Rituximab Mediates a Strong Elevation of B-Cell-Activating Factor Associated with Increased Pathogen-Specific IgG but Not Autoantibodies in Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is a severe autoimmune disease affecting the skin and mucous membranes, characterized by autoantibodies mainly against desmoglein 3 (dsg3). This study investigated the effects of different treatment options on two B-cell mediators, B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL), in 19 PV patients on immunosuppressive drugs alone or in combination with immunoadsorption and anti-CD20 antibody, respectively. Serum BAFF and APRIL levels, circulating desmoglein-reactive autoantibodies, and serum IgG specific for varicella-zoster virus (VZV) and Epstein–Barr virus (EBV) were determined by ELISA before and at different time points after initiation of the respective therapy. In contrast to immunosuppressive therapy alone and in combination with adjuvant immunoadsorption, respectively, rituximab treatment led to a strong and significant elevation of BAFF, but not of APRIL levels, which normalized upon recovery of peripheral $CD19^+$ B cells. Moreover, rituximab treatment led to a statistically significant increase of anti-VZV-IgG and anti-EBV-IgG titers, whereas anti-dsg1 and -3 specific autoantibody titers decreased significantly. Our results suggest that elevated BAFF levels might exert a differential effect on the induction of autoreactive *versus* pathogen-specific IgG antibody production in PV patients, possibly due to promotion of antibody release of pathogen-specific long-lived plasma cells.

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INTRODUCTION

Pemphigus vulgaris (PV) is a severe, potentially life-threatening autoimmune bullous disease primarily characterized by extensive blisters and erosions of the mucous membranes and the skin. IgG autoantibodies (autoab) against the desmosomal adhesion proteins desmoglein 3 (dsg3) and dsg1 have been shown to induce acantholysis of epidermal keratinocytes, a hallmark of PV (Amagai, 1999; Payne *et al.*, 2004). Although immunosuppressive medication including high-dose systemic glucocorticoids is commonly applied, PV remains a therapeutical challenge. Adjuvant settings, such as immunoadsorption (IA) and more recently the monoclonal anti-CD20 antibody rituximab, have been successfully introduced for the treatment of severe and recalcitrant PV (Eming and Hertl, 2006; Eming *et al.*, 2007). Rituximab induces long-term clinical remission that is presumably due to depletion of autoreactive memory B cells as progenitors of autoab-secreting plasma cells (PC) (Ahmed *et al.*, 2006; Joly *et al.*, 2007). Accordingly, rituximab has probably a major impact on the crucial factors for growth and survival of B lymphocytes, including the B-cell-activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL). BAFF and APRIL are mostly produced by antigen-presenting cells including dendritic cells and macrophages, monocytes, and, to a lesser extent, T cells. Moreover, BAFF is constitutively expressed by stromal cells of spleen, lymph nodes, and bone marrow (Dillon *et al.*, 2006; Sutherland *et al.*, 2006; Mackay *et al.*, 2007), determining the size of the peripheral B-cell pool, whereas inducible BAFF produced by myeloid and other cells supports the local survival of B lymphocytes (Schneider, 2005). Recent studies demonstrate that upregulation of BAFF expression *in vivo* results in the rescue of self-reactive, otherwise eliminated B-cell clones as survival of autoreactive B cells is more dependent upon BAFF than is the survival of non-autoreactive B cells (Lesley *et al.*,

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Abbreviations: APRIL, a proliferation-inducing ligand; AZA, azathioprine; BAFF, B-cell-activating factor of the TNF family; BAFF-R, BAFF receptor; BCMA, B-cell maturation antigen; dsg, desmoglein; EBV, Epstein–Barr virus; IA, immunoadsorption; IS, immunosuppressive drugs; IVIg, intravenous immunoglobulin; MMF, mycophenolate mofetil; PC, plasma cells; PV, pemphigus vulgaris; RA, rheumatoid arthritis; Rtx, rituximab; SLE, systemic lupus erythematosus; VZV, varicella-zoster virus

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2004; Thien *et al.*, 2004). Considering its function, BAFF acts as a key factor controlling B-cell survival and maturation (Mackay and Leung, 2006), but not proliferation (Mackay *et al.*, 2003), and plays an important role in enforcing B-cell self-tolerance (Mackay *et al.*, 2007). BAFF and APRIL promote an immunoglobulin switch to the IgG, IgE, and IgA subclasses (Litinskiy *et al.*, 2002; Bossen and Schneider, 2006). B-cell maturation antigen (BCMA), a receptor preferentially expressed on plasmablasts and PC (Avery *et al.*, 2003), is important for the survival of long-lived bone-marrow PC (O'Connor *et al.*, 2004), although it is not yet known whether BAFF or APRIL is critically involved in this process (Dillon *et al.*, 2006).

It is noteworthy that systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are characterized by over-expression of BAFF (SLE) or APRIL (RA), which has been shown to be unaffected by immunosuppressive therapy (Vallerskog *et al.*, 2006). In contrast, recent studies in PV did not detect significant differences in serum BAFF and APRIL levels before or after immunosuppressive treatment in comparison to healthy controls (Asashima *et al.*, 2006; Matsushita *et al.*, 2007; Watanabe *et al.*, 2007). Remarkably, several studies showed an induction of BAFF, but not of APRIL, in patients with SLE, RA (Vallerskog *et al.*, 2006), and primary Sjögren's syndrome (Seror *et al.*, 2007) on rituximab treatment.

