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## Rituximab and Corticosteroid Effect on Desmoglein-Specific B Cells and Desmoglein-Specific T Follicular Helper Cells in Pemphigus

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**TITLE: RITUXIMAB AND CORTICOSTEROID EFFECT ON DESMOGLEIN-SPECIFIC B-CELLS AND DESMOGLEIN-SPECIFIC T-FOLLICULAR-HELPER-CELLS IN PEMPHIGUS**

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**Short title :**

DSG-specific B- and TFH-cells in pemphigus

**Abbreviations**

Abs: antibodies; ASC: antibody-secreting-cells; CS: corticosteroids; DSG: desmoglein; ELISA: enzyme-linked immunosorbent assay; Ig: immunoglobulin; IL: Interleukin; mAbs: monoclonal antibodies; M: Month, PBMC: peripheral blood mononuclear cells; RTX: rituximab; TFH: T-Follicular-Helper; TH: T-Helper.

## **ABSTRACT**

Pemphigus is an autoimmune blistering disease mediated by autoantibodies directed against desmogleins. We recently showed that first-line treatment with rituximab enables more patients to achieve long-lasting remission off therapy than corticosteroids alone. To understand the immunological mechanisms that mediate the long-lasting clinical remission after rituximab treatment, we analyzed the phenotype of desmoglein-specific memory B cells and desmoglein-specific T-follicular-helper cells by flow cytometry, and measured antibody-secreting-cells by ELISPOT in patients treated with corticosteroids alone or rituximab. This post hoc analysis of the RITUX3 trial showed that rituximab induced a significant decrease of IgG-switched desmoglein-specific memory B cells. Accordingly, anti-desmoglein antibody-secreting-cells were no longer detected in patients in complete remission after rituximab. In contrast, corticosteroids did not modify the frequency or the phenotype of desmoglein-specific memory B cells, and anti-desmoglein antibody-secreting-cells were still detected after treatment, even in patients in remission. Using peptide-HLADRB1\*0402 tetramer staining, we identified desmoglein-3-specific T-follicular-helper cells, which dramatically decreased after rituximab, while remaining stable after corticosteroid treatment. Our findings suggest that the long-lasting response to rituximab in pemphigus relies on the decrease of desmoglein-specific circulating T-follicular-helper cells, which correlates with a sustained depletion of IgG-switched memory autoreactive B cells, leading to the disappearance of anti-desmoglein antibody-secreting-cells.

## **GRAPHICAL ABSTRACT**

## INTRODUCTION

Pemphigus is a life-threatening autoimmune disease of the skin and mucosae due to the production of antibodies (Abs) directed against two desmosomal proteins: desmoglein (DSG)-1 and DSG-3, which are adhesion molecules of the epidermis (Schmidt et al. 2019). We recently reported the long-lasting efficacy of rituximab (RTX), the anti-CD20 monoclonal antibody (mAb), in pemphigus patients (Joly et al. 2017). Relative to a standard regimen of corticosteroids (CS) alone, RTX associated with a short-term regimen of prednisone allowed 89% of patients to achieve complete remission off therapy at Month (M) 24. RTX is thought to act mainly by B-cell depletion in pemphigus, as in other autoimmune diseases (Alberici et al. 2015; Albers et al. 2017). Relapses have been reported to be associated with B-cell reconstitution. Up to now, the effect of RTX has mainly been assessed on total B-cells, which does not necessarily reflect its effect on autoreactive DSG-specific B-cells. In particular, we previously reported in pemphigus patients treated with RTX as second-line treatment, that long-term remission was associated with prolonged failure of anti-DSG B-cell response (Colliou et al. 2013).

Most of the anti-DSG Abs belong to the immunoglobulin (Ig) G4 subclass (Ellebrecht et al. 2018), which suggests the involvement of T-Follicular-Helper (TFH)-cells in the selection process within the germinal center. TFH-cells are characterized by the expression of CXCR5 chemokine receptor and the production of Interleukin (IL)-21, a cytokine which is essential for the generation of memory B-cells and plasma-cells (Zotos et al. 2010). Interestingly, a higher frequency of circulating TFH-cells and increased serum IL-21 levels have been recently reported in pemphigus patients relative to healthy controls (Hennerici et al. 2016). This latter study suggested the existence of DSG-3-specific autoreactive T-Helper

(TH)-cells producing IL-21 upon ex vivo stimulation with DSG-3. The aim of this post analysis of the RITUX3 trial was to investigate the effect of RTX compared to a standard CS regimen alone on the frequency and phenotype of DSG-specific B-cells and DSG-specific TFH-cells in pemphigus patients. In particular, we studied the relationships between autoreactive B- and T-cell populations, and long-lasting clinical remission, or the onset of relapses.



