Circulating T follicular helper cell and regulatory T cell frequencies are influenced by B cell depletion in patients with granulomatosis with polyangiitis

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Abstract

Objective. Granulomatosis with polyangiitis (GPA) is a rare and sometimes fatal systemic autoimmune disease. ANCAs specific for PR3 are associated with GPA. Remission in GPA can be achieved through B cell depletion (BCD) therapy. Our aim was to understand whether the frequencies of T cell subsets are influenced by BCD.

Methods. The frequencies of circulating T follicular helper cells (cTFHs) and regulatory T cells (Tregs) from 36 GPA patients including 11 rituximab-treated patients and 10 healthy controls were studied by flow cytometry. The functional capacity of Tregs was assessed by in vitro co-culture assays.

Results. We observed an increased frequency of cTFHs and a reduced frequency of antigen-experienced Tregs in peripheral blood from GPA patients on conventional therapies but not in those treated with rituximab compared with healthy controls. Furthermore, the ratio of cTFHs to Tregs was significantly higher in GPA patients on conventional therapies than in GPA patients treated with rituximab who were clinically improved or controls. Whereas Tregs were numerically reduced in GPA patients on conventional therapy, the suppressive capacity of Tregs on a per cell basis was not significantly altered in these individuals.

Conclusion. Our study illustrated increased cTFHs with decreased antigen-experienced Tregs in GPA patients on conventional therapies, but in B cell-depleted patients the levels of cTFHs and Tregs were similar to healthy controls. The negative correlation between cTFHs and Tregs implies the balance between T cell subsets and its B cell dependence impact on disease activity in GPA.

Key words: granulomatosis with polyangiitis (GPA), follicular helper T (TFH), regulatory T (Treg) cells, B cell depletion.

Introduction

Granulomatosis with polyangiitis (GPA) is an autoimmune disease that affects all ethnicities with an equal male to female ratio and a higher prevalence in Caucasians [1–3]. Serologically the majority of patients develop ANCA with specificity for PR3 (ANCA/anti-PR3) [4–7]. The autoantibodies produced in the mucosa in GPA patients are encoded by genes that are mutated from the germline by somatic hypermutation [8, 9]. This is the classic hallmark of previous transit through germinal centres, although conventional germinal centre structures are not a common feature of the inflamed mucosa in GPA [10]. GPA has benefited from B cell depletion (BCD) therapy with rituximab—a chimeric monoclonal anti-CD20 antibody that received approval from the US Food and Drug Administration and the European Medicines Evaluation Agency for use in GPA in 2011, based on two successful
non-inferiority randomized controlled clinical trials [11, 12]. Although the pathology in GPA may be autoantibody mediated, it is unlikely that disease remissions achieved by rituximab are a consequence of autoantibody depletion alone. There is increasing evidence that rituximab depletes B cells that may interact with T cell subsets that contribute to the disease process.

While autoimmune diseases are consistently linked with autoantibody production implicating B cell involvement, T cells are intrinsically associated with the generation of autoimmune pathology. Certain T cell subsets may be associated with a T cell–dependent B cell response [13–16]. T follicular helper cells (TFHs), for example, are a T cell subset that normally resides in the germinal centres of secondary lymphoid tissues [17]. Clones of TFHs are known to disseminate, so that a single TFH clone can be detected in both tonsils [18]. Small populations of TFHs in blood are referred to as circulating TFH (cTFH) [19]. Whereas TFHs are defined by their microanatomical location in the germinal centres in tissues, cTFHs can be detected in the blood by their expression of CXCR5, which facilitates migration towards CXCL13 produced in the germinal centres, and co-expression of CD57, programmed death 1 (PD-1) and inducible co-stimulator (ICOS) [20]. Although the potential for cTFH to help B cells has been demonstrated, the precise lineage of these cells and their relationship to TFHs is unknown [21, 22].

