Response to: 'Acquiring new N-glycosylation sites in variable regions of immunoglobulin genes by somatic hypermutation is a common feature of autoimmune diseases' by Visser et al

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Acquired *N*-glycosylation sites in the variable region of B-cell receptors of patients with autoimmune diseases

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We thank Visser *et al.* for their interesting correspondence to our recently published letter entitled "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" [1, 2]. Visser *et al*. performed a meta-analysis on publicly available datasets to analyse acquired *N*-glycosylation sites in the variable region of B-cell receptors (BCRs) derived from patients with different autoimmune diseases. BCR sequences of antigen-specific B cells isolated after vaccination or infection and BCR sequences of healthy donors (HD) served as comparison. The meta-analysis showed acquired *N*-glycosylation sites in 9.0% of BCR sequences derived from patients with autoimmune diseases and in 2.3% and 2.7% of sequences derived from HD and vaccine/infection-induced B cells, respectively. This enhanced frequency of acquired *N*-glycosylation sites (compared to controls) was observed for all autoimmune diseases, with the exception of ankylosing spondylitis (AS, 3%) and granulomatosis with polyangiitis (GPA, 0%).

The meta-analysis presented is a valuable addition to our work as it further highlights the relevance of variable domain *N*-glycosylation in general and its possible role in autoimmunity in particular. With regard to these data, it is important to note that our analysis referred selectively to BCR-sequences of the IgG-isotype derived from B cells specific for citrullinated antigens. We reported that up to 88% of these ACPA-IgG BCRs carry at least one acquired *N*-glycosylation site. So far, this extensive presence of *N*-glycosylation sites is unique and has not been reported for another (auto)antigen-specific B cell response. Our control dataset consisted of BCR sequences derived from 12 HD and was matched for V-region and isotype (IgG) usage. We observed a frequency of 10% of acquired *N*-glycosylation sites in HD, which is higher than the 3% in the HD datasets presented by Visser *et al*. This discrepancy in frequency might be due to the inclusion of IgM and IgA sequences in the meta-analysis, while our analysis included only IgG isotypes. Indeed, the authors acknowledge a possible overrepresentation of IgM-encoding sequences in particular in the AS and GPA datasets. IgM-encoding sequences likely contain less *N*-glycosylation sites than IgA- and IgG-encoding sequences due to a lower rate of somatic hypermutation (SHM). The latter notion is also supported by our recent observation, which suggests that ACPA-IgM molecules do not harbour additional *N*-linked glycans in the variable region [3]. Thus, matching for isotype usage might be critical for a direct comparison of *N*-glycosylation site frequency between datasets.

Extending these considerations, we observed a significant difference between the introduction of the *N*-linked glycosylation consensus sequence N-X-S/T (where X≠P) and N-P-S/T in ACPA-IgG B cell clones. Although highly similar to the consensus *N*-linked glycosylation site, the N-P-S/T sequence cannot accommodate *N*-linked glycans. In contrast to the BCRs from HD, ACPA-IgG BCRs specifically acquired the N-X-S/T-site and had not acquired the N-P-S/T sequence (Table 1, $p < 0.005$). This provides further evidence for a selective advantage of the introduction of *N*-linked glycosylation-sites in the variable region of ACPA-IgG BCRs. Of note, we selected N-X-S/T and N-P-S/T sites in ACPA-IgG and HD sequences manually because, to the best of our knowledge, the NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/) used by Visser *et al*., includes both N-X-S/T and N-P-S/T sites in the analysis. As we did not observe acquired N-P-S/T sites in ACPA-IgG clones, it would be of great interest to perform additional analyses regarding the presence of N-P-S/T sites in a multiple autoimmune disease meta-analysis to further investigate the notion that autoreactive B cells selectively introduce N-X-S/T (X≠P) sites in a non-random manner.

Irrespective of these considerations, the possibility that acquired *N*-glycosylation sites are more frequently observed in autoimmune diseases is intriguing. This is also supported by previous work of the authors on primary Sjögren's syndrome IgG-BCR sequences [4]. The findings presented by Visser *et al.* contribute to the further understanding of the introduction of *N*-glycosylation sites by autoreactive B cells and its possible impact on selective advantages in B-cell selection and/or the breach of tolerance of autoreactive B cells. Therefore, in future studies it will be important to analyse full-length BCR sequences of different antigen-specific autoreactive B cells in RA and other autoimmune diseases to elucidate whether enrichment of *N*-glycosylation sites in the variable region of BCRs is a feature of autoreactive B cell responses that share common mechanisms for tolerance escape.

Table 1. Selective preference for the introduction of *N*-glycosylation sites in ACPA-IgG compared to healthy donor IgG-expressing B cells.

	NPS/T sites	NXS/T* sites
ACPA (next-generation sequencing)	0	102
Healthy donors (next-generation sequencing)	55	660
ACPA (single cell sequencing)	0	87

ACPA = Anti-citrullinated protein antibodies

*Where X is any amino acid except Proline

Healthy donors were significantly different from ACPA (next-generation sequencing) and ACPA (single cell sequencing) using Fisher's exact test ($p < 0.005$). No significant differences were observed between ACPA (next-generation sequencing) and ACPA (single cell sequencing) using Fisher's exact test ($p = 1$).

References

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