Kv1.3 channel blockade modulates the effector function of B cells in granulomatosis with polyangiitis

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Abstract

Objectives
Granulomatosis with polyangiitis (GPA) patients are often treated with immunosuppressives or B cell depleting therapy. While B cells are involved in GPA pathogenesis as precursors of anti-neutrophil cytoplasmic antibody (ANCA) producing plasma cells, they can also exert cytokine dependent proinflammatory and regulatory functions. In GPA, treatment strategies could be improved by selective targeting of the effector function of B cells. Here, we investigated the effect of the Kv1.3 channel blocker ShK-186 on effector and regulatory B cell function.

Methods
The distribution of B cell subsets was determined in peripheral blood samples of 33 GPA patients and 17 matched healthy controls (HC). Peripheral blood mononuclear cells (PBMC) from GPA patients and HC were stimulated in vitro with CpG-ODN or a combination of CpG-ODN, B cell activating factor (BAFF) and interleukin (IL)21 in the presence and absence of ShK-186. The production levels of total IgG and PR3-ANCA IgG in culture supernatants were analysed by ELISA and Phadia EliA, respectively. In addition, the effect of ShK-186 on B cell proliferation and cytokine production was determined by flow cytometry.

Results
Circulating switched and unswitched memory B cells were relatively decreased in GPA patients as compared to HC. Treating stimulated PBMCs with ShK-186 resulted in decreased production of both total and PR3-ANCA IgG. Proliferation of B cells was not affected by Shk-186. A strong decrease in production of the proinflammatory cytokines TNFα, IL2 and IFNγ was observed with ShK-186 treatment. While IL10 production was also decreased with Shk-186 treatment in GPA patient samples, this effect was less pronounced. As such, ShK-186 modulated the TNFα/IL10 ratio among the B cells, resulting in a relative increase in the regulatory B cell pool.

Conclusions
ShK-186 clearly modulates the effector function of B cells in vitro, by decreasing autoantibody production and the release of proinflammatory cytokines. Kv1.3 channel blockade may hold promise as a novel therapeutic strategy in GPA and other B cell mediated autoimmune disorders.
Introduction

Granulomatosis with polyangiitis (GPA) is a chronic inflammatory relapsing disorder. It is part of the anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV), which mainly affect the small blood vessels in the lungs and kidneys [1]. GPA patients present with autoantibodies which, in the majority of patients, are directed against the neutrophil constituent proteinase 3 (PR3) [2]. Several observations suggest that PR3-ANCA play an important role in the pathophysiology of GPA. For example, leukocytes activated by ANCA release mediators that can injure endothelial cells in vitro [3] and activation of neutrophils by ANCA can stimulate the release of neutrophil extracellular traps that contain chromatin and proteins including PR3 [4].

As B cells are the progenitors of the ANCA producing plasma cells [5], targeting the B cells is an interesting therapeutic option for GPA. Currently, patients are usually treated with broadly acting immunsuppressives. This strategy consists of cyclophosphamide and corticosteroids for induction therapy, often followed by azathioprine or mycophenolate mofetil as maintenance treatment [6]. While the introduction of immunosuppressive treatment has significantly improved the survival of GPA patients, severe adverse events are common, such as high rates of infections, thromboembolic complications and drug toxicity [7]. Results from the Wegener’s Granulomatosis Etanercept Trial indicated that 15% of damage could be attributed to treatment rather than disease [8]. This emphasises the need for more specific and less toxic treatment regimens for GPA patients.

More recently, the anti-CD20 monoclonal antibody rituximab has been approved for induction therapy in AAV. Rituximab was found to be non-inferior to standard cyclophosphamide treatment for induction of remission [9, 10]. However, it was not possible to indicate rituximab as a clearly safer alternative to cyclophosphamide, as adverse event rates were similar [11]. Moreover, there is a risk of persistent severe hypogammaglobulinemia and associated infections after rituximab treatment, necessitating IgG replacement therapy [12]. Rituximab indiscriminately depletes all B cells, which may not be ideal as it has become evident that antibody-independent functions of B cells are also important in GPA [13]. Certain B cells can exert regulatory functions, for example through production of the regulatory cytokine interleukin (IL)10 [14, 15]. Conversely, B cells can also produce a variety of effector cytokines [16]. Therefore, selective targeting of proinflammatory B cells without impairing the regulatory function of B cells may be preferable to targeting all B cells in general.