The aim of the current observational study was to investigate the impact of rituximab-induced B-cell depletion on serum BAFF and APRIL levels in patients with PV. Moreover, serum BAFF and APRIL concentrations were compared to titers of circulating dsg3-specific IgG autoab and varizella-zoster virus (VZV)- and Epstein–Barr virus (EBV)-IgG, respectively. Our findings suggest that elevated BAFF levels might exert a differential effect on the induction of autoreactive *versus* pathogen-specific antibody production in PV.

RESULTS

Induction of BAFF serum levels in PV patients on immunosuppression and rituximab, but not on immunosuppression alone or in combination with adjuvant IA

Figure 1a demonstrates serum BAFF levels in three groups of PV patients, on immunosuppressive therapy alone or on immunosuppressive therapy with adjuvant rituximab or IA. Apparently, there were no differences in serum BAFF levels between the pre-treatment values of the three patient groups and of healthy controls. In contrast to PV patients treated with immunosuppressive drugs (IS) alone or in combination with IA, who did not show any significant alterations in serum BAFF levels over an observation period of at least 90 days (Friedman's test, IS: $P=0.323$; IA: $P=0.118$), treatment with IS plus rituximab (Rtx) resulted in a pronounced and significant elevation of serum BAFF levels beginning immediately 1 month after therapy (Wilcoxon's signed rank test, $P=0.003$) and lasting for up to 12 months after therapy (Wilcoxon's signed rank test, $P\leqslant0.007$).

Differences in BAFF levels in the three groups of patients before initiation of the respective therapy (d0) did not reach

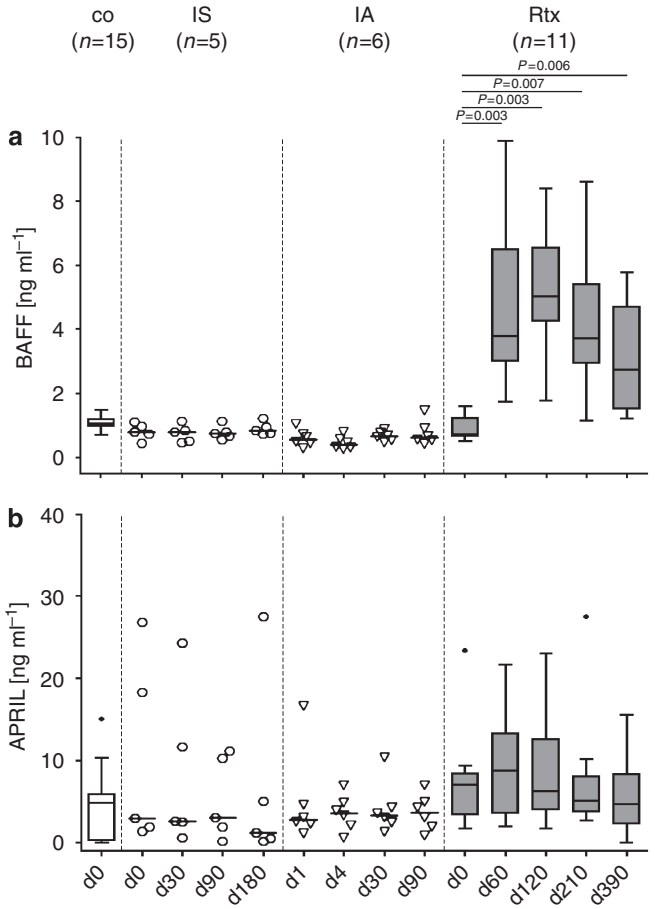

Figure 1. Serum levels of BAFF and APRIL in pemphigus vulgaris (PV) patients on three different treatment schedules. Serum BAFF (**a**) and APRIL (**b**) levels were analyzed in three different groups of PV patients. The first group of patients was treated with immunosuppressive drugs only (IS) and the second group was treated with immunosuppressives and adjuvant immunoadsorption (IA). Neither of these two groups showed significant alterations in BAFF or APRIL serum levels over the entire observation period (Friedman's test, $P>0.05$). The third group of patients was treated with immunosuppressives and rituximab (Rtx). Although serum levels of APRIL remained unchanged over the observation period of 12 months, BAFF levels were strongly and significantly elevated already 1 month (d60) after rituximab treatment, which continued up to 12 months (d390) after therapy (Wilcoxon's signed rank test, $P\leqslant0.007$). In contrast to comparative analysis before treatment, comparison of BAFF levels between the three groups of patients 1 month after the respective therapy showed statistically significant differences (Kruskal–Wallis test, $P=0.0004$) due to strong elevation of BAFF after rituximab-induced B-cell depletion at this time. Design of box plot: box—50% of data, whiskers—extend from highest to lowest values, black points—outliers. For box plot and scatter plot: lines—median.

statistical significance (Kruskal–Wallis test, $P=0.139$). In contrast, owing to a strong elevation of circulating BAFF in PV patients on Rtx, patients on different treatment options showed a statistically significant disparity in BAFF 1 month after therapy (Kruskal–Wallis test, $P=0.0004$). At this time BAFF levels of patients on IS and IA were not significantly different (Mann–Whitney *U*-test, $P=0.792$), whereas PV patients treated with IS and adjuvant Rtx exhibited statistically significant differences compared to pooled data of patients treated with IS alone or with IS and adjuvant IA

(Mann–Whitney *U*-test, $P<0.0001$). Of note, the separate *P*-values of IS *versus* Rtx and IA *versus* Rtx, respectively, are $P=0.0005$ and $P=0.0002$ (Mann–Whitney *U*-test).

