody affinity. Our aim was to elucidate the role of variable domain glycans during antigen-specific antibody responses. By analyzing B-cell repertoires by next-generation sequencing, we demonstrate that *N*-glycosylation sites are introduced at positions in which glycans can affect antigen binding as a result of a specific clustering of progenitor glycosylation sites in the germline sequences of variable domain genes. By analyzing multiple human monoclonal and polyclonal (auto)antibody responses, we subsequently show that this process is subject to selection during antigen-specific antibody responses, skewed toward IgG4, and positively contributes to antigen binding. Together, these results highlight a physiological role for variable domain glycosylation as an additional layer of antibody diversification that modulates antigen binding.

antibody diversification | variable domain glycosylation | Fab glycosylation

Immunoglobulins are glycoproteins produced by plasma cells that are crucial for protective immunity. The most abundant class of immunoglobulins in the blood is IgG, representing 75% of total serum immunoglobulins. All IgGs contain glycans that are linked to a conserved asparagine in the constant Fc tail. These glycans have a profound effect on IgG effector functions by affecting FcR and C1q binding (1, 2), underscored by the fact that therapeutic monoclonal antibodies with enhanced cytotoxic potency have been developed by glycoengineering of these Fc glycans (3). In addition, about 15% of IgGs in serum from healthy humans have been described to contain *N*-linked glycans in the variable domains of the Fab arms (4). These mainly consist of complex biantennary glycans that contain high percentages of sialic acid in comparison with Fc glycans (4–7).

The role of these Fab glycans in immunity is poorly understood. Notably, enhanced Fab glycosylation is associated with certain pathophysiological conditions, including rheumatoid arthritis (RA) (8), primary Sjögren's syndrome (9), and several types of malignancies (10–13). Nevertheless, the role of Fab glycosylation in pathogenesis has not been established for any of these conditions. In contrast to these studies, Fab glycans have also been implicated as contributing to the anti-inflammatory activity of i.v. Ig (IVIg) (14) or reducing autoimmunity by masking antigen-binding sites of autoantibodies (15). Also, elevated Fab glycosylation was reported during pregnancy (5). In addition, Fab glycans have been implicated to affect antigen binding (16–20). Importantly, a coherent model of the emergence and functional consequences of Fab glycosylation is currently lacking. Insight into the events leading to Fab glycosylation would be a prerequisite to a more detailed understanding of the role of Fab glycans in pathogenicity and immunity in general.

N-linked glycosylation is contingent on the presence of glycosylation sites, which consist of asparagine, followed by any amino acid but proline, followed by serine or threonine. The naive human B-cell antibody repertoire is almost devoid of such sites, with only few variable domain alleles containing *N*-glycosylation sites (7). The occurrence of Fab glycans in vivo is therefore expected to mainly result from somatic hypermutation (SHM) during antigen-specific immune responses (21). This implies that acquiring *N*-linked glycans might be subject to selection mechanisms responsible for affinity maturation. In this study, we demonstrate by next-generation sequencing that SHM preferentially introduces *N*-glycosylation sites in the complementarity determining regions

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Significance

Structural variation of antibodies is generally defined in terms of amino acid composition, neglecting posttranslational modifications such as *N*-linked glycosylation. Little is known about the role of the glycans that are present in about 15% of variable domains. However, recent studies suggest that variable domain glycans exhibit distinct patterns according to (patho)physiological conditions, and can have immunomodulatory effects. Here we highlight a physiological role for variable domain glycans that is predetermined in the germline antibody repertoire: We show that variable domain *N*-linked glycans are acquired during somatic hypermutation at positions predisposed in the germline and may be positively selected during affinity maturation, representing an additional mechanism of secondary antibody diversification that contributes to the extent of the B-cell antibody repertoire.

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(CDRs). Strikingly, this preference is largely predetermined by precursor nucleotide motifs found in the germline variable domain genes. Furthermore, we reveal that variable domain glycosylation is subject to selection mechanisms that depend on the nature of the antigen, resulting in significant antigen-associated selection, and show that these glycans contribute directly to antigen binding, and thereby to specificity. Collectively, our data show that variable domain glycosylation is an inherent mechanism of antibody diversification, subject to selection mechanisms, on top of V(D)J recombination and SHM, and thereby contributes to the extent of the antibody repertoire of B cells.

