# chapter 6

## The dual role of interleukin-10 in *in vitro* production of anti-neutrophil cytoplasmic antibodies

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Work in progress

# Abstract

## Introduction

Previously, we demonstrated that in the majority of granulomatosis with polyangiitis (GPA) patients isolated peripheral blood mononuclear cells (PBMC) can be stimulated *in vitro* to produce PR3-ANCA IgG. The aim of the present study was to investigate whether the induced cytokine milieu *in vitro* is a factor that drives production of PR3-ANCA. To this end, we broadly explored cytokine production in relation to *in vitro* PR3-ANCA production and maintenance treatment drugs.

## Methods

PBMC from GPA patients and controls were isolated and cultured in presence of CpG-ODN, B cell activating factor and interleukin (IL)21 for 12 days. Levels of *in vitro* produced total and PR3-ANCA specific IgG were determined. Subsequently, 29 factors were measured in a selection of samples from controls (n=14) and patients positive (n=15) and negative (n=15) for *in vitro* PR3-ANCA production by Luminex. IL10 and IL6 levels were measured in the entire patient cohort (n=84) in supernatants by ELISA. The effect of adding exogenous IL10 or the active metabolites of azathioprine (6MP) and mycophenolate mofetil (MPA) to the *in vitro* ANCA cultures was investigated. Finally, the effect of 6MP and MPA on B cell IL10 and TNFα production and plasma cell formation was investigated by flow cytometry.

## Results

Luminex results demonstrated large inter-individual variation with regard to the levels of cytokine production *in vitro*. PR3-ANCA IgG production was positively associated with several cytokines, including IL6, TNFα and IL10. For IL10 this association was confirmed in the larger patient cohort. When added exogenously to cell cultures at t=0, IL10 could inhibit the production of PR3-ANCA. Both 6MP and MPA could also inhibit the production of PR3-ANCA, as well as inhibit plasma cell formation. Moreover, addition of MPA resulted in decreased IL10 production by B cells, while addition of 6MP affected neither IL10 nor TNFα production.

## Conclusion

PR3-ANCA production *in vitro* is positively associated with production of IL10. However, IL10 is also capable of inhibiting PR3-ANCA production. It is possible that the effect of IL10 is dependent on timing and the composition of cells *in vitro*. When added early it can act to suppress plasma cell formation and IgG production, whereas when added after plasma cell formation IL10 will function as a survival factor for these cells and thus increase PR3-ANCA production.

# Introduction

Granulomatosis with polyangiitis (GPA) is part of the anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV). These are chronic inflammatory disorders that mainly affect the small blood vessels in the respiratory tracts and kidneys [1]. GPA patients are generally treated with cyclophosphamide and high dose steroid treatment to induce remission, and subsequently receive maintenance therapy. The latter consists mainly of azathioprine or mycophenolate mofetil (MMF) combined with low dose steroids [2]. The majority of patients with GPA present with autoantibodies directed against the neutrophil constituent proteinase 3 (PR3) [3]. Presence of ANCA can be measured in serum using indirect immunofluorescence and enzyme-linked immunosorbent assays, resulting in a serum ANCA titer [4]. ANCA titers have been related to disease activity, and in certain cases increase prior to relapse [5]. However, the relation between ANCA titer and disease relapse is not strong enough to be used in clinical practice [6, 7]. Production of PR3-ANCA can also be induced *in vitro*, using a combination of exogenous and endogenous factors [8], although *in vitro* responses from patient samples show a high level of variability. In some samples high levels of *in vitro* PR3-ANCA IgG are observed, whilst others are negative despite patients having a positive serum ANCA titers.

The mechanisms behind these differences are unclear, but one factor that should be considered is the induced production of cytokines. B and T cells can both produce pro- and anti-inflammatory cytokines [9, 10], which may affect B cell proliferation, differentiation to plasma cells and production of immunoglobulins *in vitro*. For example, interleukin (IL)2 is known to induce B cell proliferation [11], and it can drive activated B cells to differentiate towards plasma cells [12]. The cytokines IL5, IL6 and tumour necrosis factor (TNF)α have been identified as factors that can support survival of isolated and cultured plasma cells [13]. Moreover, IL6 was shown to promote the production of immunoglobulins by B cells [14]. Anti-inflammatory cytokines include IL10 and transforming growth factor (TGF)β, of these IL10 is considered the hallmark cytokine of the regulatory B cell ($B_{reg}$) population [15, 16]. IL10 can suppress the differentiation of proinflammatory lymphocytes, for example T helper (Th) 1 cells and Th17 cells [17]. Conversely, IL10 has also been identified as a differentiation and survival factor for plasma cells, and frequencies of CD138+CD27high cells were significantly decreased after inhibition of IL10 signalling [18].

Here we broadly explored cytokine production in relation to *in vitro* PR3-ANCA IgG production. Furthermore, we investigated the effect of the two main used maintenance treatments, azathioprine and MMF on cytokine production, *in vitro* PR3-ANCA production and plasma cell formation.

# Patients and Methods

## Study population

Eighty-four GPA patients and 33 healthy controls (HC) were included. The diagnosis of GPA was based on definitions outlined in the Chapel Hill Consensus Conference and patients fulfilled the classification criteria of the American College of Rheumatology [19, 20]. All patients were confirmed positive for PR3-ANCA at least once during their disease. All subjects gave informed consent and the study was approved by the Medical Ethical Committee of the University Medical Center Groningen.



