Platelet Biology & its Disorders

Efficacy, safety and immunological profile of combining rituximab with belimumab for adults with persistent or chronic immune thrombocytopenia: results from a prospective phase IIb trial

Matthieu Mahévas,1,2,3 Imane Azzaoui,1,3* Etienne Crickx,1,2,3* Florence Canou-Poiotrine,4 Delphine Gobert,5 Laetitia Languille,1 Nicolas Limal,2 Constance Guillaud,2 Laure Croisille,6 Mohamed Jeljeli,7 Frédéric Batteux,7 Samia Baloul,6 Olivier Fain,2 France Pirenne,3 Jean-Claude Weill,2 Claude-Agnès Reynaud,2 Bertrand Godeau1,3 and Marc Michel1,3

*IA and EC contributed equally as co-second authors

1Service de Médecine Interne, Centre National de Référence des Cytopénies Auto-Imunes de l’Adulte, Centre Hospitalier Universitaire Henri-Mondor, Assistance Publique-Hôpitaux de Paris, Université Paris Est Créteil (UPEC), Créteil; 2Institut Necker Enfants Malades, INSERM U1151 CNRS UMS 8253, Université Paris Descartes, Sorbonne Paris Cité, Paris; 3Equipe n°2 "Transfusion et Maladies du Globule Rouge", EFS Ile-de-France, IMRB U955 INSERM, Hôpital Henri-Mondor, AP-HP, Créteil; 4CEpiA (Clinical Epidemiology and Ageing), EA 7376-IMRB, Université Paris Est Créteil (UPEC), Hôpital Henri-Mondor, AP-HP Department of Public Health, Clinical Research Unit (URC-Mondor), Créteil; 5Sorbonne Université, Service de Médecine Interne, Hôpital Saint-Antoine, Assistance Publique-Hôpitaux de Paris, Paris; 6Etablissement Français du Sang, Service d’Immunologie Plaquettaire, Hôpital Henri-Mondor, Créteil and 7Service d’Immunologie Biologique, Hôpital Cochin, AP-HP, INSERM U1016, Institut Cochin, Paris, France

ABSTRACT

B-cell activating factor may be involved in the failure of B-cell depleting therapy with rituximab in immune thrombocytopenia (ITP) by promoting the emergence of splenic long-lived plasma cells. From results obtained in mouse models, we hypothesized that combining rituximab with sequential injections of belimumab could increase the rate of response at 1 year in patients with persistent or chronic ITP by preventing the emergence of these long-lived plasma cells. The study was a single-center, single-arm, prospective phase IIb trial investigating the safety and efficacy of rituximab given at a fixed dose of 1,000 mg, 2 weeks apart, combined with five infusions of belimumab, 10 mg/kg at week 0 (W0)+2 days, W2+2 days, W4, W8 and W12 for adults with primary persistent or chronic ITP. The primary endpoint was the total number of patients achieving an overall response (complete response + response) at W52 according to a standard definition. In total, 15 non-splenectomized adults, nine (60%) with persistent IPT and six (40%) with chronic ITP, were included. No severe adverse event, infection, or severe hypogammaglobulinemia was observed. Thirteen patients achieved an initial overall response. At W52, 12 (80%) patients achieved an overall response, including ten (66.7%) with complete response. When compared with a cohort of patients receiving rituximab alone, the kinetics of B-cell repopulation appeared similar, but the number of circulating T-follicular helper cells was significantly decreased with belimumab combination therapy. Combining rituximab and belimumab seems a promising strategy in ITP, with high efficacy and acceptable safety (clinicaltrials.gov. Identifier: NCT03154385).
Introduction

Primary immune thrombocytopenia (ITP) is a bleeding disorder mainly mediated by autoreactive B cells and plasma cells (PC) secreting pathogenic anti-platelet auto-antibodies, eventually leading to accelerated platelet destruction and impaired megakaryopoiesis. First-line treatments include steroids and intravenous immunoglobulins (IVIg). Because less than 40% of newly diagnosed ITP adults will achieve a spontaneous remission within 12 months after disease onset, second-line treatments are frequently needed. Over the past 20 years, the anti-CD20 monoclonal antibody rituximab (RTX) has been considered an off-label second-line option in many countries and most guidelines. RTX leads to an overall response rate of 40% at 1 year. Whereas an almost complete B-cell depletion is achieved in peripheral blood and in secondary lymphoid organs after RTX in ITP, approximately half of the patients do not respond to RTX, which raises many questions and has led to some investigations in the past years.

