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B cell phenotype and function in granulomatosis with polyangiitis

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Land, J. (2016). *B cell phenotype and function in granulomatosis with polyangiitis: Towards prediction of relapse*. [Thesis fully internal (DIV), University of Groningen]. Rijksuniversiteit Groningen.

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chapter 5

Regulatory and effector B cell cytokine production in patients with relapsing granulomatosis with polyangiitis

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Arthritis Research & Therapy 2016, 18:84

Abstract

Objectives

B cells are capable of producing regulatory and effector cytokines. In granulomatosis with polyangiitis (GPA) patients skewing of the pro- and anti-inflammatory cytokine balance may affect the risk for relapse. This study aimed to investigate differences in B cell cytokine production in (relapsing) GPA patients and controls, and determine whether this can aid in relapse prediction.

Methods

Thirteen GPA patients with an upcoming relapse were matched with non-relapsing patients and healthy controls in a retrospective design. The B cell subset distribution was determined from peripheral blood. Cryopreserved peripheral blood mononuclear cells were cultured and intracellular B cell production of regulatory (IL10) and effector (TNFα, IFNγ, IL2, IL6) cytokines was assessed. Finally, serum markers associated with B cell activation (sCD27) and migration (CCL19) were determined.

Results

GPA patient samples exhibited significantly lower percentages of TNFα+ B cells than controls, an effect that was most pronounced in patients about to relapse. B cell capacity for IL10 production was similar in patients and controls. No significant differences were observed for cytokine production in relapsing and non-relapsing GPA patients. TNFα production correlated strongly with IL2, IFNγ and the percentage of memory B cells. No change in effector cytokines occurred before relapse, while the percentage of IL10+ B cells significantly decreased. GPA patients in remission had increased serum levels of CCL19 and sCD27, and sCD27 levels increased upon active disease.

Conclusions

While differences in effector B cell cytokine production were observed between patients and controls, monitoring this in GPA could not clearly distinguish patients about to relapse. Prospective measurements of the regulatory cytokine IL10 may have potential for relapse prediction. Memory B cells appear mainly responsible for effector cytokine production. Increased migration of these cells could explain the decreased presence of TNFα+ B cells in the circulation.

Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) are associated with chronic inflammatory small vessel vasculitides such as granulomatosis with polyangiitis (GPA). Patients with GPA frequently have circulating autoantibodies directed against the neutrophil constituent proteinase 3 (PR3) [1]. GPA is a relapsing disorder and while numerous risk factors for relapse have been described no good markers are available to predict upcoming relapses in individual patients [2, 3]. B cells are important effector cells in autoimmune disease pathogenesis, not only as the producers of autoantibodies but also as antigen presenting cells and cytokine producers [4]. B cell depletion therapy using the anti-CD20 monoclonal antibody rituximab has proven to be an effective therapeutic strategy for inducing remission in GPA [5, 6], indicating a pathogenic role for these cells. Clinical improvement in rituximab treated patients can precede the reduction in autoantibody titers [7], highlighting the importance of antibody independent mechanisms of B cells. Moreover, a subset of B cells has been ascribed with regulatory function through the production of anti-inflammatory cytokines like interleukin (IL)10 [8]. These cells have been termed regulatory B cells (B_{reg}) but no commonly accepted phenotypical description exists for this subset. Several proposals made to identify B_{regs} in the circulation by their surface markers include $CD24^{high}CD38^{high}$ [9], $CD24^{high}CD27+$ [10] and CD5+ B cells [11], although B_{regs} are functionally defined as IL10 producing B cells. Several studies have examined IL10 production in patients with ANCA associated vasculitis (AAV). Results are inconclusive, as one demonstrated that active AAV patients have lower production of IL10 [12], another found decreased IL10 production in both active and remission patients [13] while the third did not detect any differences compared to healthy controls [14]. None of these studies examined whether IL10 production changes in individual patients prior to relapse or investigated production of other cytokines. This may be relevant since B cells are also capable of producing proinflammatory cytokines [15]. For these effector B cells (B_{eff}) numerous effects on the immune response have been described in mouse models [16]. Tumour necrosis factor (TNF) expressed by B cells promotes T-helper 1 (Th1) differentiation, leading to amplification of interferon (IFN)γ production by CD4+ and CD8+ T cells [17]. IFNγ produced by B cells could also support Th1 responses [18], and promote macrophage activation [19]. B cells can promote Th2 memory responses through production of IL2 [20] and produce large quantities of IL6, shown capable of increasing disease pathogenesis in experimental autoimmune encephalomyelitis models through activation of Th17 cells [7]. Moreover, several cytokines including IL6 and TNFα can support the survival of plasma cells [21]. Collectively, these observations indicate that cytokine production by B cells might be an important factor in autoimmune disease pathogenesis. In GPA patients the B_{reg} and B_{eff} cytokine production may be a factor that affects the balance between remission and relapse. However, data on human B cell cytokine production in autoimmunity is scarce. In the present study we investigated

whether production of pro- and anti-inflammatory cytokines by B cells in GPA patients deviates from that in healthy individuals and examined whether analysis of the B cell cytokine production profile could help predict upcoming relapses. As B cell cytokine production from peripheral cells may be affected by B cell migration and activation, markers for these processes were also assessed.

