SHORT COMMUNICATION

Faster IgG4 Depletion Kinetics Observed for Anti-Desmoglein 3 Autoantibodies Following Rituximab Treatment in Patients with Pemphigus Vulgaris

Katharina BOCH[1], Ewan A. LANGAN[1,2], Nina VAN BEEK[1,3], Khalaf KRIDIN[3,4], Enno SCHMIDT[1,3], Detlef ZILLIKENS[1], Ralf J. LUDWIG[3], Christoph M. HAMMERS[1,3,5#*] and Katja BIEBER[3#]

*[1]Department of Dermatology, University of Luebeck, Ratzeburger Allee 160, DE-23562 Luebeck, Germany, [2]Manchester Sciences, University of Manchester, Manchester, UK, [3]Lübeck Institute of Experimental Dermatology, University of Lübeck, Lübeck, Germany, [4]Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel, and [5]Department of Dermatology, Christian-Albrechts-University, Kiel, Germany. *E-mail: christoph.hammers@uksh.de*

[#]These authors contributed equally.

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Pemphigus vulgaris (PV) is a rare intraepidermal blistering disease affecting the skin and mucous membranes. Desmoglein (Dsg) 3 is the predominant target autoantigen, but autoantibodies to Dsg1 are also present when there is cutaneous blistering (1). These autoantibodies are predominantly of the IgG isotype and directly mediate blister formation (1). Anti-Dsg3 autoantibodies associate preferentially with the IgG4 subclass (2, 3). Moreover, patients with PV with active disease demonstrate Dsg-reactive IgG4 and IgG1, in contrast to IgG1 Dsg autoantibodies in patients whose disease is in remission (4). IgG2 and IgG3 Dsg autoantibodies have not been associated with disease (2).

The anti-CD20 antibody rituximab, systemic corticosteroids, and corticosteroid-sparing immunosuppressive agents (azathioprine, mycophenolate mofetil and mycophenolate sodium) are current first-line treatments (5). Rituximab targets $CD20^+$ B cells in general and Dsg1/3-specific $CD20^+$ B cells in particular, leading to the depletion of circulating disease-specific autoantibodies (5). It has been shown that the anti-Dsg3 IgG subclasses drive disease activity; however, they also have an impact on outcome after treatment with rituximab (6, 7). The aim of this cohort study was to longitudinally analyse IgG4- and IgG1-specific anti-Dsg3 and anti-Dsg1 autoantibodies before and after rituximab +/– immunosuppressive treatments in patients with PV.

MATERIALS, METHODS AND RESULTS

Patients with active PV ($n=10$; at the time of initial diagnosis or relapse) were enrolled into the study at least 12 months post-rituximab. The patient cohort has already been described (8) and their biomaterial used in a previous study (8). The diagnosis of PV was confirmed by clinical presentation, serology, and direct immunofluorescence microscopy (3, 7). The Pemphigus Disease Area Index (PDAI) was calculated at baseline and follow-up visits. Treatment decisions were based on disease severity and according to consensus recommendations: rituximab (1,000 mg on days 0 and 14) or rituximab (1,000 mg on days 0 and 30) plus dexamethasone pulse therapy (100 mg/day on 3 consecutive days for ongoing intervals every 4 weeks).