Results

Fab Glycosylation Sites Preferentially Emerge Near Antigen-Binding Regions. Antibody variable domains contain three hypervariable regions, which coincide with three of the four structural loops at the top of the variable domain (Fig. 1*A*). The large structural diversity reflects the importance of these loops for conferring specificity and binding to each unique antigen in the very large antigen repertoire. We investigated whether glycans are preferentially introduced in or near these regions, forming an additional layer of diversification of the antibody repertoire. Therefore, as a first approximation, the human germline V_H, V_κ, and V_λ sequences were analyzed for positions in which a single nucleotide mutation would suffice to introduce a glycosylation site (hereafter referred to as a progenitor glycosylation site; see Fig. 1*B* as an example). Interestingly, these positions were significantly clustered around the CDRs and the loop between the second and third CDR (the DE loop; Fig. 1*C* and *SI Appendix*, Fig. S1*B*), whereas analysis of the human germline C_H, C_κ, and C_λ sequences did not show a similarly skewed distribution of progenitor glycosylation sites (*SI Appendix*, Fig. S1 *A* and *B*), suggesting the germline variable domain repertoire may contain a predefined set of locations in which glycosylation sites may be easily introduced on SHM. We next extended our analysis to the mouse germline repertoire. A similar, albeit less pronounced, clustering around the CDRs and the DE loop was observed for progenitor glycosylation sites in the variable domain sequences (*SI Appendix*, Fig. S2 *A* and *C*), whereas no apparent clustering was observed for the constant domain sequences (*SI Appendix*, Fig. S2 *B* and *C*).

Next, human rearranged V_H sequences from the IMGT database (Fig. 1*D*) and from switched memory B cells isolated from seven healthy donors (Fig. 1*E*) were analyzed for the actual occurrence of *N*-glycosylation sites. This uncovered a distinct pattern in the distribution of Fab glycosylation sites in both datasets, with most sites being located either in or near the CDRs or the DE loop, such that an attached glycan has the potential to influence the binding properties of the antibody. In addition, within the IGHV genes, the majority of the sites (respectively, 79% and 86%) are in fact the actualization of a progenitor glycosylation site (i.e., are the result of a single nucleotide mutation). This distribution of Fab glycosylation sites was similar between IgG and IgA sequences (*SI Appendix*, Fig. S1 *C* and *D*), whereas frequencies of individual sites can vary substantially between donors (*SI Appendix*, Fig. S1*E*). This demonstrates that a predefined progenitor glycan repertoire is the predominant source of glycosylation sites after SHM in memory B cells. These observations were extended to patients with systemic lupus erythematosus and mouse rearranged V_H sequences of IgG-switched B cells (22) (*SI Appendix*, Figs. S3 and S2*D*), with 87% and 79% of sites matching progenitor sites. Sequences with a glycosylation site contained more replacement mutations than sequences without a glycosylation site, and sequences with two glycosylation sites contained even more mutations (*SI Appendix*, Fig. S4), in agreement with SHM being responsible for introducing these sites. Overall, almost 9% of all human rearranged V_H sequences were demonstrated to contain one or more sites, versus 4.4% for mouse, which is in line with the lower overall mutation rate in mouse (*SI Appendix*, Fig. S4) (23). Glycosylation sites are less frequent in IGHV3 (5%) than IGHV4 (19%), with IGHV1 intermediate between those (9%), a frequency similar to a previous report (15). Together, these data show that Fab glycosylation sites preferentially emerge near antigen-binding regions, and that the germline variable domain repertoire is inherently biased toward this distribution. NXS sequons were three times as common as NXT (*SI Appendix*, Fig. S5*A*). NXS sequons tend to be less frequently glycosylated (24), depending on, among other factors, the amino acid at position X. The most frequent amino acid at position X was leucine, associated with low glycosylation efficiency, but the next four most frequent amino acids predispose for more efficient glycosylation (*SI Appendix*, Fig. S5 *A* and *B*) (25). Of note, in sequons within the CDR3, the frequency of leucine at position X was low (*SI Appendix*, Fig. S5*C*). Furthermore, the introduction of a glycosylation site in a model antibody, adalimumab, at predicted progenitor positions as described earlier, demonstrated