Patients and Methods

Study population

A cohort of 84 PR3-ANCA positive GPA patients was prospectively monitored for 15-24 months. Clinical parameters including current therapy were recorded and peripheral blood mononuclear cells (PBMCs) and serum samples were stored. During the period of sample collection 16 patients relapsed. Relapses were based on clinical judgement and had to result in the decision to increase or initiate immunosuppressive therapy. For the current study, patients were selected retrospectively based on relapse, availability of PBMCs and presence of more than 3% of B cells within the lymphocyte population. Thirteen patients were selected and age and sex matched with 13 GPA patients that did not relapse for at least 1.5 years and 13 healthy controls. Median time between sampling and relapse was 74 (range 14-157) days. For 11 relapsing patients samples from an earlier time point in remission were also available, and median time between the two samples taken prior to relapse was 98 (range 38-140) days. The diagnosis of GPA was based on definitions outlined in the Chapel Hill Consensus Conference [22] and all patients fulfilled the classification criteria of the American College of Rheumatology [23]. All subjects gave written informed consent according to the Declaration of Helsinki and the study was approved by the Medical Ethical Committee of the University Medical Center Groningen (METc UMCG 2012/151). Patient and control characteristics are listed in Table 1.

Flow cytometry for analysis of the B cell phenotype

EDTA blood was collected and 100 µl was incubated with anti-human CD19-eFluor450 (eBioscience, San Diego, CA, USA), CD24-FITC (BD biosciences, Franklin Lakes, NJ, USA), CD27-APC-eFluor780 (eBioscience), CD38-PeCy5 (eBioscience), CD5-PerCp-Cy5.5 (Biolegend, San Diego, CA, USA) or the corresponding isotype controls. After 15 minutes cells were treated with FACS Lysing solution (BD Biosciences). Samples were 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 in transitional, memory, naive, $CD24^{high}CD38^{high}$ and $CD24^{high}CD27+$ B cells as described previously [14]. CD5+ B cells were gated on an isotype control.

Cell culture and intracellular B cell cytokine pattern upon *in vitro* stimulation

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. The cryopreserved PBMC were thawed, concentrations adjusted to 10^6 cells/mL in RPMI + 10% FCS and cells were seeded in 24 wells flat bottom plates (Corning, NY, USA). Cells were left untreated or stimulated using 500 ng/mL CpG-oligodeoxynucleotides (ODN) 2006 (Hycult Biotech, Uden, the Netherlands). Culture plates were incubated for 72 hours at 37 °C with 5% CO2. During the last 5 hours of incubation 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO, USA), 2mM calcium ionophore (CaI; Sigma-Aldrich) and/or 10 µg/mL Brefeldin A (BFA; Sigma-Aldrich) were added to the cell culture. Cells were harvested and stained using anti-human CD19-eFluor450 and CD22-PeCy5 (Biolegend). Subsequently, cells were fixed and permeabilised for intracellular staining using a Fix&Perm kit (Invitrogen, Life Technologies, Grand Island, NY, USA) and incubated with antibodies against human IL10-PE (Biolegend), TNFα-Alexa Fluor 488 (BD biosciences), IL6-APC (eBioscience), IL2-PeCy7 (eBioscience) and IFNγ-Alexa Fluor 700 (BD biosciences). Samples were measured with an LSR-II flow cytometer and data were analysed using Kaluza 1.2. Samples that had not been stimulated with PMA and CaI 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.

Enzyme-linked immunosorbent assay (ELISA) for CCL19 and soluble CD27

Serum samples from healthy controls and patients had been collected and stored at -80 °C on the same day as PBMC storage and B cell phenotype analysis. Moreover, from the relapsing patients serum samples were available from time of active disease. A Human CCL19/MIP-3 beta DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) and a PeliKine Compact™ human soluble CD27 ELISA kit (Sanquin, Amsterdam, the Netherlands) were performed according to manufacturers' instructions. CCL19 levels are expressed as pg/mL and sCD27 levels as units (U)/mL.

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 interquartile range, unless stated otherwise. For comparison between groups the unpaired t-test was applied for data with Gaussian distribution and the Mann-Whitney U test was used for data without Gaussian distribution. For intra-individual comparison the paired t-test or Wilcoxon matched pairs test were

performed for Gaussian and non-Gaussian data, respectively. Correlation analysis was done using the Spearman rank correlation coefficient. P-values <0.05 were considered statistically significant.

Results

B cell subset distribution in GPA patients and HC

The B cell subset distribution in GPA patients differed from healthy controls. Specifically, GPA patients presented with lower percentages of CD27+ memory B cells (*p=0.0014*), $CD24^{high}CD27+$ B cells (*p<0.001*) and higher percentages of naive B cells (*p<0.001*) than healthy controls (Table 1). Relapsing and non-relapsing patients were not significantly different.

