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Alterations in B- and circulating T-follicular helper cell subsets in immune thrombotic thrombocytopenic purpura

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Abstract:
T follicular helper (Tfh) cells regulate development of antigen-specific B-cell immunity. We prospectively investigated B-cell and cTfh subsets in 45 immune TTP patients at presentation and longitudinally after rituximab (RTX). B-cell phenotype was altered at acute iTTP presentation with decreased transitional cells and postgerminal centre (post-GC) memory B cells and increased plasmablasts compared to healthy controls. A higher percentage of plasmablasts was associated with higher anti-ADAMTS13 IgG and lower ADAMTS13 antigen levels. In asymptomatic patients with ADAMTS13 relapse, there were increased naïve B cells and a global decrease in memory subsets, with a trend to increased plasmablasts. Total circulating Tfh ($CD4^+CXCR5^+$) and $PD1^+$ Tfh cells were decreased at iTTP presentation. CD80 expression was decreased on IgD^+ memory cells and double negative memory cells in acute iTTP. Longitudinal analysis: at repopulation after B cell depletion in de novo iTTP, post-GC and double negative memory B cells were reduced compared to pre-RTX. RTX did not cause alteration in cTfh frequency. The subsequent kinetics of naïve, transitional, memory B cells and plasmablasts did not differ significantly between patients who went on to relapse vs those who remained in remission. In summary, acute iTTP is characterised by dysregulation of B- and cTfh-cell homeostasis with depletion of post-GC memory cells and cTfh cells and increased plasmablasts. Changes in CD80 expression on B cells further suggest altered interactions with T cells.

Conflict of interest: COI declared - see note

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Key Points

- **Abnormal B-cell phenotype in acute iTTP with decreased transitional and postgerminal centre memory cells and increased plasmablasts**
- **Decreased total and PD1$^+$ circulating T-follicular helper cells and changes in B-cell CD80 expression suggest altered B-T–cell interactions**

ABSTRACT

T follicular helper (Tfh) cells regulate development of antigen-specific B-cell immunity. We prospectively investigated B-cell and cTfh subsets in 45 immune TTP patients at presentation and longitudinally after rituximab (RTX). B-cell phenotype was altered at acute iTTP presentation with decreased transitional cells and postgerminal centre (post-GC) memory B cells and increased plasmablasts compared to healthy controls. A higher percentage of plasmablasts was associated with higher anti-ADAMTS13 IgG and lower ADAMTS13 antigen levels. In asymptomatic patients with ADAMTS13 relapse, there were increased naïve B cells and a global decrease in memory subsets, with a trend to increased plasmablasts. Total circulating Tfh ($CD4^+CXCR5^+$) and $PD1^+$ Tfh cells were decreased at iTTP presentation. CD80 expression was decreased on IgD+ memory cells and double negative memory cells in acute iTTP. Longitudinal analysis: at repopulation after B cell depletion in *de novo* iTTP, post-GC and double negative memory B cells were reduced compared to pre-RTX. RTX did not cause alteration in cTfh frequency. The subsequent kinetics of naïve, transitional, memory B cells and plasmablasts did not differ significantly between patients who went on to relapse *vs* those who remained in remission. In summary, acute iTTP is characterised by dysregulation of B- and cTfh-cell homeostasis with depletion of post-GC memory cells and cTfh cells and increased plasmablasts. Changes in CD80 expression on B cells further suggest altered interactions with T cells.

INTRODUCTION

Immune thrombotic thrombocytopenic purpura (iTTP) is a life-threatening thrombotic microangiopathy mediated by an immunoglobulin G (IgG) antibody against the metalloprotease ADAMTS13 that enhances its clearance or inhibits its VWF processing activity.[1] In iTTP, there is an incompletely understood loss of tolerance resulting in a shift from immune homeostasis to autoimmunity. This involves dendritic cells which acquire antigens derived from ADAMTS13 that activate cross-reactive naïve CD4+ T cells which differentiate into autoreactive effector CD4+ T cells.[2,3] Mature autoreactive B cells recirculate into the germinal centre of secondary lymph nodes where they are stimulated by compatible antigens in the presence of the autoreactive T helper cells and differentiate into autoantibody-producing plasma cells or long-lived memory B cells.[4]

B cell depletion therapy with rituximab (RTX; a chimeric monoclonal antibody against the pan-B cell marker, CD20) has been demonstrated to be effective in reducing relapse rates in iTTP and prolonging disease-free survival in acute episodes, compared to PEX and steroid alone.[5-7] Giving rituximab pre-emptively in patients at high risk of a clinical relapse (based on a fall in ADAMTS13 activity levels to<10-20%, i.e. 'ADAMTS13 relapse') reduces clinical relapse rates compared to historical controls.[8]