As illustrated in Figure 1b, in contrast to BAFF, APRIL serum levels were not significantly different between the three groups of PV patients, neither before the respective treatment (Kruskal–Wallis test, $P=0.431$) nor at the primary end point, 1 month after therapy (Kruskal–Wallis test, $P=0.349$). Furthermore circulating levels of APRIL were not considerably altered over the entire observation period, neither on IS alone (Friedman's test, $P=0.519$) nor in combination with either IA (Friedman's test, $P=0.985$) or Rtx (Friedman's test, $P=0.268$).

These results apparently suggest that the dramatic induction of serum BAFF levels was due to Rtx treatment, because systemic immunosuppressives alone or in combination with adjuvant IA did not show any effect on BAFF. The BAFF-inducing effect of Rtx treatment is illustrated in a representative patient (PV7), who initially received IA and subsequently Rtx treatment (Figure S1a).

Inverse correlation of BAFF serum levels and peripheral CD19$^+$ B cells after B-cell depletion

Figure 2a summarizes the strong inverse correlation between the numbers of peripheral CD19$^+$ B cells and serum BAFF levels of 11 studied PV patients on Rtx treatment. Rituximab led to a pronounced and significant increase in BAFF serum levels once B cells were depleted (Wilcoxon's signed rank test, $P=0.003$), which remained elevated until recurrence of peripheral B cells (repopulation) (Wilcoxon's signed rank test, $P=0.028$). Upon restoration of pre-treatment values of peripheral B-cell numbers (recovery), serum BAFF levels decreased to pre-treatment levels (''baseline'') again in the studied PV patients (Figure 2a). It is noteworthy that Rtx-induced B-cell depletion did not affect serum APRIL levels (Figure 2b).

In this regard Figure 3 illustrates the correlation of peripheral CD19$^+$ B cells and serum levels of the B-cell activating factors BAFF (Figure 3a) and APRIL (Figure 3b). Each line represents the data of an individual Rtx-treated PV patient. Shown is the inverse relationship between circulating BAFF levels and peripheral CD19$^+$ B cells (expressed as % of total lymphocytes), that is, decreasing B-cell numbers cause increasing serum BAFF levels. This correlation was not seen between APRIL serum levels and peripheral C19$^+$ B-cell numbers (Figure 3b).

B-cell depletion promotes raised titers of pathogen-specific but not of autoreactive IgG antibodies in PV patients

One goal of the present study was to investigate the influence of different treatment options on (auto) antibody production in PV. In patients on IS alone (Figure 4a) or in combination with IA (Figure 4b), there was a decline in dsg3-specific IgG

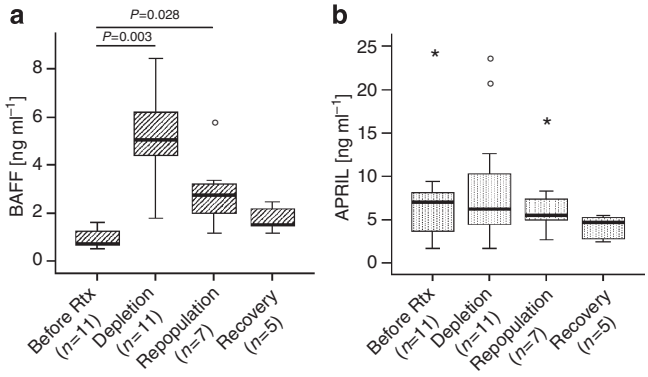

Figure 2. Effect of rituximab (Rtx) on B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) serum levels in PV patients. Shown are median ± range of serum BAFF (**a**) and APRIL (**b**) levels in 11 pemphigus vulgaris (PV) patients in relation to relative numbers of peripheral B cells (*x*-axis). BAFF serum levels were strongly and significantly elevated with highest amounts of BAFF measured after B-cell depletion. This statistically significant elevation of BAFF persisted until the time of B-cell repopulation, defined as recurrence of B cells in peripheral blood (that is, ⩾1% CD19$^+$ B cells, calculated as % of total lymphocytes) (**a**). At the time of B-cell recovery, characterized by reaching pre-treatment numbers again, BAFF levels returned to baseline. Using Wilcoxon's signed rank test, statistically significant changes compared with pre-treatment values are illustrated above the related box plots. APRIL levels remained unchanged on rituximab-induced B-cell depletion (**b**). Design of box plot: box—50% of data, center lines—median, whiskers —extend from highest to lowest values, points—outliers, asterisks—extreme values.

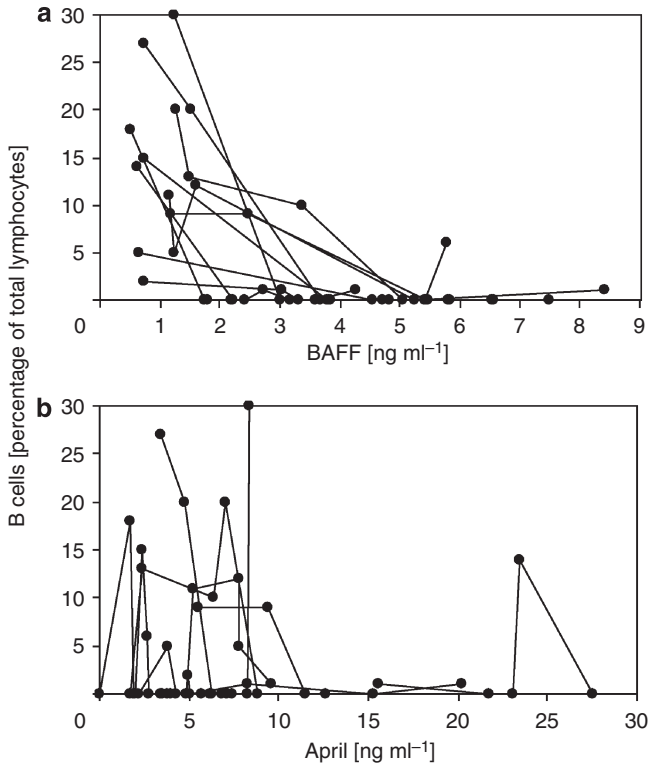

Figure 3. Inverse correlation of B-cell-activating factor (BAFF) levels and peripheral CD19$^+$ B cells. BAFF serum levels and relative numbers of peripheral CD19$^+$ B cells (expressed as % of total lymphocytes) showed an inverse correlation (**a**). In contrast, APRIL levels and peripheral B-cell numbers (**b**) were not directly related to each other. Illustrated are data of the 11 patients before and during the entire observation period of 12 months (d0, d60, d120, d210, d390) after rituximab treatment, whereas each line represents the data of these time points for one individual patient.