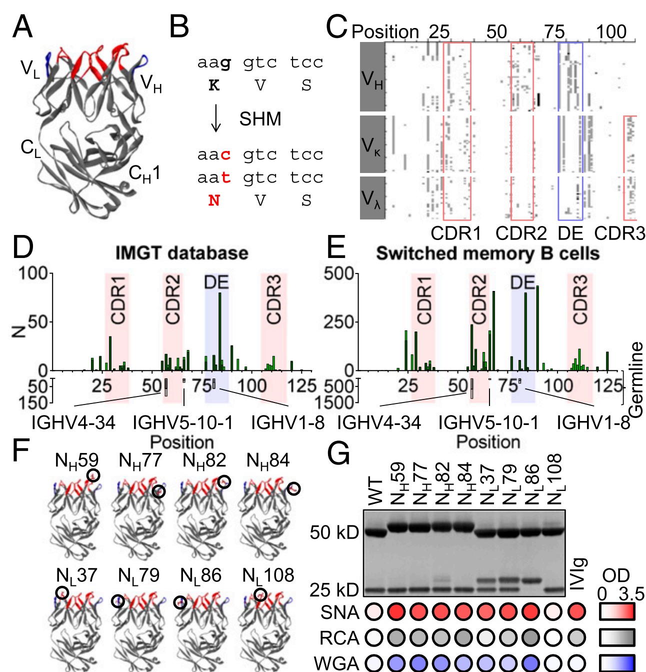

Fig. 1. Fab glycosylation sites preferentially emerge near antigen-binding regions. (*A*) Both variable domains (V_H, V_L) of an antibody Fab arm contain four top loops: three complementarity determining regions (CDR1, CDR2, CDR3, red), which usually constitute the antigen-binding site, and the adjacent DE loop (blue). (*B* and *C*) Triplets of codons were screened for the possibility of coding for a glycosylation site on mutating a single nucleotide anywhere within the triplet of codons, of which an example is shown in *B* (see *Materials and Methods* for details). (*C*) Heat map of positions in which progenitor glycosylation sites are located within human germline V_H, V_κ, and V_λ sequences (Dataset S1, only *01 alleles shown). A darker shade indicates multiple possibilities for a single nucleotide mutation to introduce a glycosylation site at that position. Numbering according to IMGT. (*D* and *E*) Number of *N*-glycosylation sites at different positions within human rearranged V_H sequences from (*D*) the IMGT database (4,026 unique sequences) and (*E*) switched memory B cells from seven healthy donors (37,077 unique, productive sequences out of a total of 82,281). Dark green bars represent sites that match predicted progenitor sites (*C*). Bars below the X axis represent germline glycosylation sites, and dark shades represent those sites that are not removed by mutations. (*F*) Eight variants of adalimumab (*SI Appendix*, Table S1) were expressed with a glycosylation site introduced at one of the indicated positions in the crystal structure of adalimumab Fab (PDB ID: 3WD5). The position of the Fab glycan is indicated (e.g., N_H59 indicates a Fab glycan at position 59 of the heavy chain). (*G*) Gel electrophoresis and lectin ELISA data for the eight adalimumab glycovariants. WGA recognizes *N*-acetylglucosamine, RCA recognizes galactose, and SNA recognizes sialic acid. Shown are representative data of two replicates.

that for seven of eight positions, the sites indeed became occupied with glycans (including all five NXS sites), as confirmed by gel electrophoresis and lectin ELISAs (Fig. 1 *F* and *G* and *SI Appendix*, Table S1). In other words, the introduction of glycosylation sites at predicted positions in general results in properly folded antibodies that express glycans in the variable domains.

Fab Glycosylation Differs Between IgG Subclasses and Specificities. Next, we investigated whether Fab glycosylation is subject to (context-dependent) selection mechanisms during specific antibody responses. We first analyzed whether there were distinct levels of Fab glycosylation between IgG subclasses. To this end, we fractionated eight healthy donor sera using *Sambucus nigra* agglutinin (SNA) affinity chromatography (Fig. 2*A*), making use of the fact that SNA enriches for Fab glycans, but not for Fc glycans (14, 26, 27) (*SI Appendix*, Fig. S6), through their terminal 2,6-linked sialic acid residues found in >90% of Fab glycans (5). A median of 11% [interquartile range (IQR), 11–14%] of total serum IgG was found to contain sialylated Fab glycans (i.e., was recovered in the enriched fraction), as measured by ELISA. Interestingly, by the same method, IgG4 Fab glycosylation (44%; IQR, 39–49%) was found to be significantly increased compared with that of total IgG, whereas Fab glycosylation of IgG1 (12%; IQR, 11–18%), IgG2 (11%; IQR, 9.6–16%), and IgG3 (15%; IQR, 11–16%) was not (Fig. 2*B*). This result can partially be explained by the ability of IgG4 antibodies to engage in Fab-arm exchange, resulting in an increased fraction of IgG4 antibodies that carry at least one glycan (Fig. 2*C*). However, by mathematically accounting for the Fab-arm exchange (for calculations see *Materials and Methods*), 25% (IQR, 22–28%) of IgG4 was calculated to contain Fab glycans, indicating that the IgG4 BCR repertoire itself is also intrinsically biased toward increased Fab glycosylation. This observation was corroborated by analysis of human rearranged IgG4 V_H sequences of healthy volunteers (28), of which 30% (87/288) contained a glycosylation site compared with 13% (264/2,063) for IgG1 reads (*SI Appendix*, Fig. S14).