A recent Genome Wide Association Study (GWAS) of iTTP confirmed associations with SNPs at the HLA locus and identified a novel association on chromosome 3.[9] The locus on chromosome 3 contains five genes, one of which is the *CD80* gene. CD80 is a co-stimulator for T lymphocyte activation. After activation through the B-cell receptor (BCR) or IL4, B cells express CD80, which interacts with CD28 on T cells to provide co-stimulation signals.[10] The HLA-DRB1*11 and DRB1*03 genes are known risk factors for iTTP, suggesting the MHC class II protein variants they encode have optimal affinity for certain ADAMTS13 peptides recognised by CD4+ T-cell receptors in iTTP patients.[11,12] Two peptides derived from the CUB-2 domain of ADAMTS13 are presented on HLA-DRB1*11 and HLA-DRB1*03 respectively and recognised by iTTP patient-derived CD4+ T cells.[13,14]

CD4+ T cells are pivotal in development of iTTP as CD4+ T cell help is required in the production and affinity maturation of ADAMTS13-directed antibodies.[3] Within the CD4+T cell population, the T follicular helper (Tfh) subset are vital for supporting antibody-mediated immune responses by providing co-stimulation signals through CD40L and IL-21 production, which promote the growth, differentiation and class-switching of antigen-activated naïve B cells.[15-17] Tfh cells constitutively express the chemokine receptor CXCR5 which facilitates

migration into germinal centres (GC).[18] Tfh cells express co-stimulatory molecules such as inducible co-stimulatory molecule (ICOS) and immune-regulatory molecules such as programmed death-1 (PD-1) as their transcription factors, which can be used to further define Tfh cells.[19] More specifically, PD-1+ICOS- Tfh cells seem to represent quiescent and PD-1+ICOS+ Tfh cells recently activated memory Tfh cells[20,21]
Circulating Tfh (cTfh) cells also express the GC homing receptor CCR7.[22] Expansion of cTfh cells has been associated with the development of several autoimmune diseases.[23-29]

The aim of this study was to investigate the B cell and cTfh subset distribution in iTTP patients, both at presentation and longitudinally after anti-CD20 therapy in relation to clinical and laboratory parameters and B cell kinetics. The role of cTfh has not previously been investigated in iTTP. The temporal relationships between B and T cell subpopulation at critical stages throughout the course of disease will improve the understanding of the pathogenesis of iTTP, potentially provide biomarkers to predict relapse and may identify new avenues for therapeutic intervention.

METHODS

Patients and controls:

We prospectively enrolled iTTP patients from September 2018 - June 2021 through the United Kingdom TTP Registry (database and Biobank of UK TTP - Multicentre Research Ethics Committee [MREC]:08/H0810/54 and MREC:08/H0716/72). The study was conducted according to the Declaration of Helsinki. Blood samples were obtained from age and sex-matched healthy controls (HC), with no history of autoimmune disease or immunosuppressant medication. Definitions of iTTP diagnosis, remission and relapse were based on previous studies.[30-32]

Patients were divided into two groups: *de novo* acute iTTP episodes and asymptomatic ADAMTS13 relapses (treated with pre-emptive RTX). Acute presentations of iTTP were treated with PEX, corticosteroids and RTX (4-8 doses 375 mg/m^2 to normalize ADAMTS13 activity). Time after RTX was defined as time since first RTX infusion. Caplacizumab was given to 18/22 of patients.

Patients with previously diagnosed iTTP who were in clinical remission (normal platelet count) but developed a new severe ADAMTS13 deficiency were referred to as ADAMTS13 relapse episodes.[32] These patients received pre-emptive RTX (four doses of

200/500/375mg/m^2 one week apart). Dosing varied at clinician discretion or as part of a separate randomised control trial (Elective Rituximab in TTP trial;[REC]17/LO/1055).

Blood samples and PBMC isolation:

Heparinised blood samples collected from patients prior to initiation of RTX or other immunosuppression and HC were analysed within 24 hours or frozen at -80˚C and stored for later analysis. Samples were also collected one month and three months post RTX and then three monthly thereafter until clinical or ADAMTS13 relapse, or end of study (whichever came first). Peripheral blood mononuclear cells (PBMC) were isolated by diluting blood (1:1) with phosphate-buffered saline(PBS) 1X and layered over Ficoll-Paque Premium 1084R (Sigma-Aldrich).[33]

Flow cytometry

PBMC (approximately 1 ×10^6/sample) were incubated with conjugated antibodies for 20 minutes in the dark at room temperature. Cells were washed twice and resuspended in PBS and samples were analysed on a Cytoflex S cytometer (Beckman Coulter) (Supplementary Materials). Each sample was divided into two antibody panels: for B cell subsets and Tfh cells (Supplementary Materials: Table 1a & 1b). Viability was determined using LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen™). Analysis was performed on FlowJo software version 10 (FlowJo LLC).

B cell immunophenotyping and gating strategy

We used the mature B cell 'Bm1–Bm5' classification to identify B cell subsets based on co-expression of IgD and CD38. B cell subsets include transitional B cells, naive B cells, memory populations including IgD+ memory cells, post germinal centre (post-GC) cells, double negative memory cells and plasmablasts.[34,35] Antibodies used for the B cell panel were CD19 PECy7, CD27 APC, IgD BV421, CD38 PE. Following on from our GWAS finding that the CD80/POGLUT1 locus is associated with iTTP,[9] we also analysed the level of CD80 expression (median fluorescence intensity or MFI) on B-cells subsets in iTTP.[36] Gating strategies are shown in Supplemental Figure 1.

