

Bacterial DNA motifs trigger ANCA production in ANCA-associated vasculitis in remission

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Abstract

Objectives. CpG motifs, which are highly prevalent in bacterial DNA, have been shown to trigger the production of ANCA *in vitro* by B lymphocytes from patients with active ANCA-associated vasculitis (AAV). *Staphylococcus aureus* is associated with relapses in AAV, and CpG motifs from staphylococcal DNA may trigger ANCA production in AAV patients in remission. We investigated the presence of ANCA-producing B lymphocytes during quiescent disease and tested the capacity of these cells to produce ANCA in response to CpG.

Methods. Expression of Toll-like receptor 9 (TLR9) by B lymphocytes from AAV patients and controls was assessed. Peripheral blood mononuclear cells were isolated from 23 PR3-ANCA and 15 MPO-ANCA patients (33 quiescent, 5 active disease) and 14 healthy controls, and cultured for 12 days in the presence of cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) and IL-2. B-lymphocyte activation, differentiation, immunoglobulin production and *in vitro* ANCA production were studied.

Results. TLR9 expression by B lymphocytes was comparable in AAV patients and controls. B lymphocytes were activated and differentiated towards a plasma cell phenotype in response to CpG-ODN and IL-2. ANCA were produced *in vitro* by 13 out of 23 PR3-ANCA patients and 3 out of 15 MPO-ANCA patients.

Conclusions. We conclude that ANCA-producing B lymphocytes can be present in the peripheral blood of AAV patients during remission. These autoreactive B lymphocytes are triggered by CpG-ODN and IL-2 to produce ANCA *in vitro*. CpG motifs may trigger the production of ANCA *in vivo*, contributing to the development of relapses in AAV.

Key words: Anti-neutrophil cytoplasmic antibodies, B lymphocytes, Bacterial DNA, Cytosine-phosphate-guanine oligodeoxynucleotide, Vasculitis.

Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitides (AAV) are a group of severe, systemic autoimmune disorders characterized by necrotizing inflammation of small- to medium-sized blood vessels. ANCA in AAV are mainly directed against PR3 or MPO

[1]. The aetiology of AAV is still unknown, although bacterial and viral infections have been linked to the onset and development of AAV and other autoimmune disorders [2–5].

In Wegener's granulomatosis (WG), 63% of patients are chronic nasal carriers of the bacterium *Staphylococcus aureus*, and the carriage is associated with an increased risk of relapses [6, 7]. Interestingly, anti-bacterial treatment with co-trimoxazole reduces the risk of relapses in WG as part of maintenance therapy [8, 9]. These findings point towards a link between infection and autoimmunity, but the underlying mechanisms are still unknown. Recent studies speculate on the role of bacterial proteins as triggers of the autoimmune response in AAV. Pendergraft *et al.* [10] found antibodies specific for a protein complementary to a part of PR3 in 21% of patients with PR3-AAV.

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This complementary protein demonstrates homology to several bacterial proteins, including two found in *S. aureus* [10]. Another study focused on antibodies against lysosome-associated membrane protein 2 (LAMP-2), a protein present in neutrophils. In 93% of patients with active focal necrotizing GN (FNGN), often associated with the presence of circulating ANCA, Kain *et al.* [11] found antibodies against LAMP-2. These LAMP-2 antibodies cross-reacted with FimH, an adhesin used by Gram-negative bacteria to enter host tissues [11–13], again feeding speculation about the role of bacterial proteins in the pathogenesis of AAV.

Besides bacterial proteins, bacterial DNA may be involved in the pathogenesis of AAV. Hurtado *et al.* [14] recently demonstrated production of ANCA *in vitro* by B lymphocytes isolated from patients in response to cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) and IL-2. CpG-ODN is short synthetic DNA that contains unmethylated CpG motifs that are highly prevalent in bacterial DNA and are recognized by Toll-like receptor 9 (TLR9), which is constitutively expressed by immune cells [15, 16]. Signalling through TLR9 can result in the production of inflammatory cytokines such as IL-6, IL-8 and Type I IFNs, contributing to inflammation. B lymphocytes respond to CpG motifs by proliferation, enhanced antigen presentation, production of cytokines and differentiation into immunoglobulin-producing cells [17, 18]. The observation that CpG-ODN triggered B lymphocytes from AAV patients to produce ANCA *in vitro*, implies that bacterial DNA may contribute to the course of AAV by stimulating ANCA production.