## **RESULTS**

### **Clinical course of patients**

Ninety patients were randomly assigned to standard CS regimen or RTX plus short-term prednisone groups (Figure 1). At the final evaluation at M36, 44 of 46 patients (96%) from the RTX group were in complete remission off therapy, including 2 patients who were secondarily treated with RTX between M24 and M36, and 2 patients had persistent disease activity. Fifteen patients from the CS group were in complete remission off therapy or still on minimal therapy at M24 and 29 patients had persistent disease activity. According to the protocol of the RITUX3 trial, 20 out of 44 patients (45%) from the CS group were secondarily treated with RTX between M24 and M36 (after assessment of the primary endpoint at M24) due to persistent disease activity or CS side effects. Since the treatments of patients from the CS group were no longer homogeneous during the third year of the trial, biological analyzes were not performed after the M24 visit in these patients from the CS group.

### **Serum autoantibodies against DSG-1 and DSG-3**

Relative to the RITUX3 trial (Joly et al. 2017), which reported the monitoring of biological parameters until M24, we have shown in Fig 2 the evolution of mean serum anti-DSG-1 and anti-DSG-3 Abs ELISA values of patients from the RTX until M36. In both treatment groups, autoantibody titers decreased from baseline to M9. They then re-increased mainly in patients from the CS group, while they remained negative or at very low levels in most patients from the RTX group. At M24 evaluation, 5% of patients from the RTX group had positive serum anti-DSG-1, *versus* 29% of patients from the CS group ( $p < 0.01$ ). Anti-DSG-3 Abs were still detected at M24 in 14% of patients from the RTX group *versus* 45% of

those from the CS group ( $p < 0.01$ ). In patients from the RTX group, positive anti-DSG-1 and anti-DSG-3 Abs were detected at the M36 evaluation in 2% and 27% of patients' sera, respectively.

### **Evolution of circulating B-cells and TFH cells, and IL-21 serum level**

The evolution of peripheral whole blood B-cells, TFH-cells and serum IL-21 level during the course of patients treated with RTX or CS alone is shown in figure 3. The TFH-cell subpopulation was defined by the phenotype  $CD3^+CD4^+CD45RA^-CXCR5^+$ . This analysis was performed longitudinally in 11 patients from the RTX group and in 9 patients from the standard CS group. Serum IL-21 levels were measured by enzyme-linked immunosorbent assay (ELISA) in all patients. As expected, a dramatic decrease in peripheral whole blood B-cells was observed after the initial infusion of RTX, followed by a transient re-increase from M9 to M12, and a further progressive re-increase after the last infusion of RTX at M18, while no significant evolution was observed in patients treated with CS alone (Figure 3, a). In patients from the RTX group, a 50% decrease in TFH-cell frequency, which paralleled B-cell depletion, was observed from M3 to M24, followed by a re-increase of TFH-cells up to baseline values, which occurred after B-cell recovery (Figure 3, b). In contrast, no variation in TFH-cell frequency was observed in patients treated with a standard CS regimen. A dramatic and persistent decrease of serum IL-21 level was observed from baseline to M36 in patients treated with RTX. Conversely, whereas only a transient decrease of IL-21 level was observed from baseline to M3 in patients from the standard CS group, IL-21 levels re-increased after M3 in these patients, once CS doses were tapered under 20mg/day (Figure 3, c).

To assess the potential prognostic value of serum level of IL-21 and TFH cell frequency, we compared these parameters between patients with long-lasting clinical remission and those who relapsed during the follow-up. Since too few patients in the RTX group had persistent disease activity, we performed these experiment in patients treated with

CS alone. We did not evidence a statistically significant difference in IL21 serum level ( $p=0.53$ ), nor frequency of whole TFH ( $p=0.80$ ) between patients with prolonged remission and those who further relapsed.

### **Immunophenotyping of DSG-1- and DSG-3-specific B-cells**

DSG-1 and DSG-3-specific circulating B-cells were detected in blood samples collected at baseline from 30 pemphigus patients at frequencies ranging from 0.11% to 0.54% (mean  $\pm$  standard error of the mean:  $0.23 \pm 0.01\%$ ) for DSG-1-specific B-cells, and from 0.1% to 0.6% (mean:  $0.21 \pm 0.01\%$ ) for DSG-3-specific B-cells, of whole CD19<sup>+</sup> B-cells (baseline frequencies in Figure S1, a and b). DSG-specific B-cells from pemphigus patients were more frequently CD27<sup>+</sup> and class-switched IgG<sup>+</sup> CD27<sup>+</sup> than whole B-cells (CD27<sup>+</sup>:  $51 \pm 2\%$  of DSG-specific B-cells *versus*  $31 \pm 3\%$  of whole CD19<sup>+</sup> B-cells,  $p<0.0001$ ; IgG<sup>+</sup> CD27<sup>+</sup>:  $15.5 \pm 1.4\%$  of DSG-specific B-cells *versus*  $8.4 \pm 1.2\%$  of whole CD19<sup>+</sup> B-cells,  $p<0.0001$ ) (Figure S1, b and c).