We next analyzed whether there were distinct levels of Fab glycosylation between antibodies specific for different antigens. For a typical IgG4-dominated immune response, namely, experienced (healthy) beekeepers that make antibodies to phospholipase A2 (PLA2) in bee venom (29), we found that levels of Fab glycosylation of anti-PLA2 IgG (of which a median of 66% was IgG4) were similar to those of total IgG4, but significantly elevated compared with those of total IgG (41% vs. 44% vs. 12%; Fig. 2*D*). Also, Fab glycosylation levels of anti-PLA2 IgG4 were similar to those of total IgG4 (*SI Appendix*, Fig. S7*A*). In contrast, antibodies to tetanus toxoid (TT), a typical vaccine antigen that mainly induces IgG1, had levels of Fab glycosylation comparable to those of total IgG (13%). To extend these findings, we also investigated sera from patients who make antibodies to adalimumab or infliximab in response to treatment with these biologicals [of which a substantial fraction is IgG4 (30, 31), a median of 44% and 32% in the samples tested]. Again, we found high levels of Fab glycosylation for anti-adalimumab (47%; Fig. 2*E*) and anti-infliximab (53%; Fig. 2*F*). However, in both cases, Fab glycosylation was in fact significantly higher than that of total IgG4 (which again was elevated compared with total IgG; Fig. 2 *E* and *F*), indicating an increased positive selection for Fab glycosylation compared with a typical IgG4-dominated immune response. This was confirmed by measuring glycosylation levels of anti-adalimumab IgG4 and anti-infliximab IgG4, which were increased compared with those of total IgG4 (*SI Appendix*, Fig. S7 *B* and *C*). We obtained similar results using the percentage of Fab glycosylation of specific IgG and total IgG4 corrected for IgG4 half-molecule exchange (*SI Appendix*, Fig. S8). In contrast, the antibody response to another biological, natalizumab, is characterized by low levels of IgG4 (median, 4%), and lower levels of Fab glycosylation were observed by analyzing serum samples from natalizumab-treated patients with multiple sclerosis (26%; Fig. 2*G*), which were still significantly higher compared with total IgG, but not compared with anti-TT. Furthermore, multiple additional antigen-specific responses were evaluated. We also analyzed sera from patients with RA containing anticitrullinated protein antibodies (ACPAs), and observed a median of 89% Fab glycosylation, in line with the recently reported high degree of Fab glycosylation of these autoantibodies (32) (Fig. 2*H*). Interestingly, for another inflammation-associated antibody response, namely, anti-hinge antibodies (33), our data also show increased Fab glycosylation (53%; Fig. 2*I*). In contrast, Fab glycosylation of antibodies against the RhD blood group antigen was found to be decreased in pooled serum IgG of RhD-immunized donors compared with