In ITP, pathogenic antibody-secreting PC are constantly generated in the spleen, mainly through the germinal center pathway. Because most of these splenic PC are short-lived and have lost CD20 expression, the clinical improvement observed after RTX is thought to result mainly from germinal center depletion, thus limiting PC generation. However, analysis of spleen samples from ITP patients with failure of RTX revealed that despite complete peripheral B-cell depletion, residual splenic PC secreting anti-platelet antibody persisted. More surprisingly, transcriptomic analysis showed that these splenic PC had acquired a long-lived program, similar to bona fide bone-marrow long-lived PC. Quantitatively, the data suggested that B-cell depletion had induced the differentiation of short-lived PC into long-lived ones, rather than the selection of pre-existing long-lived PC, thus providing clues for explaining RTX failure in the context of ITP.

By using a fate mapping mouse model, we recently demonstrated that B-cell activating factor (BAFF) played a major role in the emergence of these splenic long-lived PC. BAFF is a pro-survival key cytokine for the B-cell lineage, and elevated levels of uncostomized BAFF are observed in serum and spleen after RTX therapy in ITP patients. Combining anti-CD20 with four infusions of anti-BAFF antibodies in this mouse model significantly reduced the number of splenic PC, with little impact on bone marrow PC.

Hence, we hypothesized that combining two fixed doses of 1,000 mg of RTX with five sequential injections of belimumab (Benlysta, 10 mg/kg dose) could increase the rate of response at 1 year in patients with persistent or chronic ITP by preventing the emergence of autoreactive splenic long-lived PC. Here, we report the efficacy and safety of this new strategy in ITP during a prospective phase IIb pilot trial.

Methods

Study design and study drugs

The study was a single-center, single-arm, prospective phase IIb trial (RITUX-PLUS, clinicaltrials.gov Identifier: NCT03154385) investigating the safety and efficacy of RTX at a fixed dose of 1,000 mg, 2 weeks apart, combined with five intravenous infusions of belimumab (Benlysta, 10 mg/kg) at week 0 (W0) + 2 days, W2 + 2 days, W4, W6 and W12 (see Online Supplementary Figure S1). The rationale for the choice of this schedule was based on previous results obtained in a mouse model showing that BAFF inhibition should start early. The belimumab dosing was similar to the one approved in systemic lupus erythematosus. Research was conducted in accordance with the Declaration of Helsinki and was approved by the Comité de Protection des Personnes Ile-de-France VI.

Inclusion and exclusion criteria

Inclusion and exclusion criteria are reported the Online Supplementary Appendix.

Primary endpoint

The primary endpoint was the total number of patients achieving an overall response (complete response [CR] + response [R]) at W52. CR was defined by platelet count >100x10^9/L and R by platelet count 50-100x10^9/L with at least a 2-fold increase from baseline according to international definitions. Patients who required any other treatment for ITP including rescue therapy more than 6 weeks after inclusion were considered non-responders regardless of the platelet count.

Secondary endpoints

Secondary endpoints were the number of patients achieving an overall response (CR+R) initially and at W12, W24, W36, number of bleeding events, number of patients showing severe hypogammaglobulinemia (γ-globulin level <4 g/L at W24, W36, W52), duration of severe hypogammaglobulinemia, variation in γ-globulin subclass levels during the study, and number of severe infections requiring hospitalization during the study.

Adverse events

Adverse effects were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) v5.0.

Immunological analysis, antibody titer tests, free B-lymphocyte stimulator assay

Phenotype of circulating T- and B-cell subpopulations was analyzed by flow cytometry at W0, W4, W12, W24, W36 and W52 for every included patient and in a prospective control cohort of ITP patients not included in this trial who received two infusions of fixed-dose RTX (1,000 mg) at W0 and W2 after pre-medication with 100 mg intravenous methylprednisolone as a standard of care for ITP (see Online Supplementary Methods, Online Supplementary Table S1, and Online Supplementary Figure S2 for gating strategy). Antibody titers for pneumococcal, tetanus, measles vaccine were measured by enzyme-linked immunosorbent assay (ELISA). Serum-free BAFF level was measured using the assay developed by Glaxo Smith Kline pharmaceuticals (see Online Supplementary Appendix).