## Quantification of *in vitro* produced PR3-ANCA specific IgG

Cell isolation, cell culture, and quantification of total IgG and PR3-ANCA IgG was performed as previously described [8]. Briefly, lithium-heparinised venous blood was obtained from patients and HC. Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep (Axis-Shield, Oslo, Norway). Cells were resuspended at a concentration of $10^6$ cells/mL in Roswell Park Memorial Institute (RPMI) medium (Lonza, Basel, Switzerland), supplemented with 50 µg/mL gentamicin (GIBCO, Life Technologies, Grand Island, NY, USA) and 10% fetal calf serum (FCS, Lonza) and cultured with or without 3.2 µg/mL CpG-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. After 12 days supernatants were collected and stored at -20°C. Levels of PR3-ANCA IgG in the supernatants were measured using Phadia ImmunoCAP 250 analyser with EliA PR3$^S$ (Thermo Fisher Scientific, Waltham, MA, USA) and are expressed in response units (RU)/mL. Cryopreserved PBMC were selected that were known to be positive for *in vitro* PR3-ANCA production. These were cultured as described above with the addition of 10 ng/mL recombinant human IL10 (PeproTech), added at the beginning of the culture or after six days. Furthermore, either 600 ng/mL 6-mercaptopurine (6MP, active metabolite of azathioprine; Sigma-Aldrich, St Louis, MO, USA) or 1µM mycophenolic acid (MPA, active metabolite of MMF; Sigma-Aldrich) were added at the beginning of the culture.

## Enzyme-linked immunosorbent assay (ELISA) for IgG

Levels of total IgG in the culture supernatants were measured using an in-house ELISA. Briefly, 96 wells plates (Corning inc., Corning, NY, USA) were coated with 1.3 µg/mL goat anti-human IgG F(ab')2 fragments (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in PBS. Plates were incubated for 1h with blocking buffer (PBS with 0.05% Tween-20 and 2% bovine serum albumin (BSA)). The culture supernatants were diluted in PBS with 0.05% Tween-20 and 1% BSA. Purified human IgG (Siemens, Marburg, Germany) was used to make a standard curve. Plate-bound IgG was detected using mouse-anti-human-IgG-HRP (SouthernBiotech, Birmingham, AL, USA).

3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB; Sigma-Aldrich, St Louis, MO, USA) was used as substrate and optical density was read at 450 nm using a microplate spectrophotometer (Vmax, Molecular devices, Sunnyvale, CA, USA).

## Luminex

From the *in vitro* PR3-ANCA IgG results a patient group (n=30) was selected for analysis of cytokine levels in the culture supernatants. These were the 15 highest individual *in vitro* PR3-ANCA producers, age and sex matched with 15 patients that were negative for *in vitro* PR3-ANCA production and 14 healthy controls. Characteristics of patients and controls are listed in Table 1. For all subjects both an unstimulated and a CpG, BAFF and IL21 stimulated culture supernatant were included. Levels of granulocyte macrophage colony-stimulating factor (GS-MCF), interferon (IFN)α, IFNβ, IFNγ, IL1α, IL1β, IL1RA, IL2, IL2R, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12-p70, IL13, IL15, IL17A, IL18, IL21, IL22, IL23, IL27, IL31, TGFα, TNFα and TNFβ were measured with a Multiplex panel (ProcartaPlex, Affymetrix eBioscience, Vienna, Austria) according to the manufacturer's instructions. Samples were measured using the Luminex 100 System (Luminex, Austin, TX, USA) and data were analysed with StarStation software, version 2.3 (AppliedCytometry, Birmingham, UK). Results are expressed in pg/mL.

## ELISA for IL10 and IL6

IL10 and IL6 levels were measured in the entire GPA patient cohort (n=84) by in-house ELISA. Patient characteristics are listed in Table 1. The samples measured by Luminex were repeated by ELISA to confirm reproducibility of the results. Briefly, 96 wells Costar plates were coated with mouse-anti-human IL10 or IL6 (R&D Systems, Minneapolis, MN, USA) in PBS overnight. Plates were incubated for 1h with blocking buffer (PBS with 0.05% Tween-20 and 2% BSA). Standard curves were made utilising recombinant human IL10 or IL6 (R&D). Supernatant samples and standards were diluted in PBS with 0.05% Tween-20 and 0.2% gelatine and incubated for 2h. Polyclonal goat-anti-human IL10 or IL6 detection antibodies (R&D) were incubated for one hour and streptavidin poly-HRP for 30 min. Bound product was detected using TMB and optical density was read at 450 nm.

## B cell intracellular cytokine production

Cryopreserved PBMC from GPA patients and healthy controls were used. Concentrations were adjusted to $10^6$ cells/mL in RPMI + 10% FCS and cells were seeded in 24 wells flat bottom plates (Corning). Cells were stimulated using 500 ng/mL CpG-ODN and 600 ng/mL 6MP or 1μM MPA was added. Plates were incubated for 72 hours at 37 °C with 5% CO2. During the last five hours of incubation 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich), 2mM calcium ionophore (Sigma-Aldrich) and/or 10 μg/mL Brefeldin A (BFA; Sigma-Aldrich) were added to the cell culture. Cells were harvested, washed with PBS with 5% FCS and stained using anti-human CD19-eFluor450 (eBioscience,

San Diego, CA, USA) and CD22-PeCy5 (BD Biosciences, San Jose, CA, USA). The cells were fixed and permeabilised for intracellular staining using a Fix&Perm kit (Invitrogen, Life Technologies, Grand Island, NY, USA) and subsequently incubated with antibodies against human IL10-PE (Biolegend, San Diego, CA, USA) and TNFα-Alexa Fluor 488 (BD biosciences). Samples were analysed using an LSR-II flow cytometer (BD). Data were analysed using Kaluza 1.2 flow analysis software (Beckman Coulter, Brea, CA,