As class-switched memory B cells have a higher propensity to undergo plasma cell differentiation and are important in the amplification and maintenance of autoimmune responses [17], targeting these class-switched memory B cells may hold therapeutic promise for autoimmunity diseases in general and for GPA patients in particular. It has been demonstrated that class-switched memory B cells express a significantly higher level of voltage-gated Kv1.3 K+ channels when compared to other B cell subsets.
These Kv1.3 channels can serve as a therapeutic target for modulation of class-switched memory B cell function [18]. A potent peptide inhibitor of Kv1.3 channels termed ShK-186 has been identified and investigated for its modulatory effects on T cells [19]. Considering the significant expression of Kv1.3 channels on switched memory B cells we hypothesise that blockade of these channels would result in inhibition of B cell effector functions. To ascertain this we investigated the effect of Kv1.3 channel blockade on B cells in vitro, by determining its effect on ANCA production, B cell proliferation, and production of pro- and anti-inflammatory cytokines in GPA patients and healthy controls.

Patients and Methods

Study population

Thirty-three PR3-ANCA positive GPA patients and 17 age and sex matched healthy controls (HC) were enrolled in this study. All patients were in clinical remission at time of sampling. The majority of patients (n=20) were not treated with immunosuppressives at time of sampling, the remaining patients received low-dose maintenance treatment. The diagnosis of GPA was based on definitions outlined in the Chapel Hill Consensus Conference [20] and all patients fulfilled the classification criteria of the American College of Rheumatology [21]. Subjects provided written informed consent and the study was approved by the Medical Ethical Committee of the University Medical Center Groningen. Characteristics of GPA patients and healthy controls are listed in Table 1.

Table 1. GPA patient and HC characteristics

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<th>Healthy controls</th>
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<td>Subjects, n (% male)</td>
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<td>Age, mean (range)</td>
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<td>Treatment at time of sampling, n (%)</td>
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<td>No immunosuppressive therapy</td>
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ANCA, anti-neutrophil cytoplasmic antibody; Aza, azathioprine; BVAS, Birmingham Vasculitis Activity Score; GPA, granulomatosis with polyangiitis; MMF, mycophenolate mofetil; MTX, methotrexate; pred, prednisolone.
Antibodies and reagents

The following conjugated antibodies were used in flow cytometry: anti-human CD19-eFluor450, anti-human CD27-APC-eFluor780, anti-human IL6-APC and anti-human IL2-PeCy7 were purchased from eBioscience (San Diego, CA, USA). Anti-human IgD-PE, anti-human CD22-APC, anti-human TNFα-Alexa Fluor 488 and anti-human IFNγ-Alexa Fluor 700 were obtained from BD biosciences (Franklin Lakes, NJ, USA). Anti-human CD22-PeCy5 and anti-human IL10-PE were purchased from Biolegend (San Diego, CA, USA). The appropriate isotype matched control antibodies were obtained from eBioscience or BD biosciences. In case of peripheral blood staining, samples were lysed with FACS lysing solution from BD biosciences.

Flow cytometry for analysis of the B cell phenotype

Fresh EDTA blood samples from GPA patients and HCs were washed twice with PBS + 1% BSA to remove soluble plasma proteins. Next, 100 µL of the cell suspension was stained with anti-human CD19-eFluor450, anti-human IgD-PE, anti-human CD27-APC-eFluor780 or the corresponding isotype controls. After 15 minutes, cells were fixed and erythrocytes were lysed using FACS Lysing solution. Samples were washed and measured using an LSR-II flow cytometer (BD biosciences) and data were analysed using Kaluza 1.2 flow analysis software (Beckman Coulter, Brea, CA, USA). B cells were divided based on their surface expression of IgD and CD27. Results are expressed as percentages of total CD19+ B cells.