Direct monoclonal antibody-specific immobilization of platelet antigen assay

Anti-platelet antibodies on patient platelets were detected by using the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay (ApDia, Turnhout, Belgium).

Statistical analysis

The statistical analysis was performed as described in the Online Supplementary Methods.
Results

Baseline characteristics

We included 15 patients (12 females) with median age 50 years (range, 20-70 years). All patients had previously received corticosteroids and/or IV Ig (n=8) as first-line treatment, and ten had received a second-line treatment (Table 1). All but one had a previous transient response to corticosteroids. Within 1 month prior to inclusion, the median platelet count nadir was 16x10^9/L (range, 3-28x10^9/L). All but one patient had cutaneous bleeding manifestations and four had mucosal bleeding. Six patients had positive anti-nuclear antibody titers >1/160 with no features of systemic lupus erythematosus. When receiving the first RTX infusion, the median duration of ITP was 11 months (range, 4-52 months). Nine (60%) patients had the first RTX infusion, the median duration of ITP was 11 months (range, 4-52 months). Nine (60%) patients had persistent ITP and six (40%) had chronic ITP.

Safety

Overall, 31 adverse events were reported during the study (Table 2); five were infusion-related reactions during the first RTX administration (all grade I according to the CTCAE classification). No infusion-related reaction was reported with belimumab. All but one of 26 adverse events occurring during the study were grade I, and eight were possibly related to treatment (bronchitis, n=2; nasopharyngitis, n=3; arthralgia, n=1; candida vulvovaginitis, n=1; cystitis, n=1). One patient experienced grade II serum sickness with moderate arthralgia and rash one day after the second infusion of RTX. γ-globulin levels were systematically monitored during the study (Figure 1A to D). We observed no severe infection and no severe hypogammaglobulinemia (total serum immunoglobulin (lg) <4g/L or IgG <4.5g/L). We observed a significant decrease in IgG and IgM titers (Figure 1; Online Supplementary Table S2) between baseline and W24 (0.98 g/L and 0.42 g/L decrease in median IgG and IgM titers, respectively). One patient experienced moderate hypogammaglobulinemia (total serum Ig titer 4.9 g/dL, IgG 4.7 g/L) at W12, which was transient and recovered at W24. One patient had IgM titers <0.4 g/dl at W12 (IgM baseline 0.7 g/L) that did not recover at W52. IgA titers did not vary throughout the study.

Efficacy

Thirteen (86.7%) patients achieved an initial overall response at W12, including nine (60%) with CR (Figure 2). Two patients had a response at W7 and W8, respectively; other patients achieved a response after W4. One non-responder had bleeding symptoms at W4 and required thrombopoietin receptor agonists at W6. No other patient required ITP-directed therapy until W30. Among initial responders (R), one relapsed at W30, with moderate bleeding (cutaneous), and one eventually achieved CR at W36. At W52, the median platelet count among responders was 189x10^9/L (range, 69-416x10^9/L) and 12 of 15 (80%) patients achieved overall response (95% Confidence Interval [CI]: 52-96), including ten (66.7%) with CR (95% CI: 38-88). After a follow-up of 18 months, one patient with an initial CR eventually relapsed at 16 months (Online Supplementary Figure S3).