TFHs are involved in the progression of autoimmunity in a mouse model of SLE by lowering the threshold for B cell survival [23]. This lack of stringency in T cell–mediated B cell selection permits the evolution of autoreactive antibody-secreting cells. A correlative study has suggested that cTFHs may be involved in autoimmune responses in humans in a subset of patients with severe SLE, where the cTFH population is significantly larger than that in controls [20].

Regulatory T cells (Tregs) are critical regulators in autoimmune diseases by suppressing the proliferation and cytokine production of effector T cells [24, 25]. The failure of Tregs to suppress inflammation may facilitate the development of autoimmune pathology. Some studies have shown Treg frequencies to be reduced in GPA, and other studies have detected defects in the suppressive function of Tregs in GPA—both sets of observations implicate Tregs in the disease process [26–28]. Interestingly, Treg frequencies have been shown to increase in the blood of patients with SLE following rituximab [15]. This could be a secondary consequence of clinical remission, or if Tregs are actually associated with the disease process, these data might imply that Tregs are in some way B cell dependent.

In this cross-sectional study, we quantified cTFHs and Tregs in GPA patients who were on conventional oral nonbiologic immunosuppressive therapies or who had responded clinically to BCD with rituximab to determine if any changes in these immunoregulatory T cell subsets are associated with BCD.

Materials and methods

Ethics approval

This study was approved by the National Research Ethics Service (approval no. 10/H0715/3) and by the Guys and St Thomas’ Hospital Ethics Committee. All patients donated blood after giving fully informed consent.

Patients

GPA patients attending the systemic vasculitis clinic in the Louise Coote Lupus Unit at St Thomas’ Hospital were invited to participate in this study. GPA patients had either active or inactive disease and fulfilled the ACR classification criteria [29]. Data on disease activity using the BVAS were collected at the time of venesection [30, 31]. Information on organ involvement and treatment at the time that the sample was taken was also collected (supplementary Tables S1 and S2, available at Rheumatology Online).

Patients who underwent BCD with rituximab did so based on clinical need. These patients had all previously had induction therapy with corticosteroids and standard immunosuppressive agents and still had active or relapsing disease despite maintenance therapy. All patients gave written informed consent prior to receiving rituximab. Our protocol was to continue oral corticosteroids but to discontinue immunosuppressive agents 1 week prior to rituximab. Patients received rituximab 1 g on day 1 and day 15. Prior to each infusion, patients received i.v. methylprednisolone 100 mg and i.v. chlorpheniramine 10 mg to minimize the risk of a hypersensitivity reaction. Additional medication was sometimes required, as indicated in supplementary Table S2, available at Rheumatology Online. Some patients with active or relapsing GPA were identified as suitable for rituximab therapy but after counselling choose to have an alternative immunosuppressive therapy. These patients were therefore included in the group receiving conventional immunosuppressive therapies.

Four patients were followed longitudinally. The patients donated blood before rituximab therapy and again 1 month after the second infusion. Age- and gender-matched healthy controls [age range 22–68 years, male:female (M:F) ratio 1:1] donated blood following written informed consent.

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque from fresh blood samples donated by 36 GPA patients (M:F = 17:19, ages 25–83, average 55.3 years) including 11 rituximab-treated patients (M:F = 7:4, ages 38–73, average 51.5 years) and 10 healthy controls (M:F = 1:1, ages 22–68, average 45 years). Details of the patients’ clinical information are listed in supplementary Tables S1 and S2, available at Rheumatology Online.
Flow cytometry

PBMCs were incubated with fluorochrome-conjugated mAbs on ice for 20 min. To identify cTFHs, PBMCs were stained with CD3-APC-Cy7, CD4-Pacific Blue, CXCR5-PE and PD-1-APC. cTFHs were identified as CD4+CD127lowCD25highPD-1highCXCR5high [19]. To identify Tregs, PBMCs were stained with CD4-Pacific Blue, CD14-APC-H7, CD127-PerCP-Cy5.5, CD25-PE and CD45RA-FITC. Tregs were identified as CD4+CD127lowCD25high. Naive, CD127-PerCP-Cy5.5, CD25-PE and CD45RA-FITC were stained with CD4-Pacific Blue, CD14-APC-H7, CD127-PerCP-Cy5.5, CD25-PE and CD45RA-FITC. Naive, memory and activated Tregs (mTregs and aTregs) were further identified by the expression of CD45RA and CD25. Tregs were also examined for expression of FoxP3 using the eBioscience FoxP3 Fix/Perm buffer set according to the manufacturer’s instructions (eBioscience, San Diego, CA, USA). After staining, cells were analysed using a BD FACSCanto II (BD Biosciences, San Jose, CA, USA). All samples were analysed in a blinded manner.