Induction and measurement of total and PR3-ANCA specific IgG

Cell culture and quantification of total and PR3-ANCA IgG was performed as previously described [22] with slight modifications. Peripheral blood mononuclear cells (PBMC) were isolated and stored in RPMI 1640 (Lonzo, Basel, Switzerland) supplemented with 50 µg/mL gentamycin (GIBCO, Life Technologies, Grand Island, NY, USA), 10% fetal calf serum (FCS, Lonza) and 10% dimethyl sulfoxide (DMSO). Cryopreserved PBMC were thawed and cell suspensions were adjusted to 10⁶ cells/mL in RPMI + 10% FCS. Cells were seeded in 48 wells plates (Corning, NY, USA) and stimulated with 3.2 μg/mL CpG-oligodeoxynucleotides (ODN) 2006 (Hycult Biotech, Uden, the Netherlands), 100 ng/mL B cell activating factor (BAFF; PeproTech Inc., Rocky Hill, NJ, USA) and 100 ng/mL IL21 (Immunotools, Friesoythe, Germany) at 37°C with 5% CO₂, in the presence and absence of 1 nM ShK-186 (Kineta Inc, Seattle, WA, USA). After 12 days the culture supernatants were harvested and levels of both total IgG and PR3-ANCA IgG were determined using in-house enzyme-linked immunosorbent assay (ELISA) and Phadia ImmunoCAP 250 analyser with EliA PR3 (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Levels of PR3-ANCA IgG are expressed as response units (RU)/mL.
B cell proliferation assay
Thawed PBMCs from GPA patients and HCs were stained with 2.5 μg/mL carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Life Technologies, Grand Island, NY, USA). Cells were then cultured at a concentration of 10^6 cells/mL in RPMI + 10% FCS and stimulated using 3.2 μg/mL CpG-ODN 2006, 100 ng/mL BAFF and 100 ng/mL IL21, in the presence and absence of 1 nM ShK-186. After 4 days of incubation, cells were harvested, washed and labelled with anti-human CD19-eFluor450, anti-human CD22-APC and propidium iodide (PI; BD biosciences). Samples were measured using an LSR-II flow cytometer and data were analysed with Kaluza 1.2 software. The CFSE staining intensity of unstimulated B cells was used to determine the percentage of proliferated B cells. Proliferated B cells are expressed as the percentage of B cells that have undergone at least one round of cell division.

Cell stimulation and measurement of intracellular B cell cytokines
Thawed PBMC were seeded in 24 wells flat bottom plates (Corning) at 10^6 cells/mL in RPMI + 10% FCS. Cells were stimulated with 500 ng/mL CpG-ODN 2006, in the presence and absence of 1 nM ShK-186. Plates were incubated for 72 hours at 37°C with 5% CO2. During the last 5 hours of incubation cells were restimulated with 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) and 2mM calcium ionophore (Sigma-Aldrich). As a negative control, one sample was kept without restimulation. To inhibit cytokine release from cells, 10 µg/mL brefeldin A (BFA; Sigma-Aldrich) was added to each sample. Subsequently, cells were harvested, washed with PBS + 5% FCS and stained using anti-human CD19-eFluor450 and CD22-PeCy5. Next, cells were fixed, washed, permeabilised using an Invitrogen Fix&Perm kit and stained with anti-human IL10-PE, anti-human TNFα-Alexa Fluor 488, anti-human IL6-APC, anti-human IL2-PeCy7 and anti-human IFNγ-Alexa Fluor 700. Samples were measured using an LSR-II flow cytometer and data were analysed with Kaluza 1.2. Samples that were not stimulated with PMA + calcium ionophore were used as negative controls in order to set gates during data analysis. Data are presented as the total percentage of cytokine positive cells within the CD19+CD22+ B cell population.

Statistical analysis
Statistical analysis was performed using SPSS v22 (IBM Corporation, Chicago, IL, USA) and Graphpad Prism v5.0 (GraphPad Software, San Diego, CA, USA). Data are presented as median values with an interquartile range unless stated otherwise. Data were analysed with the D’Agostino & Pearson omnibus normality test for Gaussian distribution. For comparison between groups the unpaired t-test was used for data with Gaussian distribution and the Mann-Whitney U test for data without Gaussian distribution. For paired comparisons the paired t-test or Wilcoxon matched pairs test
were applied for Gaussian and non-Gaussian data respectively. Correlation analysis was performed using the Spearman rank correlation coefficient. P-values <0.05 were considered statistically significant.

