SHORT COMMUNICATION

Sustained CD19$^+$CD27$^+$ Memory B Cell Depletion after Rituximab Treatment in Patients with Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is a rare autoimmune disease in which autoantibodies target desmosomal adhesion proteins, resulting in intraepidermal blistering. Predominantly of the immunoglobulin G (IgG) isotype, these autoantibodies target desmoglein (Dsg) 3, but may additionally bind to Dsg1 (1, 2). Sites of pathophysiological blister formation can be explained by the anti-Dsg3/1 autoantibody profile and the tissue-specific expression pattern of Dsg3/1 (1, 2). Clinically, PV presents as a mucosal-dominant or mucocutaneous variant with erosions on mucous membranes and/or skin (1, 2). Depending on the geographical location, current first-line treatment options are administration of the anti-CD20 antibody rituximab (RTX), the combination of RTX plus systemic corticosteroids, systemic corticosteroids alone, or systemic corticosteroids combined with a corticosteroid-sparing immunosuppressive agent (e.g. azathioprine, mycophenolate mofetil, mycophenolate sodium) (3, 4). RTX leads to general B cell depletion and reduction in circulating CD20$^+$ anti-Dsg3/1 B cells in particular, i.e. of the cells primarily responsible for the generation of disease-specific autoantibodies (4, 5). A delayed reconstitution of the peripheral B cell repertoire following RTX treatment is well-known, and naïve B cells are appearing first during reconstitution. These cells then give rise to the memory B cell compartment, approximately 24–36 weeks after treatment (5, 6). The aim of this prospective cohort study was to longitudinally analyse circulating B cell subpopulations (CD19$^+$CD27$^-$, CD19$^+$CD27$^+$, Dsg3-reactive CD19$^+$CD27$^+$) and CD4$^+$ T cells via flow cytometry analysis (FACS) before and after RTX +/– immunosuppressive treatment in a defined cohort of patients with PV treated in a tertiary referral centre.

METHODS AND RESULTS

Patients with active PV were enrolled into the study at the time of diagnosis or when they developed a recurrence of disease (i.e. relapse) at least 12 months post-RTX. Diagnosis of PV was confirmed by histology as well as direct and indirect immunofluorescence microscopy (3, 7). Serum autoantibody titres were determined by commercial enzyme-linked immunosorbent assays (ELISA) (3, 7). The Pemphigus Disease Area Index (PDAI) (8) and anti-Dsg3/1 IgG levels were determined at baseline and follow-up visits. Treatment decisions were based on disease severity and patient preference, according to the consensus recommendations on pemphigus (3, 7). In addition, age- and sex-matched healthy controls were recruited. Written informed consent was obtained from all participants and controls. The study was approved by the institutional review board of the University of Lübeck, Lübeck, Germany, (study #12-178) and performed in accordance with the Declaration of Helsinki. EDTA blood samples were collected at baseline, weeks 1–4, 12, 24, and 48. Peripheral blood mononuclear cells (PBMCs) were isolated according to standard protocols (9). Staining of freshly isolated PBMCs was performed with the following, appropriately titrated monoclonal antibodies (see Table SI for concentrations): Live/Dead Aqua, CD45R(B220) PE-Cyanine7, CD27-Qdot (all ThermoScientific, Waltham, MA, USA), IgD FITC, CD19 APC-Cy7 (both BD Pharmingen, San Diego, CA, USA), and CD4 Pacific Blue (Biolegend, San Diego, CA, USA). Antigen-specific cells were detected using His-tagged human recombinant Dsg3 (provided by EUROIMMUN, Lübeck, Germany) labelled with Alexa Fluor 647 (ThermoScientific). For accurate gating fluorescence minus one (FMO) controls were performed each time. A minimum of 1,000,000 lymphocytes were acquired on a flow cytometer (BD FACSAriaII™ (BD Life Sciences, San Jose, CA, USA)) and analysed using FlowJo™ (BD Life Sciences) software. Besides forward scatter, 5% of the total dynamic range was used as thresholding parameter. Ten patients with PV were recruited (female: $n=8$; male: $n=2$), median age 57 (range 21–77) years. Mucosal-dominant PV was found in 7 patients, and the mucocutaneous variant in 3 patients. Four patients were treated with RTX (1,000 mg on days 0 and 14) and 6 patients with RTX (1,000 mg on days 0 and 30), in combination with dexamethasone pulse therapy (100 mg/day over 3 days, in ongoing 30-day intervals). Nine patients were treated with RTX for the first time, 1 patient had received a RTX cycle previously. Concomitant immunosuppressants were azathioprine ($n=4$), mycophenolate mofetil ($n=1$), and mycophenolate sodium ($n=1$). The baseline median PDAI was 6.5 (range 2–43), with median baseline anti-Dsg3 autoantibody levels measuring 137 U/ml (range 44–1,611) and anti-Dsg1 at 0 U/ml (range 0–180). All patients achieved disease control, with complete epithelialization of skin and/or mucosal lesions after a median time of 8 (range 5–12) months. Remissions were sustained in 9/10 patients during the 12-month observation period. One out of 10 patients experienced a relapse at month 11 of follow-up.