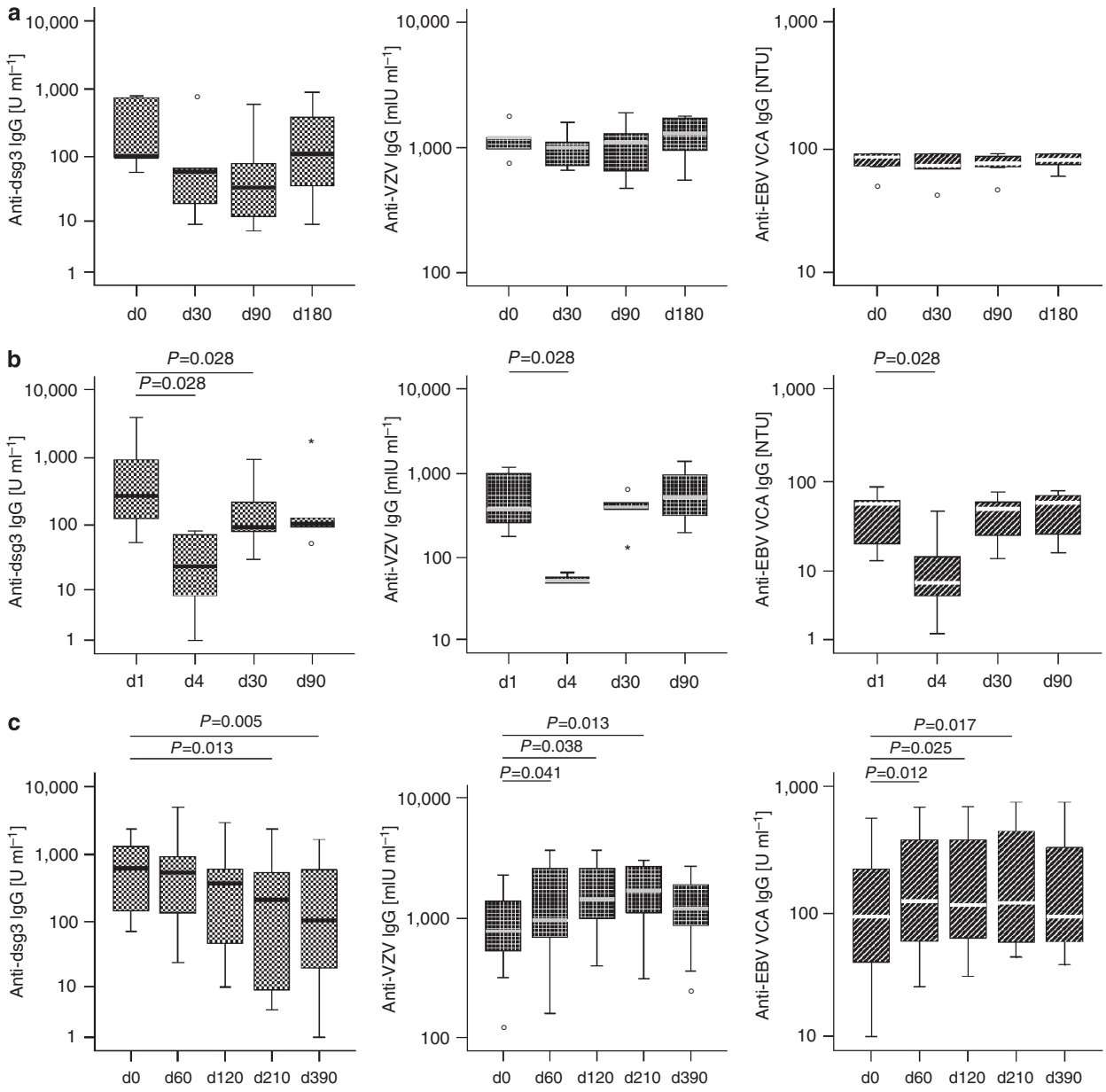

Figure 4. Effect of different treatment schedules on autoreactive *versus* pathogen-specific antibody titers. Illustrated is the change in desmoglein 3 (dsg3)-specific autoantibodies (left row), anti-VZV-IgG (center row), and anti-EBV-IgG (right row) in the sera of PV patients over the entire observation period of immunosuppressive treatment (**a**), immunosuppressives and immunoadsorption (**b**), and immunosuppressives and rituximab (**c**). Although there is a decrease in dsg3-reactive autoantibodies in each group (Figure 4, left row), pathogen-specific anti-VZV-IgG and anti-EBV-IgG antibodies were not influenced by immunosuppressives alone (**a**, center and right) and only transiently reduced, owing to intense removal of total IgG from plasma, by immunoadsorption (**b**, center and right). In contrast, there was a statistically significant increase in serum anti-VZV-IgG (**c**, center) and anti-EBV-IgG (**c**, right) up to 6 months after rituximab. Using Wilcoxon's signed rank test, statistical significance compared to pre-treatment values is presented above the respective box plots. Design of box plot: box—50% of data, center lines—median, whiskers—extend from highest to lowest values, points—outliers, asterisks—extreme values. Serum titers of anti-EBV-IgG were available only for 8 of 11 rituximab-treated PV patients (**c**, right).