Table 1. GPA patient and healthy control characteristics

	HC	GPA, no future relapse	GPA, future relapse
subjects, n (% male)	13 (46)	13 (46)	13 (46)
Age, mean (range)	55 (44-74)	52 (32-76)	52 (32-75)
PR3-ANCA titer, median (range)		1:40 (0->640)	1:80 (20->640)
Disease duration in years, median (range)		12.6 (5.5-31.5)	15.3 (2.3-24.3)
Number of previous relapses, median (range)		2 (0-5)	5 (1-10)
Time to relapse after sample in days, median (range)		n/a	74 (14-157)
Disease form, n (%)			
Localised		0 (0)	0 (0)
Early systemic		2 (15.4)	2 (15.4)
Generalised		8 (61.5)	9 (69.2)
Severe		3 (23.1)	2 (15.4)
Treatment at time of sampling, n (%)			
Aza		1 (7.7)	3 (23.1)
Pred		1 (7.7)	1 (7.7)
MMF + pred		2 (15.4)	3 (23.1)
No immunosuppressive therapy		9 (69.2)	6 (46.2)
B cell phenotype in %, median (range)			
Total CD19+ B cells	12.3 (6.6-22.24)	9.8 (6.3-28.6)	9.9 (3.4-19.9)
Transitional B cells	10.77 (3.9-17.6)	9.2 (4.8-18.1)	9.3 (3.1-11.7)
Naive B cells	63.6 (37.4-83.4)	79.0 (74.5-90.1)	79.3 (60.1-85.7)
Memory B cells	22.4 (6.6-54.0)	8.1 (4.2-29.7)	8.2 (2.3-19.5)
$CD24^{high}CD38^{high}$ B cells	6.6 (2.2-9.6)	4.6 (2.1-11.8)	5.3 (0.9-8.5)
$CD24^{high}CD27+$ B cells	15.5 (3.5-45.4)	3.3 (2.1-24.8)	3.4 (0.7-10.8)
CD5+ B cells	23.4 (12.1-57.6)	19.8 (12.1-64.2)	18.9 (4.7-49.9)

ANCA, anti-neutrophil cytoplasmic antibody; Aza, azathioprine; GPA, granulomatosis with polyangiitis; HC, healthy control; MMF, mycophenolate mofetil; n/a, not applicable; pred, prednisolone.

Intracellular B cell cytokine pattern in GPA patients and controls

PBMC were cultured with or without CpG for 3 days, followed by a PMA and CaI stimulation for 5 hours. In all samples the production of TNFα, IL2, IFNγ, IL6 and IL10 was determined by flow cytometry (Figure 1). IL10 production was only clearly detectable when samples had been stimulated with CpG, without CpG IL10 was detected in less than 0.5% of B cells. In contrast, for the effector cytokines clear positive populations were present both with and without CpG stimulation. Simultaneous production of regulatory and effector cytokines was also observed in B cells. For example median double positivity for IL10 and TNFα was 20% of the

