B cell phenotype and function in granulomatosis with polyangiitis

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Toll-like receptor 9 activation enhances B cell activating factor and interleukin-21 induced anti-proteinase 3 autoantibody production in vitro

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Abstract

Objectives
Granulomatosis with polyangiitis (GPA) is a relapsing small-vessel vasculitis characterised by circulating anti-neutrophil cytoplasmic autoantibodies (ANCA) against proteinase 3 (PR3). The mechanisms that trigger PR3-ANCA production are unknown. The aim of this study was to determine whether endogenous factors (B cell activating factor (BAFF) and interleukin (IL)-21) and exogenous factors (oligodeoxynucleotides containing CpG motifs (CpG-ODN)) synergise in stimulating PR3-ANCA production in GPA patients.

Methods
Peripheral blood mononuclear cells from GPA patients and healthy controls (HCs) were cultured in the presence of BAFF and IL-21, with or without CpG-ODN, for 12 days. PR3-ANCA production in culture supernatants was quantified by Phadia EliA. Phenotypic characterisation and the influence of CpG-ODN treatment on IL-21 receptor (IL-21R), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and BAFF receptor (BAFF-R) expression on B cells was analysed by flow cytometry.

Results
Stimulation with BAFF and IL-21 significantly increased ANCA production in patient samples, which could be augmented further by addition of CpG-ODN. Stimulation with CpG-ODN increased the percentage of IL-21R+ and TACI+ B cells, but did not affect BAFF-R expression. GPA patients had an increased percentage of circulating IL-21R+ and a decreased percentage of TACI+ circulating memory B cells when compared with HCs. Additionally, patients had decreased expression of BAFF-R on B cells, which was inversely correlated with BAFF concentrations in plasma.

Conclusions
Our data demonstrate that endogenous and exogenous factors can synergise to promote PR3-ANCA production. Mechanistically, CpG-ODN up-regulated IL-21R and TACI expression on B cells, possibly sensitising these cells for IL-21- and BAFF-mediated signals. Agents inhibiting Toll-like receptor 9, BAFF and IL-21 signalling pathways may serve as potential therapeutics for intervention in GPA patients.
Granulomatosis with polyangiitis (GPA) is characterised by necrotising inflammation of the small- to medium-sized blood vessels, frequently affecting the respiratory tract and kidneys [1]. A hallmark of GPA is the presence of circulating anti-neutrophil cytoplasmic autoantibodies (ANCA), which are mainly directed against proteinase 3 (PR3) [1, 2]. In patients, a rising serum titer of PR3-ANCA often precedes a disease relapse, supporting the notion that autoantibodies are involved in disease pathogenesis. The pathogenic potential of PR3-ANCA has been studied both in vitro and in vivo (reviewed by Land et al. [3]). Neutrophil and monocyte stimulation in vitro with PR3-ANCA induces cell activation, resulting in generation of reactive oxygen species, neutrophil degranulation and proinflammatory cytokine production [4–7]. More recently, experiments in humanised mice demonstrated that PR3-ANCA can induce acute vascular injury, providing further support for a direct pathogenic role of these autoantibodies [8].

Although the pathogenic potential of PR3-ANCA is well established, the mechanisms that trigger PR3-ANCA production and disease relapse are less clear. Based on clinical observations, infections have been proposed to play an important role in GPA pathogenesis. In GPA patients, chronic nasal carriage of Staphylococcus aureus is strongly linked to relapsing disease, and maintenance treatment with the antibiotic co-trimoxazole can prevent relapses [9, 10]. The association with infections is further supported by in vitro studies demonstrating that B cell stimulation with Toll-like receptor (TLR) 9 ligand oligodeoxynucleotides containing CpG motifs (CpG-ODN) can induce the production of PR3-ANCA [11, 12].