Circulating Tfh immunophenotyping and gating strategy

The total cTfh population was defined by CD3+ T cells that expressed CD4+CXCR5+. The different subsets within the total cTfh population were defined by expression of activation markers: PD1+ cTfh, ICOS+ cTfh and PD1+ICOS+ cTfh. In the cTfh panel, PBMC were stained with CD3 PECy7, CD4 PerCP, CXCR5 FITC, PD1 APC and ICOS BV421 antibodies. The gating strategy and representative plots are shown in Supplemental Figure 2.

ADAMTS13 assays

ADAMTS13 activity was analysed by fluorescence resonance energy transfer (FRETS (normal range: 60%-123%).[37] ADAMTS13 antigen levels were quantified using in-house developed enzyme-linked immunosorbent assay, previously described (normal range: 74% to 134%).[38] ADAMTS-13 activity and antigen levels were expressed as a percentage relative to pooled normal plasma (PNP). Anti-ADAMTS13 IgG levels were measured with in-house ELISA (normal range <6.1%) with concentration of anti-ADAMTS13 IgG calculated as a percentage relative to an index plasma from a patient with a high auto-antibody titre (assigned a value of 100%).[7]

Statistical Analysis

Mann-Whitney *U* and Wilcoxon tests were used for paired and unpaired continuous variables, respectively. The X^2 and Fisher's exact tests were performed for categorical variables. Spearman correlation test was used to measure the possible relationship between two variables of interest. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA).

Data Sharing statement

For original data, please contact jin-sup.shin@nhs.net.

RESULTS

Patient demographics and ADAMTS13 biomarkers

Demographics of iTTP patients and HC and ADAMTS13 assay results are shown in Supplemental Table 2a). There were 45 unique patients with iTTP involving 46 episodes: 22 *de novo* acute episodes and 24 asymptomatic ADAMTS13 relapses treated with pre-emptive rituximab.

In acute iTTP episodes, 82% (18/22) cases had cardiac involvement and 50% (11/22) neurological. 14% (3/22) of acute episodes required organ support on the intensive care unit. All acute episodes were treated with PEX, corticosteroids and RTX. 82% (18/22) patients received anti-VWF nanobody, Caplacizumab. Additional immunosuppression included mycophenolate mofetil (27%, 6/22) and bortezomib (9%, 2/22). All but one of ADAMTS13 relapse episodes in this study had previously received rituximab treatment, either during an acute presentation or previous ADAMTS13 relapse episode. In this cohort, the median number of previous TTP episodes was 3 (range 1-8 episodes), with a median duration since the most recent TTP episode being 21 months (range 13-191 months), (Supplemental Table 2b).

Post germinal centre memory B cells are decreased and plasmablasts increased at iTTP presentation. (Figure 1)

The total number of CD19+ B cells at presentation of acute iTTP or preceding pre-emptive rituximab for ADAMTS13 relapse episodes was not significantly different to HC. At presentation in acute iTTP episodes, post-GC memory cells were decreased compared to healthy controls (HC 13.7% (range, 3.1-28.8%) *vs* acute iTTP 7.9% (range, 1.8-27.8%), p=0.008), whereas plasmablasts were increased (HC 0.7% (range, 0.2-2.5%) vs acute iTTP 1.6% (range, 0.2-15.4%), p=0.007), (Figure 1).

B cell repopulation after RTX recapitulates ontogeny beginning with naïve B cell exit from the bone marrow followed by gradual maturation of memory subsets over time. In ADAMTS13 relapse cases, there was an increased proportion of naïve B cells compared to healthy controls and a trend to increased transitional cells and plasmablasts (Figure 1). There was a marked reduction in all memory subsets.

Altered circulating T follicular helper cell subsets at iTTP presentation: decreased total cTfh and PD1+ cTfh (Figure 2)

The relative proportions of CD4+ cTfh subsets in acute TTP and ADAMTS13 relapse were determined and compared with HC (Figure 2). The frequency of total cTfh and PD1+ cTfh were significantly reduced in acute iTTP compared to HC. This may be suggestive of migration of circulating Tfh cells into germinal centers. There were no significant differences seen in ICOS+ or PD1+ICOS+ cTfh cells. In ADAMTS13 relapses, no differences were seen in total, PD1+ or PD1+ICOS+ cTfh cells. However, ICOS+ cTfh cells were increased in patients compared to HC.

Relationship between ADAMTS13 parameters, circulating Tfh cells and B cell subsets at iTTP presentation

In the acute iTTP group, median time to achieve a normal platelet count from presentation was 4 days (range, 2-11) and median time to normalization of ADAMTS13 activity was 33 days (range 3-382; interquartile range, 22-124). At presentation in acute iTTP episodes, a higher percentage of plasmablasts appears to be associated with lower antigen levels (r= -0.41, p=0.055) (Figure 3). Indeed, a plasmablast level of >3% was associated with IgG antibody level of >50% (Table 1). This may be due to increased production of anti-ADAMTS13 IgG antibody from a larger number of plasmablasts, which in turn results in increased ADAMTS13 clearance. In both acute iTTP cases and ADAMTS13 relapses, there was no correlation between total cTfh cells and any of the B cell subsets (data not shown).