The aim of the present study was to test the hypothesis that AAV patients have ANCA-producing B lymphocytes in the circulation, also during remission, and that these cells can be triggered to produce ANCA in response to CpG-ODN *in vitro*. This could explain the link between carriage of *S. aureus* and relapsing disease. To this end, we analysed TLR9 expression on B lymphocytes and the capacity of these cells to produce ANCA *in vitro* in response to CpG-ODN and IL-2.

Patients and methods

Patients

Heparinized blood was collected from 38 consecutive AAV patients and 14 age- and sex-matched healthy controls (HCs). The study was approved by the local research ethics committee (Medical Ethical Committee of the University Medical Center Groningen) and informed consent was obtained from each participant. Table 1 describes patient characteristics. Disease diagnoses were based on the definitions of the Chapel Hill Consensus Conference [19]. Thirty-three patients were in remission, and five had a relapse at the time of inclusion. Disease activity was defined by BVAS, and a BVAS ≤ 1 was considered quiescent disease. Patients had received minimal or no treatment for at least 3 months. Two patients experienced relapses, 5 and 12 months after being tested, respectively.

TABLE 1 Patient characteristics

	PR3-ANCA	MPO-ANCA
Total number, <i>n</i>	23	15
Men/women, <i>n</i>	15/8	8/7
Age, mean (s.d.), years	55.6 (14.7)	58.4 (12.4)
Diagnosis		
WG	22	6
Microscopic polyangiitis	1	8
Necrotizing crescentic GN		1
Serum ANCA titre, median (range)	1:80 (1:20–1:640)	1:80 (1:20–1:640)
Active/remission	4/19	1/14
BVAS		
Active patients, median (range)	12 (8–22)	21
Patients in remission	≤ 1	≤ 1

PBMC isolation and culture

Heparinized blood was diluted 1:1 in PBS, layered on top of Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged for 20 min at 600 $\times g$. Peripheral blood mononuclear cells (PBMCs) were collected, washed twice with RPMI 1640 (Cambrex Bio Science, East Rutherford, NJ, USA) and cultured as described before [14]. One million PBMCs were cultured in 1 ml of RPMI 1640 supplemented with 10% fetal calf serum and 50 $\mu\text{g/ml}$ gentamycin (Gibco, Invitrogen, Carlsbad, CA, USA), in the presence of 3.2 $\mu\text{g/ml}$ CpG-ODN 2006 (Hycult Biotechnology, Uden, The Netherlands) and 10 ng/ml IL-2 (Peprotech, Rocky Hill, NJ, USA). After 12 days, cultures were harvested, cells were spun down and supernatants were collected and stored at -20°C until further analysis.

Flow cytometry

TLR9 expression by B lymphocytes was determined in 19 AAV patients (10 PR3-ANCA and 9 MPO-ANCA) with quiescent disease and 14 HCs. Membrane expression of TLR9 was assessed by incubating 100 μl of whole blood with mAbs specific for CD19 (BD Biosciences, Franklin Lakes, NJ USA), CD27 (eBioscience, San Diego, CA, USA) and TLR9 (Imgenex, San Diego, CA, USA) for 15 min at room temperature. Cells were washed with 20-fold excess of PBS/1% BSA and then analysed by fluorescence-activated cell sorter using FACSCalibur (BD Biosciences). For intracellular TLR9 staining, cells were permeabilized using a Fix & Perm kit (Invitrogen, Carlsbad, CA, USA). Briefly, cells were labelled for membrane expression of CD19 and CD27, fixed and permeabilized and incubated with either TLR9 antibody or an isotype control. Intracellular TLR9 expression is presented as δ -mean fluorescent intensity (MFI), which was calculated for each individual: $\Delta\text{MFI} = \text{MFI}_{\text{TLR9}} - \text{MFI}_{\text{isotype}}$. To study B-lymphocyte activation and differentiation in response to CpG-ODN + IL-2, membrane expression of CD69, CD27 and CD38 was assessed. PBMCs were cultured for 24 h or 7 days, labelled with mAbs against CD19, CD27, CD38 (BD Biosciences) and CD69 (BD

Biosciences), and analysed by flow cytometry. Flow cytometry data were analysed using Winlist 6.0 (Verity Software House, Topsham, ME, USA).