Results

Distribution of circulating B cell subsets in GPA
To evaluate the distribution of different B cell subsets, phenotypic characterisation of circulating B cell populations was performed on blood samples from 33 GPA patients and 17 matched HCs. B cells were identified using CD19 and surface expression of CD27 and IgD was used to distinguish four B cell subsets; IgD+CD27- transitional/naive B cells, IgD+CD27+ unswitched memory B cells, IgD-CD27+ switched memory B cells and IgD-CD27- double negative B cells (Figure 1A). The percentage of double negative B cells did not differ between GPA patients (median 4.4, interquartile range 2.6-6.7%) and HCs (4.5, 2.6-7.8%). An increased proportion of transitional/naive B cells was detected in GPA (88, 81-91%) compared to HC (74, 70-84%). Finally, GPA patients had lower percentages of both switched (3.4, 2.1-5.5% vs 7.5, 4.5-9.3%) and unswitched memory B cells (3.3, 2.4-6.0% vs 9.2, 8.0-14%) compared to HCs (Figure 1B).

ShK-186 inhibits PR3-ANCA IgG production in vitro
We next evaluated the effect of Kv1.3 channel blockade on the production of total and PR3-ANCA IgG by B cells in vitro. To this end, PBMC from 13 GPA patients and 5 HC were cultured in the presence and absence of ShK-186. Addition of 1 nM ShK-186 only at the start of the cell culture resulted in a significantly decreased production of total IgG (23, 15-40 µg/mL) compared to PBMC cultured without ShK-186 (38, 26-52 µg/mL).

Figure 1. Phenotypic characterisation of circulating B cell subsets in GPA patients and HCs. (A) Flow cytometry gating strategy to distinguish differentiation subsets within peripheral blood CD19+ B cells. CD27-IgD+ transitional/naive B cells, CD27+IgD+ unswitched memory B cells, CD27+IgD- switched memory B cells and CD27-IgD- double negative B cells were identified. (B) Relative distribution of distinct CD19+ B cells subsets from HCs (open circles) and GPA patients (grey circles). Horizontal lines indicate median values. Graphs represent data of 17 HCs and 33 GPA patients. **p<0.01, ***p<0.001.
in HC samples. IgG production from GPA patient samples (17, 11-23 µg/mL) was similarly reduced by treatment with 1 nM ShK-186 (11, 8-19 µg/mL) (Figure 2A). The effect of ShK-186 on IgG production was dose-dependent (Supplementary Figure 1A). Additionally, the PR3-ANCA specific IgG production (40, 20-100 RU/mL) was significantly inhibited by ShK-186 (29, 3-120 RU/mL) in GPA samples (Figure 2B). The effect of ShK-186 appeared to be more pronounced on PR3-ANCA IgG production (median reduction of 39%) than on total IgG production (23%) in samples from GPA patients.

Production of total IgG in GPA correlated positively with the presence of IgD-CD27+ switched memory B cells (spearman’s rho=0.62, p=0.025), while production of PR3-ANCA specific IgG did not (spearman’s rho=0.03, p=0.92), nor did production of PR3-ANCA IgG correlate with other B cell populations.

**Figure 2.** ShK-186 inhibits PR3-ANCA production in vitro. (A) IgG production after PBMC stimulation with CpG, BAFF and IL21 from 5 HCs (open circles) and 13 GPA patients (grey circles) in the presence and absence of 1 nM ShK-186. (B) PR3-ANCA production after PBMC stimulation with CpG, BAFF and IL21 from 13 GPA patients in the presence and absence of 1 nM ShK-186. *p<0.05, **p<0.01

**ShK-186 does not affect B cell proliferation**

To determine whether the decreased production of IgG and PR3-ANCA upon ShK-186 treatment occurs due to decreased B cell proliferation, the effect of ShK-186 on B cell proliferation was assessed in samples from 11 GPA patients and 5 HC. Stimulation of PBMC with CpG, BAFF and IL21 induced proliferation of B cells in GPA patients samples (76.6, 68.6-86.2%), and this was not suppressed by addition of 1 nM ShK-186 (76.6, 67.6-85.0%). The same was seen in HC samples without (68.7, 59.3-76.2%) and with 1 nM ShK-186 (69.0, 61.3-76.7%; Figure 3). These data indicate that the reduced IgG production upon ShK-186 treatment is not caused by decreased B cell proliferation.
ShK-186 suppresses proinflammatory cytokine production by B cells with minor effect on anti-inflammatory IL10 expression