**Table 1.** Patient and control characteristics.

| | | GPA – ANCA- | GPA – ANCA+ | GPA – ANCA- | GPA – ANCA+ |
|---|---|---|---|---|---|
| | HC | Luminex cohort | | ELISA cohort | |
| Subjects, n (% male) | 14 (50) | 15 (53.3) | 15 (53.3) | 49 (40.8) | 35 (45.7) |
| Age, mean (range) | 60 (47-67) | 60 (30-79) | 60 (26-83) | 60 (27-80) | 60 (27-85) |
| Unstim *in vitro* PR3-ANCA, RU median (range) | 0.12 (0-0.18) | 0.12 (0-0.34) | 1.6 (0.2-198) | 0.18 (0-1.28) | 0.72 (0.12-198) |
| Stim *in vitro* PR3-ANCA, RU median (range) | 0.28 (0.1-0.4) | 0.26 (0-0.42) | 53.6 (21-214) | 0.28 (0-2.68) | 15.06 (2.92-241) |
| PR3-ANCA titer, median (range) | | 20 (0-80) | 160 (0->640) | 20 (0-320) | 160 (0->640) |
| Disease duration in years, median (range) | | 8.7 (1.4-28.7) | 8.1 (2.5-23.3) | 9.7 (0.2-31.6) | 9.1 (0.8-42.1) |
| Number of total relapses, median (range) | | 1 (0-6) | 0 (0-10) | 1 (0-8) | 2 (0-10) |
| Disease form, n (%) | | | | | |
| Localised | | 0 (0) | 0 (0) | 3 (6.1) | 0 (0) |
| Early systemic | | 4 (26.7) | 0 (0) | 10 (20.4) | 3 (8.6) |
| Generalised | | 9 (60) | 12 (80) | 27 (55.1) | 28 (80.0) |
| Severe | | 2 (13.3) | 3 (20) | 9 (18.4) | 4 (11.4) |
| Renal involvement | | 6 (40) | 12 (80) | 24 (49.0) | 27 (77.1) |
| Treatment at time of sampling, n (%) | | | | | |
| Aza | | 2 (13.3) | 0 (0) | 4 (8.2) | 4 (11.4) |
| Pred | | 2 (13.3) | 3 (20) | 5 (10.2) | 5 (14.3) |
| Aza + pred | | 4 (26.7) | 2 (13.3) | 9 (18.4) | 6 (17.1) |
| MMF + pred | | 1 (6.7) | 1 (6.7) | 6 (12.2) | 1 (2.9) |
| MTX | | 0 (0) | 0 (0) | 0 (0) | 1 (2.9) |
| No immunosuppressive therapy | | 6 (40) | 9 (60) | 25 (51.0) | 18 (51.4) |

ANCA, anti-neutrophil cytoplasmic antibody; Aza, azathioprine; GPA, granulomatosis with polyangiitis; HC, healthy control; MMF, mycophenolate mofetil; MTX, methotrexate; PR3, proteinase 3; pred, prednisolone; stim, cell culture supernatant from CpG + B cell activating factor + Interleukin-21 stimulated cultures; unstim, unstimulated cell culture supernatants

USA). Samples not stimulated with PMA and calcium ionophore were used as negative controls to set the gates during data analysis. Data are presented as the percentage of cytokine positive B cells within the total CD19+CD22+ population.

### Plasma cell formation

Freshly isolated PBMCs from healthy controls were cultured in polypropylene tubes, $10^6$ cells/ml in RPMI + 10% FCS. Cells were cultured with or without 3.2 µg/mL CpG-ODN 2006 at 37 °C with 5% CO2 for 7 days. Furthermore, either 600 ng/mL 6MP or 1µM MPA was added at t=0. Subsequently cells were harvested and stained using anti-human CD19-eFluor450, CD22-PeCy5 (Biolegend), CD27-APC-eFluor780 (eBioscience) and CD38-PeCy7 (eBioscience). Samples were analysed using an LSR-II flow cytometer. Data were analysed using Kaluza 1.2 flow analysis software. CD19+CD22+ B cells with a $CD27^{high}CD38^{high}$ phenotype were considered plasma cells.

### Statistical analysis

Hierarchical clustering for the Luminex data was performed for cytokines with a median concentration of > 10 pg/mL, using Genesis [21] with Pearson correlation as the distance metric. Statistical analysis was performed using SPSS v22 (IBM Corporation, Chicago, IL, USA) and Graphpad Prism v5.0 (GraphPad Software, San Diego, CA, USA). 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 nonparametric Mann-Whitney U test for data without Gaussian distribution. For paired comparisons the unpaired t-test or Wilcoxon matched pairs test was performed for Gaussian and non-Gaussian data respectively. Correlation analysis was done using the Spearman rank correlation coefficient. Data are presented as median values with the interquartile range, unless stated otherwise. P-values <0.05 were considered statistically significant.

## Results

### *In vitro* PR3-ANCA production

PR3-ANCA IgG levels were determined in samples from 84 GPA patients and 33 healthy controls. A positive cut-off was calculated using the mean + 3 times the standard deviation of the healthy controls. Samples from 15 patients with high levels of *in vitro* PR3-ANCA IgG production were selected and matched with samples from 15 patients that were negative for *in vitro* PR3-ANCA production and 14 healthy controls.

### Luminex for 12 day cell culture supernatant samples

In the selected unstimulated and CpG, BAFF and IL21 stimulated cell culture supernatants 29 factors were determined using a Luminex assay. First, unstimulated samples were compared between healthy controls and GPA patients. Levels of

a number of factors were significantly higher in healthy control samples than GPA patients samples, specifically TNFα (*p=0.02*), TNFβ (*p=0.045*), IFNγ (*p=0.005*), GM-CSF (*p=0.003*), IL2R (*p=0.01*), IL1RA (*p=0.08*), IL5 (*p=0.003*) and IL13 (*p=0.008*) (Table 2). However, no significant differences were observed when samples were divided based on *in vitro* ANCA production in stimulated PBMC.

In samples stimulated with CpG, BAFF and IL21 fewer differences were observed between patients and controls, with only TNFβ being significantly higher in control samples (*p=0.01*). Again ANCA positive and negative samples were not significantly different for any cytokine measured, although several trends were observed. Samples from patients positive for *in vitro* ANCA production showed higher levels of TNFα, IL10, IL6 and IL8 in the culture supernatants (Table 3). Comparing unstimulated and stimulated samples, levels of IL10 and IL22 in the supernatants were increased with CpG, BAFF and IL21 stimulation in both controls and patients. IFNγ, TNFα and IL6 levels were lower with stimulation in control samples but increased in patient samples, while IL2R levels demonstrated the exact opposite.

Hierarchical clustering analysis did not reveal a clear clustering of the two patient groups or healthy controls in unstimulated (Figure 1A) or CpG, BAFF and IL21 stimulated samples (Figure 1B).