autoab (Figure 4a and b, left row), associated with clinical remission as illustrated in Table S1. In contrast, serum concentrations of anti-VZV-IgG and anti-EBV-IgG were not significantly changed during the entire observation period in both groups of PV patients. As expected, IA treatment led to a strong and transient reduction in both pathogen-specific and autoreactive IgG due to the dramatic removal of circulating total IgG from the patients' plasma (d4, Figure 4b). To analyze the potential effect of Rtx-induced B-cell depletion on antibody production, we studied the serum titers of dsg1- and dsg3-specific IgG autoab and those of VZV- and EBV-reactive IgG in Rtx-treated PV patients. Figure 4c demonstrates that anti-dsg3-IgG titers decreased slowly but continuously, starting 3 months (d120) after Rtx treatment, finally leading to 20–30% of the pre-treatment values 12 months after B-cell depletion (Figure 4c, left; Wilcoxon's

signed rank test, $P = 0.005$). In 6 of the 11 PV patients, serum autoab titers against dsg1 were also detectable before Rtx treatment. Along with a decrease in serum anti-dsg3 autoab titers there was a decline of dsg1-specific IgG from a median of 168 U ml^{-1} before therapy to a median of 16 U ml^{-1} 6 months after treatment (data not shown). In contrast, there was a statistically significant increase in VZV- and EBV-specific IgG antibodies in the sera of all investigated PV patients up to 6 months after Rtx therapy (Figure 4c; Wilcoxon's signed rank test, $P \leqslant 0.041$ (VZV), $P \leqslant 0.025$ (EBV)). Between 6 and 12 months after Rtx, the titers of pathogen-specific IgG went back to baseline levels again (Figure 4c, center and right, Wilcoxon's signed rank test, $P = 0.374$ (VZV), $P = 0.208$ (EBV)).

Interestingly, there were no differences in the decline in autoab and elevation of BAFF levels, or of anti-EBV-IgG and anti-VZV-IgG titers, between PV patients with ongoing clinical remission 12 months after Rtx treatment (9 of 11 patients) and the two PV patients who relapsed at this time (data not shown).

In addition, the pronounced Rtx-mediated inducing effect on anti-VZV- and anti-EBV-IgG is illustrated for one representative PV patient in Figure S1b. This patient showed a strong increase in both anti-VZV and anti-EBV antibody titers after B-cell depletion, whereas dsg3-specific autoab remained unchanged up to 6 months after Rtx treatment (d210) and decreased slightly afterwards (Figure S1b).

Elevated BAFF levels seem to correlate with raised titers of pathogen-specific but not of autoreactive IgG antibodies in PV patients

Figure 5 shows data of all investigated PV patients treated with Rtx, correlating serum BAFF levels with serum anti-dsg3-IgG (Figure 5a), anti-VZV (Figure 5b), and anti-EBV (Figure 5c) titers. As the primary end point was defined as alteration of BAFF and circulating antibody titers immediately after B-cell depletion, Figure 5 illustrates the relation of serum BAFF and antibody levels before Rtx treatment and 1 month (d60) after B-cell-depleting therapy. At this time peripheral B cells were completely eliminated in all PV patients. In contrast to autoreactive, dsg3-specific IgG (Figure 5a), elevation of pathogen-specific anti-VZV- (Figure 5b) and anti-EBV-IgG (Figure 5c) seemed to be related to strongly increased levels of circulating BAFF in most of the studied PV patients 1 month after B-cell depletion. Thus, elevated BAFF levels may possibly exert a differential effect on the production of dsg3-reactive IgG autoab *versus* pathogen-specific IgG antibodies.