In addition to antibody production, B cells can also participate in orchestrating the immune response by producing a wide range of pro- and anti-inflammatory cytokines. Therefore, it was determined whether cytokine production by circulating effector B cells (TNFα, IFNγ, IL2, IL6) or regulatory B cells (IL10) was influenced by blockade of the Kv1.3 channel (Figure 4A). Samples from 21 GPA patients and 12 HC were stimulated in the presence and absence of ShK168 and B cell cytokine production was assessed. It appeared that B cells from HC produce higher levels of TNFα, IL2 and IFNγ as compared to B cells from GPA patients, whereas levels of IL6 and IL10 were similar. As shown in figure 4B, addition of ShK-186 to cell cultures significantly reduced the production of TNFα, IL2, IFNγ and IL6 in samples from both HCs and GPA patients. The effect of ShK-186 on production of IL2, TNFα and IFNγ was dose-dependent in both GPA and HC samples (Supplementary Figure 1B). For IL10 no significant effect of ShK-186 was observed in HC samples while in B cells from GPA patients a significant reduction was observed (Figure 4B). Remarkably, the suppressive effect of ShK-168 on IL6 and IL10 production was less pronounced than that on TNFα, IL2 and IFNγ.

Next, we assessed whether ShK-168 can modulate the ratio of effector B cells to regulatory B cells. In this analysis we defined effector B cells as TNFα+IL10− and regulatory B cells as TNFα−IL10+ cells. The B effector : B regulatory ratio was significantly decreased after treatment with ShK-186 in GPA patients (Figure 4C).
Figure 4. Effect of ShK-186 on intracellular cytokine production by circulating B cells. (A) Representative flow cytometry dot plots of cytokine production in CD19+CD22+ B cells from a GPA patient. Upper panel represents cytokine production after CpG stimulation, lower panel represents cytokine production after CpG stimulation in the presence of 1nM ShK-186. (B) Percentages of cytokine producing B cells after stimulation with CpG in the presence and absence of 1 nM ShK-186 from HCs (upper panel) and GPA patients (lower panel). Horizontal lines indicate median values. Graphs represent data of 12 HCs and 21 GPA patients. (C) For all GPA patients the single-positive B cells for either TNFα or IL10 were determined in the presence and absence of 1 nM ShK-186 (left panel) and the TNFα/IL10 ratio was calculated (right panel). *p<0.05, **p<0.01, ***p< 0.001
Discussion

GPA is a relatively rare but deadly disease when left untreated. Current treatment is unsatisfactory as it includes strong immunosuppressive treatment with many severe side effects and is not always effective [23]. Therefore, selective targeting of pathogenic pathways may hold therapeutic promise for GPA patients. Given the proposed pathogenic role of B cells and ANCA in GPA, total B cell depletion with rituximab seems a logical strategy for treatment. However, as a fraction of B cells exert regulatory functions, it is important to identify a targeting strategy that can specifically inhibit effector functions of B cells, leaving suppressor aspects intact. Here, we evaluated the effect of blocking Kv1.3 channels, that are highly expressed by switched memory B cells, on (auto)antibody production, proliferation and production of pro- and anti-inflammatory cytokines in B cells from GPA patients.

We found that ShK-186 inhibits the production of both total and PR3-ANCA specific IgG in B cells from GPA patients. The mechanisms behind this effect are not fully understood. It has been shown that Kv1.3 channels on B cells are mainly expressed on IgD-CD27+ switched memory B cells [18], and total IgG production in vitro was indeed associated with this B cell subset. This association indicates that direct inhibition of switched memory B cells may explain the decreased production of total IgG. However, production of PR3-ANCA specific IgG was not positively associated with the IgD-CD27+ B cell subset. Nevertheless, addition of ShK-186 did result in inhibition of PR3-ANCA IgG production. It is possible that the inhibitory effect is indirect, through inhibition of T cell activation. Up to date Kv1.3 channel blockade has mainly been investigated for its effects on effector memory T cells (T\textsubscript{EM}) resulting in inhibition of proinflammatory cytokine production and proliferation of T\textsubscript{EM} cells in vitro, with little or no effects on other T cell populations [19, 24]. T cell assistance is important for the production of immunoglobulins [25], and reduced activity of T cells could affect production of IgG. Another option is that Kv1.3 channel blockade reduces the number of plasma cells formed in vitro, thus affecting the number of cells producing (PR3-ANCA) IgG. However, total B cell proliferation was not affected by ShK-186, so it seems that reduced proliferation is not the underlying cause for the lower levels of IgG observed.