Expression of CD80 on B cell subsets at acute presentation and post RTX

In acute iTTP episodes, CD80 MFI was decreased in IgD+ memory cells and double negative memory cells compared to HC (Figure 4). However, in ADAMTS13 relapse cases, CD80 MFI was significantly increased in post-GC and double negative memory cells compared to HC). A possible explanation for this difference between acute iTTP and asymptomatic ADAMTS13 relapse may be that in asymptomatic falls in ADAMTS13 activity prior to an acute clinical relapse, it is possible to detect activated memory B cell populations due to less 'background noise' or before they marginate.

A summary of the B and T follicular helper cell immunophenotyping results at acute iTTP presentation and ADAMTS13 relapse is shown in Table 2.

Longitudinal analysis: effect of RTX

Flow cytometric analysis of B cell subsets and cTfh cells was performed for patients pre-RTX and longitudinally 1 month, 3 months, 6, months, 9 months, 12 months etc until end of study/relapse. After RTX treatment, all 46 episodes achieved B cell depletion (defined by a laboratory CD19+ count <0.005 $\times 10^9$/L), except 1 patient in the ADAMTS13 relapse cohort (Supplemental Figure 3 (a) – (f) for acute iTTP cases; data not shown for ADAMTS13 relapse episodes). In contrast, RTX did not cause any significant alterations in cTfh numbers (Supplemental Figure 3 (g) – (k) for acute iTTP cases; data not shown for ADAMTS13 relapse episodes). B cell return occurred at median 10 months (range 6-14) months) in the

acute iTTP group and 8 months (range 0.25-15 months) in ADAMTS13 relapse group. The timepoint of B cell return was not associated with ADAMTS13 or clinical relapse.

Comparison of B cell subsets frequencies prior to RTX treatment and at B cell return

We then compared two specific timepoints: pre-RTX and B cell return. Repopulation following B cell depletion in the acute iTTP group demonstrated relatively higher proportion of plasmablasts but a reduction of in post-GC and double negative memory B cells (Figure 5a). In the ADAMTS13 relapse group where all except 1 patient had received historical RTX, plasmablasts were increased in frequency at B cell return compared to pre-treatment levels but the distribution of B cell subsets was otherwise similar before and after re-treatment (Figure 5b).

Longitudinal follow up: relapses vs sustained remissions

We prospectively investigated changes in B-cell subsets after RTX in 20 iTTP patients (16 from our initial cohort and 4 additional patients) longitudinally until a subsequent clinical or ADAMTS13 relapse, and compared to patients who remained in remission over an equivalent period.

There were 10 patients in the relapse cohort (3 followed after an acute episode, 7 after elective rituximab) and 10 in the remission cohort (4 acute and 6 elective episodes at t=0). Median age was 51y (range 38-81) and 45.5y (21-68) respectively. Median follow up was15 months (range 6-24). One patient in the relapse group did not achieve B-cell depletion (CD19+ count 0.005x10^9/L). Time to B-cell return was similar: 8 months (4-13 months) in relapses vs 8months in remission (0.25-15.5 months). All subsequent relapses were ADAMTS13 relapses and occurred at a median of 14 months (9-25 months).

Longitudinal analysis showed that in both groups B-cell return after rituximab develops along normal B-cell ontogeny. The kinetics of naïve, transitional, memory B-cells and plasmablasts did not differ significantly between patients who subsequently went on to relapse *vs* those who remained in sustained remission. (Figure 6) No alterations in B-cell subsets were identified prior to a relapse.

DISCUSSION

Interactions between T and B cells which occur within germinal centres (GC) of secondary lymphoid organs (SLO) are critical for the development of humoral immune responses. Tfh cells, a distinct subset of T helper cells, have been recognised in recent years as a crucial regulator of GC formation, B cell development and long-term humoral memory generation, and have a significant role in the pathogenesis of autoimmune diseases.[17] The mechanism involved in the loss of tolerance and subsequent development of anti-ADAMTS13 antibodies in iTTP patients is still largely unknown and we currently lack the ability to predict relapse accurately. The observed association between the MHC class II allele HLA DRB1*11 and development of iTTP implies a role for helper CD4+ T cells in the initiation of autoimmune reactivity against ADAMTS13.[11,12,39]

This is the first prospective study to perform a comprehensive analysis of B cell subsets and cTfh cell changes in iTTP before and after immunosuppressive therapy. B cell phenotype is altered at acute iTTP presentation with decreased post-GC memory B cells and an increase in plasmablasts in iTTP compared to healthy controls. Potential mechanisms for the reduced proportion of memory B cells among circulating B cells at presentation of iTTP include 1) some B cells dying in the early memory B cell stages and never becoming memory B cells, 2) hyperactivation of T and B cells in iTTP resulting in an elevated differentiation of memory B cells into antibody-producing plasma cells or 3) migration of memory B cells into lymphoid tissue.[40-42] Similar reductions in the memory B cell compartments have been described in sarcoidosis, systemic sclerosis and Sjogren's syndrome.[40,43-48]