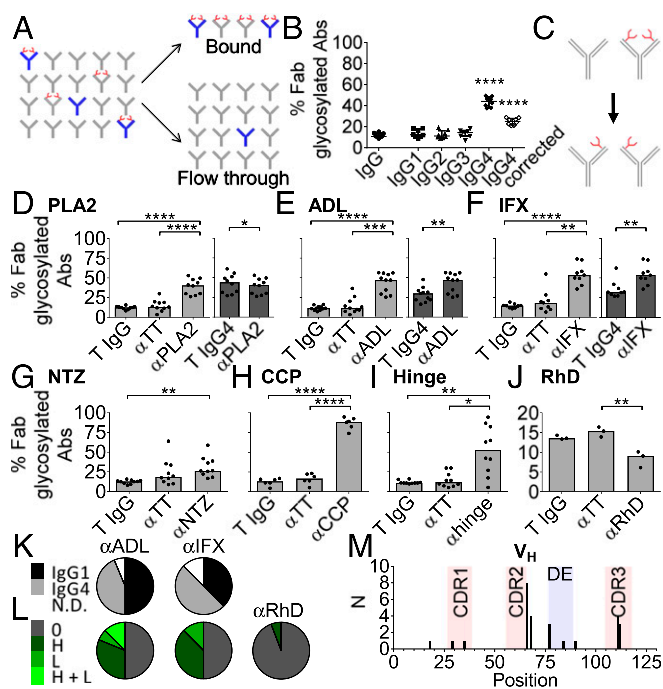

Fig. 2. Fab glycosylation differs between IgG subclasses and specificities. (*A*) SNA affinity chromatography of IgG results in SA^+ IgG (enriched for Fab glycosylated IgG) and SA^- IgG (almost devoid of Fab glycosylated IgG) (4, 24, 27, 28) (*SI Appendix*, Fig. S6). Total and specific IgG is measured in both fractions by ELISA, RIA, or agglutination (*Materials and Methods*). (*B*) Percentage of Fab glycosylated antibodies (Abs) for total IgG and IgG subclasses in healthy donor sera ($n = 8$). Symbols represent means of at least six replicates. ♦, before, and ◇, after correction for IgG4 half-molecule exchange. (*C*) IgG4 molecules can exchange half-molecules, resulting in a fraction of IgG4 molecules carrying a Fab glycan on only one of the two Fab arms. (*D*–*J*) Percentage of Fab glycosylated Abs for total (T) IgG/IgG4, anti-tetanus toxoid (TT), and (*D*) anti-PLA2 [in sera from experienced beekeepers, $n = 10$, of which 66% (58–86%) is IgG4], (*E*) anti-adalimumab [ADL; $n = 11$, of which 44% (14–97%) is IgG4], (*F*) anti-infliximab [IFX; $n = 9$, of which 32% (20–73%) is IgG4], (*G*) anti-natalizumab (NTZ; $n = 10$, *E*–*G* in sera from patients with a respective anti-biological antibody response), (*H*) anti-cyclic citrullinated peptide (CCP; in sera from patients with RA, $n = 6$), (*I*) anti-hinge (in sera from random subjects, $n = 10$), and (*J*) anti-RhD (in pooled, fractionated plasma preparations of RhD-immunized donors, $n = 3$). Symbols represent means of at least four replicates. Bars indicate medians. One-way ANOVA, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and $****P < 0.0001$ or paired *t* test, $*P < 0.05$; $**P < 0.01$. (*K*–*M*) Single B cells specific for ADL, IFX, or RhD from patients who made anti-ADL or anti-IFX antibodies or from RhD-immunized donors. (*K*) Distribution of IgG1 and IgG4 among monoclonal anti-ADL ($n = 16$) and anti-IFX ($n = 8$) antibodies. (*L*) Absence (0) or presence of glycosylation sites in the heavy (H) or light (L) chain or in both (H + L) chains of monoclonal anti-ADL, anti-IFX, and anti-RhD ($n = 294$) antibodies. (*M*) Number of *N*-glycosylation sites at different positions within human rearranged V_H sequences from monoclonal anti-ADL, anti-IFX, and anti-RhD antibodies.

that of total IgG or anti-TT (Fig. 2*J*). Together, these data demonstrate that sialylated Fab glycans are differentially expressed in a variety of immune responses, ranging from as low as 9.0% for anti-RhD antibodies to more than 80% for ACPAs compared with approximately 14% for anti-TT (median, 56 serum samples).

To confirm our findings at the DNA/monoclonal level, single B cells specific for adalimumab or infliximab were isolated from patients, cultured, and screened for specificity (34, 35). The original isotypes were either IgG1 or IgG4, both approximately 50% (Fig. 2*K*). We found that 50% of the anti-adalimumab and anti-infliximab B-cell clones contained Fab glycosylation sites (Fig. 2*L*), in close agreement to the 47% and 53% Fab glycosylation of serum-derived anti-adalimumab and anti-infliximab antibodies, respectively (Fig. 2 *E* and *F*). We confirmed that, of the examined clones (7/8 anti-adalimumab and 4/4 anti-infliximab), all glycosylation sites were occupied by glycans when expressed recombinantly as IgG antibodies (*SI Appendix*, Tables S2 and S3). We also analyzed variable domain sequences of B cells specific for RhD obtained from hyperimmunized anti-D donors by the same method. Analysis of these anti-RhD sequences showed a low frequency of glycosylation sites (5.8%; Fig. 2*L*), consistent with the low degree of glycosylation of pooled plasma-derived anti-RhD antibodies (Fig. 2*J*). In all but one case, the sites in these antigen-specific antibodies correspond to a predicted predefined site, and they are located in or near the CDRs or the DE loop (Fig. 2*M*). Taken together, we found different levels of Fab glycosylation between specific IgG subsets (subclasses and specificities), indicating that Fab glycosylation is not a random process but is, rather, subject to context-dependent selection mechanisms (e.g., antigen binding) during specific antibody responses.

Modulation of Antigen Binding by Fab Glycans. Next, we systematically investigated the effects of Fab glycosylation on antigen binding. We started by evaluating the effects of Fab glycans that were introduced at predicted predisposed sites in adalimumab (Fig. 1*F*). For five of seven adalimumab glycovariants (N_H59, N_H77, N_H82, N_H84, and N_L37), binding to its antigen (TNFα) was reduced to various degrees, as measured by competition ELISA (Fig. 3 *A*–*C*). Unexpectedly, one of the other two glycovariants (N_L86) showed a slightly enhanced TNFα binding. For three variants, reduced binding to TNFα was (at least partially) mediated by the glycans rather than the amino acid difference, as demonstrated by the significantly less reduced binding of the same clones expressed in the presence of tunicamycin, which blocks *N*-linked glycosylation (Fig. 3*C* and *SI Appendix*, Fig. S9*A*). Thus, introducing Fab glycans at predicted sites may affect antigen binding, which in this model system in which no selection mechanisms are operative is expected to predominantly result in decreased binding strength, in line with our observations. We also investigated TNFα binding by surface plasmon resonance (SPR). Although overall similar results were obtained in comparison with the ELISA data, clear differences compared with the inhibition ELISA were found for two clones (N_H77 and N_L86; Fig. 3*D*), indicating that antigen binding can be differentially influenced by Fab glycans, depending on the context in which antigen is presented (i.e., free vs. bound to another protein/antibody).