Given that ANCA predominantly belong to IgG1 and IgG4 subclasses, this suggests that an antigen-driven T cell-dependent immune response mediates ANCA production [13, 14]. In particular, a specific T-helper cell subset characterised by production of interleukin (IL)-21 provides signals to B cells, supporting generation of high-affinity clones and differentiation to plasma cells [15]. We have recently reported increased frequencies of IL-21-producing CD4+ T-helper cells in the circulation of GPA patients during remission [16]. IL-21 signalling is environment dependent, and co-stimulatory signals have a critical influence on the resultant biological effect. In combination with CD40 signalling, IL-21 stimulates naive B cell proliferation and differentiation, whereas together with B cell activating factor (BAFF), IL-21 can stimulate plasma cell formation and antibody production by antigen-experienced B cells [17, 18]. BAFF is crucial for B cell survival, proliferation and differentiation, and overexpression of BAFF is strongly associated with the development of systemic autoimmunity [19]. Increased BAFF levels have been reported in patients with various autoimmune disorders, including systemic lupus erythematosus (SLE) and Sjogren’s syndrome (SS), as well as GPA [20–23].

The observations that in GPA the proportion of IL-21+ helper T cells is increased and that BAFF concentrations are elevated even during clinical remission suggest that in these patients an environment exists that facilitates survival and activation of
autoreactive B cells. As the relapsing nature of GPA has been associated with infections, we hypothesised that infectious agents can synergise with BAFF and IL-21 present in the endogenous environment to stimulate autoantibody production in GPA patients. To test this hypothesis, we studied the effect of IL-21, BAFF and the TLR9 agonist CpG-ODN on PR3-ANCA production, B cell proliferation and plasma cell formation. Furthermore, we evaluated the expression of receptors for IL-21 and BAFF on circulating B cells and studied the effect of CpG-ODN stimulation on the expression of these receptors in GPA patients and healthy controls (HCs).

**Patients and Methods**

**Study population**

Patient and HC characteristics are described in Table 1. Two cohorts of GPA patients and HCs were included in the study. Cohort 1 was used to study the effects of BAFF, IL-21 and CpG-ODN on ANCA production in vitro. Cohort 2 was recruited to study the expression of receptors for IL-21 and BAFF and to reveal whether CpG-ODN stimulation affected the expression of IL-21R, BAFF receptor (BAFF-R) and transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) on B cells. The diagnosis GPA was based on the definitions of the Chapel Hill Consensus Conference, and patients fulfilled the classification criteria of the American College of Rheumatology (ACR) [24, 25]. Samples were obtained in compliance with the Declaration of Helsinki. All subjects provided informed consent, and the study was approved by the Medical Ethics Committee of the University Medical Center Groningen, University of Groningen (the Netherlands).

**Peripheral blood mononuclear cell isolation and culture**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood as described before [12]. Cells were cultured at a concentration of 1 x 10⁶ cells/mL in the presence of 100 ng/mL BAFF (PeproTech Inc., Rocky Hill, NJ, USA), 100 ng/mL IL-21 (Immunotools, Friesoythe, Germany), 3.2 μg/mL CpG-ODN 2006 (Hycult Biotech, Uden, the Netherlands) and combinations at 37°C with 5% CO₂ (as previously described [11, 12]). After 12 days of stimulation, the culture supernatants were collected and stored at -20 °C.

**Quantification of PR3-ANCA**

The concentration of PR3-ANCA was measured with a Phadia ImmunoCAP 250 analyser using EliA PR3® (Thermo Fisher Scientific, Waltham, MA, USA) and the levels of PR3-ANCA IgG were expressed in response units (RU). To determine the number of patients that produced significant levels of PR3-ANCA in vitro, the signal measured in HCs samples was used to calculate a cut-off value (mean level in HCs + 3x S.D.).
Enzyme-linked immunosorbent assay (ELISA) for measurement of IgG

IgG in PBMC culture supernatants was quantified using an in-house ELISA. Briefly, microtiter plates were coated with 1.3 μg/mL goat anti-human IgG F(ab’)2 fragments (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The supernatants were diluted in incubation buffer (PBS with 0.05% Tween-20 and 1% BSA). Purified human IgG (Siemens, Marburg, Germany) was used as a standard sample. The bound IgG was detected with mouse-anti-human-IgG-horseradish peroxidase (SouthernBiotech, Birmingham, AL, USA). Tetramethylbenzidine dihydrochloride (Sigma-Aldrich, St Louis, MO, USA) was used as the substrate, and optical density was read at 450 nm using an Emax microplate reader (Molecular Devices, Silicon Valley, CA, USA).