Comparing immunophenotype results between different studies is complex due to the variety of staining and gating protocols used, and clinical and demographic characteristics of the patient cohort, but some general conclusions can be made regarding iTTP in comparison to other autoimmune diseases. We found an increased ratio of naïve to switched memory B cells and higher plasmablasts in the acute patients. In contrast, in patients with active SLE there is an apparent expansion of memory *vs* naïve B cell subsets correlating in part with disease activity, but this is due to a naïve B cell lymphopaenia.[49] Expansion of plasmablasts also occurs in SLE but is relatively higher than we found in iTTP. In Sjogrens syndrome, transitional and naïve B cells seem expanded compared to memory subsets, thus closer to the iTTP result, but in RA patients naïve and memory populations are generally similar to healthy age matched controls.[50] More recently, attention has focussed on the double negative (DN) (IgD-CD27-) B cell subset. In systemic sclerosis, SLE and RA, the relative importance of this subset, especially in relation to therapy resistance, has been recognised.

The DN B cell population contains both activated and antibody secreting post germinal centre B cells and very early switched B cells (with fewer mutated Ig transcripts).[51,52] This population was not different from HC in the iTTP patients in relation to relapse, but additional studies would be needed to assess functional properties of B cells within this population in iTTP.

The relative contribution of different naive and antigen-experienced B cell subsets to pathogenesis and response to therapies will depend on the position and circumstances underlying specific breaches of tolerance and thus ultimately depend on the specificity of the autoreactive B cell receptor(s). The different patterns we have described in iTTP compared to other systemic autoimmune diseases indicate a lack of apparent expansion of memory populations in the peripheral blood and thus the probability of rapid sequestration or expansion of autoantibody producing clones within tissues or lymphoid organs.

At acute iTTP presentation, we also demonstrate a novel association between higher plasmablast frequency and higher ADAMTS13 IgG antibody level and a trend towards reduced ADAMTS13 antigen levels. This suggests a potential underlying mechanism for iTTP development, where rapid differentiation to auto-antibody producing plasmablasts leads to increased production anti-ADAMTS13 IgG which in turn results in increased ADAMTS13 clearance.

In asymptomatic patients with ADAMTS13 relapse, alterations in B cell subsets prior to pre-emptive RTX therapy were pronounced with significantly increased naïve B cell population, global decrease in all memory subsets and trend towards increased plasmablasts. These subset alterations most likely represent changes related to historic RTX therapy with long-term suppressive effects of RTX on memory cell subsets. This also explains the largely similar distribution of B cell subsets before and after pre-emptive RTX treatment. Following B cell depletion therapy, B cells repopulate primarily from bone marrow-derived naive B cells, with delayed regeneration of memory B cells.[53,54] Poor memory B cell reconstitution may reflect long-term effects of RTX on B cells, described both after organ transplantation and in other autoimmune diseases.[55-57]

Total cTfh (CD4+CXCR5+) and PD1+ Tfh cells were decreased in acute iTTP patients at presentation compared to HC. A similar decrease in cTfh has been observed in sarcoidosis with infiltration of Tfh into skin lesions, suggesting that cTfh are recruited into affected sites.[47] In acute iTTP, a potential explanation for the concomitant decrease in post-GC memory cells and cTfh cells may be that they are localising in germinal centres within lymphoid tissue. In

contrast, in asymptomatic patients with ADAMTS13 relapse, there were no significant differences in total cTfh cells and PD1+ cTfh. This may be because this is a different stage of the disease process or possibly that the effect of previous Rituximab alters the interplay of B and cTfh cells. Tfh helper T cells utilise CXCR5 positivity to access germinal centres (GC). In GC, they produce IL21 which plays a key role in class switch and affinity maturation leading the generation of memory B cells and plasma cells from antigen-activated naïve B cells. Other peripheral blood helper T cells (CXCR5-; CXCR2 and PD1+) can also produce IL21 and have been identified as a source of extrafollicular T cell help to B cells in patients with other autoimmune diseases.[58] Although there are no frank inflammatory sites or lymphoid clusters in iTTP, it is possible that aberrant interactions between ADAMTS13-specific memory B cells and other T helper cells may occur in extrafollicular sites in spleen for example.

We also compared B and cTfh frequencies at 2 different timepoints following RTX infusion: prior to RTX and at B cell return. B cell depletion was achieved in all patients by 1 month. Subsequent B cell reconstitution occurred at 10 months in the acute iTTP cohort and 8 months in the asymptomatic ADAMTS13 relapse cohort. At B cell return after a *de novo* acute iTTP episode, plasmablast levels were significantly raised with a trend towards increased transitional and naïve cells. Importantly, no patients relapsed at the time of B cell return. The time interval between B cell return and relapse suggests that additional differentiation and selection of specific autoreactive B cell clones from naive populations may play a key role, although expansion of ADAMTS13 specific memory B cell populations in lymphoid tissues to critical levels cannot be ruled out. RTX did not cause any significant alterations in cTfh frequency.