To further examine the modulating capacity of Fab glycans for differential binding to related antigens, we determined how the Fab glycans on the different adalimumab variants affect binding to a panel of anti-adalimumab anti-idiotype antibodies. Here, the anti-idiotype antibodies serve as surrogate antigens of adalimumab, each having a slightly different mode of binding (34, 36) (Fig. 3*E*). Overall, the adalimumab glycovariants that bound TNFα with lower affinity also showed less binding of these anti-idiotype antibodies (Fig. 3*F* and *SI Appendix*, Fig. S9*B*). However, the glycans on adalimumab glycovariants N_H59, N_H82, N_L37, and N_L86 had substantial differential effects on the binding to these surrogate antigens (Fig. 3*F*). For instance, although the glycan on variant

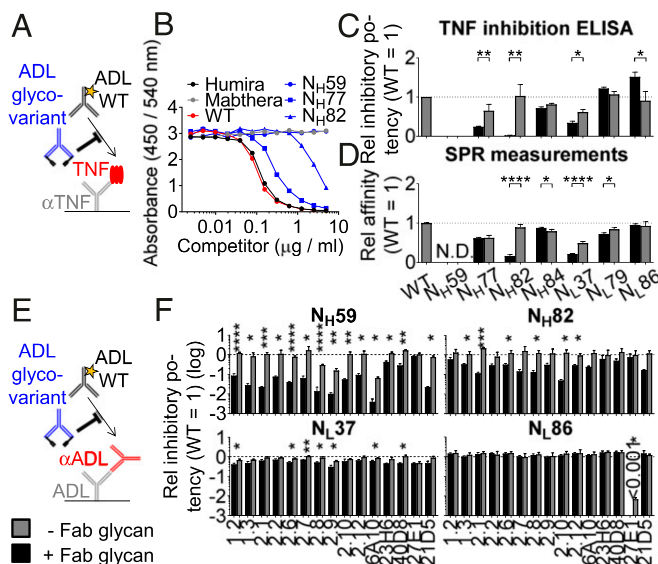

Fig. 3. Fab glycans can modulate antigen binding. (*A*) Setup of the TNF inhibition ELISA for assessing relative (rel) binding strengths of adalimumab (ADL) glycovariants to TNF. (*B*) TNF binding by in-house expressed ADL (WT), ADL glycovariants (+Fab glycan, black bars, notation as in Fig. 1*F*), and these variants expressed in the presence of tunicamycin or depleted using SNA (−Fab glycan, gray bars) was determined. Humira (ADL) and Mabthera (rituximab) were used as positive and negative controls. (*C*) Inhibitory potency relative to WT (means of at least four replicates with SEM). (*D*) TNF binding by ADL WT and glycovariants (+/−Fab glycan) determined by biosensor (SPR) experiments. Affinity relative to WT (means of at least four replicates with SEM). (*E*) Setup of the ADL-anti-ADL competition ELISA. (*F*) Binding of ADL glycovariants N_H59, N_H82, N_L37, and N_L86 (+/−Fab glycan) to the anti-ADL clones was determined as inhibitory potency relative to WT (means of at least four replicates with SEM). Unpaired *t* test, legend as in Fig. 2.

N_H59 left binding to clone 27E1 fully unaffected, this glycan decreased binding to clone 6A10 more than 100 times. Similarly, the glycan on variant N_H82 decreased binding to clone 1.2 only two times, whereas this glycan decreased binding to clone 2.10 more than 20 times. This demonstrates that the introduction of a glycan can selectively and differentially influence binding of an antibody (in this case adalimumab) toward a panel of antigens (in this case the anti-idiotype antibodies), suggesting a potential to modulate the specificity of the antibody.

Naturally Occurring Fab Glycans Can Enhance Antigen Binding. We also investigated the consequences of naturally occurring Fab glycosylation for antigen binding. We tested antigen binding of several of the anti-adalimumab and anti-infliximab antibody clones with Fab glycosylation sites and mutants in which we removed these naturally occurring Fab glycosylation sites by mutating these sites back to germline (*SI Appendix*, Table S2). We confirmed the removal of glycans by gel electrophoresis and lectin ELISAs (Fig. 4 *A* and *B*). We then examined binding of the anti-adalimumab and anti-infliximab antibodies with and without Fab glycans to their respective antigens (adalimumab and infliximab) by SPR (Fig. 4*C*). Three of seven tested clones (anti-adalimumab 2.2 and 2.6 and anti-infliximab 1.3) showed no effect of these Fab glycans on antigen binding (Fig. 4 *D* and *E*). In contrast, for anti-adalimumab clone 1.3 and anti-infliximab clones 1.4 and 2.1, the presence of Fab glycans resulted in up to a twofold higher affinity (Fig. 4 *D* and *E*), suggesting that the B cells carrying BCRs with these Fab glycans were positively selected during affinity maturation. Tunicamycin controls confirmed that the glycans, and not the change in amino acid sequence, caused the increase in affinity (Fig. 4 *D* and *E*). We were not able to produce the mutant of anti-infliximab clone 2.3