IgG and IgM ELISA

Costar ELISA plates were coated with goat anti-human-Ig antibody (Southern Biotec, Birmingham, AL, USA) in Carbonate buffer (pH=9.6) at a concentration of 2 µg/ml. Plates were washed (washing buffer: 0.025 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20) and blocked for 1 h (blocking/incubation-buffer: 0.05 M Tris-HCl, 0.3 M NaCl, 0.05% Tween-20, 1% BSA). Supernatants were diluted 1:80 and 1:160 in incubation buffer. Immunoglobulin G (IgG) binding was detected with goat-anti-human-IgG antibody conjugated with alkaline phosphatase (Sigma, St Louis, MO, USA). Purified human IgG with a known concentration was used as standard sample. Immunoglobulin M (IgM) was detected by alkaline phosphatase-conjugated mouse anti-human-IgM antibody (Southern Biotec). N Protein Standard SL (Dade Behring, Deerfield, IL, USA) was used as IgM standard curve. P-nitrophenyl-phosphate disodium was used as substrate and optical density was read at 405 nm using an Emax microplate reader (Molecular Devices, Silicon Valley, CA, USA).

PR3-ANCA and MPO-ANCA ELISA

Presence of ANCA in the PBMC culture supernatants was determined by ELISA as previously described [20]. Supernatants were tested undiluted and ANCA units were calculated relative to an ANCA-positive standard sample. Cut-off levels for significant ANCA production were determined by calculating the mean reactivity plus three times the s.d. [$\text{mean} + (3 * \text{s.d.})$] from HC supernatants. Patients who produced ANCA levels above cut-off were considered producers (PR3-ANCA patients: >0.61 U, MPO-ANCA patients: >1.61 U), and patients with ANCA levels below cut-off were considered non-producers.

Measurement of ANCA titres

Serum ANCA titres were determined by IIF on ethanol-fixed human granulocytes according to standard procedures, as previously described [21]. Titres were considered positive if $\geq 1:20$.

Statistics

Statistical analyses were performed using Graphpad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The non-parametric Mann-Whitney U-test was used to compare the number of circulating B lymphocytes and the proportion of CD27⁺ B lymphocytes in patients and controls. TLR9 expression levels were compared using unpaired (patients vs controls) or paired (CD27⁻ vs CD27⁺ B lymphocytes within individuals) *t*-tests. Chi-square test was used to compare the number of ANCA-producing patients among PR3-ANCA and MPO-ANCA patients. $P < 0.05$ (two-tailed) was considered statistically significant.

Results

B-lymphocyte numbers, phenotype and TLR9 expression

We first characterized peripheral blood B lymphocytes from AAV patients and HCs. Circulating B lymphocytes were quantified and naïve (CD27⁻) and memory (CD27⁺) subsets were analysed. AAV patients had lower absolute numbers of circulating CD19⁺ B lymphocytes (Fig. 1A, $P=0.01$) and lower proportions of circulating CD27⁺ memory B lymphocytes than HCs (Fig. 1B, $P=0.001$). Next, intracellular and membrane expression of TLR9 by CD19⁺ B lymphocytes was assessed. Membrane expression of TLR9 by B lymphocytes was comparable between AAV patients and HCs (the median percentage of TLR9-positive cells was 2.3% for AAV patients and 3% for HCs). In both AAV patients and HCs, within the B-lymphocyte population, CD27⁺ memory cells expressed higher levels of intracellular TLR9 than CD27⁻ naïve cells (Fig. 1C and D, $P < 0.001$). Intracellular expression of TLR9 by CD27⁻ and CD27⁺ B lymphocytes was comparable between AAV patients and HCs (Fig. 1D). Membrane and intracellular expression of TLR9 in PR3-ANCA- and MPO-ANCA-positive patients was comparable (data not shown).

PBMC cultures

To study the presence of circulating autoreactive B lymphocytes in AAV patients and the capacity to produce ANCA *in vitro*, PBMCs were isolated and cultured in the presence of CpG-ODN and IL-2. Comparable numbers of B lymphocytes were cultured from AAV patients and controls (Fig. 2A). Total levels of IgG and IgM, produced after 12 days, were measured by ELISA. AAV patient culture supernatants contained lower levels of IgG (Fig. 2B, $P=0.01$) and IgM (Fig. 2C, $P=0.01$) than HC supernatants. Production of both IgG ($r=0.61$, $P < 0.001$) and IgM ($r=0.47$, $P=0.02$) correlated with the quantity of CD27⁺ memory B lymphocytes in culture.