Our data also reveals altered expression of CD80 on B cells in iTTP, with decreased expression in IgD+ memory cells and double negative memory cells in acute iTTP episodes and increased expression in post-GC memory and double negative memory cells in ADAMTS13 relapses, prior to RTX therapy. CD80 and CD86 are expressed on antigen presenting cells such as dendritic cells and activated B cells, and have the capacity to stimulate or inhibit T cell responses through their receptors CD28 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) respectively.[10] Dendritic cells exposed to ADAMTS13 have previously been shown to present ADAMTS13 peptides, with preferential HLA-DRB1* 11–dependent presentation of CUB2-derived peptides, which the authors hypothesised may contribute to the onset of acquired TTP by stimulating low-affinity self-reactive CD4+ T cells.[2,3]

Manipulation of the CD80/CD86 pathway has shown efficacy in the clinical setting, with Abatacept (a CTLA-4 immunoglobulin which targets autoimmune B cells by reducing CD80/CD86 expression) approved for the treatment of rheumatoid arthritis.[59] Of interest, the CD80 locus was identified in the GWAS for iTTP and contained a SNP in the 3' UTR that was in high linkage with the lead SNP.[9] Further investigation into the link between possession of this SNP with translation and expression of CD80 on different cell types is ongoing. Additional studies are required to understand how these differences in expression could translate into functional properties of CD80/86 in iTTP patients.

In the longitudinal analysis, no alterations in B-cell subsets were identified prior to a relapse, suggesting temporal relationships between B sub-populations cannot be used as a biomarker to better predict relapses.

Taken together, our findings give a novel insight into the role of B and cTfh cells in the development of iTTP. We propose that *de novo* acute iTTP is characterised by dysregulation of B and cTfh cell homeostasis with decreased circulating subsets of GC memory cells and cTfh cells and an increased frequency of plasmablasts in the circulation, perhaps reflecting the increase in cognate interaction between antigen-specific T and B cell populations in secondary lymphoid tissue. Changes in the frequency of CD80 on B cells suggests altered interactions with T cells. The finding of alteration in CD80 expression further supports the recent novel association between iTTP and five alleles within a haploblock on chromosome 3 – one of which encodes CD80.[9] Although our studies did not address the questions of antigen selectivity and exquisite specificity of the autoimmune response to ADAMTS13 in iTTP, the novel findings will direct further functional analysis, and provide potentially interesting targets for further research and therapeutics in iTTP.

AUTHORSHIP CONTRIBUTIONS

Contributions: J.S. designed research, recruited patients, performed laboratory testing, collected data, analysed data and wrote the manuscript. M.O.S. designed research, recruited patients, performed laboratory testing, collected data, analysed data and wrote the manuscript. G.C. designed research, analysed data and wrote the manuscript. Y.G. performed laboratory testing, collected data and reviewed the manuscript. R.dG. designed research, analysed data and wrote the manuscript. M.S. designed research, recruited

patients, analysed data and wrote the manuscript. M.T. designed research, recruited patients, analysed data and wrote the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

M.S. has received speaker's fees and honoraria from Alexion, Sanofi, Novartis, and Takeda and has received research funding from Takeda. M.T. has received speaker's fees and honoraria from Sanofi and Bayer. The remaining authors declare no competing financial interests.

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TABLES

Table 1: A high plasmablast frequency is associated with a higher ADAMTS13 IgG antibody level at acute iTTP presentation.

	Plasmablasts <3% (n=5)	Plasmablasts >3% (n=17)	*p* value *
ADAMTS13 antigen % (median (range))	5 (0.4 - 86)	1.9 (0.6 - 2.2)	0.4
ADAMTS13 IgG antibody % (median (range))	32 (2-113)	64 (52 - 127)	**0.02**

*Mann Whitney *U* test

Table 2: Summary of B and cTfh cell subsets at acute iTTP presentation and ADAMTS13 relapse compared to healthy controls

B and cTfh cell subsets at iTTP presentation compared to healthy controls (HC)	Acute iTTP presentation (n=22)	ADAMTS13 relapse (n=24)
B cell subsets (% $CD19^+$ B cells):		
Transitional cells (IgD^+CD38^{++})	↓	→
Naive cells (IgD^+CD38^+)	→	↑
IgD^+ memory cells (IgD^+CD38^-)	→	↓
Post GC memory cells (IgD^-CD38^+)	↓	↓
Double-negative memory cells (IgD^-CD38^-)	→	↓
Plasmablasts (IgD^-CD38^{++})	↑	→
CD80 expression on B cell subsets:		
IgD^+ memory cells (IgD^+CD38^-)	↓	→
Post GC memory cells (IgD^-CD38^+)	→	↑
Double-negative memory cells (IgD^-CD38^-)	↓	↑
Circulating T follicular helper cells (% $CD3^+CD4^+$ cells):		
$CD4^+$ $CXCR5^+$ (Total cTfh cells)	↓	→
$CD4^+$ $CXCR5^+$ $PD1^+$	↓	→
CD4+ CXCR5+ ICOS+	→	↑