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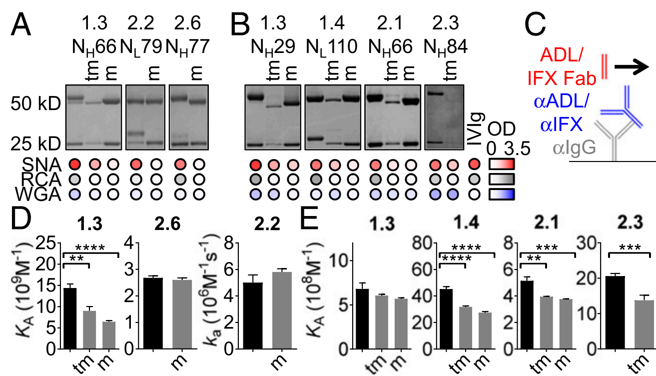

Fig. 4. Naturally selected Fab glycans can enhance antigen binding. (*A* and *B*) Gel electrophoresis and lectin ELISA data for (A) anti-adalimumab clones 1.3, 2.2, and 2.6 and (*B*) anti-infliximab clones 1.3, 1.4, 2.1, and 2.3 (notation as in Fig. 1*F*), these clones expressed in the presence of tunicamycin (tm), and mutants (m) thereof lacking the Fab glycosylation site (experiments as in Fig. 1*G*). Shown are representative data of two replicates. (*C*) Setup of the biosensor (SPR) experiments. (*D* and *E*) Using surface plasmon resonance, (*D*) adalimumab (ADL) or (*E*) infliximab (IFX) binding affinity of the anti-ADL/IFX clones and mutants was determined. Shown are means of at least four replicates with SEM. For anti-ADL clone 2.2, the k_a instead of the K_A was analyzed because the k_d was too low to be measured (42). One-way ANOVA, legend as in Fig. 2.

(N84K), suggesting the glycan on this clone may be important for its stability. However, tiny amounts of this clone were expressed in the presence of tunicamycin, and a nearly twofold higher affinity was found for the glycosylated variant (Fig. 4*E*). For anti-adalimumab 1.3, the influence of sialylation was investigated, suggesting a modest contribution to affinity (*SI Appendix*, Fig. S10). Taken together, these results demonstrate that Fab glycosylation can enhance affinity of antibodies toward their cognate antigens.

Discussion

Although it has been known for quite some time that glycans can be present on the variable domains of antibodies, the precise function of these Fab glycans remains poorly understood. Here, we present three important findings. First, Fab glycosylation sites preferentially emerge near antigen-binding regions because of an inherent bias in the germline variable domain repertoire. Second, different levels of Fab glycosylation exist between IgG subclasses and specificities, demonstrating that Fab glycosylation is not a random process but, rather, is subject to (antigen-associated) positive and/or negative selection mechanisms. Third, we found that Fab glycans can modulate antigen binding and contribute to affinity. Together, these data highlight a physiological role for Fab glycosylation as an additional mechanism of diversification of the antibody repertoire that modulates antigen binding, subject to selection mechanisms to optimize the antibody response. Indeed, we show high levels of Fab glycosylation for several antigen-specific immune responses, and we obtained evidence for positive selection via antigen binding for multiple naturally occurring antigen-specific antibody clones.

In contrast to a positive contribution to antigen binding, we show that the level of Fab glycosylation of IgG against RhD is quite low (i.e., lower than that of total IgG), suggesting that Fab glycans would strongly negatively influence binding. As the extracellular loops of RhD are close to the negatively charged plasma membrane of red blood cells, binding by antibodies carrying (negatively charged) sialylated Fab glycans may explain the apparent negative selection for the introduction of these glycans. Similarly, whereas the effects of Fab glycosylation on antigen binding found for naturally occurring antibodies are mainly positive, probably as a result of subsequent rounds of proliferation and selection in vivo, introducing Fab glycans bypassing this selection is expected to predominantly have negative or no effects, which is in line with our findings for the glycovariants of adalimumab we generated, and also consistent with similar findings for introducing follicular lymphoma-associated Fab glycans into anti-NIP and anti-HEL mouse monoclonal antibodies (37). Also, introducing glycans in the CDR3 of the heavy chain might more often be disruptive for antigen binding compared with the other loops because of steric constraints, in line with the relative absence of sites in this region.

Nevertheless, Fab glycans were found to leave antigen binding unaffected in more than one case, suggesting it is possible that Fab glycans may also accumulate in part as a neutral byproduct during SHM. However, despite a correlation between the number of mutations and the frequency of glycosylation sites, which is expected, it is unlikely that this fully accounts for the large differences in Fab glycosylation between various affinity-matured, antigen-specific antibody repertoires. Alternatively, positive selection of B cells carrying BCRs with Fab glycans may also involve (endogenous) lectins. In this respect, it is interesting to note that follicular lymphoma B cells may carry BCRs that are heavily Fab glycosylated (11), and it has been suggested that lectins bind to the high mannose glycans on these BCRs, and thereby stimulate B cells (12, 37–39). Whether this mechanism of selection also occurs for the more common highly sialylated complex biantennary Fab glycans is unknown.

