B-cell receptor sequencing of anti-citrullinated protein antibody (ACPA) IgG-expressing B cells indicates a selective advantage for the introduction of *N*-glycosylation sites during somatic hypermutation

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The majority of patients with rheumatoid arthritis (RA) harbours immunoglobulin G (IgG) antibodies targeting citrullinated protein antigens (ACPA). Recently, we showed that >90% of ACPA-IgG in serum are glycosylated in the variable domain.[1] *N*-linked glycosylation requires a consensus sequence in the protein backbone (N-X-S/T, where X is not proline), which is scarce in germline-encoded Ig variable region genes.[2, 3] Accordingly, hyperglycosylation of ACPA-IgG requires either clonal expansion of B cells expressing B cell receptors (BCR) containing germline-encoded *N*-glycosylation sites or generation of *de-novo* sites through somatic hypermutation (SHM).[4]

Here, we analysed the BCR repertoire of ACPA-expressing B cells to understand the molecular basis of this remarkable glycosylation. ACPA-expressing B cells were sorted as pools (10 cells per pool) from PBMC of 8 ACPA-positive RA patients.[5] ARTISAN PCR-based BCR-sequencing[6] followed by full-length variable region IgG transcript analysis revealed high nucleotide mutation rates in 97 unique ACPA-IgG heavy chains (HC; mean±SD: 52.86±16.73; figure 1A). 81% of these contained one or more *N*-glycosylation sites.

To replicate these findings and to acquire additional information on paired heavy and light chains (LC), Ig transcripts of 87 single cell-sorted ACPA-IgG clones (6 donors) were analysed, again revealing high nucleotide mutation rates in the HC variable region (mean±SD: 48.55±16.05; figure 1B). Significantly lower mutation rates were observed for 31 single cell-sorted tetanus toxoid (TT)-specific clones (mean±SD: 25.15±18.92; figure 1B). TT-specific clones contained no *N*-glycosylation sites, in contrast to 79% of HC and 88% of paired HC/LC sequences from the ACPA-IgG clones. Additionally, both pool- and single-sorted cell sequence analyses revealed similar high nucleotide mutation rates for ACPA-LC (mean±SD: 36.18±15.09 and mean±SD: 34.51±16.79, respectively; not shown). Furthermore, 59% of ACPA-LC contained one or more *N*-glycosylation sites compared to 4-5% of healthy control LC. Further analyses of HC revealed that all sites in pool/single cell-sorted ACPA-IgG clones were introduced by SHM; furthermore, the degree of SHM did not correlate with the frequency of sites (figures 1C and 1D). Moreover, no accumulation of N-P-S/T sites (chosen as reference due to its similarity to N-X-S/T) was observed in ACPA-IgG, in contrast to the *N*-glycosylation tripeptide N-X-S/T. In fact, no N-P-S/T sequences were identified by either ACPA-IgG sequencing approach (pool/single cell-sorted). Finally, we observed a relative increase of sites in the complementarity-

determining region (CDR) 1 and a relative absence in CDR3 compared to healthy controls (figure 2A). Together, these findings indicate that the remarkable frequency of *N*-glycosylation sites is not the result of random accumulation of mutations but of a selective process during maturation of ACPA-expressing B cells. Intriguingly, modelling of the spatial positioning of the sites revealed that most sites are located on the exterior of the antibody molecule (figures 2B-D).

In conclusion, we provide the first in-depth analysis of the presence of *N*-glycosylation sites in the variable region of ACPA-IgG. The distribution pattern of sites across the ACPA-IgG variable domain and the spatial localization of *N*-glycosylation sites on the exterior of the molecule suggest that their function in selection processes is not primarily related to antigen recognition. Our data favour the concept that introduction of *N*-glycosylation sites generates selective advantages which allow ACPA-expressing B cells to escape from classical selection mechanisms in germinal centers. This is in contrast to the selection of B cells against recall antigens, which is primarily driven by affinity for cognate antigens.[7] In fact, the overall low-avidity of secreted polyclonal ACPA-IgG is in line with this hypothesis.[8] Possibly, ACPA-IgG variable domain glycans interact with glycan receptors in the vicinity of the BCR. These glycans are highly sialylated, suggesting siglecs as potential receptors.[2] Thus, these findings and considerations have important implications for understanding citrulline-specific immunity in RA.

Acknowledgements and affiliations

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Figure legends

Figure 1. A high degree of somatic hypermutation in ACPA-IgG clones which does not correlate with the frequency of N-glycosylation sites. Pool and single cells were sorted as described.[5] All independent clones are defined as having identical V, D, J genes and CDR3 regions.