## Associations between cytokine levels and (PR3-ANCA) IgG levels

Correlation analysis was performed between those cytokines that were found at a median concentration of > 10pg/mL in stimulated samples from GPA patients and (PR3-ANCA) IgG levels. This demonstrated several positive correlations between the level of PR3-ANCA and levels of IL10, TNFα, IL6 and IL8 in cell culture supernatants. When total IgG levels were associated with the cytokine levels positive correlations were detected as well, with IL10, TNFβ and TGFα. IL10 was the only cytokine that had a positive association with both total and PR3-ANCA specific IgG production *in vitro* (Table 4).

## Increased IL10 levels in samples positive for PR3-ANCA

IL10 and IL6 levels were measured by ELISA in CpG, BAFF and IL21 stimulated samples from 84 GPA patients. As a cut-off value for high PR3-ANCA production 2.8RU was used. Levels of IL10 and IL6 were compared for the samples that were measured by Luminex and ELISA. Results from these two assays showed strong positive correlation, spearman's rho=0.92, *p<0.0001* for both IL10 and IL6 (Figure 2A). However, IL6 results as measured by ELISA were considerably lower (median 5-fold) than those from Luminex analysis.

IL6 levels were not significantly different in samples with high (median 65.5, interquartile range 25.4-196.2 pg/mL) and low (49.6, 27.5-136.8 pg/mL) *in vitro* PR3-ANCA production (Figure 2B). Moreover, no correlation between IL6 and PR3-ANCA IgG or total IgG levels was detected. IL10 levels were significantly higher in samples from high PR3-ANCA producers (476.6, 163.4-791.3 pg/mL) compared to low producers (250.3,

113.8-484.2 pg/mL) (Figure 2B). Supernatant IL10 and PR3-ANCA levels also showed a moderate positive correlation (spearman's rho=0.29, *p=0.008*) while a stronger positive correlation was seen for supernatant IL10 and total IgG production (spearman's rho=0.53, *p<0.0001*).

**Table 2.** Cytokine levels in unstimulated culture supernatants

| | Controls | GPA patients | *in vitro* ANCA - | *in vitro* ANCA + |
|---|---|---|---|---|
| GM-CSF | **124 (32-395)** | **13 (0.0-76)** | 13 (2.0-65) | 23 (0.0-84) |
| IFNα | 0.2 (0.0-0.6) | 0.0 (0.0-0.3) | 0.1 (0.1-1.3) | 0.1 (0.1-0.2) |
| IFNβ | 0.3 (0.3-0.6) | 0.6 (0.3-1.4) | 0.9 (0.6-1.7) | 0.6 (0.3-1.2) |
| IFNγ | **574 (82-1652)** | **22 (5.0-310)** | 58 (3.8-369) | 13 (5.0-331) |
| IL1α | 0.9 (0.6-3.6) | 0.6 (0.3-1.5) | 0.6 (0.2-0.8) | 1.2 (0.4-1.7) |
| IL1β | 1.2 (0.5-2.0) | 0.5 (0.2-1.0) | 0.7 (0.5-0.7) | 0.5 (0.2-1.2) |
| IL1RA | **36200 (9845-46645)** | **5531 (1327-28605)** | 9911 (2342-22514) | 2935 (1005-32460) |
| IL2 | 14 (8.9-17) | 8.9 (5.0-19) | 12 (5.0-18.8) | 8.9 (5.0-19) |
| IL2R | **16333 (870-22843)** | **342 (77-9394)** | 422 (77-7838) | 195 (103-10804) |
| IL4 | 10 (5.1-17) | 3.8 (2.0-7.6) | 3.9 (2.1-5.6) | 3.9 (2.1-8.8) |
| IL5 | **45 (6.6-105)** | **2.8 (1.2-21)** | 4.4 (1.2-12) | 2.3 (1.2-58) |
| IL6 | 1197 (79-2811) | 96 (15-1436) | 98 (14-1056) | 91 (20-2326) |
| IL7 | 0.3 (0.1-0.6) | 0.3 (0.1-0.4) | 0.3 (0.1-0.4) | 0.3 (0.1-0.4) |
| IL8 | 20030 (7325-26714) | 11016 (3093-15420) | 6197 (2332-15320) | 14636 (3259-20209) |
| IL9 | 23 (14-51) | 21 (14-31) | 21 (14-28) | 19 (12-37) |
| IL10 | 31 (7.2-69) | 12 (5.4-41) | 14 (5.5-41) | 9.5 (5.0-57) |
| IL12-p70 | 0.8 (0.4-0.9) | 0.2 (0.0-0.4) | 0.2 (0.1-0.4) | 0.1 (0.1-0.4) |
| IL13 | **217 (35-464)** | **2.7 (0.8-115)** | 21 (0.8-107) | 2.6 (0.8-142) |
| IL15 | 1.9 (0.8-2.3) | 1.6 (0.8-1.9) | 1.2 (0.8-1.6) | 1.6 (0.8-2.3) |
| IL17A | 8.7 (3.5-35) | 1.7 (0.1-25) | 12 (0.4-30) | 0.8 (0.4-23) |
| IL18 | 32 (15-56) | 20 (6.2-37) | 20 (6.2-30) | 23 (6.1-43) |
| IL21 | 20 (8.2-27) | 9.1 (7.3-15) | 8.2 (6.4-14) | 9.1 (7.3-20) |
| IL22 | 239 (91-377) | 135 (32-181) | 135 (47-223) | 113 (32-181) |
| IL23 | 23 (12-39) | 12 (12-25) | 12 (12-25) | 12 (12-25 |
| IL27 | 3.8 (2.0-5.6) | 2 (2-3.8) | 2 (2-4.7) | 2 (2-3.8) |
| IL31 | 3.3 (1.2-5.9) | 2.6 (1.2-5.5) | 2.6 (0.8-4.0) | 4.0 (1.2-5.5) |
| TGFα | 20 (6.7-26) | 8.5 (5.1-20) | 12 (5.2-19) | 6.4 (4.7-23) |
| TNFα | **96 (24-206)** | **6.9 (2.7-63)** | 7.2 (2.7-54) | 5.0 (2.7-97) |
| TNFβ | **86 (34-325)** | **21 (4.8-105)** | 30 (4.2-105) | 15 (5.0-117) |