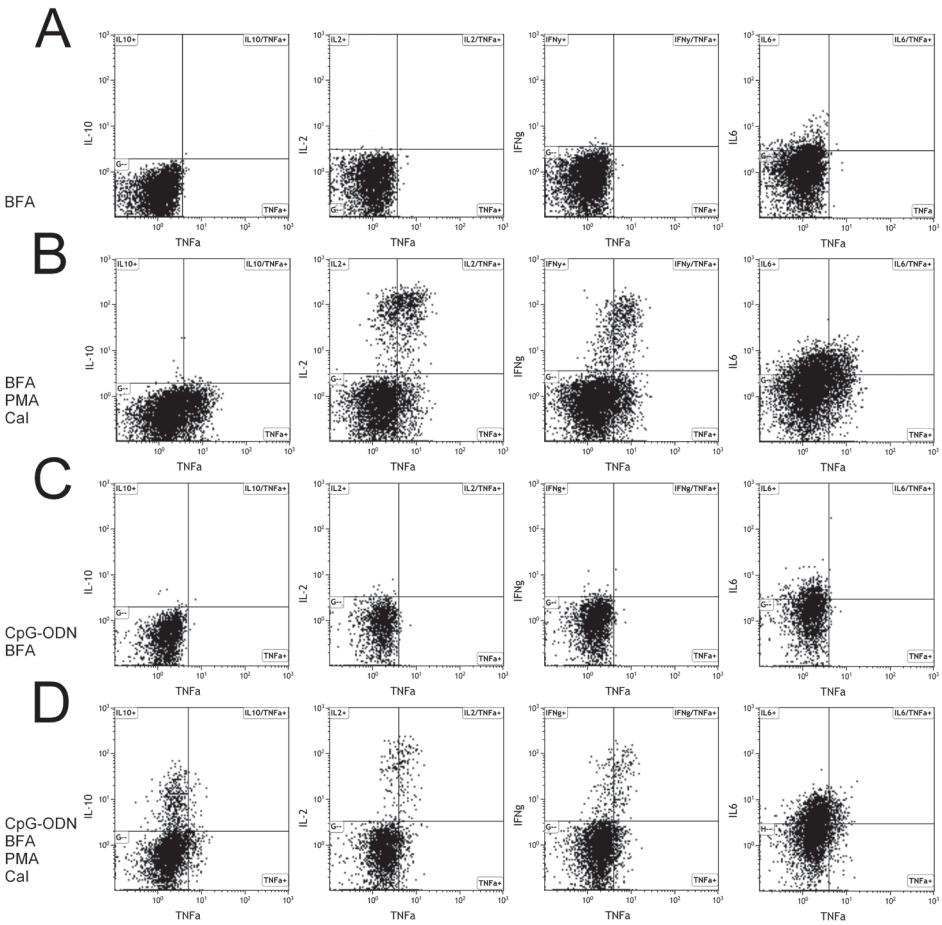

Figure 1. Flow cytometry gating example. Representative flow cytometry dot plots of CD19+CD22+ B cells. **(A)** BFA treated samples. **(B)** PMA and CaI stimulated, BFA treated samples. **(C)** CpG-ODN stimulated, BFA treated samples. **(D)** CpG-ODN stimulated, PMA and CaI stimulated, BFA treated samples. To determine the percentage of B cells positive for cytokine production A and C were used as negative controls to set the gates for B and D, respectively.

total IL10 positive B cell population, this proportion was similar for GPA patients and controls. Moreover, the B_{eff} cytokines IL2, IFNγ and TNFα were regularly produced together by B cells.

In samples not incubated with CpG a lower percentage of TNFα producing B cells was present in samples from GPA patients (median 7.3%, interquartile range 5.5-10.0%) compared to healthy controls (12.3, 8.7-18.8%, *p=0.003*). Expression of the other cytokines was not significantly different between controls and patients, although numerically the percentages of B cells positive for IL2 (6.7, 4.7-8.2% in HC vs 5.1, 2.7-6.8% in GPA) and IFNγ (6.0, 3.8-6.7% vs 4.2, 3.3-4.9%) were lower in patients, while IL6 (21.2, 15.6-26.3% vs 26.2, 20.9-31.1%) appeared mildly increased in GPA patients. In samples stimulated with CpG no significant differences were observed for B cell production of IL10 between healthy controls (6.7, 3.8-9.7%) and GPA patients (6.5, 3.6-9.6%), or for the effector cytokines (data not shown). Stimulation with CpG also affected the production of IL6, by inducing a significant increase in IL6+ B cells in both patient and control samples *(p=0.0014)*. Furthermore, a significant decrease of TNFα+ B cells was seen upon CpG stimulation in healthy control samples (*p=0.017*), whereas in patients a more diverse response existed. For the remaining analyses results of samples not stimulated with CpG were used, with the exception of IL10 results.

Changes in B cell cytokine production before relapse

Samples from patients who were diagnosed with a relapse after sampling were compared with samples from patients that had not relapsed for at least 1.5 years after sampling. There was no significant difference between patients that did or did not relapse with regard to production of the regulatory cytokine IL10. The differences we observed in the effector cytokine production for GPA patients and controls were most notable in GPA patients that had relapsed after sampling (Figure 2). There were fewer TNFα+ B cells in the non-relapsing patient samples (7.9, 5.5-10.0%, *p=0.045*) compared to the control samples, but when comparing relapsing patients (6.7, 4.6-8.0%, *p=0.002*) to controls this difference was clearly more pronounced. Relapsing patients also appeared to have fewer IL2+ B cells than controls, albeit not significantly (3.6, 2.3-7.2%, *p=0.058*). The same was seen for the increased proportion of IL6+ B cells in relapsing patients (27.7, 20.9-35.9%, *p=0.10*) compared to HC. However, when compared directly, the relapsing and non-relapsing patients were not significantly different in production of any cytokine. For 11 patients two consecutive samples before relapse were available and changes in cytokine production levels could be analysed. No clear change in the production of effector cytokines occurred prior to relapse (Figure 3A-D), while there was a significant decrease in IL10+ B cells (Figure 3E). As there was a discrepancy in the proportion of treated patients in the relapse and non-relapse groups we compared all patients with current immunosuppressive treatment (n=11) to those without (n=15). Percentages of TNFα, IL2 and IFNγ producing cells were