Production of cytokines by B cells was also clearly inhibited when samples were treated with ShK-186. This effect appeared to be more pronounced on the effector cytokines TNF\textalpha, IL2 and IFN\gamma than on the regulatory cytokine IL10. Indeed when the TNF\textalpha/IL10 ratio was determined, this significantly decreased upon treatment with ShK-186. The decrease in the TNF\textalpha/IL10 ratio suggests that Kv1.3 channel blockade results in a relative increase of regulatory B cell phenotype compared to effector B cells. This may have positive effects in the treatment of patients with autoimmunity, as ShK-168 modulates the B cell response towards a suppressive phenotype. Results from several mouse models have indicated an important role for IL10 in controlling autoimmunity. In experimental autoimmune encephalomyelitis (EAE) absence of IL10...
production by B cells led to a persistent immune response and lack of recovery in mice [26]. In a collagen type II induced arthritis (CIA) model, mice that expressed IL10 under the control of an inflammation dependent promoter developed a reduced severity of arthritis compared to control mice [27]. Conversely, TNFα has numerous inflammatory effects on the immune response, since it can for instance act as a growth factor for B cells inducing production of IL1 and IL6, enhance T cell proliferation and promote dendritic cell maturation [28]. Inhibition of TNFα by anti-TNF biological agents has shown promise in several autoimmune disorders, including rheumatoid arthritis [29] and ankylosing spondylitis [30]. Although this therapeutic strategy has also been associated with complications which include cases where development of additional autoimmunity was observed [31]. Moreover, anti-TNF strategies appear to have limited benefit for AAV patients [32, 33].

The importance of targeting memory B cells is highlighted by the (lack of) efficacy of new treatment strategies as exemplified by the disappointing results from atacicept trials in systemic lupus erythematosus and multiple sclerosis patients [34, 35]. The fusion protein atacicept binds BAFF and a proliferation-inducing ligand (APRIL), cytokines involved in B cell proliferation and survival. While atacicept targeted mature B cells and short-lived plasma cells, the memory B cells were spared [36], and especially switched memory B cells appear to be resistant to BAFF depletion [37]. No results from BAFF targeted therapy trials are currently available for GPA patients, however, the lack of effect on (switched) memory B cells is concerning. The humanised monoclonal IL6 receptor antibody tocilizumab was demonstrated to be effective for treatment of rheumatoid arthritis patients. One result of tocilizumab treatment was a reduction of IgD-CD27+ switched memory B cells [38]. This treatment strategy may be promising for GPA, especially since several studies have found increased IL6 levels in active AAV patients, and IL6 production has been detected at AAV lesion sites [39]. However, the efficacy of tocilizumab has not yet been established in GPA.

In summary, considering the important role of switched memory B cells in autoimmune responses, specific targeting of this B cell subset is an interesting option for treatment. One method to achieve this is Kv1.3 channel blockade. Here we demonstrate that ShK-186 is capable of inhibiting (auto)antibody and proinflammatory cytokine production, whereas production of the regulatory cytokine IL10 was less affected. Selective targeting of Kv1.3 channels using ShK-186 may hold therapeutic promise for GPA.

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## References


Supplementary Figure 1. Dose-dependency. (A) IgG production after PBMC stimulation with CpG, BAFF and IL21 from 13 GPA patients in the presence and absence of 0.1 nM and 1 nM ShK-186. (B) Percentages of B cells producing TNFα, IL2 and IFNγ after stimulation with CpG in the presence and absence of 0.1 nM and 1 nM ShK-186 from HCs (upper panel) and GPA patients (lower panel). Similar results were obtained for TNFα and IFNγ. Horizontal lines indicate median values. Graphs represent data of 12 HCs and 21 GPA patients. *p<0.05, **p<0.01, ***p<0.001