Treg cell functional study

Regulatory (CD4+CD127lowCD25high) and effector (CD4+CD127+CD25low) T cells were isolated using a BD FACS-Aria II flow cytometer (BD Biosciences) for functional studies. In vitro co-culture suppression assays were performed by culturing effector T cells (2.5 x 10^5/well) in the presence or absence of autologous Tregs at the ratios indicated. Cells were activated by Dynabeads Human T-Activator anti-CD3/anti-CD28 beads (Life Technologies, Carlsbad, CA, USA) at a bead:conventional cell ratio of 1:1, 1:2 or 1:4. All assays were conducted in triplicate. After 5 days of culture, proliferation was assessed by the addition of 0.5 μCi/well [3H]thymidine for the final 18 h of co-culture. The percentage of suppression was calculated using the following formula: % suppression = 100 – (cpm with Tregs ÷ cpm without Tregs x 100). All analyses were performed in a paired manner with one GPA and one control individual. A total of three pairs were studied.

Statistical methods

Data sets are expressed as mean (s.d.) or average (range) and analysed using the Student’s t-test for normally distributed data and the Mann-Whitney U test for non-normally distributed data. Paired data for four patients before and after rituximab treatment were compared using a paired Wilcoxon signed-rank test (non-parametric). Statistical analysis was carried out using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

Results

Patient population

Twenty patients who had active GPA and nine patients who had inactive GPA (BVAS = 0) had all received corticosteroids and standard immunosuppressive therapies such as i.v. CYC as induction therapy and MMF, oral MTX and AZA as maintenance agents. Eleven patients who had severe active or refractory GPA despite standard immunosuppressive therapies were treated with rituximab. In total, 36 patients were studied, as 4 rituximab-treated patients were classified as both active GPA and rituximab treated. Patients classified with active GPA had blood samples taken before increasing their standard immunosuppressive therapy. Details are summarized in supplementary Tables S1 and S2, available at Rheumatology Online. BVASs improved significantly following rituximab in GPA patients (average 18.2, range 4–35 vs average 6.4, range 0–13) (supplementary Fig. S1, available at Rheumatology Online; P = 0.0009).

No difference was observed in the clinical manifestations of the most severe diseases between the two groups of GPA patients: renal manifestations (n = 5 in BCD GPA, n = 6 in active GPA), severe lung manifestations (n = 5 in BCD GPA, n = 5 in active GPA), neurological manifestations (n = 6 in BCD GPA, n = 10 in active GPA) and tracheal stenosis (n = 0 in BCD GPA, n = 1 in active GPA). The median vasculitis damage index score was 2 (range 0–5) and 1 (range 0–4) in the BCD GPA group and active GPA on conventional therapy group, respectively. At the time of blood sampling, average ESR values were 11.8 mm/h (range 4–24) and 13.5 mm/h (range 2–85) and average CRP values were 11.1 mg/l (range <5–20) and 26.6 mg/l (range <5–134) for the groups of patients after rituximab infusion and on conventional therapies, respectively.