In order to assess the differential effects of CS alone and CS combined with RTX, we analyzed the evolution of DSG-1- and DSG-3-specific B-cells at M36 (corresponding to 18 months after the last infusion of RTX) in patients from the RTX group, and between M9 and M12 (when prednisone doses were tapered below 20mg/day) in patients from the CS group. Surprisingly, DSG-1-and DSG-3-specific B-cells were still detected at M36 evaluation in patients in complete remission after RTX treatment at mean frequencies of  $0.23 \pm 0.01\%$  and  $0.23 \pm 0.02\%$  of B-cells, respectively, which were close to the frequencies measured at baseline (Figure 4, a and b). Interestingly, the mean frequency of DSG-1-and DSG-3-specific CD27<sup>+</sup> memory B-cells decreased from baseline to M36 after RTX treatment (DSG-1<sup>+</sup> B-cells:  $55 \pm 4\%$  *versus*  $36 \pm 4\%$ ,  $p<0.001$ ; DSG-3<sup>+</sup> B-cells:  $48 \pm 4\%$  *versus*  $36 \pm 5\%$ ,  $p<0.05$ ) (Figure 4, c). Additionally, the frequency of class-switched DSG-specific CD27<sup>+</sup> IgG<sup>+</sup> B-cells

dramatically decreased from baseline to M36 after RTX (DSG-1<sup>+</sup> B-cells:  $13.5 \pm 1.6\%$  *versus*  $3.8 \pm 1.0\%$ ,  $p < 0.001$ ; DSG-3<sup>+</sup> B-cells:  $13.0 \pm 2.4\%$  *versus*  $4.5 \pm 0.9\%$ ,  $p < 0.01$ ) (Figure 4, d). Interestingly, the one patient whose blood sample was available out of two patients with persistent disease activity after RTX had the highest frequency of class-switched DSG-3-specific CD27<sup>+</sup>IgG<sup>+</sup> B-cells (25.0%, Figure 4, d, black arrow). In contrast with the changes observed in the RTX group, we did not observe any modification in the frequency of DSG-specific class-switched CD27<sup>+</sup>IgG<sup>+</sup> B-cells from baseline to M12 in patients treated with CS alone (DSG-1<sup>+</sup> B-cells:  $20.9 \pm 2.7\%$  *versus*  $15.9 \pm 3.9$ ;  $p = 0.17$ ; DSG-3<sup>+</sup> B-cells:  $18.7 \pm 4.3\%$  *versus*  $20.0 \pm 5.3$ ;  $p = 0.83$ ) (Figure 4, e, f, g and h).

**Assessment of circulating anti-DSG-1 and anti-DSG-3 IgG secreting B-cells using**

**ELISPOT assay.**

DSG-1 and DSG-3-specific antibody-secreting-cells (ASC) were measured in 11 patients from the RTX group (including 10 patients in complete remission off treatment and the one patient with persistent disease activity) and in 11 patients from the standard CS group. At baseline, anti-DSG-1 and/or anti-DSG-3 IgG-ASC were detected both in patients from the RTX group ( $0.10 \pm 0.07\%$  and  $0.43 \pm 0.16\%$ , respectively) and in those from the standard CS group ( $0.10 \pm 0.05\%$  and  $0.32 \pm 0.10\%$ , respectively) (Figure 5, a and b). After B-cell recovery at M36 evaluation, anti-DSG-1 and anti-DSG-3 IgG-ASC were no longer detectable in 10 patients from the RTX group who were in clinical remission ( $p < 0.01$  *versus* baseline samples), whereas 0.17% anti-DSG-3 IgG-ASC were still detected in the one patient with persistent mucosal lesions (Figure 5, a, black arrow). In contrast, anti-DSG-1 and/or anti-DSG-3 IgG-ASC were still detected after treatment in patients from the CS group, even in those in clinical remission although at a lower frequency than at baseline (DSG-1 ASC:  $0.10 \pm 0.05$  at baseline *versus*  $0.01 \pm 0.01$  at M12,  $p = 0.5$ ; DSG-3 ASC:  $0.32 \pm 0.10$  at baseline *versus*  $0.089 \pm 0.050\%$  at M12;  $p < 0.05$ ).

## Effect of treatments on frequencies and phenotype of circulating DSG-3-specific

### TH-cells and TFH-cells

Since anti-DSG-1 and anti-DSG-3 IgG<sup>+</sup>ASC were no longer detected in patients in complete remission after RTX treatment, while non switched DSG-1- and DSG-3-specific naive and to a lesser degree memory B-cells were still detected after RTX treatment, we further investigated DSG-3-specific TH-cells and TFH-cells.