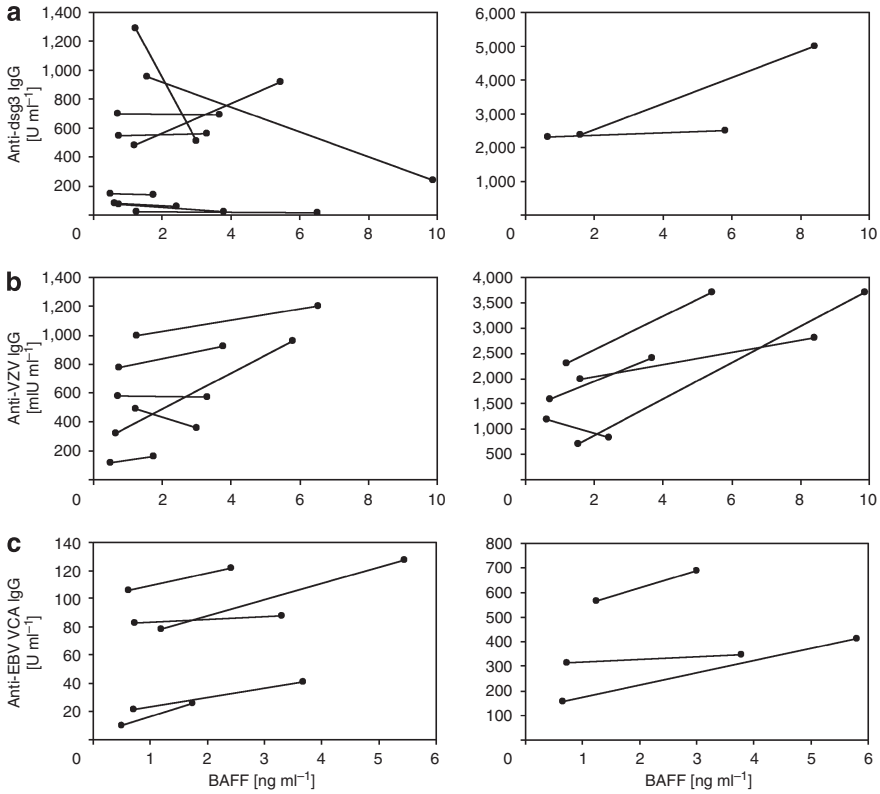

Figure 5. Different effects of B-cell-activating factor (BAFF) on autoreactive *versus* pathogen-specific antibody titers. Shown is the relation between rituximab-induced elevation of BAFF and anti-desmoglein 3 (dsg3)-IgG (**a**), anti-VZV-IgG (**b**), and anti-EBV-IgG (**c**) before and 1 month after B-cell-depleting therapy in PV patients. In contrast to dsg3-specific autoantibodies, which did not show any consistent relation to BAFF (**a**), elevation of BAFF correlated with increased anti-VZV- and anti-EBV-IgG in most of the PV patients at this time (**b** and **c**). Illustrated are data of all investigated patients, whereas each line represents the data of one patient. Owing to the broad range of individual (auto)antibody titers, PV patients were divided into two groups regarding their (auto)antibody levels (Figure 5, left and right). Serum titers of anti-EBV-IgG were available only for 8 of 11 rituximab-treated PV patients (**c**).

DISCUSSION

In this study, we investigated the effect of systemic immunosuppression alone or in combination with IA or Rtx on the B-cell-activating factors BAFF and APRIL in 19 PV patients. Upon treatment with the B-cell-depleting anti-CD20 monoclonal antibody rituximab, we observed an inverse correlation of BAFF and relative numbers of peripheral $CD19^+$ B cells. Rituximab-dependent B-cell depletion induced a dramatic increase in serum BAFF levels. Moreover, dsg3-specific IgG autoab titers declined over time, whereas VZV- and EBV-specific IgG titers showed a statistically significant increase immediately after Rtx treatment. Elevation of BAFF seems to be associated with the observed increase in pathogen-specific IgG antibodies, possibly due to promoting effects of BAFF on antibody production by long-lived pathogen-specific PC.

In contrast to other B-cell-mediated autoimmune disorders, such as SLE (Vallerskog *et al.*, 2006) and systemic sclerosis (Matsushita *et al.*, 2006), overexpression of BAFF or APRIL has not yet been described in PV. Our present results show that neither treatment with IS alone nor IS in combination with IA interferes with serum levels of these two B-cell-activating factors in PV. Instead, administration of Rtx induces a strong elevation of BAFF, but not of APRIL. Nonetheless, in contrast to BAFF, circulating APRIL levels showed a broad variability in PV patients. Therefore it is possible that therapy-mediated effects on APRIL could not be detected primarily owing to the small number of investigated patients.

The increase in BAFF is most likely due to the B-cell-depleting effect of Rtx as BAFF-R, the main receptor of BAFF, is mostly expressed on B cells (Schneider and Tschopp, 2003; Mackay *et al.*, 2007). Thus, elevation of serum BAFF in Rtx-treated PV patients may partly be explained by its reduced consumption by B cells. In addition, a direct Rtx-mediated induction of BAFF through its positive transcriptional regulation was shown in RA, SLE, and primary Sjögren's syndrome (Lavie *et al.*, 2007).

As 2 of 11 investigated PV patients on Rtx showed a clinical relapse 12 months after treatment, a combination of Rtx with BAFF antagonists may provide a promising synergistic therapeutic approach for long-term clinical remission in PV. Mouse models of SLE using lupus-prone mice showed clinical improvement after administration of BAFF-R-Fc alone and a total depletion of splenic B lymphocytes after concomitant treatment with BAFF-R-Fc and a monoclonal anti-hCD20-antibody (Gong *et al.*, 2005). Thus, BAFF antagonists, administered with Rtx, probably operate by depleting B cells more efficiently and for a longer time owing to the reduced *de novo* generation of B lymphocytes. This effect may apply specifically to down-regulating autoreactive B cells, as very high serum levels of BAFF, as seen on Rtx, might interfere with the elimination of autoreactive B cells that are more dependent on BAFF for survival than non-autoreactive B cells (Lesley *et al.*, 2004; Thien *et al.*, 2004). Several BAFF antagonists, such as belimumab, TACI-Ig, BAFF-R-Fc, and AMG 623, are currently tested in clinical trials (Stohl and Looney, 2006). Interestingly, a recent study identified anti-BAFF and anti-APRIL antibodies in two commercial intravenous immunoglobulin (IVIg) preparations (Le Pottier *et al.*, 2007). As administration of IVIg is an effective therapeutic approach in PV (Ahmed, 2001; Segura *et al.*, 2007), a combination therapy of Rtx and IVIg may act synergistically in down-regulating the autoimmune response in PV. Recently, a therapeutic combination of Rtx and IVIg has been shown to induce a significant clinical improvement in patients with recalcitrant PV (Ahmed *et al.*, 2006). However, studies comparing both treatment regimens in PV are still lacking.