Vaccine response

All patients had received vaccination with pneumococcal polysaccharide vaccine (Pneumovax-23®, n=2) or conjugate vaccine PCV13 (Prevenar 13®, n=13) at least 15 days before inclusion (range, 15-90 days). When considering a protective threshold ≥1 μg/mL for anti-pneumococcal antibodies,13 15 patients were protected for at least 11 of the 13 serotypes tested (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19E, 23F), and two patients (who had received Prevenar 13®) were protected for eight serotypes at baseline (Online Supplementary Table S3). At W52, eight (53%) patients had no change (n=6) or <2 serotypes (n=2) loss in protection among the 13 serotypes tested. Two patients who were protected for eight serotypes at baseline had lost two and four other serotypes, respectively, at W52. Finally, five patients (30%) had lost protection for a medi-

Table 1. Baseline characteristics of patients with immune thrombocytopenia receiving rituximab and belimumab.

| Age/Sex | ITP duration (months) | Bleeding manifestations | Treatments received before inclusion | Nadir platelet count during the month before inclusion (x 10^9/L) |
|---------|----------------------|------------------------|-------------------------------------|---------------------------------------------------------------|
| 25/F    | 11                   | Cutaneous              | CST                                 | 16                                                            |
| 29/F    | 4                    | Cutaneous + mucosal    | CST                                 | 3                                                             |
| 42/F    | 15                   | No                     | CST, IVlg, hydroxychloroquine       | 3                                                             |
| 51/F    | 15                   | Cutaneous              | CST, Dapsone                        | 28                                                            |
| 31/F    | 5                    | Cutaneous              | CST, IVlg                           | 16                                                            |
| 57/F    | 52                   | Cutaneous              | CST, IVlg                           | 27                                                            |
| 39/F    | 44                   | Cutaneous              | CST, hydroxychloroquine             | 18                                                            |
| 33/F    | 11                   | Cutaneous              | CST, hydroxychloroquine             | 18                                                            |
| 70/F    | 3                    | Cutaneous              | CST, hydroxychloroquine             | 18                                                            |
| 66/M    | 42                   | Cutaneous              | CST, IVlg, dapsone, hydroxychloroquine | 9                                                            |
| 66/M    | 5                    | Cutaneous + mucosal    | CST, IVlg, dapsone                  | 15                                                            |
| 20/F    | 4                    | Cutaneous              | CST, IVlg, romiplostim              | 15                                                            |
| 50/M    | 51                   | Cutaneous + mucosal    | CST, IVlg                           | 6                                                             |
| 54/F    | 4                    | Cutaneous              | CST, eltrombopag                     | 7                                                             |
| 57/F    | 4                    | Cutaneous + mucosal    | CST, IVlg, vinblastin, romiplostim, eltrombopag | 17                                                            |

CST: corticosteroids, IVlg: Intravenous immunoglobulin. F: female; M: male.
an of seven serotypes (range, 4-8). Overall, at W52, 11 (73%) patients had protective titers for at least 11 serotypes. All patients had received vaccination with tetanus and measles at different times before enrollment. Fourteen patients (93%) had no significant change in anti-tetanus antibody titers between W0 and W52. There were also no significant changes in anti-measles antibody titers, which remained at protective level >200 UA/m (protective >16.5 UA/m) for all patients at W52.

**Antiplatelet antibody testing**

Direct MAIPA was performed at inclusion in all but one patient and was positive in ten (71%) patients, including nine with glycoprotein IIb/IIIa (GpIIb/IIIa) specificity, and one with GpIb/IX specificity (Online Supplementary Table S4). Among these patients, seven (70%) achieved response and had negative MAIPA results at W52, two achieved response (one CR and one R) and still had anti-GpIIb/IIIa antibodies at W52, and one did not respond but had negative MAIPA results at W52.

**Immunological analysis**

In order to precisely assess the impact of blocking BAFF concomitantly with B-cell depletion on B- and T-cell subsets, we analyzed in parallel a prospective cohort of 12 ITP patients who received RTX without belimumab as a standard of care (Online Supplementary Table S5). As previously reported, we observed a significant increase in BAFF serum levels in patients receiving RTX alone at W12, W24, W36 and W52 (all P<0.001) after RTX, as compared to baseline (1,210±248 vs. 90±38 pg/mL, P<0.0001). BAFF levels started to return to baseline at W24 (730±294 pg/mL, P<0.01), then strongly increased at W36 (2,199±1,498 pg/mL, P<0.0001) and reached a plateau at W52 (2957±1561 pg/mL, P<0.0001) (Figure 3A). BAFF levels at W36 and W52 did not significantly differ between patients receiving belimumab + RTX in the study and control patients receiving RTX alone.

