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SHORT REPORT

Systemic immunosuppression depletes peripheral blood regulatory B cells in patients with immune thrombocytopenia

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Summary
Regulatory B (Breg) cells are potentially implicated in the pathogenesis of immune thrombocytopenia (ITP). We analysed a prospective cohort of newly diagnosed steroid naïve ITP patients enrolled in the multicentre FLIGHT trial and found that the numbers of Bregs in their peripheral blood were similar to healthy controls. In contrast, Breg numbers were significantly reduced in ITP patients treated with systemic immunosuppression (glucocorticoids or mycophenolate mofetil). We also demonstrate that glucocorticoid treatment impairs Breg interleukin-10 production via an indirect T-cell-mediated mechanism.

KEYWORDS
Bregs, cytokines, glucocorticoids, ITP

INTRODUCTION

Regulatory B (Breg) cells play an important role in suppressing pathological immune responses, primarily through the production of the immunoregulatory cytokine interleukin (IL)-10. In humans, IL-10-producing B cells are enriched within the transitional B-cell population ($CD19^{+}CD24^{hi}CD38^{hi}$) in peripheral blood (PB).[1] Breg IL-10 production can be induced through B-cell activation via toll-like receptor and B-cell receptor ligation, or indirectly through T-cell-mediated activation via CD40:CD40 ligand (CD40L) interactions. Loss of Breg-mediated suppression, either through numerical deficiency or loss of function, has been implicated in the pathogenesis of several autoimmune and inflammation-mediated diseases.[2–4]

Immune thrombocytopenia (ITP) is an autoimmune blood disorder characterised by a low platelet count ($<100 \times 10^9$/L) and bleeding risk. Dysregulated autoreactive T and B cells mediate an autoimmune attack against platelets and megakaryocytes resulting in the increased consumption and reduced production of platelets. Reduced numbers of circulating Bregs have been reported in patients with chronic ITP.[2] However, it remains unclear whether this change is a cause or effect of ITP, or a consequence of treatment.[3,4] Glucocorticoids are the recommended first-line treatment for adults with ITP,[5] but the effect of these on Bregs is not

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well understood with literature currently limited to heterogenous groups of patients with chronic ITP.[2]

We aimed to assess circulating Breg numbers in patients with ITP and to evaluate how these are influenced by disease activity, chronicity and treatment. We also aimed to assess the effect of glucocorticoids on B-cell IL-10 production and to understand the mechanism of action of this effect.

METHODS

Blood samples from 75 newly diagnosed ITP patients recruited to the FLIGHT trial (ClinicalTrials.gov number: NCT03156452) were taken at baseline (when randomised; 47 men, 28 women; median age 60 years, range 17–87 years) and at 2 months follow-up ($n = 61$; 35 men, 26 women; median age 60, range 18–87 years). Full methods for FLIGHT have been previously published[6]—in brief, patients were randomised to receive glucocorticoid alone or in combination with mycophenolate mofetil. A healthy control (HC) cohort (UK NHS Research Ethic Committee reference: 04/Q2002/84) of 20 donors was recruited (3 men, 17 women; median age 27.5 years, range 19–53 years) alongside 21 chronic ITP patients (11 men, 10 women; median age 50 years, range 31–85 years; ISRCTN95606674). The chronic ITP group included patients who were not receiving treatment ($n = 8$, all with platelet counts between 20 and 100×10^9/L), or were receiving glucocorticoids ($n = 5$), mycophenolate mofetil ($n = 4$) or thrombopoietin receptor agonists (TPO-RAs; $n = 4$).

Breg phenotyping

PB from patients recruited to the FLIGHT trial was collected in TransFix®/ethylenediaminetetraacetic acid (EDTA)-containing vacutainers (allow processing by flow cytometry up to 14 days at 4°C after collection). Six hundred microlitres of whole blood was stained for 30 min at 4°C with the following antibodies: anti-CD3-FITC (clone: UCHT1; BD Biosciences), CD19-PE.Cy7 (clone: HIB19; BioLegend), CD24-PE (clone: ML5; BioLegend), CD27-BrilliantViolet 605™ (clone: O323; BioLegend) and CD38-BrilliantViolet 421™ (clone: HIT2; BioLegend). Cells were washed with phosphate-buffered saline (PBS) and 10% fetal calf serum (FCS) then lysed with red blood cell lysis buffer before a further two washes. Cell pellets were resuspended in 600 µL PBS + 10% FCS before analysis. Samples were run on a BD Fortessa X-20, and 10 000 $CD19^+$ cell events per sample were collected.