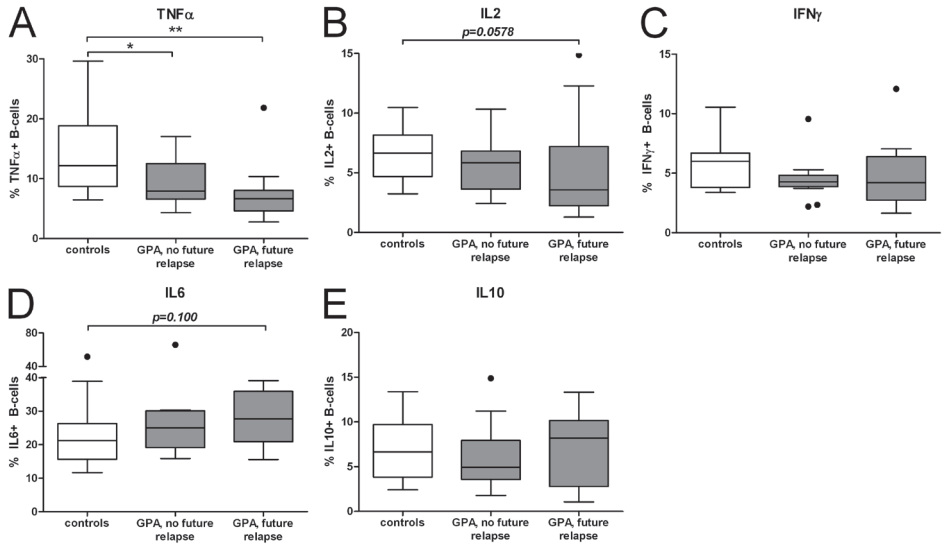

Figure 2. B cell cytokine production in (relapsing) GPA patients and controls. PBMCs were cultured (**A-D**) without CpG-ODN and (**E**) with CpG-ODN. Total percentages of cytokine producing B cells were determined within the CD19+CD22+ B cell population. Patients were divided in those that relapsed soon after the sampling (future relapse) and those that did not relapse for at least 1.5 years (no future relapse). Graphs represent data from 13 relapsing patients, 13 non-relapsing patients and 13 healthy controls. Box and whiskers plots (Tukey), boxes represent median values and interquartile range. *p<0.05, ***p<0.001.

significantly higher in patients that were currently being treated (Figure 4A-C), as more patients were treated in the relapse group this is unlikely to affect the significance of our results. IL10 and IL6 production was not significantly different in treated and untreated patients (Figure 4 D-E).

Correlation analysis for the B cell phenotype and cytokine production

The results from the correlation analysis for the B cell subset distribution and B cell cytokine production are summarised in Table 2. Results are from the combined cohorts of GPA patients (n=26) and healthy controls (n=13). The effector cytokines TNFα, IL2 and IFNγ correlated strongly with each other. Production of these cytokines, in particular TNFα, also demonstrated a strong positive correlation with the percentage of circulating CD27+ memory B cells. These results were also observed when GPA patients were analysed separately. A positive correlation for the production of IL6 and IL10 was detected in CpG stimulated samples. These cytokines did not correlate with any specific B cell subset. Interestingly no correlation could be found between the production of IL10 and any of the proposed B_{reg} populations.

CCL19 and sCD27 serum levels are increased in GPA patients

As results were obtained using peripheral blood cells, differences in B cell migration and activation were investigated by analysing the B cell trafficking chemokine CCL19 [24, 25] and soluble CD27 serum levels by ELISA. Levels of CCL19 were significantly increased in GPA patients in remission (144, 105-281 pg/mL) compared to healthy controls (102, 81-131 pg/mL; Figure 5A). When remission patients were divided based on future relapse, no significant difference was observed in CCL19 serum levels between patients that did (153, 99-402 pg/mL) and did not (142, 105-187 pg/mL) relapse after sampling (Figure 5C). Within the relapsing patient group CCL19 levels did not change