Table 1. Characteristics of the study population

<table>
<thead>
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<th>Cohort 1</th>
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<th>GPA - Remission</th>
<th>GPA - Active</th>
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<td>n (% of males)</td>
<td>15 (67%)</td>
<td>15 (58%)</td>
<td>6 (64%)</td>
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<td>21 (38%)</td>
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<td>Treatment</td>
<td>AZA (100 mg/day)</td>
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<td></td>
<td>Pred (5 – 10 mg/day)</td>
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<td></td>
<td>AZA (100 mg/day) / Pred (5 mg/day)</td>
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<td></td>
<td>MMF / Pred</td>
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<td>No immunosuppressive therapy</td>
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B cell proliferation
Freshly isolated PBMCs from HCs were stained for 10 minutes with 500 ng/mL of carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Life Technologies, Grand Island, NY, USA). After washing, the cells were seeded in a plate at a concentration of 10^6 cells/mL and treated with 3.2 μg/mL CpG-ODN, 100 ng/mL BAFF, 100 ng/mL IL-21, combinations of the stimuli, or were left without stimulation. After 4 days of incubation the cells were harvested, washed and labelled with anti-human CD19-eFluor-450 (eBioscience, San Diego, CA, USA) and anti-human CD22-PE-Cy5 (BD Biosciences, San Jose, CA, USA). Cells were washed and analysed using a BD LSR-II flow cytometer (BD, Franklin Lakes, NJ, USA). The data were analysed using FlowJo Analysis Software v7.6.5 (Tree Star, Inc., Ashland, OR, USA). The percentage of proliferated B cells was determined based on the CFSE staining intensity and data are expressed as the percentage of B cells that have undergone at least one round of cell division.

Plasma cell formation
Freshly isolated PBMCs from HCs were suspended in culture medium at a concentration of 10^6 cells/mL and treated with 3.2 μg/mL CpG-ODN, 100 ng/mL BAFF, 100 ng/mL IL-21, combinations of the stimuli, or were left without stimulation. After 7 days, the cells were harvested, washed and labelled with anti-human CD19-eFluor-450, anti-human CD22-PE-Cy5, anti-human CD27-APC-eFluor-780 (eBioscience), and anti-human CD38-PE-Cy7 (eBioscience). After washing, the cells were measured using a BD LSR-II flow cytometer. The data were analysed using Kaluza 1.2 Flow Analysis Software (Beckman Coulter, Brea, CA, USA), and cells with a CD19+CD27^highCD38^high phenotype were considered plasma cells.

Characterisation of IL-21R, BAFF-R and TACI expression of B cell subsets by flow cytometry
Freshly drawn EDTA blood was washed twice with PBS and 1% BSA, and 100 μl of the cell suspension was incubated with anti-human CD19-PerCP, anti-human CD27-FITC, anti-human CD38-APC (all from BD Biosciences), and either anti-human IL-21R-PE (BD Biosciences), anti-human BAFF-R-PE (BioLegend, San Diego, CA, USA), anti-human TACI-PE (BioLegend), or the corresponding isotype control antibodies for 15 minutes. Erythrocytes were lysed with FACS Lysing Solution. Cells were washed and analysed with a FACSCalibur. The data were analysed using Kaluza 1.2 Flow Analysis Software. Alternatively, freshly isolated PBMCs were suspended to a concentration of 10^6 cells/mL. Some of the cells were used directly for staining to measure the baseline expression of IL-21R, BAFF-R and TACI. Cells were stimulated with CpG-ODN (3.2 μg/mL) for 4 or 24 h. At the end of the experiment, cells were washed with PBS plus 5% fetal calf serum. Then cells were stained for surface expression of IL-21R, BAFF-R and TACI using the staining protocol described above for the whole blood samples.
ELISA for quantification of BAFF concentrations in plasma

BAFF concentrations in plasma were measured using Human BAFF Quantikine ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The plasma sample was collected on the same day as the blood sample used for measuring IL-21R, TACI and BAFF-R expression by flow cytometry.