PB was independently collected from chronic ITP patients and HCs into EDTA-coated vacutainers and analysed the same day. Blood was layered over a density medium (Ficoll-Paque PLUS; GE Healthcare) in Leucosep™ tubes (Greiner Bio-One) and centrifuged at 250 *g* for 10 min. Peripheral blood mononuclear cells (PBMCs) were isolated over a density gradient and washed twice in 10% (vol/vol) FCS-containing PBS (ThermoFisher Scientific) before staining with the Breg phenotyping panel. Cells were washed with PBS + 10% FCS, resuspended in 200 µL PBS + 10% FCS with 7AAD (1:400 dilution), and analysed on a BD LSR-II. 10 000 $CD19^+$ cells were collected per sample.

B-cell activation

PB was collected from HC into EDTA-coated vacutainers, and PBMCs were isolated using a density gradient. $CD19^+$ B cells and $CD4^+$ T cells were isolated from PBMCs by fluorescence-activated cell sorting and cultured at 1:1 ratio in the presence of anti-CD3 (1 µg/mL), or CpG (3 µg/mL) and anti-IgM (10 µg/mL), with or without dexamethasone (Dex) (1×10^{-6} M). After 72 h of culture, protein transport was inhibited with GolgiStop (2 µM) for 4 h and cell surface stained with Breg phenotyping panels. Intracellular cytokine staining was performed to examine IL-10 production.

Statistical analysis

Data analysis was performed using FlowJo software (TreeStar Software). Live singlets were gated, and the percentage of cytokine positive cells determined using fluorescence minus one controls (Figure 1A). Statistical analysis was carried out using GraphPad Prism software. Mann–Whitney *U*-tests were performed between groups with a differential analysis for paired data. A value of $p < 0.05$ was considered significant.

RESULTS

To investigate the effect of immunosuppressive treatments on Breg proportions (%Bregs), we analysed samples from newly diagnosed ITP patients recruited to the FLIGHT clinical trial. For this newly diagnosed cohort, there was no statistically significant difference in the number of B regs in treatment naïve patients and HCs. Compared to baseline bloods, at 2 months follow-up marked reductions in %Breg were observed following a course of glucocorticoid treatment, either alone or in combination with mycophenolate mofetil (Figures 1B and S1A).

To further investigate the effects of immunosuppression, we also analysed samples from a smaller cohort of chronic patients with ITP. Chronic patients who were not receiving treatment showed no significant decrease in %Bregs compared to HC, whereas %Bregs were lower in patients receiving glucocorticoid or mycophenolate mofetil monotherapy. In contrast, the %Bregs in chronic ITP patients who received TPO-RAs (without glucocorticoid or mycophenolate mofetil) were similar to treatment naïve ITP patients (Figure 1C). All patients for whom repeat samples were available demonstrated reduced %Bregs after glucocorticoid treatment (Figure S1B).