Increased frequency of cTFHs in peripheral blood from GPA patients on conventional therapies but not in GPA patients treated with rituximab

No significant difference was observed in total lymphocyte count between GPA patients on conventional therapies and those following rituximab (Fig. 1A). cTFHs were identified as CD3+, CD4+ lymphocytes expressing high levels of CXCR5 and PD-1 (Fig. 1C). Consistent with other studies, cTFHs comprised an average 0.2% of CD4+ T cells in the blood of healthy donors [20]. We observed a higher frequency of cTFHs from two groups of GPA patients on conventional therapies (active and inactive) when compared with healthy controls (Fig. 1D; P = 0.001, P = 0.0003, respectively). No difference was observed between the inactive GPA patient group (BVAS = 0) and the active GPA patient group (median BVAS = 8, range 3–30) (Fig. 1D; P = 0.3). When we measured the frequency of cTFHs in GPA patients following rituximab who were confirmed to be B cell depleted (Fig. 1B) [32], we observed no significant difference compared with healthy controls (Fig. 1D; P = 0.16), while there was a significant decrease in cTFH frequencies compared with patients with active GPA on conventional therapies (Fig. 1D; P = 0.04).

Reduced frequency of peripheral blood Tregs in individuals with GPA on conventional therapies but not in GPA patients treated with rituximab

As described by Liu et al. [33], Tregs were identified as CD4+CD127low, CD25high (Fig. 2A). Co-staining of this population for expression of FoxP3 confirmed that this population, but not control populations
of effector cells, consisted primarily of FoxP3+ cells (Fig. 2B). For further validation of the gating strategy used, see supplementary Fig. S2, available at Rheumatology Online.

Consistent with other studies, Tregs comprised on average 7.7% of CD4+ T cells in healthy controls [34]. We observed a significantly reduced frequency of Tregs in GPA patients on conventional therapies (average 4.67%) compared with healthy controls (Fig. 2C; \( P < 0.0001 \)). No difference was observed between active and inactive GPA patients (Fig. 2C; \( P = 1 \)). The frequency of Tregs in GPA patients following rituximab (average 8.36%) was similar to that of healthy controls (Fig. 2C; \( P = 0.75 \)), while Treg frequencies were significantly lower than in GPA patients on conventional therapies (Fig. 2C; \( P = 0.0004 \)).

Prospective analysis of cTFH and Treg frequencies in patients with GPA before and after rituximab

Blood samples were taken from four GPA patients before the first infusion and 1 month after the second infusion of rituximab and the frequencies of cTFHs and Tregs were quantified. The patients responded to the therapy with a significant decrease in BVASs (Fig. 3A; \( P = 0.0017 \)). There was a significant decrease in cTFH frequencies after treatment (Fig. 3B; \( P = 0.021 \)). A consistent trend for Treg frequencies to increase was also observed (Fig. 3C).

Reduced frequencies of aTreg and mTreg in individuals with GPA on conventional therapies but not in GPA patients treated with rituximab

Sakaguchi et al. [35] delineated distinct populations of Tregs based on expression of CD25 and CD45RA, which represent different stages of differentiation: (i) resting Tregs (rTregs) expressing CD45RA and intermediate levels of CD25, (ii) mTregs expressing intermediate levels of CD25 but not CD45RA and (iii) aTregs expressing high levels of CD25 but not CD45RA. We therefore divided Tregs into three populations based on expression of CD25 and CD45RA as shown in Fig. 4A and...
These analyses revealed that the reduced frequency of Tregs observed in active GPA patients on conventional therapy was only observed in CD45RA+CD127− Tregs (both in the mTreg and aTreg cell populations) (Fig. 4B and C). In contrast, we only observed a reduced frequency of CD45RA+ naive Tregs in the inactive GPA patient group compared with healthy controls (Fig. 4D; \( P = 0.044 \)), but not with any of the other groups.

Peripheral blood Tregs from GPA patients were functionally suppressive

We investigated Treg functionality in individuals with active GPA on conventional therapy by conventional Shevach suppression assays using a range of stimulation strengths. Consistent with the observations described above, Tregs isolated from individuals with GPA on conventional therapy for functional studies had a reduced frequency compared with healthy controls studied in parallel. We did not observe a significant difference between Treg-mediated suppression of autologous effector T cells whether Tregs were derived from a patient with GPA or an age- and gender-matched control (Fig. 5). Taken together, these data suggest that, whereas Tregs are numerically reduced in individuals with GPA on conventional therapy, the suppressive capacity of Tregs on a per cell basis was not significantly altered in these individuals.