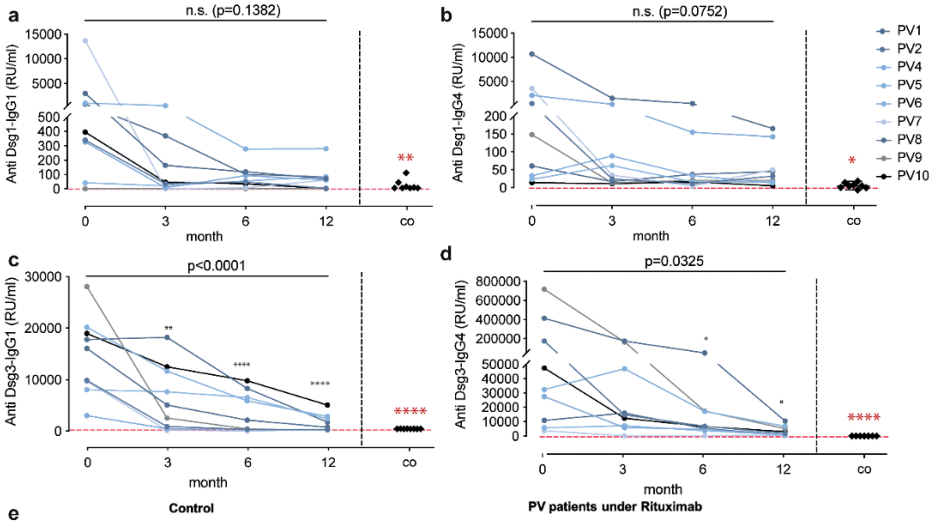
Serum samples of patients with initial manifestation, relapsing, or treatment-resistant PV were collected at baseline, weeks 1–4, 12, 24, and 48. IgG4- and IgG1-Dsg1/3 autoantibody titres were determined by enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Lübeck, Germany) according to the manufacturer's protocol with the following modifications: to determine the isotype of serum anti-Dsg3 IgG1/IgG4 antibodies as well as anti-Dsg1 IgG1/IgG4 antibodies, sheep anti-human IgG1 horseradish peroxidase (diluted 1: 2,500; Binding Site, Schwetzingen, Germany) or mouse anti-human IgG4 Fc (diluted 1: 50,000; Southern Biotech, Birmingham, AL, USA) were used as secondary antibodies. After halting the enzymatic colour reaction, the change in optical density was measured with a GlowMax Multi Detection System (Promega Corp., Madison, WI, USA) at 450 nm. Serum samples were diluted 1:100 ($n=10$ for patients with PV at months 0, 3, 6 and 12 and $n=8$ for healthy controls). Standard curves for IgG1 and IgG4 were calculated by using internal controls delivered by the manufacturer (range 2–200 RU/ml). In addition, the LEGENDplex™ Human Th Cytokine Panel (BioLegend Inc., San Diego, CA, USA) was performed, following the manufacturer's protocol, for simultaneous quantification of 12 human cytokines, including interleukin (IL-)2, 4, 5, 6, 9, 10, 13, 17A, 17F, and 22, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, which are collectively secreted by Th1, Th2, Th9, Th17, and Th22 cells.

Results

Rituximab acted preferentially on IgG4 reduction kinetics of antigen-specific autoantibodies **(Fig. 1**a–d). Longitudinal comparison of IgG4- and IgG1-Dsg3 showed a significant reduction in anti-Dsg3 IgG autoantibodies over the observation period. Here, a significant difference could be detected for IgG1-Dsg3 starting from month 3 and for IgG4-Dsg3 in month 6 and 12. In contrast, no significant reduction could be observed for IgG1-Dsg1 or IgG4-Dsg1 using the same patient cohort ($p=0.1382$ and $p=0.0752$, respectively).

Multiplex immunoassay analysis demonstrated that at baseline (active disease) IL-5 levels were elevated and IL-17 levels reduced. Longitudinal follow-up from active disease to clinical remission showed a decrease in IFN-γ over time. Beside a significant decrease in IFN-γ in serum levels of patients with PV after rituximab treatment, no significant changes in serum levels of other cytokines (IL-5, IL-13, IL-2. IL-6, IL-9, IL-10, TNF-α, IL-17, IL-4 and IL-22) could be observed by rituximab.

Anti-Dsg3 IgG1 and IgG4 levels at baseline correlated with disease activity (**Fig. 2**a,b), whereas anti-Dsg1 IgG1 and IgG4 levels did not reflect PDAI scores. Of note, this may reflect the Dsg3-dominant PV variant in this cohort (mucosal-dominant PV, $n=7$; mucocutaneous PV, $n=3$). Nevertheless, with 9/10 patients being in clinical remission at a 12-month follow-up time-point, these results may demonstrate that IgG4 depletion is associated with reduced clinical activity of pemphigus in this population. Recently, we reported sustained $CD19^+CD27^+$ memory B cell depletion after rituximab treatment (8). Here, we investigated the correlation between B and T cell subpopulations and levels of anti-Dsg autoantibodies. Interestingly, levels of $CD19^+CD27^+$ memory B cells were significantly and robustly correlated with anti-Dsg1 IgG1 ($r=0.844$; $p=0.008$) and anti-Dsg1 IgG4 ($r=0.731$;