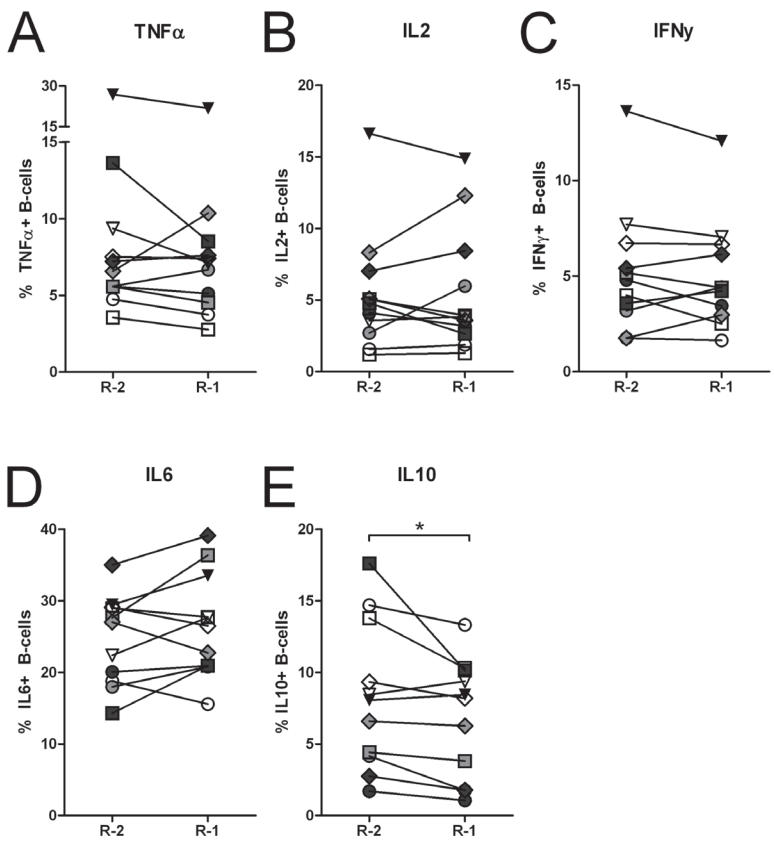

Figure 3. Changes in the B cell cytokine production before relapse. For eleven relapsing patients two consecutive time points before relapse were analysed simultaneously, to determine whether changes in cytokine production occurred prior to relapse. PBMCs were cultured (A-D) without CpG and (E) with CpG. Total percentages of cytokine producing B cells were determined within the CD19+CD22+ B cell population. Graphs represent data from 11 patients, each connected line represents an individual patient. The different symbols indicate the individual patients in each graph. R-1 indicates the sample taken closest to relapse and R-2 the sample taken before. *p<0.05.

between the two time points before relapse, or upon active disease (Figure 5E). Serum levels of CCL19 did not correlate with the B cell phenotype distribution.

Table 2. Correlation analysis results

		% TNFα+	% IL6+	% IL2+	% IFNγ+	% IL10+
% memory	Spearman's rho	**0,864**	-0,063	**0,586**	**0,576**	0,053
	p-value	**< 0,0001**	0,702	**< 0,0001**	**0,0001**	0,747
% $CD24^{high}CD38^{high}$	Spearman's rho	-0,237	-0,292	**-0,364**	-0,168	0,049
	p-value	0,146	0,071	**0,023**	0,308	0,768
% $CD24^{high}CD27+$	Spearman's rho	**0,834**	-0,115	**0,553**	**0,467**	0,043
	p-value	**< 0,0001**	0,487	**< 0,001**	**0,003**	0,797
% CD5+	Spearman's rho	-0,199	0,021	-0,132	-0,214	0,047
	p-value	0,291	0,914	0,488	0,257	0,804
% TNFα+	Spearman's rho		-0,012	**0,733**	**0,663**	0,237
	p-value		0,940	**< 0,0001**	**< 0,0001**	0,146
% IL6+	Spearman's rho			0,100	0,124	**0,364**
	p-value			0,545	0,451	**0,023**
% IL2+	Spearman's rho				**0,527**	-0,061
	p-value				**0,001**	0,714
% IFNγ+	Spearman's rho					-0,085
	p-value					0,607

IFN, interferon; IL, interleukin; TNF, tumour necrosis factor. Significant results are indicated in bold text.

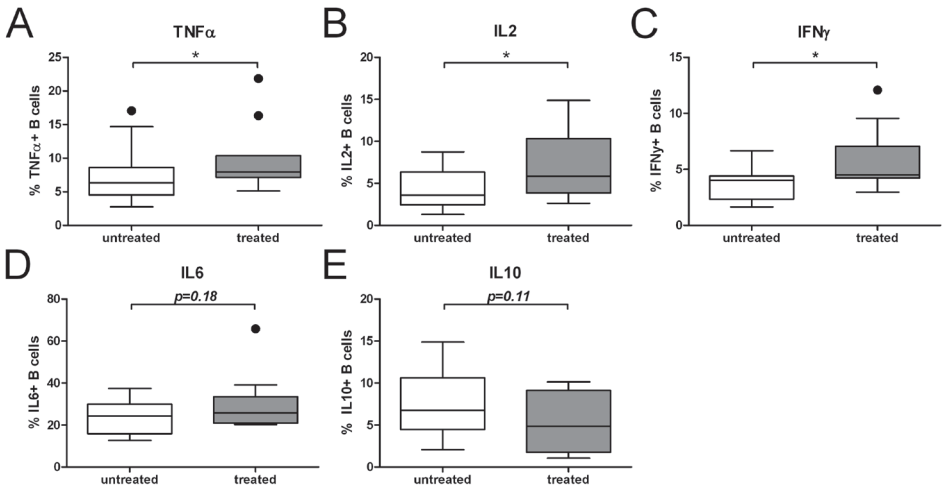

Figure 4. Current medication use and B cell cytokine production. (A-E) All GPA patients were divided based on whether they were receiving immunosuppressive treatment at time of sampling. Graphs represent data of 11 patients that were treated at time of sampling and 15 that were not treated. Box and whiskers plots (Tukey), boxes represent median values and interquartile range. *p<0.05.