Discussion

We observed that the peripheral blood of GPA patients on conventional therapies contains a higher frequency of cTFHs compared with that of controls. This has previously been seen as a feature of a subset of patients with the most severe presentation of SLE [20]. TFHs are physiologically associated with B cell activation, survival and selection for specificity during the germinal centre response. Interestingly, we observed that the frequency of cTFHs was increased in GPA patients on conventional therapies, while in GPA patients following rituximab, cTFH frequencies were statistically no different from those seen in healthy controls.

Failure of Tregs to effectively suppress immune responses either through a reduction in frequency or...
functional capacity may predispose to autoimmunity [36–38]. While some studies have observed a decrease of Tregs in GPA [28], others have not [26, 27], and these differences are likely to be technical and a result of differences in the gating strategy and may also have been due to different patient characteristics and therapies. Consistent with the findings of Rimbert et al. [28], we observed a significantly reduced frequency of Tregs in GPA patients on conventional therapies compared with healthy controls. Interestingly, this was only found in the mTreg and aTreg populations, which are generated in vivo as a consequence of antigen-driven activation and are the Treg populations responsible for immune resolution [35, 39]. The frequency of Tregs in the blood of B cell-depleted patients was similar to that of healthy controls, implying that BCD may restore Treg frequencies. A study of patients with X-linked agammaglobulinemia (XLA) who are B cell deficient identified defects in CD4 T cell development in these patients, implying an association between B cells and T cell maturation [40]. We therefore tested to see if B cell numbers were associated with Treg and cTFH frequencies in patients not taking rituximab. However, we saw no association between B cell frequencies and either of these parameters (data not shown). Restored Treg frequencies by BCD have been observed previously in SLE patients and animal models of arthritis, although the nature of B cell dependence of Tregs and whether restoration of normal levels could be the cause or consequence of disease resolution remains unclear [15, 41].

Differences in cTFH and Treg frequencies in GPA patients are not purely reflections of differences in disease activity because patients on conventional therapies, irrespective of BVASs or treatments, tended to have altered Treg and cTFH frequencies compared with healthy controls. This was despite the heterogeneity in the patient populations studied. The changes observed in cTFH and Treg frequencies in four GPA patients in response to rituximab in a preliminary longitudinal study support the hypothesis that these immunomodulatory subsets vary in frequency in a B cell-dependent way and may be involved in achieving clinical improvement. Longitudinal studies with longer follow-up after BCD in GPA patients are ongoing.

Remissions in GPA could be achieved by conventional immunosuppression therapies or rituximab through different pathways. The frequencies of cTFHs and Tregs in inactive GPA patients who achieved remission (BVAS = 0) by standard immunosuppression therapies showed no difference from those in patients with active disease, while only GPA patients clinically improved through
rituximab had changes in cTFH and Treg frequencies similar to those in the healthy controls. This suggests that achievement of remission by rituximab but not conventional immunosuppression is associated with the restoration of normal physiological proportions of immune modulatory T cell subsets. Of 11 rituximab-treated GPA patients, 4 were sampled 1 month after the second infusion, 3 were sampled 6 months after treatment and the other 4 were sampled 1 year after rituximab therapy. Despite this heterogeneity, the population of rituximab-treated patients, all of whom had lower BVASs and could be considered to have benefited from rituximab treatment, showed a similar pattern in Treg and cTFH frequencies.

We studied Treg function in three GPA patients, two of whom were ANCA positive, while one was ANCA negative. In contrast to previous studies [26, 27], the suppressive efficiency of Tregs in our study was equivalent to that of healthy controls regardless of the ANCA status of the GPA patients and where Treg frequencies were confirmed to be low. This difference between our data and previous studies may be due to different strategies of isolating Tregs and effector T cells. Previous studies did not include CD127 in the sorting criteria, so some T effector cells in those studies may have been sorted as Treg cells. This issue remains to be resolved.