In contrast to peripheral B cells expressing both CD20 and BAFF-R on their surface, long-lived bone-marrow PC are typically CD20-negative (Silverman and Weisman, 2003; Stohl and Looney, 2006). Therefore, a direct effect of Rtx on long-lived PC can be excluded. Possibly, there is an indirect effect through Rtx-induced elevation of BAFF, probably mediated by BCMA expression on PC. This hypothesis is supported by our results showing a statistically significant elevation of anti-VZV- and anti-EBV-IgG titers in all investigated rituximab-treated PV patients. Although BAFF and APRIL bind equally well to BCMA, the increase in pathogen-specific IgG seen in our PV patients seems to be most likely mediated by BAFF owing to its significant induction after Rtx-induced B-cell depletion and the apparent correlation of increased BAFF levels with increased titers of pathogen-specific antibodies (Figure 5). This finding is in line with recent reports demonstrating that BAFF but not APRIL induces the differentiation of memory marginal zone analog B cells to PC (Ettinger *et al.*, 2007) and that BAFF enhances T-cell-dependent humoral immune responses in mice by increasing antigen-specific PC (Do *et al.*, 2000). Thus, our observations in PV confirm previous studies reporting elevated or stable serum IgG titers against tetanus toxoid or pneumococcal capsular polysaccharide in patients on Rtx (Cambridge *et al.*, 2003, 2006; Cutler *et al.*, 2006; Eming *et al.*, 2008; Ferraro *et al.*, 2008).

Recent reports described long-lived (presumably pathogen-specific) PC maintaining specific humoral antibody memory in the absence of persistent antigenic stimulation in contrast to short-lived (probably autoreactive) PC generated by persistent antigenic stimulation (Manz *et al.*, 2005; Browning, 2006; Radbruch *et al.*, 2006). Long-lived bone marrow PC expressing BCMA have been reported to be strongly dependent on BAFF for survival through upregulation of anti-apoptotic Mcl-1 (O'Connor *et al.*, 2004). To our knowledge, there is no insight into the effect of BAFF on short-lived PC in B-cell-mediated diseases including PV. As in humans there is no evidence for differential half-lives of autoantigen *versus* pathogen-specific IgG, our data suggest a differential effect of rituximab and probably of elevated serum BAFF concentrations on pathogen-specific *versus* autoreactive PC possibly due to (i) differences in the expression of BAFF binding receptors, (ii) direct Rtx-mediated reduction in short-lived $CD20^+$ plasmablasts/PC, as CD20 expression has been shown in up to 20% of bone marrow PC (Terstappen *et al.*, 1990; Dorner and Lipsky, 2007), (iii) the need for T cell help in evolving proliferative B-cell responses and autoab production, which is impaired after treatment with anti-CD20 monoclonal antibody (Bouaziz *et al.*, 2007;

Eming *et al.*, 2008), and (iv) reduction in short-lived PC, resulting from Rtx-induced depletion of peripheral B cells as precursors. Finally, elevated antibody production of long-lived plasma cells, due to directly promoting effects of BAFF on IgG release by these cells, cannot be excluded.

To our knowledge, the comparison of serum BAFF and APRIL levels in PV patients on different immunosuppressive treatment regiments (alone or in combination with IA or Rtx) is previously unreported. Interestingly, in contrast to commonly applied treatment schedules, such as IS and IA, we found a strong induction of BAFF serum levels with adjuvant Rtx 1 month after treatment accompanied by upregulation of pathogen-specific but not of autoreactive IgG. As this was a monocenter observational cohort study lacking randomization and statistical independence, the main objective was focused on generating a hypothesis. Thus, further multicenter, randomized trials are mandatory to confirm our present findings. Further characterization of the possibly different effects of BAFF on autoreactive *versus* pathogen-specific PC will be most helpful to better understand the regulation of autoreactive PC and autoab in PV.

MATERIALS AND METHODS

Patients

A total of 19 PV patients (8 males, 11 females, mean age: 53 ± 19 years) were included in the present study. Diagnosis of PV was confirmed by (1) flaccid blisters and erosions of skin and/or mucous membranes, (2) histopathology revealing suprabasal acantholytic blisters, (3) direct and indirect immunofluorescence microscopy of perilesional skin biopsies and of monkey esophagus substrate, respectively, demonstrating epidermal intercellular IgG and complement deposits, and (4) detection of serum IgG autoab against dsg3 (with/without anti-dsg1 autoab) by ELISA. Patients were divided into three groups depending on treatment with IS alone, IS with adjuvant IA, and IS with Rtx. All patients treated with IA or Rtx demonstrated disease activity refractory to combined systemic immunosuppressives, including high-dose glucocorticoids, and at least one adjuvant immunosuppressive agent for more than 3 months (Table S1). According to a consensus statement on definitions of disease, end points, and therapeutic response for pemphigus (Murrell *et al.*, 2008), clinical response during follow up was defined as follows: (i) partial remission: presence of transient new lesions healing within 1 week while the patient is receiving therapy, (ii) complete remission: absence of new or established lesions while the patient is on therapy, and (iii) complete remission off therapy: absence of new or established lesions while the patient is off all systemic therapy for at least 2 months (Murrell *et al.*, 2008).

Each patient gave written consent prior to inclusion in the study, which was approved by the Ethics Committee of the Medical Faculty of Marburg. The study was conducted according to the Declaration of Helsinki Principles. Fifteen healthy individuals served as controls.

As this was a monocenter observational study, a relatively small number of PV patients were included in groups I and II (see below), owing to the rareness of the disease.

Group I: PV patients on immunosuppressive treatment (IS)

Five PV patients (1 male, 4 females, mean age: 53 ± 26 years) were treated with standard immunosuppressive drugs including high-dose systemic prednisolone (initial dose: 0.5–1.0 mg kg^{1} per day) and azathioprine (AZA; 1.5–2.5 mg kg^{1} per day, provided thiopurine-methyltransferase-activity was normal) or mycophenolate mofetil (MMF; 2–3 g per day). Depending on the clinical response, the dose of prednisolone was gradually tapered (Table S1).