Statistical analysis

Data are presented as median values, unless stated otherwise. Data from HCs and GPA patients were compared using Student’s unpaired t-test or a Mann-Whitney U-test for data with Gaussian or non-Gaussian distributions, respectively. For intra-individual comparison of more than two groups, repeated-measures analysis of variance was used if data were normally distributed and a Friedman test was used if data had a non-Gaussian distribution as determined by the D’Agostino and Pearson omnibus normality test. If a significant difference was found, further testing was done using a paired Student’s t-test or a Wilcoxon matched pairs test for data with Gaussian or non-Gaussian distributions, respectively. The significance of correlations was assessed using Spearman’s rank correlation coefficient. A value of $p < 0.05$ was considered statistically significant.

Results

CpG-ODN, BAFF and IL-21 promote PR3-ANCA production in vitro

First, we evaluated the effect of BAFF, IL-21 and CpG-ODN on IgG production in vitro. Although treatment with CpG-ODN significantly increased IgG levels when compared with unstimulated cells, the combination of BAFF and IL-21 stimulated IgG production more potently than treatment with CpG-ODN alone (Figure 1A). Furthermore, the stimulatory effect of BAFF and IL-21 could be further enhanced by addition of CpG-ODN (Figure 1A). As BAFF, IL-21 and CpG-ODN had a strong stimulatory effect on IgG production, we then tested their effect on PR3-ANCA production in vitro. No significant increase of PR3-ANCA was observed with BAFF, IL-21 or CpG-ODN alone compared with unstimulated cells (Figure 1B). The combination of BAFF and IL-21 significantly increased PR3-ANCA production, and this effect was further significantly enhanced by addition of CpG-ODN (Figure 1C). In combination with CpG-ODN, IL-21 rather than BAFF was the main potentiating factor leading to increased autoantibody production (supplementary Figure 1). Eighteen of 21 patients produced significant amounts of PR3-ANCA in vitro (Figure 1E).

To determine whether cells from patients and HCs have similar capability to respond to the stimulation, total IgG levels were measured in the supernatants. The amount of IgG produced by GPA patients and HCs upon stimulation with CpG-ODN, BAFF and IL-21 was comparable (Figure 1D).
Figure 1. BAFF, IL-21 and CpG-ODN promote PR3-ANCA production in vitro. (A-B) The effect of CpG-ODN, BAFF, IL-21 or combinations of these stimuli was tested initially on a limited number of GPA patients. Stimulation with BAFF and IL-21, with CpG-ODN or the combination of the three, induced production of (A) IgG (n=12) and (B) PR3-ANCA (n=8). (B-C) Stimulation with BAFF and IL-21 significantly increased PR3-ANCA production. CpG-ODN further significantly enhanced the BAFF and IL-21 effect. (D) Patients and HCs produced similar amounts of IgG in vitro. (E) HC samples were used to calculate a cut-off level, and patients above the cut-off were considered to produce significant amounts of PR3-ANCA. (F) PR3-ANCA production in vitro significantly correlated with the PR3-ANCA serum level. The figure demonstrates data from subjects included in cohort 1. *p<0.05; **p<0.001; ***p<0.0001.
To investigate whether in vitro ANCA production was related to the PR3-ANCA serum level, we measured PR3-ANCA levels in serum samples from the corresponding patients collected at the time of cell isolation. A significant positive correlation was found between PR3-ANCA serum levels and the amount of PR3-ANCA produced in vitro (Figure 1F). As non-specific systemic inflammation in GPA patients could affect the autoantibody levels measured in serum or in culture supernatants, we tested whether PR3-ANCA levels were associated with C reactive protein (CRP) or creatinine concentrations in serum. There was no significant correlation between the serum or in vitro levels of PR3-ANCA and CRP or creatinine (supplementary Figure 2).

**CpG-ODN, BAFF and IL-21 induce B cell proliferation and plasma cell formation**

CpG-ODN is known to promote formation of plasma blasts [26] and, together with BAFF, it induces B cell activation, proliferation, and immunoglobulin secretion [27, 28]. As treatment with BAFF, IL-21 and CpG-ODN lead to significantly more (auto)antibody production than stimulation with BAFF and IL-21, we compared the effect of BAFF, IL-21 and CpG-ODN with that of BAFF and IL-21 on B cell proliferation and plasma cell formation (Figure 2). Stimulation with BAFF, IL-21 or the combination of both did not induce B cell proliferation when compared with unstimulated cells. In contrast, CpG-ODN alone significantly induced B cell proliferation, and this effect was further significantly enhanced when CpG-ODN, BAFF and IL-21 were used together (Figure 2A). As the combined treatment of CpG-ODN, BAFF and IL-21 strongly stimulated IgG production in vitro, we also evaluated the effect of these stimuli on plasma cell formation. The combination of BAFF and IL-21 significantly promoted B cell differentiation towards plasma cells when compared with unstimulated cells. Treatment with CpG-ODN alone induced significantly more plasma cells than BAFF and IL-21 treatment, and this effect was comparable to that of CpG-ODN, BAFF and IL-21 together, demonstrating that plasma cell formation in this in vitro system is primarily driven by CpG-ODN (Figure 2B).