↓ decreased compared to HC; → no difference compared to HC; ↑ increased compared to HC

FIGURE LEGENDS

Figure 1: Percentages of (a) transitional cells (IgD+CD38++), (b) naïve cells (IgD+CD38+) (c) IgD+ memory B cells (IgD+ CD38-), (d) post germinal centre memory B cells (IgD-CD38+) and (e) double negative memory B cells (IgD-CD38-) and (f) plasmablasts (igD-CD38++) in patients with acute iTTP episodes (n=22), ADAMTS13 relapse (n=24) and HC (n=27).
A13 relapse, ADAMTS13 relapse; Post-GC memory, post germinal centre memory cells; DN memory cells, double negative memory cells

Figure 2: Percentages of (a) total cTfh (CD4+CXCR5+), (b) PD1+ cTfh (CD4+CXCR5+PD1+), (c) ICOS+ cTfh (CD4+CXCR5+ICOS+) and (d) PD1+ICOS+ cTfh (CD4+CXCR5+PD1+ICOS+) in patients with acute iTTP episodes (n=34), ADAMTS13 relapse (n=27) and HC (n=27). A13 relapse, ADAMTS13 relapse.

Figure 3: Relationship between ADAMTS13 antigen levels and plasmablast frequency. Spearman correlation analysis was performed and $p<0.05$ indicates that the difference is statistically significant.

Figure 4: Analysis of CD80 expression (median fluorescence intensity) on B cell subsets defined by IgD/CD38. (a) Acute iTTP cases and (b) ADAMTS13 relapse cases.

Figure 5: Pairwise comparison of B cell subset frequencies before and after rituximab by Wilcoxon signed–rank test. a) Acute iTTP episodes, pairwise comparison of 10 patients, b) ADAMTS13 relapse episodes, pairwise comparison of 13 patients.

Figure 6: Longitudinal changes in B-cell subsets after RTX therapy for an acute iTTP episode or ADAMTS13 relapse (ADAMTS13 activity 15%) until subsequent ADAMTS13 relapse, and compared to patients who remained in remission over an equivalent period.