Levels of cytokines measured in unstimulated cell culture supernatant samples (pg/mL). Significant differences between patients and controls are indicated in bold text.
ANCA; anti-neutrophil cytoplasmic antibodies, GM-CSF; granulocyte-macrophage colony-stimulating factor, GPA; granulomatosis with polyangiitis patients, HC; healthy controls, IFN; interferon, IL; interleukin, TGF; transforming growth factor, TNF; tumor necrosis factor

**Table 3.** Cytokine levels in CpG, BAFF and IL21 stimulated supernatant samples

| | Controls | GPA patients | *in vitro* ANCA - | *in vitro* ANCA + |
|---|---|---|---|---|
| GM-CSF | 10 (4.4-16) | 4.9 (2.8-10) | 7.7 (2.8-10) | 4.9 (2.8-14) |
| IFNα | 0.6 (0.1-4.3) | 0.8 (0.4-2.3) | 1.3 (0.8-2.3) | 0.5 (0.3-1.4) |
| IFNβ | 1.2 (0.6-1.8) | 1.7 (1.2-2.2) | 1.7 (1.2-3.1) | 1.7 (1.2-1.7) |
| IFNγ | 204 (114-596) | 87 (36-304) | 65 (31-181) | 122 (37-374) |
| IL1α | 0.5 (0.2-1.2) | 0.6 (0.1-1.2) | 0.4 (0.1-1.2) | 0.6 (0.2-1.4) |
| IL1β | 1.3 (0.9-2.9) | 1.0 (0.7-3.2) | 1.0 (0.5-2.2) | 1.2 (1.0-3.2) |
| IL1RA | 9047 (5348-19145) | 10859 (5215-29243) | 10836 (5345-25581) | 10883 (3054-29262) |
| IL2 | 8.9 (5.0-8.9) | 12 (8.9-12) | 12 (8.9-14) | 8.9 (8.9-12) |
| IL2R | 5825 (3331-9993) | 3806 (671-9236) | 2974 (658-8002) | 5042 (1584-13100) |
| IL4 | 3.9 (2.1-6.0) | 3.9 (2.1-5.6) | 3.9 (2.1-7.2) | 3.9 (2.1-5.6) |
| IL5 | 3.6 (2.3-3.6) | 2.3 (2.3-3.6) | 2.3 (2.3-3.4) | 2.3 (2.3-4.4) |
| IL6 | 225 (122-397) | 344 (75-830) | **165 (65-605)** | **474 (91-876)** |
| IL7 | 0.6 (0.3-0.7) | 0.6 (0.4-0.8) | 0.6 (0.4-0.7) | 0.7 (0.6-0.9) |
| IL8 | 16042 (10594-19247) | 15958 (6835-24395) | **15035 (5277-21834)** | **22251 (11036-31357)** |
| IL9 | 24 (21-30) | 26 (22-30) | 26 (22-34) | 26 (22-29) |
| IL10 | 222 (151-442) | 284 (86-486) | **154 (62-314)** | **367 (154-542)** |
| IL12-p70 | 0.5 (0.3-0.6) | 0.2 (0.1-0.3) | 0.3 (0.1-0.3) | 0.2 (0.1-0.3) |
| IL13 | 1.3 (1.1-1.7) | 1.1 (0.8-2.0) | 1.1 (0.8-2.0) | 1.1 (0.8-2.0) |
| IL15 | 1.2 (0.8-2.3) | 1.6 (1.6-2.3) | 1.6 (0.8-2.3) | 1.6 (1.6-2.9) |
| IL17A | 9.9 (2.6-17.5) | 6.6 (2.6-19) | 8.2 (3.9-23) | 5.6 (1.5-18) |
| IL18 | 12 (5.9-24) | 12 (5.8-21) | 9.2 (6.0-20) | 15 (4.4-22) |
| IL21 | n/a | n/a | n/a | n/a |
| IL22 | 1707 (1477-1863) | 1734 (1349-1930) | 1812 (1364-1977) | 1546 (1291-1773) |
| IL23 | 34 (30-41) | 39 (30-47) | 39 (30-47) | 39 (30-47) |
| IL27 | 5.6 (5.6-10.8) | 5.6 (2.0-11) | 5.6 (5.6-16) | 5.6 (2.0-11) |
| IL31 | 5.5 (4.0-13) | 6.2 (4.0-12) | 7.0 (4.0-13) | 5.5 (5.5-8.5) |
| TGFα | 8.9 (5.8-12) | 11 (8.2-14) | 11 (8.3-13) | 11 (7.7-16) |
| TNFα | 10 (8.4-19) | 16 (5.8-31) | **10 (5.0-25)** | **18 (5.8-38)** |
| TNFβ | **35 (20-43)** | **18 (8.9-25)** | 20 (9.0-24) | 18 (8.3-33) |

Levels of cytokines measured in CpG, BAFF and IL21 stimulated cell culture supernatant samples (pg/mL). Significant differences between patients and controls are indicated in bold text. While there were no significant differences between patients positive and negative for *in vitro* ANCA production, trends are also indicated in bold text.

ANCA; anti neutrophil cytoplasmic antibodies, BAFF; B cell activating factor, GM-CSF; granulocyte-macrophage colony-stimulating factor, GPA; granulomatosis with polyangiitis patients, HC; healthy controls, IFN; interferon, IL; interleukin, TGF; transforming growth factor, TNF; tumor necrosis factor

For 16 patients two supernatant samples were measured, one with high PR3-ANCA production and the other negative or with substantially lower production (median 11%, range 1-49%). When comparing these two time points, it was observed that the IL10 production increased concomitantly with the PR3-ANCA IgG levels (Figure 2C). In two patients a sharp decrease of IL10 was seen upon increase of PR3-ANCA levels, one of these patients subsequently relapsed.

Patients were then divided on whether they received immunosuppressive treatment at time of sampling. Those that were currently treated had significantly lower concentrations of IL10 in the cell culture supernatants (Figure 2D). However, as there was a similar proportion of treated patients in the ANCA positive (48.6%) and ANCA negative (49%) groups, this is unlikely to explain the difference in IL10 production we observe.