Longitudinal FACS analysis in these cohorts showed a significant reduction in CD19$^+$CD27$^+$ and Dsg3$^+$CD19$^+$CD27$^+$ memory B cells in pemphigus patients, beginning in the first month after RTX, without reconstitution up to week 48 (**Fig. 1**). This delayed reconstitution is considerably longer than reported previously (5, 6). In addition, no evidence was found that CD19$^+$CD27$^-$ naïve B cells or CD4$^+$ T cells counts were affected by rituximab treatment in patients (Fig. S1).

DISCUSSION

The observed low number of circulating Dsg3-specific B cells in the study cohort is in line with the literature showing mean counts of $48 \pm 20/10^7$ Dsg3-specific B

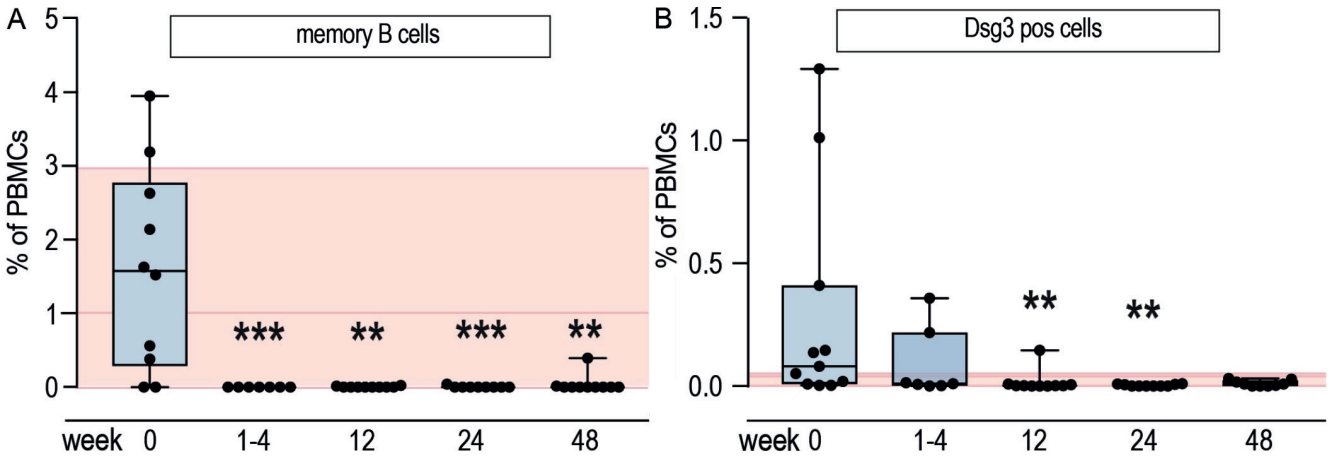

Fig. 1. Protracted depletion of memory B cells and antigen-specific memory B cells in pemphigus after rituximab therapy. (a) $CD19^+CD27^+$ memory B lymphocytes. (b) $Dsg3^+CD19^+CD27^+$ memory B lymphocytes. *Red area* represents measurements for age-/sex-matched controls for the respective cell type (median, 25%/75% percentile). Data were compared using the Kruskal–Wallis – post-hoc test after Dunn. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. GraphPad Prism 9 was used to perform all statistical analyses.

cells in pemphigus patients with active disease (5). To exclude that these low counts potentially biased results shown in Fig. 1b, we tested for outliers using Grubbs' test and could not detect significant deviations (Z-score: 0.29), validating our analyses.

A possible explanation for the observed sustained depletion and delayed reconstitution of memory B cells after RTX therapy (Fig. 1a) may be a retention of naïve progeny B cells in the bone marrow, due to strong myelosuppressive effects by the additional immunosuppressive treatments that were used. These results increase the awareness for significantly protracted B cell reconstitution after RTX plus additional immunosuppression, implying careful and extended monitoring of this patient population over more than 48 weeks and appropriate reduction in adjuvant immunosuppressive therapy. Of note, there were no serious adverse events during follow-up in the study cohort; however, larger patient cohorts are necessary to confirm this. The apparently small sample size may be a limitation of our study; however, at the same time, the rather low incidence of pemphigus and the appropriate matching of healthy controls needs to be taken into account here.

In light of the current pandemic situation, these findings have also important implications for implementing vaccinations against SARS-CoV2 that need to be coordinated with therapy and B cell status.

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The authors have no conflicts of interest to declare.

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