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Cytokine (pg/ml)		0 months	3 months	6 months	12 months
IL-5	307.43 ± 186.02 (**)	241932.63 ± 141309.69 (**)	235890.00 ± 91197.22	271245.60 ± 96767.86	182178.94 ± 143057.39
IL-13	851.76 ± 381.76	871.90 ± 567.81	503.68 ± 493.64	695.17 ± 709.58	486.81 ± 518.50
IL-2	119.95 ± 89.14	183.66 ± 187.42	63.02 ± 107.84	160.54 ± 166.58	88.75 ± 126.75
IL-6	465.53 ± 602.85	552.76 ± 427.61	380.04 ± 352.30	614.89 ± 504.06	211.60 ± 181.79
IL-9	514.20 ± 471.67	1046.57 ± 831.10	605.72 ± 538.94	801.09 ± 702.62	492.66 ± 425.09
IL-10	189.32 ± 93.70	142.40 ± 92.66	88.73 ± 86.99	124.49 ± 112.47	63.24 ± 86.04
IFN-γ	534.34 ± 335.51	609.03 ± 319.38 (*)	268.61 ± 232.81	370.19 ± 328.59	164.99 ± 213.81 (*)
TNF-α	2612.97 ± 2387.00	1747.09 ± 1407.33	626.09 ± 735.51	1146.18 ± 900.41	643.48 ± 646.97
IL-17A	122.82 ± 91.77	160.44 ± 110.30	104.04 ± 139.54	126.23 ± 124.74	73.98 ± 88.04
IL-17F	455.75 ± 169.28 (*)	261.87 ± 143.59 (*)	200.14 ± 142.64	238.07 ± 183.23	114.21 ± 116.81
IL-4	395.69 ± 197.51	633.56 ± 684.02	502.50 ± 703.26	567.58 ± 553.30	309.28 ± 424.39
IL-22	303.92 ± 225.41	333.72 ± 120.60	248.80 ± 176.41	311.24 ± 140.69	245.80 ± 183.74

Fig. 1. Serum desmoglein (Dsg)-specific autoantibodies of the IgG 1 and IgG4 subclasses before rituximab and in a longitudinal follow-up. (a) Anti-Dsg1-IgG1; (b) anti-Dsg1-IgG4; (c) anti-Dsg3-IgG1; (d) anti-Dsg3-IgG4. GraphPad Prism 9 was used to perform all statistical analyses. Co: controls. Two-way analysis of variance (ANOVA) with Dunnet's post hoc test was performed for the analysis of IgG concentrations over time (*black asterisks*). Mann–Whitney *U* test was performed for comparison of control samples vs day 0 in patients with pemphigus vulgaris (PV) (*red asterisks*). (e) Multiplex immunoassay analysis showing modulation of cytokines and interleukins by rituximab. Significant differences from pair-wise comparisons are shaded grey and highlighted with *stars*. For context, see main text. $n = 8$ (controls), $n = 9$ (patients with PV), $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