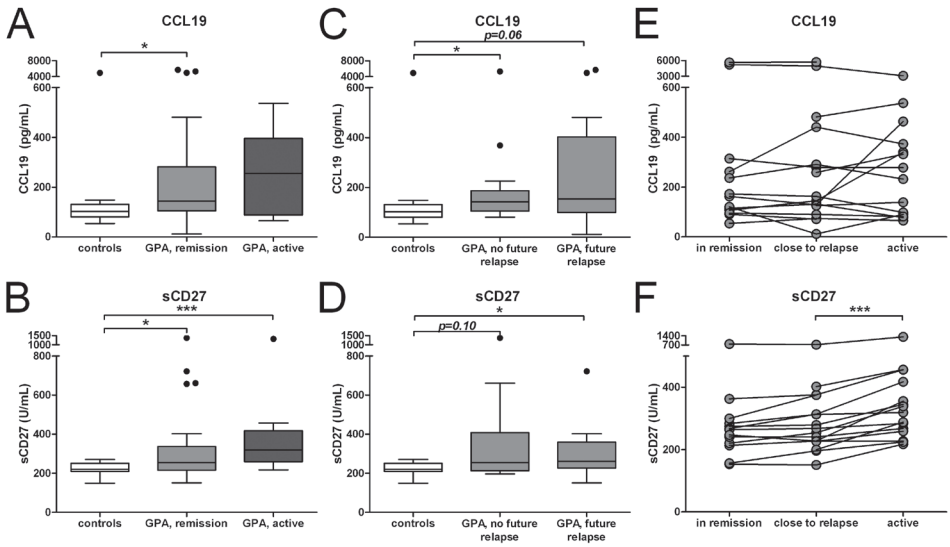

Figure 5. Serum CCL19 and sCD27 levels in (relapsing) GPA patients and controls. Serum levels of CCL19 and sCD27 were determined by ELISA. (**A-B**) Patients in remission and with active disease were compared to healthy controls. (**C-D**) Remission patients were divided in those that relapsed soon after the sampling (future relapse) and those that did not relapse for at least 1.5 years (no future relapse). Box and whiskers plots (Tukey), boxes represent median values and interquartile range. (**E-F**) The relapsing patients were compared for two consecutive time points in remission and the ensuing active disease time point. Each line represents an individual patient. *p<0.05, ***p<0.001.

Serum levels of sCD27 were significantly increased in patients in remission (255, 216-337 U/mL) and patients with active disease (319, 259-418 U/mL) compared to healthy controls (221, 210-251 U/mL; Figure 5B). Concentrations were not different when patients were divided in those with (261, 227-360 U/mL) and without (255, 213-407 U/mL) an upcoming relapse (Figure 5D). While no clear change in sCD27 levels occurred prior to relapse, when patients presented with active disease a significant increase of sCD27 (median 21%) was observed (Fig 5F). sCD27 serum levels did not correlate with the percentage of circulating CD27+ memory cells, or other B cell subsets in remission patients and controls.

Discussion

The antibody independent functions of B cells are of increasing interest. This includes the production of both proinflammatory and anti-inflammatory cytokines. In particular the production of IL10 and the presence of B_{regs} in autoimmune disease are currently under investigation. Scant information is available about B cell cytokine production in patients with GPA, especially B_{eff} cytokine production, and how this may relate to an upcoming relapse. Here we describe the B cell cytokine production profile in GPA

patients in remission, patients about to relapse and matched healthy controls.
The most prominent result of this analysis is that GPA patients exhibit a significantly lower percentage of TNFα producing B cells in the circulation, and this is especially visible in patients that are about to relapse. TNFα producing B cells correlated very strongly with the presence of CD27+ memory B cells in the circulation. We observed here that GPA patients have a decreased proportion of CD27+ memory B cells in peripheral blood, confirming our previously described results [14]. Moreover, we have found that CD27+ memory B cells are even further decreased in patients prior to relapse (unpublished data). In patients with rheumatoid arthritis (RA) CD27+ memory B cells had a greater capacity to produce TNFα than naive B cells [26]. It is likely that the reduced proportion of CD27+ memory cells in GPA patients [14] explains the lower percentage of TNFα producing cells we observe. Both IL2 and IFNγ production correlated strongly with the production of TNFα and the percentage of CD27+ memory B cells, suggesting that effector B cells produce several proinflammatory cytokines simultaneously.
One potential explanation for the decreased presence of TNFα producing CD27+ memory B cells in the circulation is a higher rate of B cell trafficking in GPA patients that mainly affects the memory B cells. As a first attempt to investigate this option the B cell trafficking chemokine CCL19 was measured in serum samples. CCL19 has been described to especially enhance the migration of CD27+ memory B cells [25]. Levels of CCL19 were indeed increased in GPA patients compared to controls, suggesting that B cell migration is intensified in these patients. In RA patients the level of serum CCL19 demonstrated a weak inverse correlation with the frequency of circulating CD27+ memory B cells [27] and increased levels of CCL19 were found in synovial tissue [28]. Moreover, CCL19 is involved in the formation of ectopic lymphoid structures [24]. We did not observe a correlation between CCL19 and the percentage of CD27+ cells in the GPA cohort or the healthy controls. However, the correlation described in RA did not have a high correlation coefficient, and the number of subjects in our study is substantially lower. Increased migration of CD27+ memory B cells is a viable option to explain their decreased proportion in the circulation of GPA patients. To prove this investigation of tissue biopsies is warranted. Concentrations of CCL19 did not change before or at time of relapse, indicating that it cannot distinguish active disease from remission, and is not a useful marker to predict relapses.
Soluble CD27 is a marker for both B and T cell activation [29, 30]. Levels of sCD27 were significantly increased in GPA compared to controls. In contrast to CCL19, the concentration of sCD27 in serum significantly increased with active disease. The increased levels of sCD27 may be a reflection of lymphocyte activation in GPA, which increases upon relapse. While not predictive for disease relapse, sCD27 could potentially be a marker for active disease. Another explanation for increased sCD27 levels is increased shedding of the receptor. Surface CD27 can be cleaved through the action of matrix metalloproteinases (MMPs) [31]. As patients with active GPA