Using immunodominant peptide of DSG-3 (190-204)/HLA-DRB1\*0402 tetramer staining, we studied the frequency of DSG-3 specific TH-cells and TFH-cells at baseline and at M36 in 7 patients treated with RTX, and at baseline and at M9-12 in 6 and 9 patients from the CS group respectively, who all carried the HLA-DRB1\*0402 PV susceptibility allele. DSG-3-specific circulating TH-cells and TFH-cells were detected in blood samples collected at baseline at frequencies ranging from TH: 0.8% to 4.5% (mean:  $3.0 \pm 0.27\%$ ) and from TFH: 0.07% to 0.68 % (mean:  $0.24 \pm 0.05\%$ ) of whole CD4<sup>+</sup> T-cells. The frequency of DSG-3-specific circulating TH-cells and TFH-cells dramatically decreased after RTX treatment (TH:  $3.0 \pm 0.31\%$  at baseline *versus*  $0.4 \pm 0.12\%$  at M36;  $p < 0.0001$ ; TFH:  $0.25 \pm 0.08\%$  at baseline *versus*  $0.05 \pm 0.009\%$  at M36;  $p = 0.02$ ) (Figure 6, a and b). In contrast, while DSG-3-specific TH-cells from patients treated with CS alone decreased after CS treatment ( $2.94 \pm 0.51\%$  at baseline *versus*  $0.97 \pm 0.17\%$  at M9-12,  $p = 0.0009$ ), DSG-3-specific TFH-cells were still detected after CS treatment at similar levels as at baseline, even in patients in clinical remission ( $0.22 \pm 0.06$  at baseline *versus*  $0.35 \pm 0.10\%$  at M9-12,  $p = 0.34$ ) (Figure 6, c and d).

## DISCUSSION

This study shows that the long-lasting efficacy of RTX in pemphigus patients is associated with the prolonged disappearance of circulating anti-DSG-1 and anti-DSG-3 IgG secreting B-cells, and consequently the dramatic decrease of serum anti-DSG-1 and anti-DSG-3 IgG<sup>+</sup> Abs, as demonstrated by ELISPOT and ELISA, respectively. These findings contrast with the presence of circulating DSG-1- and DSG-3-specific B-cells in patients in complete remission after RTX therapy, at frequencies that were in fact very close to those observed at baseline before treatment, corresponding to around 0.2% of total B-cells. This discrepancy was likely related to the phenotype of the reappearing B-cells after the initial RTX-induced B-cell depletion, among which most of the DSG-3-specific B-cells were naïve non-switched CD27<sup>-</sup> B-cells. The decrease in the CD27 expression by DSG-specific B-cells is in accordance with our previous findings which also reported a decrease in the frequency of DSG-specific memory B-cells after RTX treatment (Hébert et al. 2019; Pollmann et al. 2019). It is also in accordance with the decrease in the transcription of the CD27 gene that we recently reported in patients treated with RTX. Interestingly, the only patient who still had active disease after RTX treatment had a high frequency of memory class-switch IgG B-cells among DSG-3 specific B-cells (25%), and accordingly, a high frequency of circulating anti-DSG-3 IgG secreting B-cells (0.17%) and high titers of serum anti-DSG-3 IgG<sup>+</sup> Abs (1500 U/mL).

Due to their capacity to regulate the maturation of B-cells into memory B-cells and IgG secreting plasma-cells, TFH-cells have been suspected to be involved in several autoimmune disorders, such as systemic lupus erythematosus (Choi et al. 2015), rheumatoid arthritis (Arroyo-Villa et al. 2014) and IgG4 related diseases (Chen et al. 2018). A decrease of circulating TFH-cells has been described in patients with Sjögren's syndrome or type 1

diabetes mellitus after RTX treatment (Verstappen et al. 2017; Xu et al. 2013). An effect of RTX on DSG-specific T-cells from pemphigus patients was previously reported by the group of Hertl et al. by using ELISPOT assay, who observed a decrease in the number of DSG-3-specific CD4<sup>+</sup> Th1 and Th2 cells in patients treated with RTX (Eming et al. 2008). Using tetramer technology, we demonstrated the *in vivo* presence of DSG-3-specific TH-cells and TFH-cells in pemphigus patients. Strikingly, we observed here a dramatic decrease in DSG-3-specific TFH-cells in the blood of patients after RTX treatment, which remained at very low levels up to M36 after RTX treatment, while the whole circulating TFH-cell compartment returned to baseline values.

In addition, we observed a dramatic decrease of serum IL-21 levels, which paralleled B-cell depletion after RTX treatment. IL-21, which is highly produced by TFH-cells, plays a crucial role in B-cell maturation in particular the production of memory B-cells. It has been recently reported that T-cells produced IL-21 when cultured with recombinant DSG, suggesting that the decrease of serum IL-21 levels observed after RTX treatment in the present study might be at least partly explained by the decrease of circulating DSG-3-specific TFH-cells (Hennerici et al. 2016).