**CpG-ODN up-regulates IL-21R and TACI, but not BAFF-R expression on B cells**

As the combined treatment with BAFF, IL-21 and CpG-ODN was a more potent inducer of PR3-ANCA production compared with BAFF and IL-21, we questioned whether stimulation with CpG-ODN sensitises B cells for stimulation with IL-21 and BAFF. To this end, we evaluated the effect of CpG-ODN on the expression of the receptors for IL-21 and BAFF. IL-21 signals through IL-21R, which is mainly expressed on transitional and naive B cells, and to a lesser extent on memory B cells [17]. BAFF is known to signal through three receptors, namely BAFF-R, TACI, and B cell maturation antigen. As B cell maturation antigen is mainly expressed on terminally differentiated plasma cells and is almost absent on B cells [18, 29], we only studied the expression of BAFF-R and TACI, which are abundantly expressed on B cells.
PBMCs were stimulated with CpG-ODN and already after 4 h of stimulation a moderate increase in IL-21R+ B cells was observed when compared with baseline expression (Figure 3A-B). IL-21R expression increased further after 24 h (Figure 3A-B). The IL-21R expression level, which was induced after 24 h stimulation with CpG-ODN, was comparable between HCs and GPA patients (Figure 3C). After 24 h of stimulation, CpG-ODN significantly up-regulated TACI expression (Figure 3A-B). Increased TACI expression was observed in all HCs and five of six GPA patients (Figure 3C). The median percentage of TACI-positive B cells after CpG-ODN treatment was not different between HCs and GPA patients. In the majority of donors, all B cells expressed BAFF-R; therefore, its expression level was characterised based on mean fluorescence intensity (MFI). Unlike IL-21R and TACI, the expression of BAFF-R was not significantly affected by treatment with CpG-ODN (Figure 3).
Figure 3. CpG-ODN increases IL-21R and TACI, but not BAFF-R expression on B cells. (A) Representative histograms from one HC, demonstrating increase in IL-21R+ and TACI+ B cells after 24 h of stimulation with CpG-ODN in comparison to the baseline expression. (B) PBMCs were stimulated with CpG-ODN for 4 or 24 h, and the expression of IL-21R, TACI, and BAFF-R was measured by flow cytometry. Graphs show data from seven HCs, and the data are plotted as mean ± S.D. (C) PBMCs from seven HCs and six GPA patients were stimulated with CpG-ODN for 24 hours and the alterations in IL-21R, TACI, and BAFF-R expression were measured by flow cytometry. (A-C) The figure demonstrates data from subjects included in cohort 2.

Altered IL-21R and TACI expression on naive and memory B cells and decreased BAFF-R expression in GPA patients

We evaluated the expression of IL-21R, TACI and BAFF-R on total B cells and on naive and memory B cell subsets in HCs and GPA patients. As reported previously [16], the median percentage of IL-21R+ B cells was comparable between HCs and patients (71.6% and 73.5% in HCs and GPA patients, respectively). However, when subdivided...
into naive and memory B cell populations, GPA patients had increased percentages of IL-21R+ memory B cells when compared with HCs (25.6% in HCs and 38.6% in patients; \( p=0.0050 \); Figure 4A).