B-lymphocyte activation and differentiation

We studied activation and differentiation of B lymphocytes in response to CpG-ODN and IL-2 in subpopulations of both patients and controls. Representative dot plots are shown. After 24 h of stimulation, an increased proportion of CD19⁺ B lymphocytes within lymphocytes was observed (Fig. 3A and B), and between 60 and 70% of B lymphocytes expressed CD69 on the cell surface after stimulation, whereas in the unstimulated condition, ~5% of B lymphocytes expressed CD69 (Fig. 3B). Furthermore, we assessed expression of CD27 and CD38 by B lymphocytes after 7 days of stimulation, in order to determine the presence of plasma cells (CD27⁺⁺/CD38⁺⁺) in the cultures. In all cultures, of both patients and HCs, a population of CD27⁺⁺/CD38⁺⁺/CD19⁺ B lymphocytes was observed (Fig. 3C).

Fig. 1 B-lymphocyte numbers and phenotype and expression of TLR9 in AAV patients and HCs as determined by flow cytometry. B-cell numbers and the proportion of CD27⁺ B cells are presented as box-and-whisker plots. **(A)** AAV patients had lower numbers of CD19⁺ B lymphocytes in the peripheral blood, and within the B lymphocyte population, AAV patients had lower proportions of CD27⁺ memory cells than HCs **(B)**. **(C)** Representative histograms of intracellular TLR9 expression by CD27⁻ (black line) and CD27⁺ (filled histogram) B lymphocytes from one donor. The grey line represents the isotype control. **(D)** Mean intracellular TLR9 levels in CD27⁻ and CD27⁺ B lymphocytes from AAV patients and HCs are plotted. In both AAV patients and HCs, CD27⁺ B lymphocytes expressed higher levels of intracellular TLR9 than CD27⁻ B lymphocytes. Intracellular levels of TLR9 in CD27⁻ and CD27⁺ B lymphocyte subsets were comparable between AAV patients and HCs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

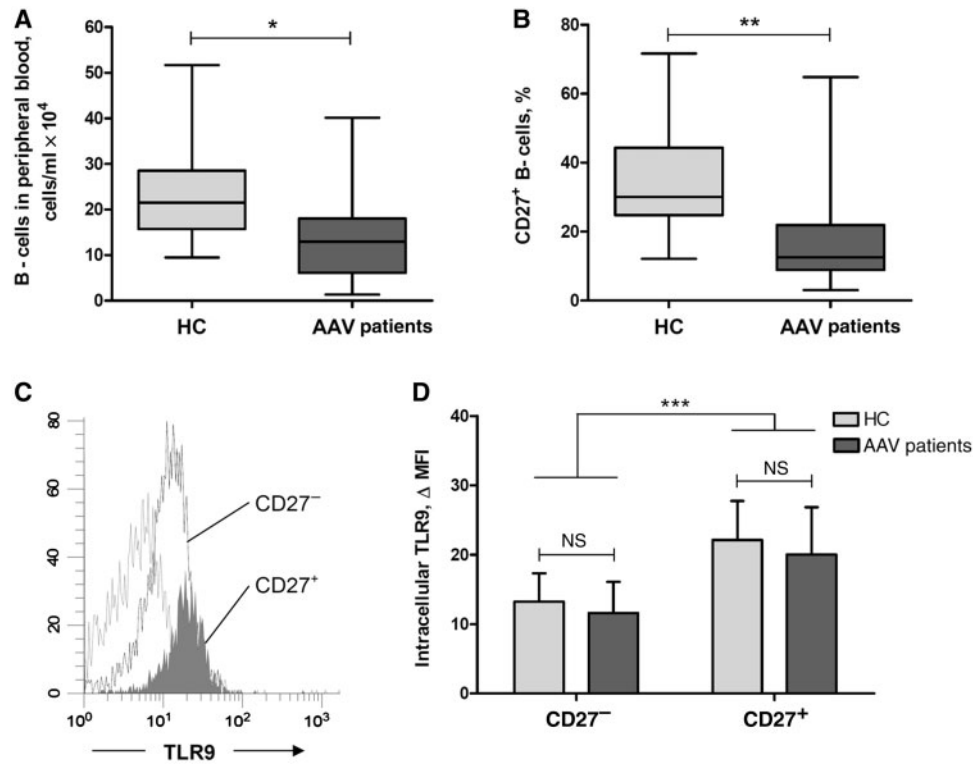


Fig. 2 B-lymphocyte numbers and production of immunoglobulins *in vitro*. PBMCs from 38 AAV patients and 14 HCs were isolated and cultured in the presence of CpG-ODN and IL-2. Data are presented as box-and-whisker plots with a range from min. to max. **(A)** Total numbers of CD19⁺ B lymphocytes in the PBMC cultures were calculated. Comparable numbers of B lymphocytes were cultured from both AAV patients and HCs. **(B and C)** After 12 days, supernatants were harvested and total levels of IgG **(B)** and IgM **(C)** were determined by ELISA. HCs produced higher levels of IgG and IgM *in vitro* than AAV patients. *, $P < 0.05$; ***, $P = 0.001$; NS, not significant.