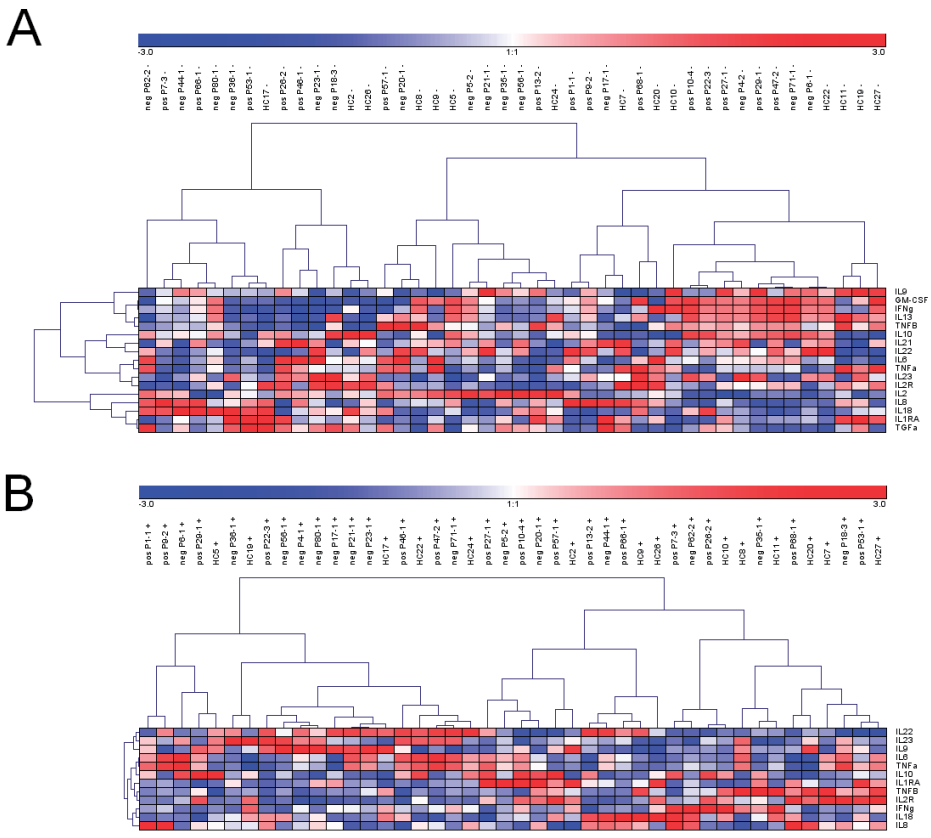


**Figure 1. Hierarchical clustering.** Cytokines were selected from Luminex analysis that were clearly present in the supernatants, with median concentrations of >10 pg/mL in (A) unstimulated culture supernatants and (B) supernatants from CpG, BAFF and IL21 stimulated samples. Hierarchical clustering was performed with Pearson correlation as distance metric.

**Table 4.** Associations between (PR3-ANCA) IgG and cytokine production *in vitro*

| | | Total IgG | PR3-ANCA IgG |
|---|---|---|---|
| IL10 | ρ | **0.413** | **0.432** |
| | p-value | **0.023** | **0.017** |
| TNFα | ρ | 0.248 | **0.377** |
| | p-value | 0.186 | **0.040** |
| IL6 | ρ | 0.247 | **0.391** |
| | p-value | 0.189 | **0.032** |
| IL8 | ρ | 0.209 | **0.410** |
| | p-value | 0.267 | **0.025** |
| TGFα | ρ | **0.459** | 0.294 |
| | p-value | **0.011** | 0.115 |
| TNFβ | ρ | **0.526** | 0.158 |
| | p-value | **0.003** | 0.405 |

In cell culture supernatants stimulated with CpG, BAFF and IL21, cytokines were measured by Luminex assay and total and PR3-ANCA specific IgG production determined. Significant correlations are indicated in bold text. ANCA; anti-neutrophil cytoplasmic antibodies, IL; interleukin, TGF; transforming growth factor, TNF; tumor necrosis factor



## IL10 can suppress PR3-ANCA IgG *in vitro*

To determine if IL10 could suppress or increase production of PR3-ANCA exogenous IL10 was added to the *in vitro* ANCA assay, either at the start of cell culture or after 6 days. When IL10 was added at t=0, a significantly lower production of PR3-ANCA IgG was observed on group level (Figure 3A) and a clear majority of patient samples responded with a decrease of PR3-ANCA production. When IL10 was added at t=6 days more variation in responses was observed, including a strong increase of PR3-ANCA production in some patients but overall no significant effect of IL10 on PR3-ANCA production was observed (Figure 3B).

## 6MP and MPA influence B cell function

The effect of the active metabolites of azathioprine (6MP) and MMF (MPA) on several B cell functions was investigated. First, the effect on the production of the proinflammatory cytokine TNFα and the anti-inflammatory cytokine IL10 by B cells was evaluated. Adding 6MP to the cell culture did not affect IL10 or TNFα production in samples from GPA patients or healthy controls. Conversely, addition of MPA resulted in significantly decreased production of the regulatory cytokine IL10, while leaving TNFα production unaffected (Figure 4A). The same effect was observed in patients and controls. Adding 6MP or MPA to cell cultures resulted in a decrease of PR3-ANCA production *in vitro* with MPA having a significantly larger effect (Figure 4B). Similarly addition of 6MP significantly decreased the formation of plasma cells, while MPA abrogated *in vitro* plasma cell formation (Figure 4C).