**Figure 4. IL-21R, TACI and BAFF-R expression on B cells in GPA patients and HCs.** The percentages of (A) IL-21R+ and (B) TACI+ B cells and the receptor distribution on naive and memory B cell subsets were assessed in HCs and GPA patients. (C) The BAFF-R expression level on B cells is expressed as MFI. (D) Plasma BAFF levels in GPA patients (n=21) were measured by ELISA; the plasma samples were collected on the same day when BAFF-R level on B cells was measured. (A-C) The scatter plots show data from 15 HCs and 21 GPA patients. The horizontal lines indicate the median value. (A-D) The figure demonstrates data from subjects included in cohort 2. *\( P<0.05 \); **\( P<0.001 \).
Likewise, the median frequency of TACI+ CD19+ cells was not significantly different between HCs and GPA patients (33.2 and 39.7%, respectively); however, GPA patients had an increased frequency of TACI-expressing naive B cells (12.5% in HCs and 19.1% in GPA; p=0.0147) and a decreased percentage of TACI+ memory B cells when compared with HCs (91.7% and 79.3%, respectively; p=0.0147; Figure 4B). Finally, we assessed BAFF-R expression on B cells. Although BAFF-R expression levels were highly variable in both HCs and GPA patients (MFI range 14.4-76.5 in HCs and 0.5-52.7 in GPA patients), the median expression level of BAFF-R on B cells in GPA patients was significantly lower when compared with HCs (p=0.0113; Figure 4C). Previous studies have reported internalisation of BAFF-R upon binding to its ligand, and circulating BAFF levels were shown to be inversely correlated with BAFF-R expression on peripheral lymphocytes [30]. Given that GPA patients have elevated levels of circulating BAFF [22, 23, 31], we hypothesised that the decreased BAFF-R expression is related to the increased circulating BAFF levels. Therefore, we measured circulating BAFF levels in plasma of patients and correlated it with the BAFF-R level on B cells. We found a significant negative correlation between the level of circulating BAFF and the level of BAFF-R expressed on the peripheral B cells (Spearman’s r=-0.6431, p=0.0017; Figure 4D). In GPA patients, the expression levels of IL-21R, TACI and BAFF-R on B cells were not correlated with the serum concentrations of CRP or creatinine, indicating that the alterations in the receptor expression are not associated with a non-specific inflammatory response (supplementary Figure 3).

Discussion

In GPA pathogenesis, ANCA are considered pivotal mediators of the vasculitic damage. In the present study, we studied factors potentially involved in the production of PR3-ANCA. Here, we show that the TLR9 agonist CpG-ODN, together with BAFF and IL-21, enhances the production of PR3-ANCA, demonstrating that both endogenous and exogenous factors are effectively involved in (auto)antibody production in GPA patients. We confirm the previously reported effect of CpG-ODN on B cell proliferation [32–34] and show that this effect can be enhanced further by addition of BAFF and IL-21. The combined treatment with BAFF, IL-21 and CpG-ODN also efficiently induced plasma cell formation when compared with unstimulated cells. However, this effect was mediated mainly by CpG-ODN, because stimulation with BAFF, IL-21 and CpG-ODN was not superior to stimulation with CpG-ODN alone. The absence of increased plasma cell formation with CpG-ODN, BAFF and IL-21 compared with CpG-ODN seems to conflict with the increased total IgG production that was observed. However, the number of B cells does increase owing to the induction of B cell proliferation. Moreover, as recently suggested by Liu et al. [35], there may also be an increased IgG production per cell, rather than only an increase in the number of IgG-producing cells.
We observed that stimulation with CpG-ODN up-regulated IL-21R on B cells, which is in concordance with a study by Ruffin et al. [36], who demonstrated TLR-mediated induction of IL-21R on B cells. In our study, the increase in IL-21R was mainly due to increased expression on memory B cells, which, in a non-activated state, have a moderate expression level of IL-21R when compared with naive B cells. Interestingly, GPA patients had an increased percentage of circulating IL-21R+ memory B cells, suggesting that these antigen-experienced B cells might be more susceptible for stimulation through the IL-21R.

CpG-ODN also affected TACI, but not BAFF-R expression on B cells. TACI was mainly expressed on memory B cells, whereas it was up-regulated on naive B cells after stimulation with CpG-ODN. This is in line with previous studies reporting induction of TACI expression on activated B cells [37, 38]. Remarkably, GPA patients displayed a decreased frequency of circulating TACI-expressing memory B cells. TACI is an inhibitory receptor, which in physiological conditions provides negative feedback signals to activated cells to circumvent their excessive expansion [38]. Thus, one might speculate that in GPA patients the memory B cells receive insufficient inhibitory signals and might be more prone to uncontrolled activation.