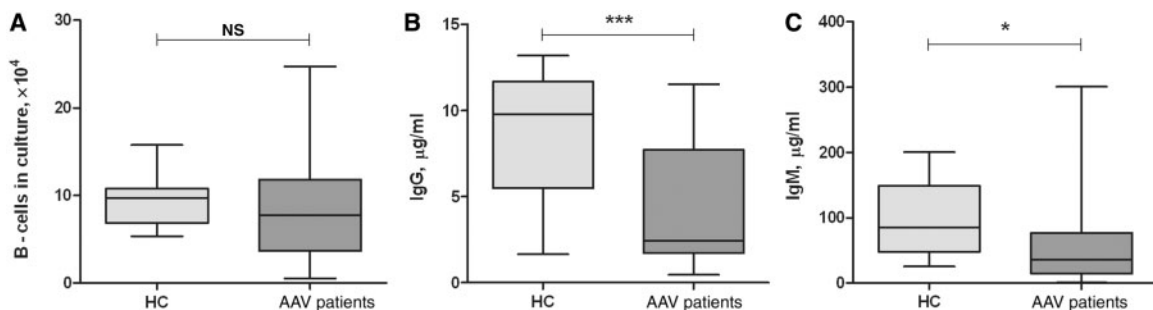
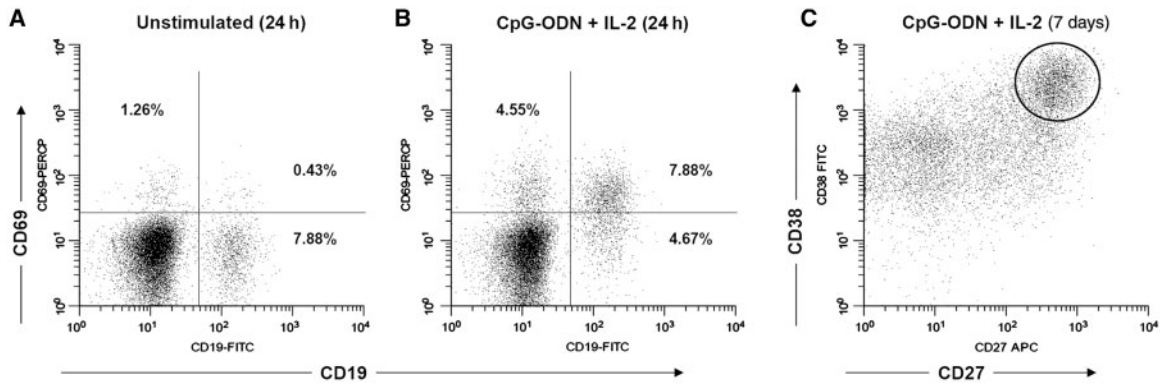


Fig. 3 Activation and differentiation of B lymphocytes in response to CpG-ODN and IL-2. Representative dot plots are shown. PBMCs were isolated and cultured either unstimulated (**A**) or in the presence of CpG-ODN and IL-2 (**B**). After 24 h, the proportion of CD19⁺ B lymphocytes within the lymphocyte population increased from 8.31% (0.43 + 7.88%, **A**) to 12.15% (7.48 + 4.67%, **B**). Sixty-two per cent of CD19⁺ B lymphocytes expressed CD69 after stimulation (**B**), whereas 5.2% of CD19⁻ lymphocytes became CD69⁺ (**B**). In the unstimulated condition, 5% of CD19⁺ B lymphocytes, and 1.4% of CD19⁻ lymphocytes expressed CD69. (**C**) After 7 days in the presence of CpG-ODN and IL-2, B lymphocytes were phenotyped, using flow cytometry. A representative CD27/CD38 plot is shown, gated from CD19⁺ lymphocytes. CD19⁺/CD27⁺⁺/CD38⁺⁺ plasma cells were present in culture (circled).