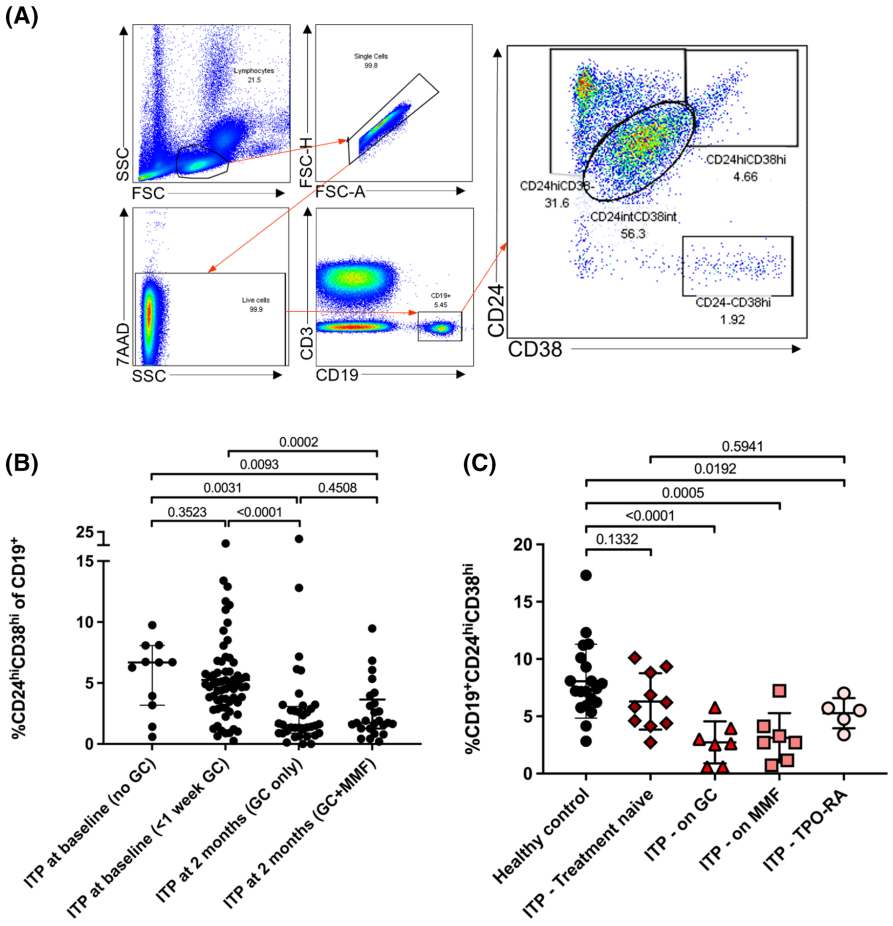

FIGURE 1 (A) Gating strategy and representative flow cytometry plots showing the expression of the cell surface markers CD24 and CD38 on $CD19^+$ cells isolated treatment naïve immune thrombocytopenia (ITP) patients. (B) Cumulative data from the FLIGHT trial newly diagnosed patient cohort showing a reduction in %Bregs in patients who have received up to 1 week of glucocorticoid compared with truly steroid naïve patients. More marked reductions are demonstrated after 2 months of immunosuppressive treatment. (C) Cumulative data showing proportion of $CD19^+CD24^{hi}CD38^{hi}$ Bregs in healthy control compared with patients with chronic ITP that are treatment naïve and chronic ITP patients on glucocorticoid (GC), mycophenolate mofetil (MMF) and thrombopoietin receptor agonist (TPO-RA).

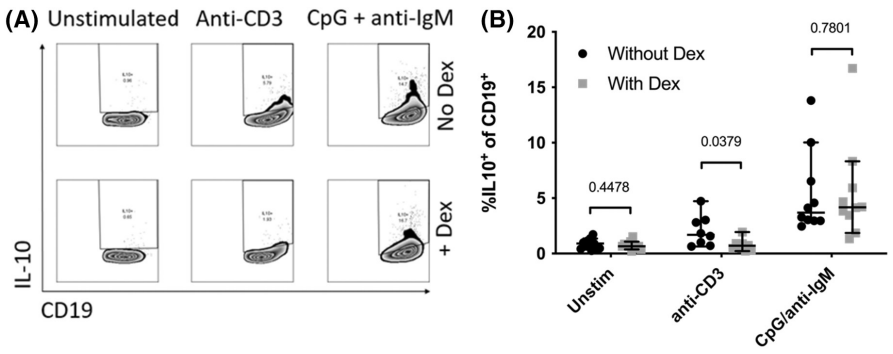

FIGURE 2 (A) Representative flow cytometry plots showing interleukin (IL)-10 expression from $CD19^+$ B cells following 72 h of co-culture with autologous $CD4^+$ T cells in the presence of either anti-CD3 (T-cell receptor activation; 'indirect B-cell activation') or a combination of CpG and anti-IgM (toll-like receptor and B-cell receptor activation; 'direct B-cell activation'). Cells were cultured in the presence or absence of Dex. Positive staining was gated on individual fluorescence minus one controls. (B) Dex can significantly inhibit IL-10 production by $CD19^+$ B cells when they are activated indirectly through anti-CD3-mediated $CD4^+$ T-cell activation. However, Dex had no effect on the production of IL-10 by $CD19^+$ B cells activated directly with CpG and anti-IgM. $N = 8–12$ healthy control (mean ± SD).

To investigate the effect of glucocorticoid treatment on Breg function, we cultured blood samples from HC ($n = 10$) in vitro with Dex, a relatively pure glucocorticoid. We found that Dex significantly inhibited IL-10 production by $CD19^+$ B cells activated indirectly through anti-CD3-mediated $CD4^+$ T-cell activation. However, Dex had no effect on the

production of IL-10 by $CD19^+$ B cells activated directly with CpG and anti-IgM (Figure 2).