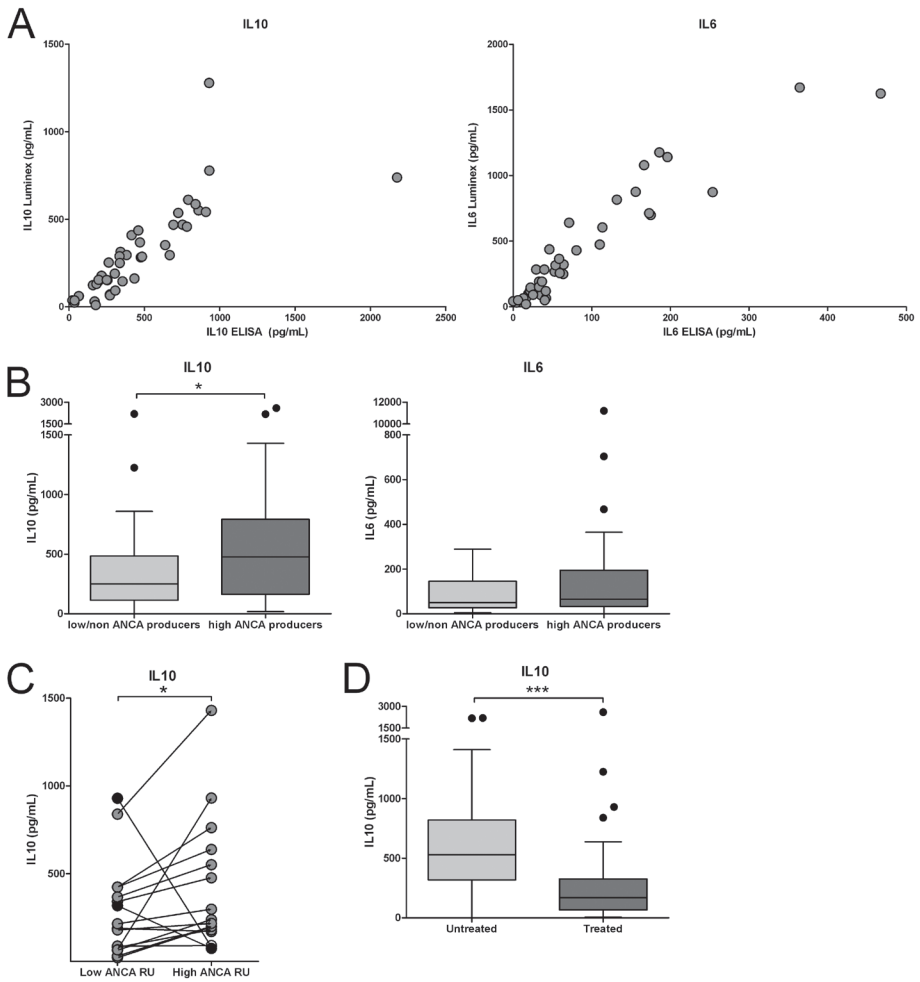


**Figure 2. IL10 and IL6 levels in the *in vitro* ANCA assay supernatants.** IL10 and IL6 levels were determined in 84 GPA patients by ELISA. (A) Results were compared with those from Luminex analysis. (B) Patients were divided in high ANCA producers (> 2.8 Response Units) and low/non ANCA producers. (C) For 16 patients two supernatant samples were tested for IL10, one with high and one with low levels of PR3-ANCA IgG. Each connected line represents an individual patient. (D) Patients were divided based on whether they were currently receiving immunosuppressive therapy. Box and whiskers plots (Tukey), boxes represent median values and interquartile range. *p<0.05, ***p<0.001.

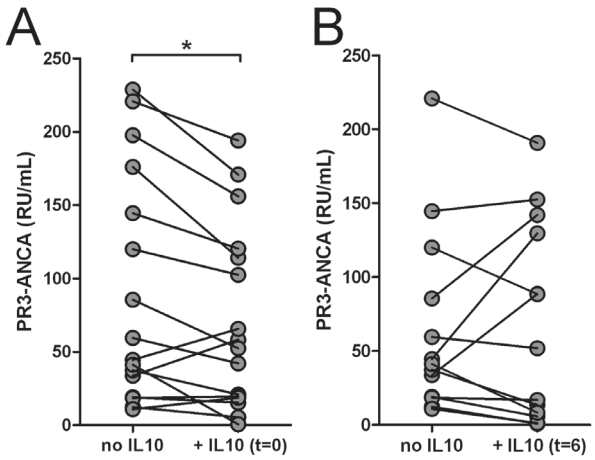


**Figure 3. Addition of IL10 to the *in vitro* ANCA assay.** PBMC were cultured for 12 days with CpG-ODN, BAFF and IL21 and levels of PR3-ANCA IgG were determined in the culture supernatants. Exogenous IL10 was added at (**A**) t=0 or (**B**) t=6 days. Each connected line represents an individual patient. *p<0.05.