ANCA production *in vitro*

To study whether ANCA were produced *in vitro*, culture supernatants were tested for the presence of PR3-ANCA and MPO-ANCA using ELISA. Thirteen (57%) out of 23 PR3-ANCA patients and 3 (20%) out of 15 MPO-ANCA patients produced significant levels of ANCA *in vitro* (Fig. 4A and B). Two out of four PR3-ANCA patients and one MPO-ANCA patient with active disease produced ANCA *in vitro* (open circles). In comparison with MPO-ANCA patients, significantly more PR3-ANCA patients produced ANCA *in vitro* ($P=0.04$, chi-square test). The production of ANCA *in vitro* did not relate to serum ANCA titres at the time of blood sampling (Fig. 4C), and no significant ANCA titre changes were observed during the follow-up of patients who produced ANCA *in vitro*. Two PR3-ANCA-associated vasculitis patients experienced relapses, 5 and 12 months after *in vitro* ANCA production assessment. These patients produced significant levels of PR3-ANCA *in vitro* (Fig. 4A, arrows). We compared patients who produced ANCA *in vitro* (producers) and non-producers for clinical and immunological characteristics, but found no significant differences (Table 2).

Discussion

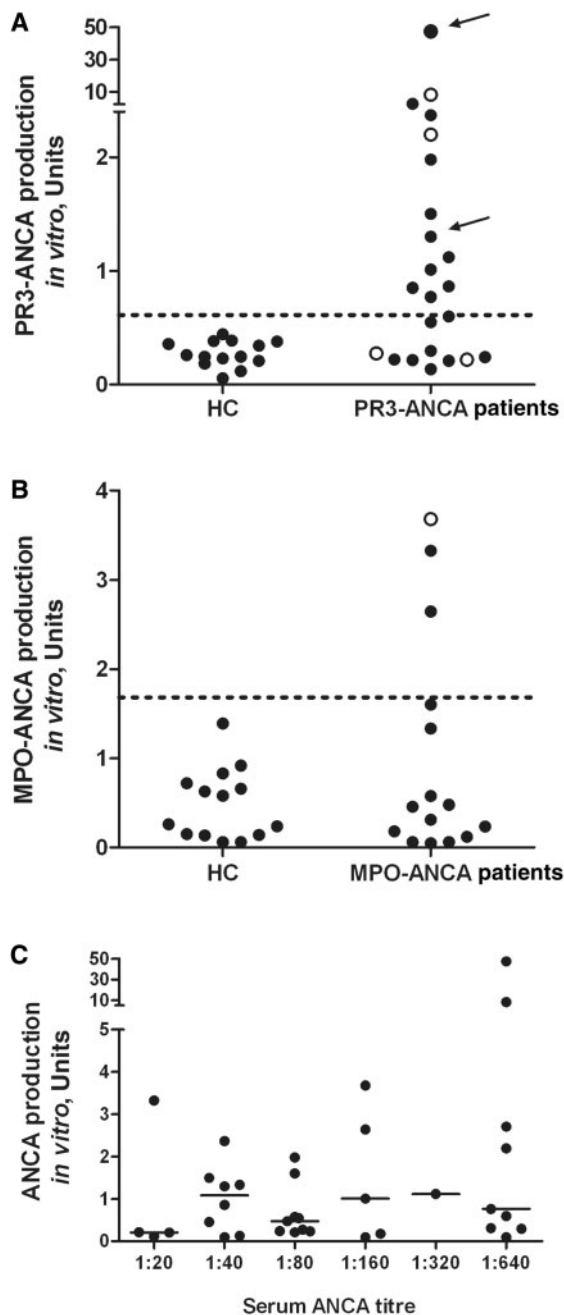
CpG motifs, which are highly prevalent in bacterial DNA, have strong stimulatory effects on the human immune system. Bacterial infections are often associated with autoimmune phenomena and particularly *S. aureus* is associated with AAV [4–6]. A recent study demonstrated that CpG motifs from bacterial DNA may be involved in the pathogenesis of AAV [14]. Here, we tested the hypothesis that AAV patients have circulating autoreactive B lymphocytes during quiescent disease and that CpG-ODN can stimulate the production of ANCA *in vitro*. In addition to

recent data from Hurtado *et al.* [14], describing the presence of circulating autoreactive B lymphocytes, mainly in patients with active AAV, we demonstrate the presence of ANCA-producing B lymphocytes in the circulation of AAV patients with quiescent disease. Interestingly, ANCA production was triggered *in vitro* by CpG-ODN and IL-2. CpG motifs from bacterial DNA may therefore contribute to relapses by stimulating the production of ANCA by autoreactive B lymphocytes *in vivo*.