Separating the effects of disease activity and treatment is complex and this complexity is increased by the heterogeneity in disease phenotypes in GPA that measurements such as the BVAS are based on. However, BCD alone is unlikely to be the sole basis for the success of rituximab in generating favourable clinical outcomes in GPA. The frequencies of Treg cell subsets are also likely to impact on disease activity. The balance between T cell

Fig. 4 Delineation of Treg subpopulations

(A) CD25⁺CD127⁻ Tregs were identified as in Fig. 2 and then divided into rTregs (CD25⁺CD45RA⁻), mTregs (CD25⁺CD45RA⁻) and aTregs (CD25⁺CD45RA⁻). (B) Percentage of mTregs in CD4⁺ T cells in 20 active GPA patients [mean 2.53 (s.d. 1.17)], 9 inactive GPA patients [mean 2.79 (s.d. 0.94)] (P = 0.0001 and P = 0.0002 compared with healthy, respectively), 10 healthy controls [mean 4.72 (s.d. 0.60)] and BCD GPA patients [mean 4.30 (s.d. 1.78)] (P = 0.0041 compared with active GPA). (C) Percentage of aTregs in CD4⁺ T cells in 20 active GPA patients [average 0.24, range 0.0061–0.91], 9 inactive GPA patients [mean 0.27 (s.d. 0.085)] (P = 0.0001 and P < 0.0001 compared with healthy, respectively), 10 healthy controls [mean 0.68 (s.d. 0.23)] and 9 BCD GPA patients [average 0.70, range 0.24–2.15] (P = 0.0016 compared with healthy). (D) Percentage of rTregs in CD4⁺ T cells in 10 healthy controls [mean 2.27 (s.d. 0.75)], 20 active GPA patients [mean 1.89 (s.d. 1.33)], 9 inactive GPA patients [mean 1.53 (s.d. 0.68)] (P = 0.044 compared with healthy) and 11 BCD GPA patients [mean 3.05 (s.d. 2.46)]. ***P < 0.001; **P < 0.005; *P < 0.05.
activity and its potential B cell dependence is intriguing and demonstrates that understanding mechanisms of distortion of the integrated networks in autoimmune disease is essential to the understanding of disease pathogenesis.

**Rheumatology key messages**

- Abnormally high circulating T follicular helper cell (TFH) and low regulatory T cell (Treg) frequencies are observed in granulomatosis with polyangiitis.
- Rituximab normalises the frequencies of TFHs and Tregs in granulomatosis with polyangiitis.
- Conventional immunosuppression does not restore the frequencies of regulatory T cell subsets as rituximab does.

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**Supplementary data**

Supplementary data are available at *Rheumatology* Online.

**References**

B cell depletion in patients with GPA


Clinical vignette

Sitagliptin-induced bilateral Achilles tendinitis

Sitagliptin (Januvia) is a widely used oral dipeptidyl peptidase-4 (DPP-4) inhibitor for the treatment of diabetes. Studies suggest that the drug is well-tolerated and no musculoskeletal side effects have been reported [1]. A 56-year-old female developed bilateral Achilles tendinitis 4 months after restarting sitagliptin. There was no trauma to the Achilles tendons, but she had recently started a low-impact exercise programme; she stopped exercising with no relief. She had no history of SpA, inflammatory arthritis or quinolone exposure and her HLA-B27 status was unknown. Physical therapy did not resolve her symptoms. She then tried a controlled ankle motion boot, ice application, AchilloTrain braces, ViscoHeel lifts and two additional courses of physical therapy over the ensuing 9 months without improvement. Bilateral ankle MRI confirmed insertional Achilles tendinosis on the left and low-grade insertional tendinitis of the right Achilles tendon (Fig. 1). Eventually her sitagliptin was discontinued because of the temporal relationship of her symptoms to restarting sitagliptin. After 4 weeks the patient reported complete resolution of her Achilles pain on the left and >50% improvement on the right. She has remained off sitagliptin and has had no further problems with tendinitis since that time.

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