(A) Immunoglobulin heavy variable region (IGHV) mutations in 97 ACPA-IgG clones obtained with pool-sequencing (n=8 donors). (B) IGHV mutations in 87 ACPA-IgG clones that were positive in CCP2-ELISA compared to 31 TT-IgG clones obtained from sequencing of cultured single cells (n=8 and n=3 donors, respectively). P-value was calculated using Mann-Whitney U test for unpaired data (**** $p < 0.0001$). (C) Correlation of the number of IGHV mutations with the number of N-glycosylation sites of 97 ACPA-IgG clones. Non-parametric Spearman correlation, $r = 0.10$, $p = 0.32$. (D) Correlation of the number of IGHV mutations with the number of *N*-glycosylation sites of 87 ACPA-IgG single cell-derived clones. Non-parametric Spearman correlation, $r = 0.19$, $p = 0.071$. All *N*-glycosylation sites were introduced by SHM. No association between IGHV-gene usage and number of N-glycosylation sites was observed using either method.

Figure 2. Distribution and spatial localisation of *N*-glycosylation sites in ACPA-IgG clones. (A) Percentage of *N*-glycosylation sites located in framework (FR) 1, CDR1, FR2, CDR2, FR3 and CDR3 regions of IGHV. Distribution of 102 sites in 97 ACPA-IgG clones obtained with pool-sequencing (left panel, black), 87 sites in 87 ACPA-IgG clones obtained with sequencing of cultured single cells (right panel, gray), both compared to 660 sites in 6724 IGHV sequences from 12 healthy donors (V-region matched, red). (B) Structural model of the top view on the antigen binding pocket of ACPA-IgG clones. (C) Front view of ACPA-IgG heavy chain structures containing *N*-glycosylation sites (asparagine residues colored in green). (D) Front view of ACPA-IgG light chain structures containing N-glycosylation sites (asparagine residues colored in red). All models contain variable regions of 58 ACPA-IgG clones with paired heavy and light chain sequences had a confidence score of 100% with a sequence identity of 47.7%±7.96 and protein coverage of 98.55%±0.63.