Circulating B cells from GPA patients had lower expression of BAFF-R when compared with HCs. In line with previous work in SLE and SS patients [30], the decreased BAFF-R expression on B cells in GPA patients was strongly associated with increased BAFF levels in the circulation. Elevated BAFF levels, increased frequency of IL-21-producing T cells or circulating IL-21 have been reported in various autoimmune conditions, including GPA [16, 39, 40]. Overexpression of BAFF or IL-21 in animal models leads to the development of a lupus-like condition, whereas blockade of these cytokines ameliorates the disease severity [19, 41]. Hence, BAFF, IL-21 and/or their receptors are considered potential therapeutic targets for treatment of autoimmune disorders.

A monoclonal antibody inhibiting BAFF has been approved for the treatment of SLE patients [42], and ongoing clinical trials also aim to test the efficacy of anti-BAFF therapy in vasculitis patients [43]. As the level of circulating BAFF is known to increase after B cell depletion therapy [44], which is also being used in GPA patients [45] treatment that regulates BAFF levels in the circulation might be useful to prevent aberrant activation of newly formed B cells. More recently, a monoclonal antibody against IL-21 has been developed and is being tested as a possible treatment for RA [46].

Our study also demonstrates that exogenous factors, such as the TLR9 agonist CpG-ODN enhance the effects of BAFF and IL-21. This is of particular interest when considering that the majority of ANCA-associated vasculitis (AAV) patients are chronic carriers of S. aureus [9], and staphylococcal DNA is also recognised by TLR9 [47]. Interestingly, some drugs, such as hydroxychloroquine, work through inhibition of TLR9 signalling and are used in autoimmune disorders, mainly SLE [48]. Treatment improves patient survival and remission rates, as well as reducing disease activity [49]. Reduction
of infectious agents in patients may be equally effective, and antibiotic treatments have been shown to reduce the risk for relapse in GPA [10]. The data of the present study provide additional evidence that blockade of TLR9, BAFF and/or IL-21 might be beneficial strategies for amelioration of autoantibody-mediated diseases. Our study primarily focused on patients in clinical remission and only a limited number of patients with active disease (n=6) were included to establish ANCA production in vitro. Also, owing to limited access to material from patients with active disease, the expression of IL-21R, BAFF-R and TACI was characterised only in patients during clinical remission. Additional studies are needed to elucidate whether the expression of these receptors is affected by disease activity.

Another limitation of our study is that experiments were performed on lymphocytes derived from peripheral blood, whereas the generation and activation of ANCA-specific autoreactive B cells is most likely to occur in secondary lymphoid organs or tertiary lymphoid structures in the affected organs. Nevertheless, it is intriguing that ANCA-specific lymphocytes can be found in the circulation of GPA patients in clinical remission, which suggests ongoing generation of ANCA-specific B cells even during quiescent disease. As ANCA have a major role in the effector phase of GPA [2], the factors that contribute to the production of autoantibodies potentially could exacerbate disease activity or promote the occurrence of relapses. Currently, we are performing a prospective study to test whether in vitro ANCA production could serve as a predictor of relapse in AAV patients.

In conclusion, we have demonstrated that endogenous B cell stimuli and bacterial products can contribute to PR3-specific B cell activation in GPA patients in remission, emphasising the contribution of infectious agents in reactivation of autoantibody production. Therapeutic strategies that regulate B cell activation might be useful to sustain stable remission in GPA patients.

Key Messages
- Granulomatosis with polyangiitis patients have PR3-specific B cells in circulation even during clinical remission.
- PR3-specific B cells in granulomatosis with polyangiitis patients can be reactivated in vitro by endogenous and exogenous signals.

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Supplementary data

Supplementary Figure 1. PBMCs from three patients were treated with the indicated stimuli for 12 days. PR3-ANCA was quantified in the culture supernatants using ImmunoCap.

Supplementary Figure 2. CRP and creatinine levels do not correlate with PR3-ANCA levels in serum, total IgG or PR3-ANCA IgG measured in PBMC culture supernatants from patient cohort 1.
Supplementary Figure 3. CRP and creatinine do not correlate with IL-21R, TACI or BAFF-R expression on B cells in patient cohort 2.