The disappearance of DSG-1- and DSG-3-specific IgG-switched CD27<sup>+</sup> B-cells perfectly correlated with the disappearance of DSG-3-specific TFH-cells and the decrease of serum IL-21 levels, which are both known to play a major role in B-cell maturation (Zotos et al. 2010).

In mice, TFH differentiation is initiated early when dendritic-cells prime naïve CD4<sup>+</sup> T-cells (Kitano et al. 2011). However, this priming is not sufficient to complete full TFH-cell differentiation, but instead drives the production of pre-TFH-cell, a partially differentiated intermediate that expresses CXCR5 and the transcriptional repressor Bcl6 (Goenka et al. 2011). Thus, the absence of B-cells or the presence of B-cells with irrelevant antigen-



specificity impair TFH-cell generation (Johnston et al. 2009). Moreover, maintenance of the TFH-cell phenotype requires sustained antigenic stimulation by B-cells (Baumjohann et al. 2013). In a mouse model of pemphigus, adoptive transfer of DSG3-specific B-cells was not sufficient to induce the disease in recipient mice deficient for both T- and B-cells, while the co-transfer of similar B-cells with activated ICOS<sup>+</sup> TFH-cells was pathogenic (Kim et al. 2020).

Our observations in pemphigus patients are in accordance with this model in which the absence of DSG-specific B-cells following RTX treatment impacts the maturation and/or maintenance of DSG-specific TFH-cells, which in turn blocks the isotype switch of DSG-specific B-cells.

Thus, our results collectively suggest that the blockage of reappearing B-cells at the naïve stage and the blockage of the class switch from IgM to IgG might play a major role in the long-lasting effect of RTX in pemphigus patients.

These findings seem likely related to a specific effect of RTX since we did not observe such variations in the number of whole and DSG-specific TFH-cells, nor any reversal in the balance between memory and naïve DSG-specific B-cell subpopulations in patients treated with CS alone. Indeed, the persistence of DSG-specific TFH-cells in these latter patients might have favored the persistence of circulating DSG-specific CD27<sup>+</sup>IgG<sup>+</sup> B-cells and anti-DSG IgG-ASC, which were responsible for the re-increase of serum anti-DSG Abs observed once CS doses had been tapered.

Overall, this study shows that the therapeutic effect of RTX in pemphigus is not only due to a long-lasting depletion of DSG-specific B-cells, but rather relates to an inefficient maturation and class switching process of DSG-specific B-cells, which likely involves the long lasting disappearance of DSG-specific TFH, a cell population which is closely associated with the anti-DSG3 antibody response in pemphigus (Kim et al. 2020).

## **MATERIALS AND METHODS**

### **Patients**

Ninety patients, 74 with pemphigus vulgaris and 16 with pemphigus foliaceus, were included in a randomized clinical trial (RITUX3: ClinicalTrials.gov number, NCT00784589). The Ethics Committee (CPP Nord-Ouest1) approved the study. Patients with a newly diagnosed pemphigus were assigned to receive either 1 to 1.5mg/kg/day of oral prednisone with a progressive tapering of prednisone doses over M12 to M18, or 2 infusions of 1g of RTX on day 0 and day 14 and 500mg at M12 and M18, combined with a short-term prednisone regimen, 0.5mg/kg/day for moderate pemphigus and 1mg/kg/day for severe pemphigus. The initial prednisone dose was gradually reduced after achievement of disease control, with the aim to stop prednisone after M3 in patients with moderate pemphigus and after M6 in patients with severe pemphigus. Patients were followed for up to 3 years.

### **Clinical and immunological evaluations**

Complete remission and relapse were defined according to the consensus statement definition for pemphigus end points (Murrell et al. 2008). Blood samples of healthy donors were from The French Blood Donor Service. Participants gave their written informed consent. Blood samples of patients were collected at baseline and at each follow-up visit until the end of the study at M36.

### **Serum auto-antibody titers**

Titers of serum anti-DSG-1 and anti-DSG-3 IgG Abs were measured using an ELISA (EUROIMMUN, Lübeck, Germany).

### **Cytokine levels**

Serum levels of IL-21 were measured by ELISA (BioLegend, San Diego, United States), according to the manufacturer's protocol.

### **Phenotyping analysis**

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Paque gradients (GE Healthcare Lifescience, Chicago, United States) to assess B and T lymphocyte markers. The phenotype of PBMCs was determined by flow cytometry with mAbs against CD19, CD24, CD27, CD38, IgD, IgM and IgG (Beckman Coulter, Brea, United States and BD Biosciences, Franklin Lakes, United States). Whole circulating TFH-cells were further characterized by using a combination of different mAbs against CD3, CD4, CD45RA, CXCR5 (Miltenyi Biotec, Bergisch Gladbach, Germany and BD Biosciences). The percentages of each subpopulation were determined using FlowJo™ software (BD Biosciences).