Enhanced Fab glycosylation was found for follicular lymphoma B cells (11), but was also associated with RA (8) and primary Sjögren's syndrome (9). The role of Fab glycosylation in pathogenesis has not been established. However, we confirmed the recently reported high degree of Fab glycosylation of ACPAs, autoantibodies present in the majority of patients with RA (32). Because ACPAs are a diagnostic and prognostic biomarker in RA, further research on the possible association between Fab glycosylation of ACPAs and severity of the disease may provide more mechanistic insight into the role of ACPAs in RA, such as the role of antigens (citrullinated proteins) in provoking and/or sustaining these responses, and might help to improve diagnosis and prognosis.

Fab glycosylation of IgG4 was found to be increased compared with that of the other IgG subclasses, even after accounting for Fab-arm exchange. This might in part be explained by a slightly higher mutation rate in IgG4 than in the other subclasses (40), although overall, mutation rates and Fab glycosylation across subclasses correlate poorly, if at all (Fig. 2*B* and *SI Appendix*, Fig. S14). IgG4 is considered a more matured response, associated with prolonged/repeated antigen exposure and a T_H2/T_{REG}-like T-cell phenotype (41), which might also affect clonal selection. The question arises whether the relative enrichment of Fab glycosylation of IgG4 has consequences for the function of IgG4. IgG4 antibody responses are associated with tolerance, as they are poor activators of effector functions because of weak C1q/FcγR binding, and their inability to form large immune complexes because of Fab-arm exchange (41). Interestingly, Fab glycans have also been associated with immunomodulation of IVIg, of which the sialylated fraction is highly enriched in Fab glycans and may contribute to the immunomodulatory effects of IVIg treatment (7, 14). Analogously, increased Fab glycosylation of IgG4 antibodies might be another feature that contributes to the tolerogenic phenotype of this particular IgG subclass. A limitation of the current study is that the glycosylation profiles of the recombinant antibodies, although generally high in sialic acid content, may not be entirely representative of the in vivo profiles (*Materials and Methods*).

The present study also indicates that next to enhancing antigen binding, Fab glycans may also modulate the specificity of an antibody by eliminating unwanted secondary reactivities. Indeed, in our model system of anti-idiotype antibodies with highly similar, but not identical, binding characteristics serving as surrogate antigens, we demonstrated substantial differential effects of Fab

glycans on the binding to these surrogate antigens. This illustrates the potential for enhancing antibody selectivity by acquiring Fab glycans that would negatively affect off-target binding rather than enhancing on-target binding. Indeed, a recent study suggested that Fab glycosylation may provide a mechanism to prevent autoreactivity (15). ACPAs, RA-associated autoantibodies, were found to be highly Fab glycosylated, possibly as a strategy of the immune system to prevent autoreactivity, although it was also shown that Fab glycans on ACPAs did not always negatively affect binding to citrullinated peptides (32). In addition, patients with primary Sjögren's syndrome, an autoimmune disease, show increased levels of Fab glycosylation, and there is little evidence for positive (auto)antigen selection (9). It remains to be investigated how antibodies with increased specificity would be selected.

In conclusion, we provide evidence that the introduction of glycans in the variable domains is an inherent, additional layer of diversification of the antibody repertoire, dependent on SHM and subject to selection mechanisms to optimize the antibody response.

Materials and Methods

Blood samples were obtained on written informed consent with approval from local ethics committees of the Academic Medical Centre, Amsterdam; Reade, Amsterdam; Leiden University Medical Center, and Erasmus Medical Center, Rotterdam, The Netherlands, as detailed in *SI Appendix, SI Materials and Methods*. B-cell repertoires were analyzed by next-generation sequencing. For analysis of germline variable domain sequences, datasets of rearranged variable domain sequences, and analysis of rearranged variable domain sequences, see *SI Appendix, SI Materials and Methods*. Multiple human polyclonal and monoclonal (auto)antibodies were analyzed for levels of variable domain glycosylation. For samples, lectin (SNA) affinity chromatography, total and specific IgG immunoassays, monoclonal antigen-specific antibodies, and analysis of glycosylation site occupancy, see *SI Appendix, SI Materials and Methods*. Effects on binding were evaluated by recombinantly removing or introducing *N*-glycosylation sites. Details on antibody glycovariants, gel electrophoresis, surface plasmon resonance measurements, and MALDI-TOF-MS *N*-linked glycan analysis given in *SI Appendix, SI Materials and Methods*.

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