DISCUSSION

Previous studies reported reduced Breg numbers in patients with chronic ITP.[2] Our results suggest that this reduction is predominantly driven by immunosuppressive treatment rather than ITP itself, regardless of disease duration. This replicates findings in myasthenia gravis and neuromyelitis optica patients,[3,4] where Bregs are depleted in patients receiving glucocorticoid treatment. The underlying mechanism behind this depletion is not yet understood and studies have postulated that Dex might induce B-cell apoptosis or a change in the balance of B-cell populations.[4,7] However, we did not observe any evidence to support these hypotheses when analysing our cohorts. In addition, our in vitro studies in HCs suggest that glucocorticoids impair Breg function via CD40L-mediated T-cell-dependent mechanisms, resulting in a failure to stimulate Breg IL-10 production in vivo which is consistent with the previous finding that glucocorticoids inhibit CD40 ligand expression of peripheral CD4+ lymphocytes.[8]

Our study is the first to examine PB Breg numbers before and after 2 months treatment in a newly diagnosed, treatment naïve cohort of ITP patients recruited to the FLGHT clinical trial. Two months follow-up is considered sufficient to assess the effect of immunosuppressive treatment on circulating B reg numbers. No further blood samples were taken from these patients although they were clinically followed in the trial up for a median of 18 months and up to 24 months.[9] We found a reduction in circulating Breg numbers in newly diagnosed patients treated with immunosuppressive agents. The consistent observation in the smaller chronic ITP cohort is additional supporting evidence of treatment effect on circulating B regulatory cell numbers. This reduction was not seen in treatment naïve newly diagnosed patients, or those with chronic ITP on non-immunomodulatory TPO-RAs or not receiving treatment. These findings highlight the need to consider the influence of previous or current treatments when describing immunological changes in patients with ITP.

Our study is limited by its small sample size for some subgroups, observational nature and our cohorts were neither age nor sex matched although we did not observe any association between B regulatory cell numbers and either age or sex. Furthermore, it is important to note that all changes described in this study were observed in PB only. It is therefore feasible that Breg numbers in the spleen, lymph nodes or other tissues may differ.

In conclusion, we demonstrate that in newly diagnosed ITP patients, immunosuppression either with systemic glucocorticoid or mycophenolate mofetil, is associated with reduced Breg numbers. This reduction is evident by 2 months. A similar association with immunosuppression and reduced B reg numbers was found in a smaller chronic ITP cohort. We also present preliminary data suggesting glucocorticoids affect Breg function, with reduced IL-10 production mediated indirectly via the effects of glucocorticoids on T cells. These data highlight the need for research to disentangle immune changes associated with treatment from those that drive or are a consequence of the underlying disease. It is currently unclear whether the reduction in Breg numbers and function associated with glucocorticoid treatment is of any adverse consequence. Similarly, it is unknown whether restoration of Breg numbers with TPO-RAs may contribute to the sustained responses off treatment observed in up to a third of patients.[10]

AUTHOR CONTRIBUTIONS

Charlotte A. Bradbury was the lead clinical investigator and CI for the FLIGHT trial. Richard W. J. Lee jointly led the laboratory studies with Charlotte A. Bradbury. Madeleine L. Stimpson conducted the laboratory work and initial analysis of the data. Philippa J. P. Lait, Emily L. Williams and Lauren P. Schewitz-Bowers contributed to sample analysis and Julia Wolf contributed to data analysis. Julia Wolf, Madeleine L. Stimpson and Bruno Charbit wrote the manuscript, which was reviewed and amended by all authors. All authors have made valuable contributions to the research design, delivery and provided feedback on the manuscript.

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CONFLICT OF INTEREST STATEMENT

RWJL and LPS-B are inventors of an IL-17-based method to identify patients likely to be resistant to glucocorticoid treatment (US Patent App. 15/106,411). The other authors have no conflict to declare.

DATA AVAILABILITY STATEMENT

De-identified data may be available to researchers subject to investigator and sponsor approval of a proposal.

ETHICS STATEMENT

Ethical approval was granted for the Flight Trial (ClinicalTrials.gov number NCT03156452, EudraCT number 2017-001171-23; REC Ref: 17/SW/0127), the 'low platelet study' (REC ref: 15/LO/2088) and for the healthy control cohort (UK NHS Research Ethic Committee reference: 04/Q2002/84).

PATIENT CONSENT STATEMENT

All patients and healthy volunteers provided written informed consent.

CLINICAL TRIAL REGISTRATION (INCLUDING TRIAL NUMBER)

Flight Trial: ClinicalTrials.gov number NCT03156452, EudraCT number 2017-001171-23. Low platelet blood study: ISRCTN95606674.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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