### **Anti-DSG B-cell analysis**

In order to analyze the phenotype of B cells specific for DSG-1 and DSG-3, B cells were isolated and labeled as described by Hebert et al (Hébert et al. 2019) with recombinant DSG-1 or DSG-3 kindly provided by Prof. M. Herlt (Eming et al. 2008). The percentage and phenotype of DSG-1 and DSG-3 specific B cells were determined using FlowJo™ software.

### **Anti-DSG-3 TFH-cell analysis**

In order to verify that our patients with pemphigus had the HLADRB1\*0402 genotype of pemphigus vulgaris susceptibility, peripheral blood from patients was collected after obtaining informed consent. Genomic DNA from PBMCs was isolated using the NucleoSpin® tissue kit (Macherey-Nagel, Düren, Germany). The specific analysis of HLADRB1\*04 was performed using the Olerup SSP® DRB1\*04 PCR Typing Kit (Bionobis, Guyancourt, France) according to the manufacturer's protocol. Auto-reactive T-cells were only characterized in pemphigus vulgaris patients with the HLADRB1\*0402 genotype. Frequency and phenotype of DSG-3-specific TH-cells and TFH-cells were determined by flow cytometry using the following mAbs against CD3, CD4, CD45RA, CXCR5 (Miltenyi

Biotec and BD Biosciences) and using tetramer-based detection system using HLADRB1\*0402-tetramers loaded with the DSG-3 190-204 immunodominant peptide (LNSKIAFKIVSQEPA, obtained from NIH Tetramer core facility, Emory University, United States ). The percentages of DSG-3-specific TH-cells and TFH-cells were determined using FlowJo™ software.

### **DSG-specific B-cell detection using ELISPOT assay**

The frequencies of circulating total IgG and DSG-specific IgG-ASC were determined by human IgG ELISPOT Basic (Mabtech, Nacka Strand, Sweden). PBMC from pemphigus patients and healthy donors were pre-stimulated with R848 (1µg/mL) and rhIL2 (10ng/mL) in complete medium (RPMI-1640 supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100U/mL penicillin and 100µg/mL streptomycin (Thermo Fisher Scientific)) in separate plates for 72 hours. Plates of ELISPOT MAIPS-4510 (Merckmillipore, Darmstadt, Germany) were coated overnight at 4°C with anti-IgG human Abs. Plates were washed and blocked with complete medium before use. Pre-stimulated PBMCs were washed, resuspended in complete medium and transferred to plate, and then incubated for 24 hours with  $1 \times 10^5$ - $4 \times 10^5$  PBMCs per well to detect anti-DSG-1 and DSG-3 IgG-secreting ASC, and with  $2.5 \times 10^3$ - $1 \times 10^4$  PBMCs per well to detect total IgG-ASC. IgG-ASC were detected by addition of biotinylated mouse IgG anti-human IgG. Frequency of anti-DSG-1 or anti-DSG-3 IgG secreting ASC was calculated after incubation for 2 hours with histidine-tagged recombinant DSG-1 or DSG-3 proteins (1µg/mL) in phosphate-buffered saline with calcium (Eurobio, Les Ulis, France). Biotinylated anti-histidine (0.5µg/mL) (Abcam, Cambridge, United Kingdom) was then added. The streptavidin enzyme and substrate tetramethylbenzidine were used to detect spots. The number of spots was determined with ELISPOT Plate Readers and ImmunoSpot software (CTL Europe GmbH, Bonn, Germany). Results were expressed as frequencies of DSG-specific IgG-ASC among total IgG-ASC. The sensitivity and specificity

of our ELISPOT assay were estimated at 1 DSG-3 specific ASC/10<sup>5</sup> total ASC, and 100%, respectively.

### **Statistical analysis**

Data are presented as means ± standard error of the mean. Prism software (Graph Pad, San Diego, United States) was used for statistical analysis. Statistical analysis was performed by Fisher exact test to compare the frequencies of patients with DGS Abs between RTX and CS groups. The paired t-test was performed to compare patients before and after treatment. For multiple analyses, one-way ANOVA with Dunnett post-test or two-way ANOVA with Sidak's multiple comparisons test were used. A p value of less than 0.05 was considered significant for all analyses.

## **CONFLICT OF INTERESTS**

Michael Hertl declares Topas Therapeutics, Hamburg, Germany. Pascal Joly is consultant for Roche, GSK, Lilly, Principabio, Sanofi Aventis, Argenx. All other authors declare no competing interests.