First, we quantified circulating B lymphocytes in AAV patients and HCs and analysed naïve and memory subsets. AAV patients had lower absolute numbers of circulating CD19⁺ B lymphocytes, and a decreased proportion of CD27⁺ memory B lymphocytes, in comparison with HCs. Lower B lymphocyte numbers in patients may have resulted from immunosuppressive treatment in the past. Induction therapy or treatment of relapses with CYC may have depleted B lymphocytes in these patients [22]. Although tapering of immunosuppressive treatment enables repopulation of B lymphocytes, repopulation of memory B lymphocytes can be delayed [23, 24].

CpG motifs act primarily through TLR9, and, therefore, we analysed membrane and intracellular expression of TLR9 on circulating B lymphocytes [17]. Membrane expression of TLR9 was comparable in AAV patients and HCs. As described by others, we found higher levels of intracellular TLR9 in CD27⁺ B lymphocytes, compared with CD27⁻ B lymphocytes [17]. When comparing intracellular TLR9 levels in the memory and naïve B-lymphocyte subsets from AAV patients and HCs, no differences were found. In other autoimmune diseases, increased levels of intracellular TLR9 in circulating B lymphocytes have been described [25–27]. TLR9 expression by B lymphocytes increases after antigen recognition, CD40 ligation and exposure to TLR9 ligands, and increased TLR9 levels

Fig. 4 Production of ANCA *in vitro*. PBMC from AAV patients and HCs were cultured in the presence of CpG-ODN and IL-2. After 12 days, the presence of ANCA was determined by PR3-ANCA or MPO-ANCA ELISA. Thirteen out of 23 PR3-ANCA patients (A) and 3 out of 15 MPO-ANCA (B) patients produced significant levels of ANCA *in vitro*. Two out of four PR3-ANCA patients and one MPO-ANCA patient with active disease produced ANCA *in vitro* (open circles). Two patients with PR3-AAVs experienced relapses after our study. These patients had ANCA-producing B lymphocytes in the circulation at the moment of inclusion (arrows). (C) No significant relationship was found between ANCA production *in vitro* and the serum ANCA titre at the moment of blood sampling.



were hypothesized to be the result of a higher activation status of B lymphocytes. We did not find increased levels of TLR9 in B lymphocytes, indicating no particular activated B lymphocyte phenotype in our patients.

To investigate whether ANCA-producing B lymphocytes were present in the peripheral blood of AAV patients with quiescent disease, we isolated and cultured PBMCs for 12 days in the presence of CpG-ODN and IL-2 as described before [14]. Comparable B lymphocyte numbers were cultured from AAV patients and HCs. Both IgG and IgM production was lower in the AAV patient cultures, likely due to a reduced presence of CD27⁺ memory B lymphocytes in AAV patients. Bernasconi *et al.* [17] have demonstrated that memory B lymphocytes, but not naïve B lymphocytes, proliferate and differentiate into immunoglobulin-secreting cells upon stimulation with CpG-ODN and IL-2. In accordance with these data, we found that levels of both IgG and IgM correlated with the number of CD27⁺ B lymphocytes in culture. Our data confirm that B lymphocytes are triggered by CpG-ODN and IL-2, by expressing the activation marker CD69. CD69 expression was not increased to such an extent on CD19⁺ lymphocytes, indicating a specific triggering of B lymphocytes by these stimuli. Furthermore, we found an increased proportion of CD19⁺ lymphocytes after 24 h of stimulation, indicating B-lymphocyte proliferation. In addition, after 7 days, a population of B lymphocytes had differentiated towards a CD27⁺⁺/CD38⁺⁺ plasma cell phenotype [28, 29].