Group II: PV patients on IS and adjuvant immunoadsorption (IA)

Six PV patients (2 males, 4 females, mean age: 52 ± 18 years) who were resistant to standard immunosuppressive therapy (Table S1) received adjuvant IA, which is characterized by high-affinity adsorption of IgG and circulating immune complexes from the patients' plasma. Briefly, one treatment cycle consisted of IA on 4 consecutive days. During and after the entire IA treatment, patients continued to receive immunosuppressive medication (Table S1) consisting of high-dose systemic prednisolone (initial dose: 0.5–1.0 mg kg^{-1} per day) and AZA (1.5–2.5 mg kg^{-1} per day) or MMF (2–3 g per day), according to the extent of the disease (Eming and Hertl, 2006; Eming *et al.*, 2006).

Group III: PV patients on IS and adjuvant rituximab (Rtx)

This group comprised 11 patients (6 males, 5 females, mean age: 52 ± 17 years) with severe PV defined as at least 30% involvement of the body surface and/or at least 25% involvement of the oral/genital mucosa. Furthermore, all patients demonstrated disease activity refractory to combined systemic immunosuppressives, and three patients underwent IA prior to Rtx (Table S1). These patients were included in both the IA and the Rtx groups, so differences between these groups were not statistically independent. Rituximab (MabThera, Roche, Grenzach-Wyhlen, Germany) treatment consisted of 4 weekly i.v. infusions of 375 mg m^{-2} body surface area on days 1, 8, 15, and 22. Standard immunosuppressive therapy included systemic prednisolone (initial dose: 0.5–1.0 mg kg^{-1} per day) and AZA (1.5–2.5 mg kg^{-1} per day) or MMF (2–3 g per day). Prednisolone doses were logarithmically reduced according to the clinical response. After tapering of prednisolone, AZA or MMF treatment was continued for an additional 6 months and then gradually reduced upon long-term clinical remission (Table S1).

Serological and cellular analyses

The numbers of peripheral CD19^{+} B cells in the studied patients were determined by flow cytometry and calculated as relative numbers of B cells, expressed as % of total lymphocytes, at the Department of Hematology and Immunology. Analysis of VZV- and EBV-virus capsid-antigen (VCA)-specific IgG was performed by ELISA and titers were expressed as mIU ml^{-1} (VZV) and U ml^{-1} or nephelometric turbidity units (NTU) in the case of EBV. For detection of anti-dsg3-IgG autoab, which were expressed as protein index value in U ml^{-1}, sera of the PV patients were subjected to ELISA (Mesacup Desmoglein-Test, MBL, Naka-ku, Nagoya, Japan). Serum levels of BAFF (R&D Systems, Wiesbaden-Nordenstadt, Germany) and APRIL (Bender MedSystems, Vienna, Austria) were also determined by ELISA according to the manufacturers' instructions. All analyses were performed at the following time points: in patients receiving IS, prior to treatment (d0) and 1 (d30), 3 (d90), and 6 (d180) months after initiation of immunosuppressive therapy. PV patients undergoing adjuvant IA were tested prior to (d1) and immediately after IA (d4) as well as 1 (d30) and 3 (d90) months later. Serum

samples of Rtx-treated PV patients were analyzed before (d0) and 1 (d60), 3 (d120), 6 (d210), and 12 (d390) months after the final rituximab infusion, which was given on day 22.

Statistical analysis

The main objective of the study was to investigate the effect of B-cell-depleting antibody, rituximab, on B-cell-activating factors (BAFF, APRIL) and antibody titers (anti-dsg3-, anti-VZV-, anti-EBV-IgG). As the generated data are non-normally distributed, continuous variables are shown as the median with quartiles and range, illustrated mostly as box-whisker plots, and non-parametric tests were used. The box contains 50% of the data (25th and 75th percentiles), whereas the center line represents the median. The whiskers restrict the minimum and the maximum of the data set. Outliers (distance from 25th or 75th percentiles $>1.5\times$ length of the box) are illustrated as points and extreme values (distance from 25th or 75th percentiles $>3\times$ length of the box) as asterisks.

Regarding the main objective of this study the immediate impact of Rtx treatment was of primary interest. Therefore, the primary end point of this study was alterations in the two B-cell-activating factors and the three antibody titers at 1 month after initiation of therapy. For comparison of paired samples of patients before and at different time points during treatment, the two-sided Wilcoxon signed rank test was used. Level of significance α was defined as less than 0.05 ($P<0.05$). The *P*-values for pairwise comparisons were not adjusted for multiple testing. Before applying the Wilcoxon signed rank test Friedman's test was used as an omnibus test for repeated measures (paired data).

To support the hypothesis of an effect of Rtx on our target values the differences in the before/after-analyses were compared to two commonly applied treatment options, that is, IS and IA. For a comparison of the three different patient groups before (d0) and 1 month after therapy the Kruskal–Wallis test was performed at first. In the case of $P<0.05$, the differences in the two groups, IS and IA, were compared. Depending on this *P*-value, $P>0.05$ or $P<0.05$, the differences were then compared between Rtx and the pooled data of IS and IA groups or separately between Rtx and IS and Rtx and IA, using the two-sided Mann–Whitney *U*-test.

Statistical analysis was performed using SPSS and GraphPad Prism.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Patients data before respective treatment (day 0).

Figure S1. Serum levels of B-cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL), and different antibodies, after immunoadsorption (IA) and rituximab (Rtx) treatment in a representative pemphigus vulgaris (PV) patient.

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