demonstrate upregulation of several MMPs [32] this could explain the increased sCD27 in active disease samples. Moreover, the presence of CD27 negative memory B cells has been described and it was speculated that the CD27- memory compartment contains autoreactive B cells that have downregulated their activation molecules in an attempt to limit autoreactivity [33]. While these cells are scarce in healthy individuals, they are significantly increased in patients with systemic lupus erythematosus (SLE) [34], and serum levels of sCD27 are also increased in SLE patients compared to controls [35]. The CD27- B cells could be a source of sCD27 present in the serum, however, as T cells can also express CD27 it is not the only possible source.

With regard to the B_{reg} cytokine IL10 there were no differences in total percentages of IL10 producing B cells between controls and (relapsing) patients. This result is in line with our previous observations which showed a similar capacity for IL10 production by B cells in AAV patients and controls [14]. However, there was a significant decrease in IL10 production in individual patients approaching a relapse, indicating that B cell regulatory capacity may decrease in individual patients prior to relapse. To fully confirm these changes further investigation is warranted.

The production of IL10 did not correlate with any of the proposed surface marker phenotypes for B_{regs}. However, none of these phenotypes have been fully confirmed as clear B_{reg} populations. Moreover the stimulation with CpG for three days may affect the phenotype of B cells in culture, resulting in a different subset distribution. For one, CpG has been described to induce naive B cell proliferation, without causing maturation into memory cells [36] and it is capable of prolonging the lifespan of mature-naive B cells *in vitro* [37]. Such changes could help explain a lack of correlation between a phenotype analysis in the peripheral blood and cytokine expression after CpG stimulation, although one study has shown that 66 hours of CpG stimulation did not significantly affect the B cell subset distribution [38]. Regardless, additional phenotypical analysis post CpG stimulation may give a clearer picture of which B cells are responsible for the production of IL10 in this setting.

IL10 production did correlate with the production of IL6 in Toll-like receptor (TLR) 9 activated B cells, even though IL6 is generally regarded as a proinflammatory cytokine and overproduction has been related to onset of autoimmunity [39]. Moreover, blockade of the IL6 receptor has proven to be an effective therapeutic target in several autoimmune diseases, including RA [40] and giant cell arteritis [41]. Marginal zone B cells that produced high levels of IL6 in response to TLR4 stimulation simultaneously produced increased levels of IL10 [7]. These cytokines can be separated, for example calcineurin antagonists were demonstrated to selectively inhibit IL10 release without affecting secretion of IL6 in TLR9 stimulated B cells [42]. In addition, we demonstrate here that B cells can produce IL10 and TNFα concomitantly, which has been described previously with $CD24^{high}CD27+$ B cells having the largest fraction of TNFα/IL10 dual positivity [43]. The capacity of certain B cells to demonstrate both regulatory and effector roles indicates that the *in vivo* context is crucial in determining the B cell

function. While we demonstrate here that GPA patients and healthy individuals have a similar capacity for B cell IL10 production after TLR9 activation, differences in the *in vivo* environment could result in different B cell cytokine production profiles.
In conclusion, GPA patients exhibit a decreased percentage of TNFα producing B cells compared to controls. This is most prominent in those patients that are about to relapse. However, effector B cell cytokine production could not clearly distinguish which patients were about to relapse. IL10 production by B cells did decrease before relapse, and monitoring this may have potential for relapse prediction. Production of the effector cytokines TNFα, IL2 and IFNγ by B cells is highly correlated with the presence of CD27+ B memory cells in the circulation. Increased CCL19 levels suggest a higher B cell migration in GPA which may particularly affect memory B cells. The resulting changes in the B cell subset distribution in GPA are likely responsible for the aberrant proinflammatory cytokine production.

Acknowledgements

This work was supported by funding from the Dutch Arthritis foundation [Reumafonds project number 11-1-405].

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