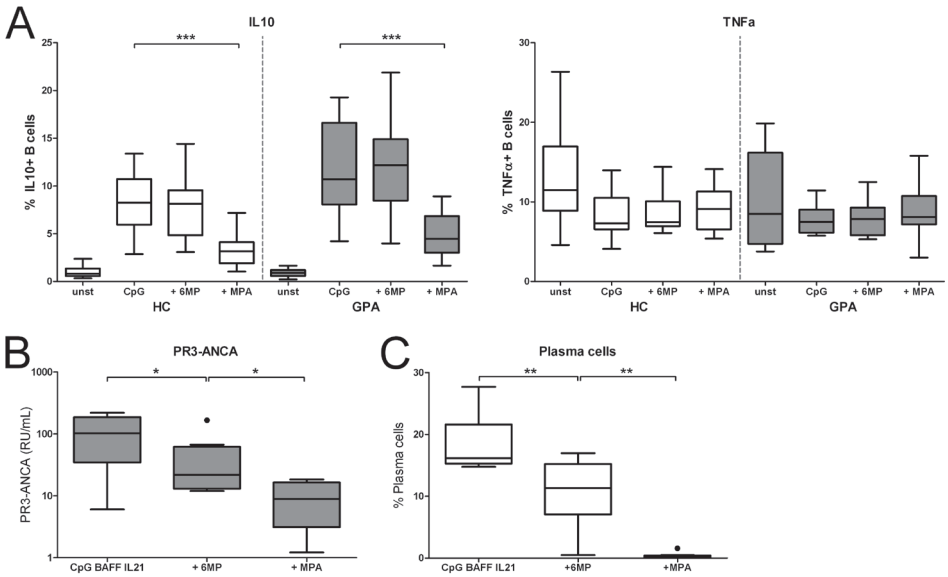


**Figure 4. Effect of treatment of B cell function.** (**A**) PBMC from GPA patients (n=17) and healthy controls (n=12) were stimulated with CpG-ODN for 72 hours and production of IL10 and TNFα was determined intracellularly in B cells. (**B**) PBMC from GPA patients (n=7) were cultured for 12 days with CpG-ODN, BAFF and IL21 and levels of PR3-ANCA IgG were determined in the culture supernatants. (**C**) PBMC from healthy controls (n=8) were cultured for 7 days with CpG-ODN and percentages of plasma cells within the B cell population were determined as CD19+CD27$^{high}$CD38$^{high}$ cells. 6MP and MPA were added once at the start of cell culture. Box and whiskers plots (Tukey), boxes represent median values and interquartile range. *p<0.05, **p<0.01, ***p<0.001.

## Discussion

We have previously demonstrated that PBMCs derived from the majority of GPA patients can be stimulated to produce PR3-ANCA *in vitro*. However, in some patients *in vitro* ANCA production is not detected, despite positive serum ANCA titers. Moreover, changes in PR3-ANCA levels can occur over time and patients may become positive or negative for *in vitro* ANCA production. Here, we hypothesised that the cytokine milieu induced *in vitro* upon PBMC stimulation is an important factor in the production of total and PR3-ANCA specific IgG. To investigate this, we measured a wide variety of cytokines in cell culture supernatants and related this to the production of PR3-ANCA. The Luminex analysis did not identify any clear differences between samples with high and no PR3-ANCA IgG production. However, for this analysis only a small number of patients and controls could be included, and the variation between individuals was substantial. There were several positive correlations between cytokines and PR3-ANCA production. This included several cytokines known to be involved in plasma cell formation and survival, IL10, IL6 and TNFα [13, 18]. Interestingly, not all cytokines that showed an association with PR3-ANCA IgG production were also associated with the production of total IgG levels. In fact, IL10 was the only cytokine to demonstrate a positive correlation with both total and PR3-ANCA positive IgG production. These results for IL10 were confirmed in a larger cohort, where IL6 did not appear increased in ANCA positive samples.

Levels of IL6 were much lower when measured by ELISA than by Luminex. The main difference here is an additional freeze-thaw cycle for the supernatant samples. It appears that this severely affected the concentration of IL6 in the samples. Previous investigation has indicated that levels of IL6 are stable in serum after 4 freeze-thaw cycles [22]. The effect of multiple freeze-thaw cycles on cytokines levels in culture supernatants needs to be further investigated.

IL10 is currently known for its anti-inflammatory properties, but the presence of high levels of IL10 produced *in vitro* did not appear to suppress the production of (PR3-ANCA) IgG. Conversely, when exogenous IL10 was added to cell cultures it was capable of reducing the production of PR3-ANCA. This effect was most prominent when added at the start of culture, with variable responses when added at a later stage. It is possible that the effect of IL10 on ANCA depends on the stage of plasma cell formation. When present prior to the formation of plasma cells, the formation of these cells may be inhibited. However, when plasma cells have already been formed, IL10 may act as a survival factor for these cells. To fully investigate the relation between IL10 and plasma cells in this culture system, the direct effect of depleting IL10 or adding IL10 at different time points on the plasma cell formation process needs to be investigated.

Using this *in vitro* culture system for ANCA production, it remains unclear whether increased levels of cytokines after 12 days of culture are the result of more production or less consumption. PR3-ANCA IgG and total IgG levels will reflect an accumulation over time, but this is not necessarily the case for cytokines. The increased IL10 in

ANCA positive samples may then also indicate a lack of usage, which could explain the increased production of ANCA. Similarly, the higher cytokine levels in samples from healthy controls compared to GPA in unstimulated samples could be due to differences in either cytokine production or consumption. This could potentially be caused by a different activation state of the immune cells from patients, an effect that is no longer seen when PBMC from patients and controls are stimulated.

We observed that in patients on current maintenance treatment the *in vitro* production of IL10 was significantly decreased. Therefore, we investigated the direct effect of the active metabolites of the two main used treatment options on cytokine production. Aside from IL10, we investigated the proinflammatory cytokine TNFα. While adding 6MP affected neither B cell IL10 nor TNFα production *in vitro*, addition of MPA significantly inhibited the production of IL10 by B cells. TNFα production was not affected by MPA, resulting in a skewing towards a proinflammatory cytokine environment. MPA also abrogated plasma cell formation *in vitro* and likely as a direct result from this significantly inhibited PR3-ANCA production. 6MP similarly, but to a lesser extent, inhibited plasma cell formation and PR3-ANCA production, even though IL10 production was not affected. The fact that MMF appears to inhibit production of IL10 whereas azathioprine does not is especially interesting in the context of the efficacy of these treatments. In a randomised controlled trial in AAV patients, treatment with MMF was proven to be less effective than azathioprine at maintaining disease remission [23]. It is possible that reduction of IL10 levels, and subsequent reduction of regulation may play a role in this difference. To further investigate this it would be interesting to compare cytokine production in B cells from patients on long-term azathioprine or MMF therapy. Moreover, serum IL10 levels could be compared between these patients groups. Finally, other current treatment strategies in AAV [24] could similarly be investigated *in vitro* for their effect on B cell cytokine and IgG production.

In summary, the data presented here indicate that the production of PR3-ANCA IgG is associated with cytokines known to be involved plasma cell formation and/or survival. The clearest association was seen with IL10. Conversely IL10 was also capable of suppressing PR3-ANCA production *in vitro*. We hypothesise that IL10 has a dual role in the production of (PR3-ANCA) IgG. It can exert a regulatory effect and suppress IgG production, but it can also increase formation and survival of plasma cells and thus increase IgG production. The actual effect will depend on timing and cell composition *in vitro*. Further research is needed to confirm the effect of IL10 on the formation of plasma cells.



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