In order to evaluate the effect of the RTX and belimumab combination on B-cell depletion and re-appearance, we analyzed circulating CD19+ B cells in both cohorts at baseline, W4, W12, W24, W36, and W52 (Figure 3B). All patients showed complete depletion of circulating CD19+ B cells at W4 and W12 (Figure 3C).

Reappearance of B cells in the peripheral blood varied among patients. We observed no significant delay in B-cell reconstitution in patients receiving belimumab plus RTX versus controls receiving RTX alone, despite a slight difference at W36. Transitional B cells (CD19+IgD+CD24+CD38+CD10+), which are precursors of naïve B cells in peripheral blood,14,15 emerged early during B-cell reconstitution (Figure 3D to E). Indeed, two of 15 patients receiving belimumab showed transitional B cells in the peripheral blood at W24 as compared with five of 12 patients receiving RTX alone. All but one patient in both groups showed naïve and transitional B cells at W52. The absolute number of CD19+ cells remained significantly decreased at W52 in both groups as compared to baseline, and B-cell depletion mainly affected memory B cells and IgD-CD27+ B cells (Figure 3F and G).

As previously reported, the number of circulating CD27highCD38high cells (mainly plasmablasts expressing
Table 2. Adverse events reported in the study.

| Patients | Infusion related reactions | Adverse events, CTCAE grade, imputability |
|----------|----------------------------|-------------------------------------------|
| 1        | Throat itching, grade I, (RTX W0) | Arthralgia (W2- W8), grade I, possible |
| 2        | Sore throat, grade I, (RTX W0)   | No                                        |
| 3        | No                           | Transient hypereosinophilia < 1000 /mm³ (W40) treated with zentel/vistronicel, grade I, not related |
| 4        | No                           | Gluteus medius tendinitis (W8-W12), grade I, not related Nasopharyngitis (W10), grade I possible Supraspinatus tendinitis (W24), grade I, not related |
| 5        | Throat itching, grade I, (RTX W0) | Serum thickness (W2), grade I, related Candida vuvovaginitis (W8), grade I, not related |
| 6        | No                           | Rhinorrhea (W2) and (W12), grade I, not related Bronchitis (W5), possible |
| 7        | Headache, grade I, (RTX W0)    | Pain extensor muscles of the forearm, (W24), grade I, not related Acute cystitis (W4), grade I, possible |
| 8        | No                           | Arthralgia (W3), thoracic pain (W3), grade I, not related Viral conjunctivitis (W4), grade I, not related Arthralgia (W52), grade I, not related |
| 9        | No                           | Rhinorrhea (W8), grade I, not related Pharyngitis (W4), grade I, possible Bronchitis (W58), grade I, possible |
| 10       | No                           | Gout arthritis (W44), grade I, not related |
| 11       | No                           | Increased number of monocytes after TPO-RA, grade I, not related |
| 12       | Abdominal discomfort grade I, (RTX W0) | Nasopharyngitis (W20), myalgia (W20), grade I, possible Knee pain (W52), grade I, not related |
| 13       | No                           | Bronchitis (W9), grade I, possible Memory problems W14, grade I, not related Erectile dysfunction W36, grade I, not related |
| 14       | No                           | Low back pain (W12), grade I, not related |
| 15       | No                           | Bronchitis (W0-W2), grade I, possible |

CTCAE: National Cancer Institute Common Terminology Criteria for Adverse Events; RTX: rituximab; W: week.

Because changes in T-cell homeostasis have been described with RTX, we investigated peripheral T-cell compartments before and after treatment. We observed no significant changes in the distribution of CCR7⁺CD45RA⁻ naive (TN), CCR7⁻CD45RA⁺ memory (TEM), and CCR7⁻CD45RA⁻ central memory (TCM) in CD4⁺ and CD8⁺ cells. The expression of CD38 and HLA-DR activation markers on CD4⁺ or CD8⁺ T cells was not modified with treatment. Finally, we observed no significant change in CD4 T-cell polarization (TH1/TH2/TH17) based on the expression of CXCR3, CXCR5, CCR6 (Online Supplementary Figure S6). By contrast, the activated subset of circulating follicular helper T cells (activated cTfh) identified as CD4⁺CD45RA⁻CXCR5⁺ inducible T-cell costimulator (ICOS)⁺ programmed death1 (PD1)⁺ cells (Figure 4A) was increased at baseline in ITP patients as compared with healthy controls (Online Supplementary Figure S4). A marked reduction of plasmablasts/PC was observed from W4 and lasted until W52, when the number of circulating plasmablasts was low and comparable to that in healthy donors (Figure 3H, Online Supplementary Figure S5).