FIGURE 1

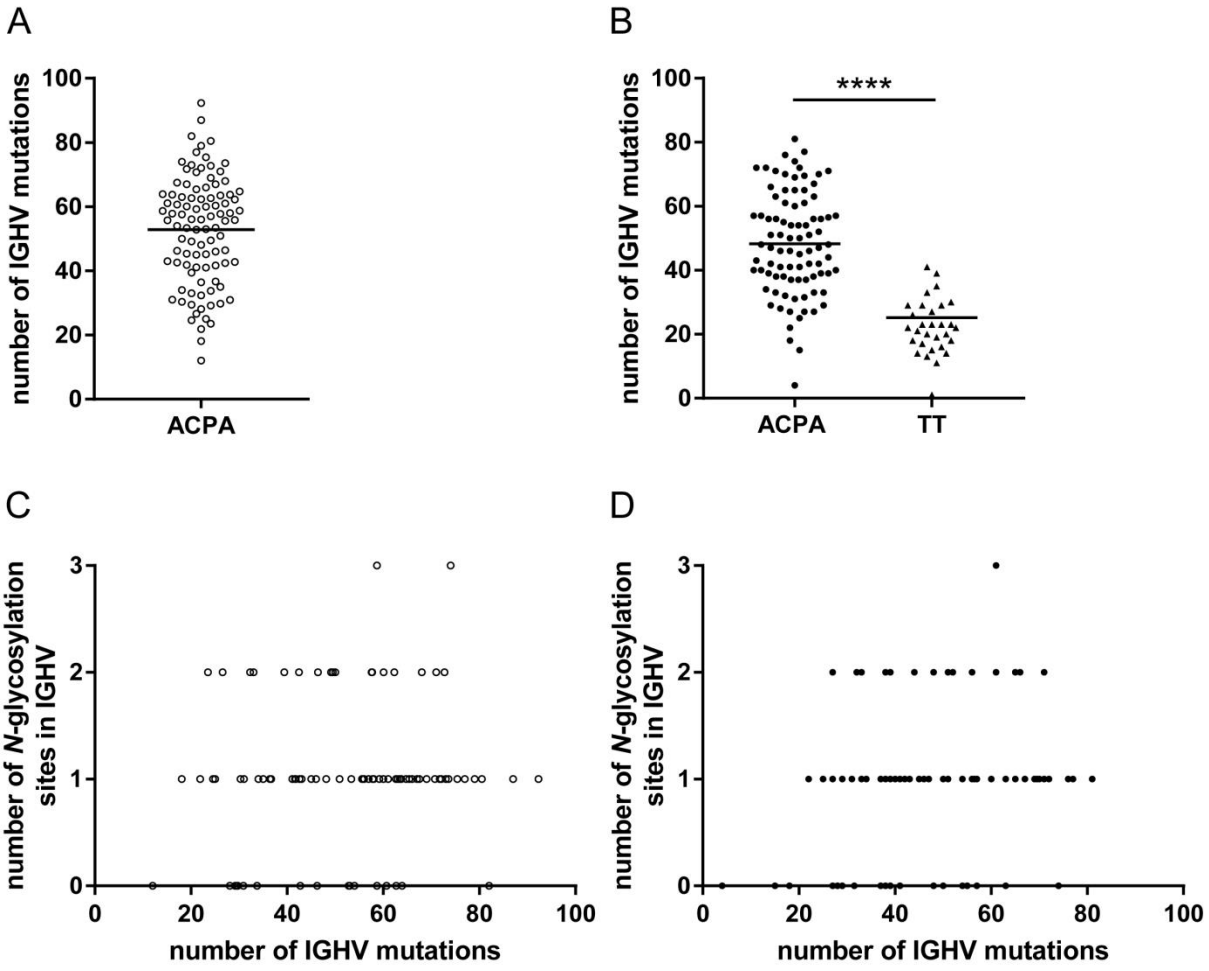
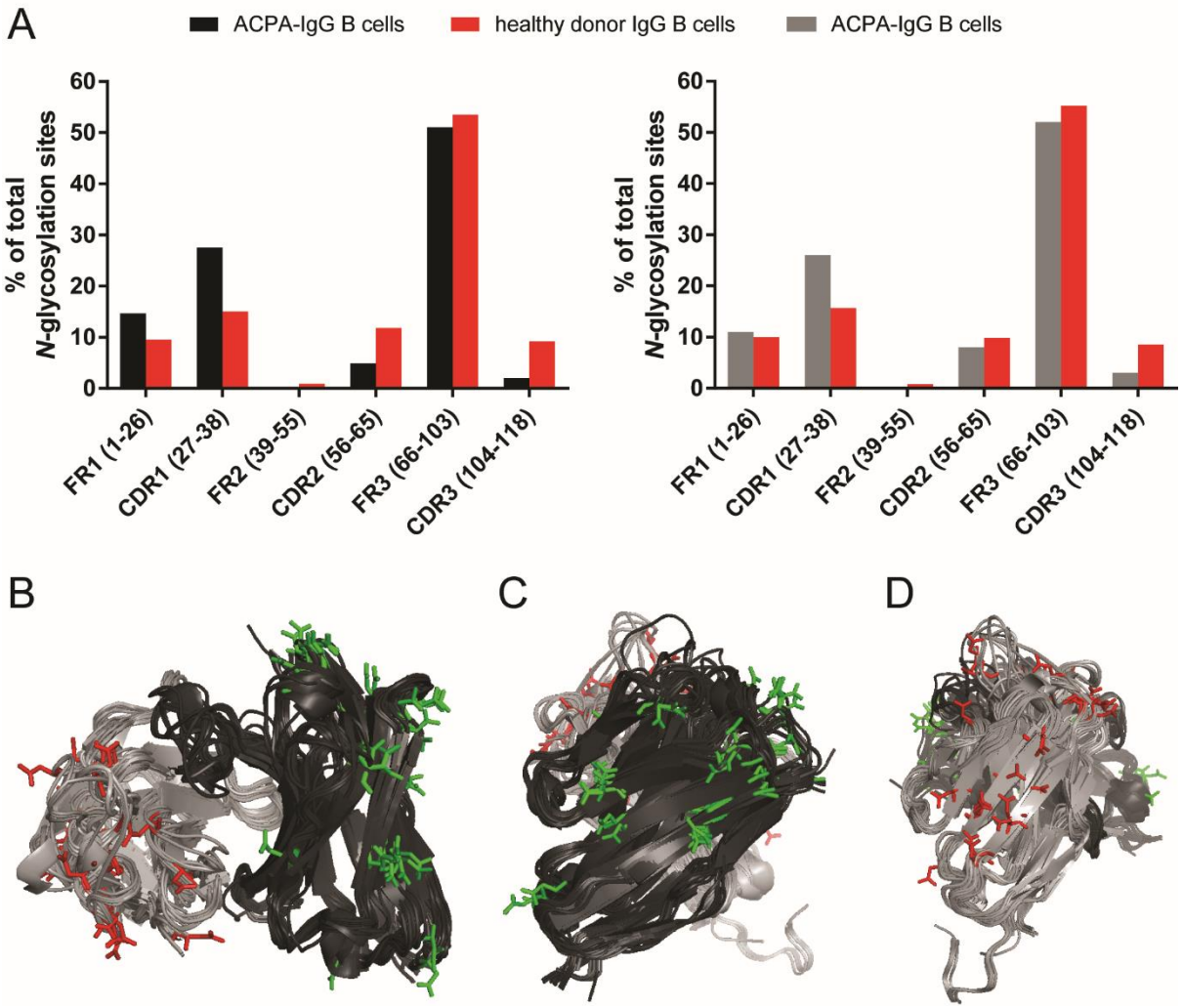

A
number of IGHV mutations
100
80
60
40
20
0
ACPA
B
number of IGHV mutations
****
ACPA
TT
C
number of N-glycosylation sites in IGHV
3
2
1
0
0
20
40
60
80
100
number of IGHV mutations
D
number of N-glycosylation sites in IGHV
number of IGHV mutations

FIGURE 2


A
ACPA-IgG B cells
healthy donor IgG B cells
ACPA-IgG B cells
% of total N-glycosylation sites
60
50
40
30
20
10
0
FR1 (1-26)
CDR1 (27-38)
FR2 (39-55)
CDR2 (56-65)
FR3 (66-103)
CDR3 (104-118)
B
C
D