Of particular importance, we analysed the production of ANCA by patient PBMCs *in vitro*. Despite lower IgG levels in the AAV patient cultures, significant ANCA production by 13 out of 23 PR3-ANCA-positive patients and 3 out of 15 MPO-ANCA-positive patients was observed. This indicates the presence of autoreactive B lymphocytes in the circulation of these patients. Hurtado *et al.* [14] studied 10 AAV patients, of whom 9 had active disease, and found *in vitro* ANCA production by all patients, and Clayton *et al.* [30] demonstrated *in vitro* ANCA production by active AAV patients as well. Our data demonstrate that not only active patients, but also a proportion of patients in remission have circulating autoreactive B lymphocytes. In particular, PR3-ANCA patients produced ANCA *in vitro*, in line with the observations by Clayton *et al.* [30], although patient numbers were low in their study. PR3-ANCA-positive AAV patients have an increased risk of relapse, compared with MPO-ANCA patients, and increased numbers of ANCA-producing B lymphocytes in PR3-ANCA-positive AAV patients may reflect this risk [31]. Two patients in our study experienced relapses shortly after the time of blood sampling, and, interestingly, these patients had ANCA-producing B lymphocytes. However, to determine whether *in vitro* ANCA production could predict relapses, a longer follow-up period will be needed. Although the highest levels of ANCA were produced by two patients with ANCA titres >1:640, no significant relationship was found between ANCA production *in vitro* and serum ANCA titres at the time of sampling, or

TABLE 2 Clinical and immunological characteristics of *in vitro* ANCA producers and non-producers

	Producers	Non-producers
Total number, <i>n</i>	16	22
PR3-ANCA/MPO-ANCA patients, <i>n</i>	13/3	10/12
Serum ANCA titre, median (range)	1 : 160 (1 : 20–1 : 640)	1 : 80 (1 : 20–1 : 640)
Active/remission, <i>n</i>	3/13	2/20
B lymphocytes		
In peripheral blood, mean (s.d.), cells/ml × 10 ⁴	24.3 (23.9)	13.6 (9.1)
In culture, cells/ml × 10 ⁴ (s.d.)	10.4 (7.3)	7.7 (3.6)
Proportion memory B lymphocytes, mean (s.d.), (% CD27 ⁺)	21.0 (16.7)	20.2 (13.4)
ANCA production <i>in vitro</i> , median (range), U	2.09 (0.77–47.5)	0.24 (0.05–1.60)
IgG production <i>in vitro</i> , median (range), µg/ml	3.29 (1.45–11.5)	2.05 (0.96–10.6)
Relapses within 12 months after testing	2	0

during follow-up, which is in line with the observations by Hurtado *et al.* [14]. We compared patients who produced ANCA *in vitro* with non-producers for clinical and immunological characteristics, but found no differences between these groups. Furthermore, we found no difference between patients with active disease and patients in remission in their capacity to produce ANCA *in vitro*, but the sample size of active patients in our study was low. Inclusion of a larger group of active patients is needed to reach a definitive conclusion.

Although B lymphocytes are the producers of ANCA, they have not been studied extensively in AAV. A number of reports demonstrate involvement of B lymphocytes in AAV pathogenesis. CD20⁺ B lymphocytes have been found in nasal mucosal biopsies derived from patients with localized WG and in the kidneys of patients with crescentic GN [32, 33], and B lymphocyte-rich follicular structures, containing plasma cells in close proximity to PR3-expressing cells, have been found in endonasal granulomatous lesions from WG patients [32, 34]. Furthermore, targeting of B lymphocytes in AAV patients seems promising and treatment with the anti-CD20 chimeric mAb rituximab has shown its capacity to induce remission [35–38].

In this study, we show that circulating autoreactive B lymphocytes are present not only in active patients, but also in patients with quiescent disease. Particularly, PR3-ANCA-positive patients produced ANCA *in vitro* in response to CpG-ODN and IL-2. These stimuli specifically activate B lymphocytes, induce the formation of CD27⁺⁺/CD38⁺⁺ plasma cells and trigger the production of autoantibodies in other autoimmune diseases as well [14, 39]. Therefore, this *in vitro* culture system may provide a useful tool to investigate autoreactive B lymphocytes in more detail. In patients with quiescent AAV, circulating autoreactive B cells may home to inflamed areas in the upper airways or the kidney, possibly as the result of infection with *S. aureus*. The pro-inflammatory milieu, the presence of autoantigens and additional stimulation by bacterial CpG motifs may trigger ANCA production, thereby contributing to the pathogenesis of ANCA-associated vasculitides.

Rheumatology key messages

- ANCA-producing B lymphocytes can be present in the circulation of patients with quiescent AAV.
- CpG motifs trigger the production of ANCA *in vitro*.
- Bacterial DNA may contribute to the pathogenesis of AAV by triggering ANCA production in patients.

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