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PJ ; Supervision: SC, NF, PJ ; Validation: MM-V, CP ; Visualization: MM-V, SC ; Writing – Original Draft Preparation: MM-V , SC, NF, PJ ; Writing - Review and Editing: MM-V, CP, MLG, VH, FC, CM, GR , MP , MV, NH, OB, SC, NF, PJ.

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## FIGURES LEGENDS

### **Figure 1. Flow chart of the study.**

### **Figure 2. Evolution of anti-Desmoglein (DSG)-1 and anti-DSG-3 antibody ELISA values in sera from pemphigus patients, according to treatment regimens.**

Blue line = patients assigned to the rituximab (RTX) group. Red line = patients assigned to the standard corticosteroids (CS) group. The dashed line represents the cut-off values proposed by the manufacturer for anti-DSG-1 (Panel a) and anti-DSG-3 (Panel b) antibody ELISA values. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  *versus* baseline).

### **Figure 3. Evolution of peripheral blood whole B-cells and T-Follicular-Helper (TFH)-cells subpopulation of CD4<sup>+</sup> T-cells, and serum IL-21 level during the follow-up of patients according to treatment regimen.**

Blue line = patients assigned to the rituximab (RTX) group. Red line = patients assigned to the standard corticosteroids (CS) group. The frequency of whole B-cells (Panel a) and TFH-cell subpopulation of CD4<sup>+</sup> T-cells (Panel b) were calculated by flow cytometry. Serum IL-21 level was measured by ELISA (Panel c) (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  *versus* baseline).

### **Figure 4. Frequency and phenotype of peripheral blood whole B-cells and Desmoglein (DSG)-1 and DSG-3 specific B-cells evaluated by flow cytometry in pemphigus patients at baseline (D0) and after treatment.**

Blue = patients assigned to the rituximab (RTX) group. The one patient with persistent disease activity is indicated by an arrow. Red = patients assigned to standard corticosteroids (CS) group. Evolution of the frequency of DSG-1 and DSG-3-specific B-cells among purified B-cells between D0 (plain symbols) and after treatment (open symbols) at month (M) 36 after

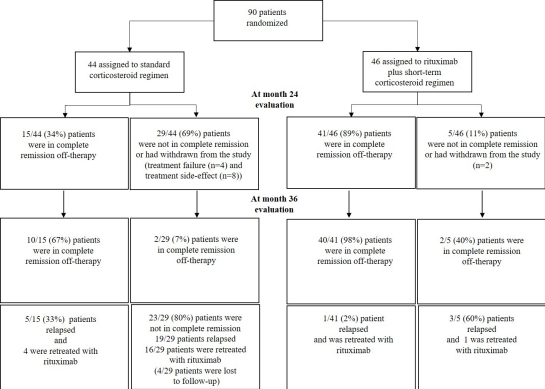
B-cell recovery for the RTX group (Panels a and b) or at M9-12 when prednisone doses were tapered under 20mg/day for the CS group (Panels e and f). Evolution of the memory B-cell subpopulation (CD19<sup>+</sup> CD27<sup>+</sup>) and class switched memory B-cells (CD19<sup>+</sup>CD27<sup>+</sup>IgG<sup>+</sup>) among whole B-cells and DSG-specific B-cells at D0 and after RTX treatment (Panels c and d) or after CS treatment (Panels g and h) (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).

**Figure 5. Frequency of peripheral blood Desmoglein (DSG) -1 and DSG-3 specific antibody-secreting-cells (ASC) evaluated by ELISPOT assay in pemphigus patients at baseline and after treatment.**

Panel a in blue = patients assigned to the rituximab (RTX) group. The one patient with persistent active disease (AD) after RTX treatment is indicated by an arrow. Panel b in red = patients assigned to the standard corticosteroids (CS) group. Panel c: Total IgG-ASC (top) and DSG-IgG ASC (bottom) were detected using ELISPOT in peripheral blood mononuclear cells of representative pemphigus patients and one healthy donor (HD). The number of spots detected showed that DSG-IgG ASC, but not total Ig-ASC, were depleted in pemphigus patients in clinical remission (CR) after RTX treatment. Analyses were performed at baseline (D0) (plain symbols) and post treatment samples (open symbols) at month (M) 36 after B-cell recovery in patients from the RTX group and at M9-12 when prednisone doses were tapered under 20mg/day in patients from the standard CS group. Result are presented as frequencies of DSG-1 and DSG-3 specific IgG-ASC among total IgG-ASC. (\*p<0.05; \*\*p<0.01).