Discussion

The rationale of this study was based on three observations: i) the presence of anti-platelet long-lived PC in the spleen of patients who did not respond to RTX; ii) the increased BAFF level in the spleen and serum of these patients, and iii) the observation that combining an anti-BAFF antibody with anti-CD20 treatment induced a major depletion of long-lived PC in a mouse model. The results of this prospective phase IIb pilot trial of ITP showed that combining RTX with five infusions of belimumab led to an overall response rate of 80% with a 66.7% CR rate at 1 year. Although the sample size was limited, these response rates were higher than expected with RTX alone (overall response rates of 40% to 50% at 66.7% CR rate at 1 year, with 30% of CR, according to most previous studies conducted in ITP). The response rates were also higher but closer to those obtained with RTX and dexamethasone or RTX associated with high-dose dexamethasone and ciclosporin. Hence, these results are promising and provide a real proof of concept for this new combination.

Most consecutive patients included in this study were women with persistent ITP. Disease duration <12 months, young age and female sex have been found associated...
with better outcomes,\textsuperscript{4,5,17,18} which could represent a bias although these factors were not associated with the overall long-term response in our large French prospective registry study.\textsuperscript{21}

The combination strategy was well tolerated, with no severe adverse events and in particular no severe infection. Despite a significant decrease in IgG and IgM titers, we did not observe severe hypogammaglobulinemia. This finding contrasted with the results of the phase Iia study conducted in severe systemic lupus erythematosus, in which severe hypogammaglobulinemia developed in three of 16 patients, with IgG titers <4.5 \text{g/L}.\textsuperscript{22} However, these patients had previously received immunosuppressive therapies, which was not the case for our patients. No significant changes in IgG or IgM titers were observed in patients receiving two infusions of 1,000 mg of RTX or 375 mg/m\textsuperscript{2} once weekly for 4 weeks.\textsuperscript{5,23,24,25} Therefore, the slight decrease in IgG/IgM titers may reflect the impact on splenic PC, because anti-tetanus and anti-measles antibody titers, which are secreted by bone-marrow long-lived PC, remained stable over time. The study was not specifically designed to assess the vaccine response; indeed, the timing of pneumococcal vaccination was heterogeneous, and two patients received T-cell–independent vaccines. Half showed no decrease in serological protection for most serotypes with treatment, and only four patients had lost protection for more than seven serotypes. In the absence of a control cohort, it was not possible to measure the specific impact of combination therapy versus rituximab given alone. Vaccine-induced antibody titers against measles and tetanus toxoid were not reduced at 1 year, suggesting that bone marrow long-lived PC were not affected by the combination therapy. Altogether, these results suggest that belimumab and RTX did not induce significant immunodepression.

From an immunological perspective, achieving a sustained CR indicates that pathogenic PC were efficiently targeted. This is exemplified by the disappearance of platelet autoantibodies in all but one patient with an initial positive test. Despite the absence of a control cohort to clearly assess the impact of this combination on PC, our results support previous results obtained in mouse models showing that combination of RTX and belimumab inhibits the emergence of pathogenic splenic long-lived PC and anti-platelets antibodies.\textsuperscript{10} Of note, the addition of belimumab had no significant impact on residual circulating PC/plasmablasts, which were mainly expressing IgA and have been described as originating from resident mucosal B cells.\textsuperscript{26} The kinetics of B-cell repopulation seemed similar regardless of belimumab exposure. We observed a slight delay in the beginning of B-cell reconstitution (i.e., reappearance of naïve and transitional B cells), but at W52, all but one patient had detectable B cells in peripheral blood. As previously reported, the memory B-cell pool was profoundly depleted until W52 in both cohorts.