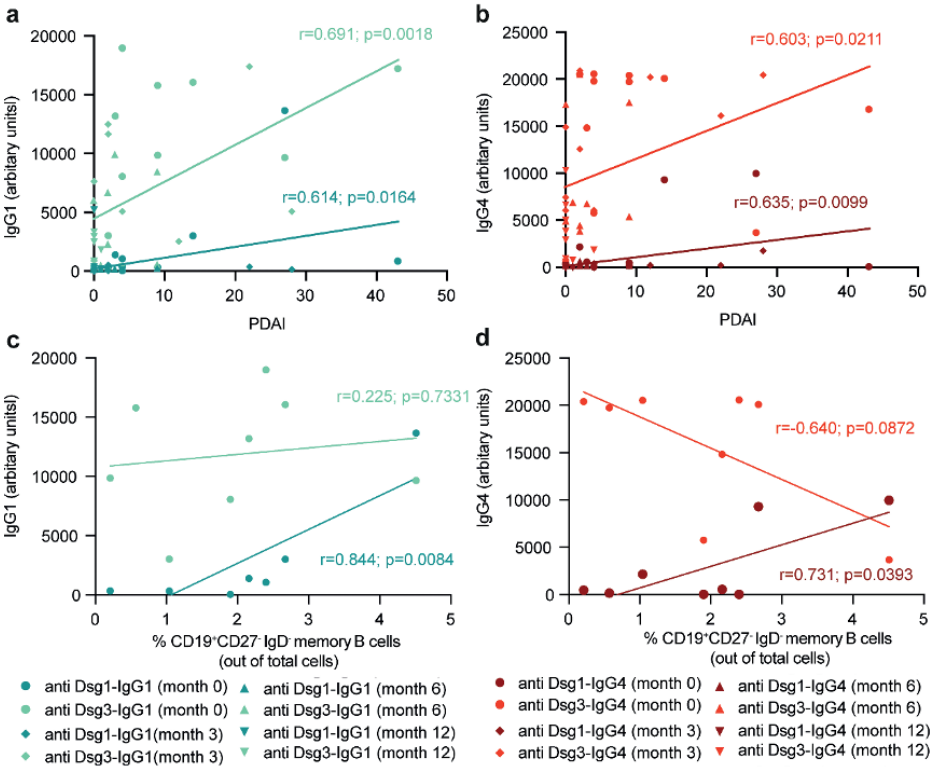

Fig. 2. Pearson correlation of Pemphigus Disease Area Index (PDAI) with serum desmoglein (Dsg)-specific autoantibodies of the IgG1 and IgG4 subclasses. (a) Dsg3/1-specific autoantibodies of the IgG1 subclass. (b) Dsg3/1-specific autoantibodies of the IgG4 subclass. Pearson correlation of $CD19^+CD27$-IgD- memory B cells with Dsg specific autoantibodies. (c) Dsg3/1-specific autoantibodies of the IgG1 subclass at baseline (r=0.844; $p = 0.008$ for anti-Dsg1-IgG1). (d) Dsg3/1-specific autoantibodies of the IgG4 subclass at baseline (r=0.731; $p = 0.039$ for anti-Dsg1-IgG4). GraphPad Prism 9 was used to perform statistical analyses. (a, b) $n = 40$ (10 patients with pemphigus vulgaris (PV) with 4 different time-points each (0, 3, 6 and 12 months); (c, d) $n = 8$ (patients with PV at baseline).

$p = 0.039$) at baseline (Fig. 2c,d). Longitudinal follow-up did not demonstrate any correlation between distributions of B- and T-cell subpopulations and levels of anti-Dsg autoantibodies at 3, 6, and 9 months after administration of rituximab (data not shown).

DISCUSSION

These results indicate that, compared with the antigen-specific IgG1 autoantibody fraction, the antigen-specific

IgG4 autoantibody fraction is more rapidly and more robustly reduced after the administration of rituximab, pointing towards enhanced IgG4 depletion kinetics.

IgG4-mediated autoimmune diseases are rare (9); however, this data underlines the importance of B cell depleting therapies in this autoimmune disease entity. A promising approach to treating pemphigus may be novel anti-B cell agents that target CD19/20 (10); obexelimab is an anti-CD19 antibody with an Fc portion engineered for increased affinity, whereas obinutuzumab is a glycoengineered anti-CD20 antibody with a greater antibody-dependent cellular cytotoxicity (compared with rituximab). It was shown that both concurrently activate the inhibitory FcγRIIb receptors, which have a low binding affinity to IgG4, employing a prolonged anti-B cell activity (9). Furthermore, antibody therapies targeting FcRn may hold promise, as anti-FcRn inhibitors effectively catabolize the IgG4 antibody subclass and may ameliorate disease activity (1, 10, 11). To prove the efficacy of rituximab on the antigen-specific IgG4 autoantibody fraction further studies on the tissue level might be useful. Multiplex immunoassay analysis showed that cytokines and interleukins are modulated by rituximab, highlighting their potential use as biomarkers of disease activity (12).

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The study was approved by the institutional review board (University of Lübeck, #12-178) and performed in accordance with the Declaration of Helsinki.

The authors have no conflicts of interest to declare.

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