**Figure 6. Frequency of peripheral Desmoglein (DSG)-3 specific T-cells and T-Follicular-Helper (TFH)-cells evaluated among whole CD4<sup>+</sup> T-cells in pemphigus patients at baseline and after treatment.**

Blue = patients assigned to the rituximab (RTX) group. The one patient with persistent active disease at month (M) 36 is identified by an arrow. Red = patients assigned to the standard corticosteroids (CS) group. The frequencies of DSG-3 specific T-cells (Panels a and c) and TFH-cells (Panels b and d) were calculated by flow cytometry using DSG-3 peptide/HLADRB1\*0402 tetramer. Analyses were performed at baseline (D0) and M36 after B-cell recovery in patients from the RTX group and at M9-12 when prednisone doses were tapered under 20mg/day in patients from the standard CS group. Baseline samples correspond to plain symbols and post treatment samples to open symbols (\* $p < 0.05$ ; \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).



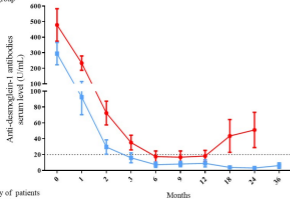
a)

RTX group : % (n)

Standard CS

group

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Frequency of patients  
> cut-off values

Standard CS group

RTX group

Months	0	1	2	3	6	9	12	18	24	36
Standard CS group	79	68	49	38	15	12	15	19	19	-
RTX group	65	50	24	11	7	9	5	4	5	2

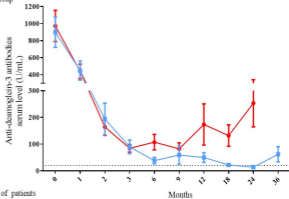
b)

RTX group : % (n)

Standard CS

group

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Frequency of patients  
> cut-off values

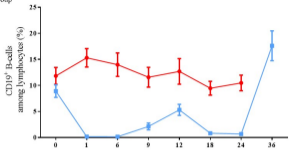
Standard CS group

RTX group

Months	0	1	2	3	6	9	12	18	24	36
Standard CS group	81	73	74	63	56	52	47	45	45	-
RTX group	74	75	61	49	29	35	33	20	14	27

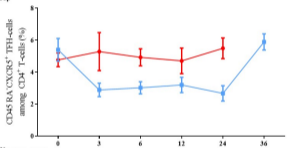
a) RTX group : Vs.D0

Standard CS : Vs.D0  
group



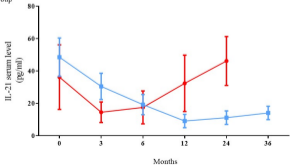
b) RTX group : Vs.D0

Standard CS : Vs.D0  
group

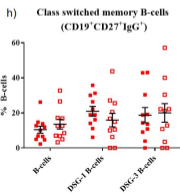
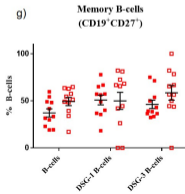
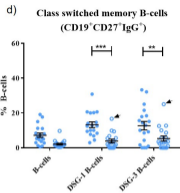
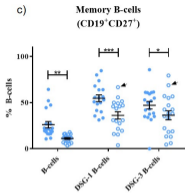
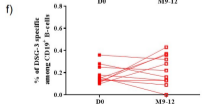
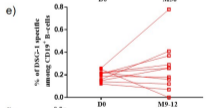
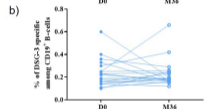
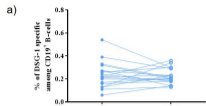


c) RTX group : Vs.D0

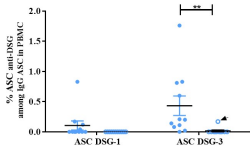
Standard CS : Vs.D0  
group



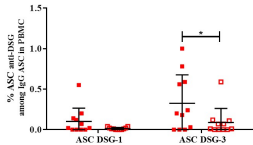




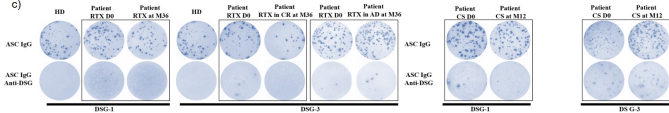
a)



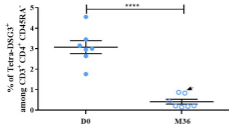
b)



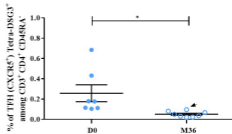
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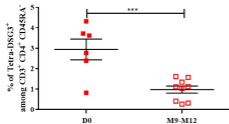
a)



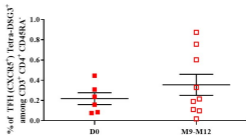
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c)

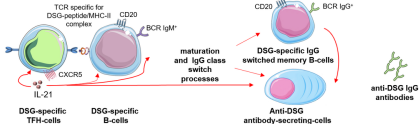


d)





**Pemphigus patient  
treated with  
corticosteroids alone**



**Pemphigus patient  
treated with  
Rituximab**