Elevated BAFF levels may lower the stringency of the B-cell selection and allow for rescuing autoreactive cells.\textsuperscript{27} This hypothesis is supported by studies showing that negative selection of high-affinity DNA-reactive B cells was impaired by increased levels of BAFF during B-cell depletion in an auto-immune mouse model.\textsuperscript{28} This was the basis for studies conducted in systemic lupus erythematosus in which belimumab is maintained for more than 52 weeks after RTX. In the present study, the last belimumab infusion was administered at W12 and resulted in a complete blockade of BAFF at least until W24. Therefore, B-cell reconstitution occurred in a milieu with increased BAFF levels. Maintaining belimumab for 6 to 9 months after RTX may allow for dampening BAFF levels during B-cell reconstitution and improve the stringency of B-cell selection, thus limiting the risk of relapse after B-cell reconstitution.

Our results also suggest that RTX followed by belimum-
ab had an unexpected effect on activated cTfh cells, which are essential for germinal center formation, B-cell affinity maturation and plasmablast generation. In humans, the majority of cTfh are central memory T cells expressing PD1 but no ICOS, but germinal center recruitment and support for B-cell differentiation requires up-regulation of ICOS. Splenic Tfh cells can contribute to ITP pathogenesis, and as previously reported, the number of activated cTfh cells was increased at baseline in the peripheral blood of ITP patients. Although some cTfh cells were shown to express BAFF-R in systemic lupus erythematosus, this was not the case in ITP (data not shown), so their sensitivity to belimumab remains unexplained so far. Of note, local BAFF production by Tfh cells has been identified as an important factor for promoting germinal center B-cell survival. Belimumab might also prevent, through such pleiotropic effects, the re-emergence of germinal centers in lymphoid organs at the time of B-cell repopulation, an effect that would be further strengthened by extending the duration of its administration. These preliminary results support a new rationale for the addition of BAFF blockade to B-cell depletion in autoimmune diseases.

Figure 3. B-cell activating factor and B-cell subsets in immune thrombocytopenia patients receiving the combination therapy or rituximab alone. (A) B-cell activating factor (BAFF) concentrations were assessed by enzyme-linked immunosorbent assay in serum of patients receiving rituximab and belimumab (in blue) or rituximab alone (in red) at week 0 (W0), W12, W24, W36 and W52. Data are mean ± standard error of the mean (pg/mL). (B) Gating strategy of B-cell subpopulations. Single lymphoid cells on peripheral blood mononuclear cells (PBMC) were gated by using scatter parameters, and dead cells were eliminated by using zombie violet. Plasma blasts/plasma cells (PB/PC) were defined as CD27⁺CD38⁺ cells among CD3⁻CD14⁻CD16⁻ cells. After excluding CD3⁻CD14⁻CD16⁻ positive cells and PB/PC from the CD19⁺ gate, B-cell subsets were separated according to their expression of CD27 and IgD and defined as memory B cells (CD27⁺IgD⁺), CD27⁺IgD⁻ B cells, and naive B cells (CD27⁻IgD⁺). Transitional B cells were defined as CD38⁺CD24⁺CD10⁺ cells among naive B cells. (C) Circulating B-cell subset count per million PBMC at W0, W4, W12, W24, W36 and W52. ****P<0.0001

In conclusion and despite these limitations, in adult ITP, adding belimumab to RTX at the initial phase of B-cell
depletion seems to be a promising strategy with high efficacy and acceptable safety.

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**Contributions**
M. Maheva designed the study and initiated this work; M. Maheva, BG, MMi, IA and EC wrote the report; all authors made substantial contributions to acquisition of data, revised the article critically and gave final approval of the manuscript to be submitted.

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**Figure 4.** Rituximab and belimumab combination affects activated circulating T- follicular helper cells. (A) Gating strategy for circulating T- follicular helper cells (cTfh) cells. After gating on CD4+CD45RA– memory T-CD4+ cells in whole blood, cTfh cells were defined as CXCR5+PD1+, and activated cTfh cells as CXCR5+PD1+ICOS+. (B) Percentages of cTfh and (C) activated Tfh cells at week 0 (W0), W4, W12, W24, W36 and W52 in patients receiving rituximab and belimumab or rituximab alone. *P<0.05, **P<0.01.
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