Figure 1

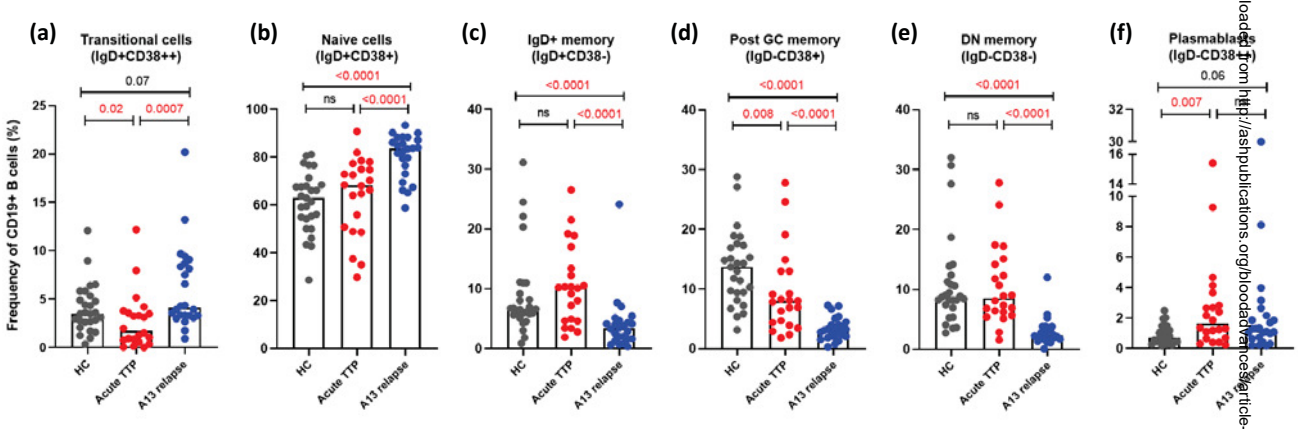


Figure 2

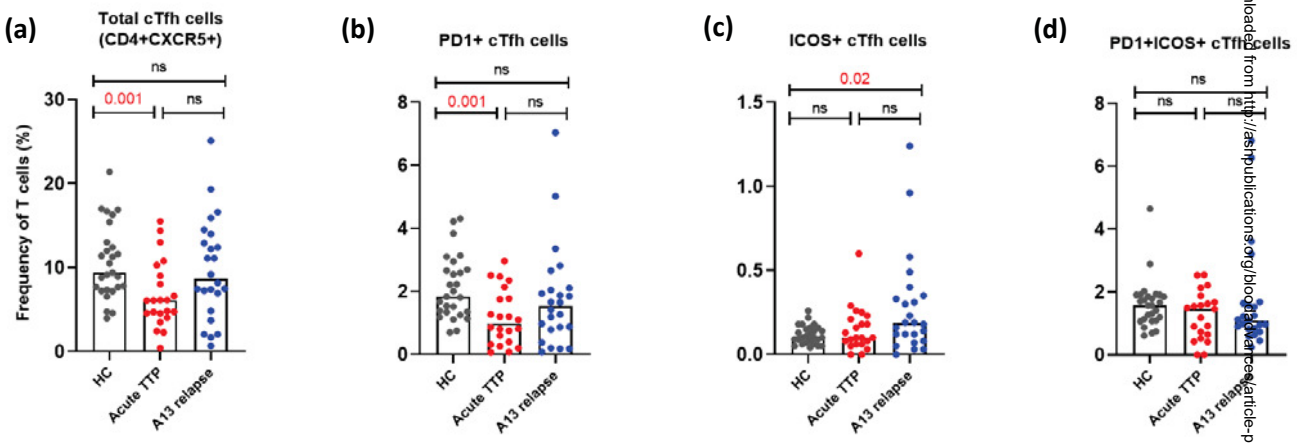


Figure 3

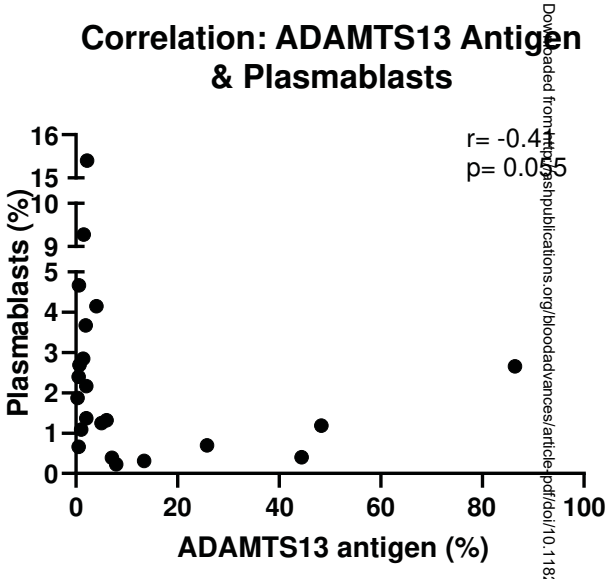
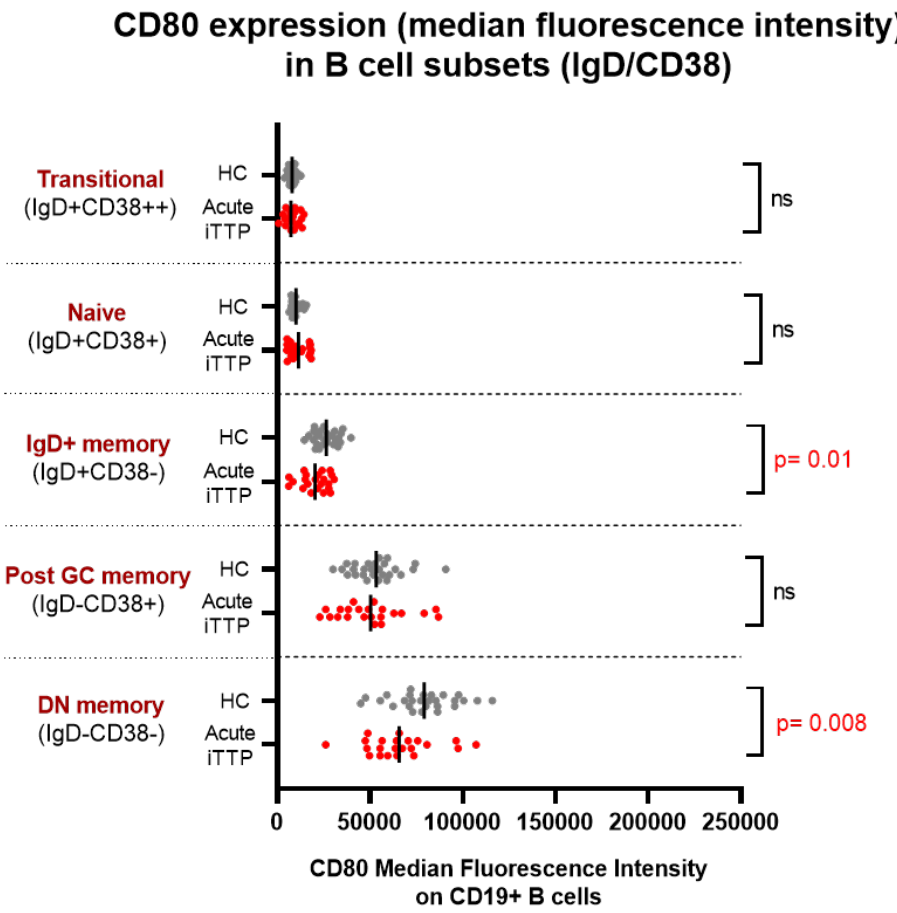


Figure 4

Figure 4

(a) Acute iTTP episodes



(b) ADAMTS13 relapse

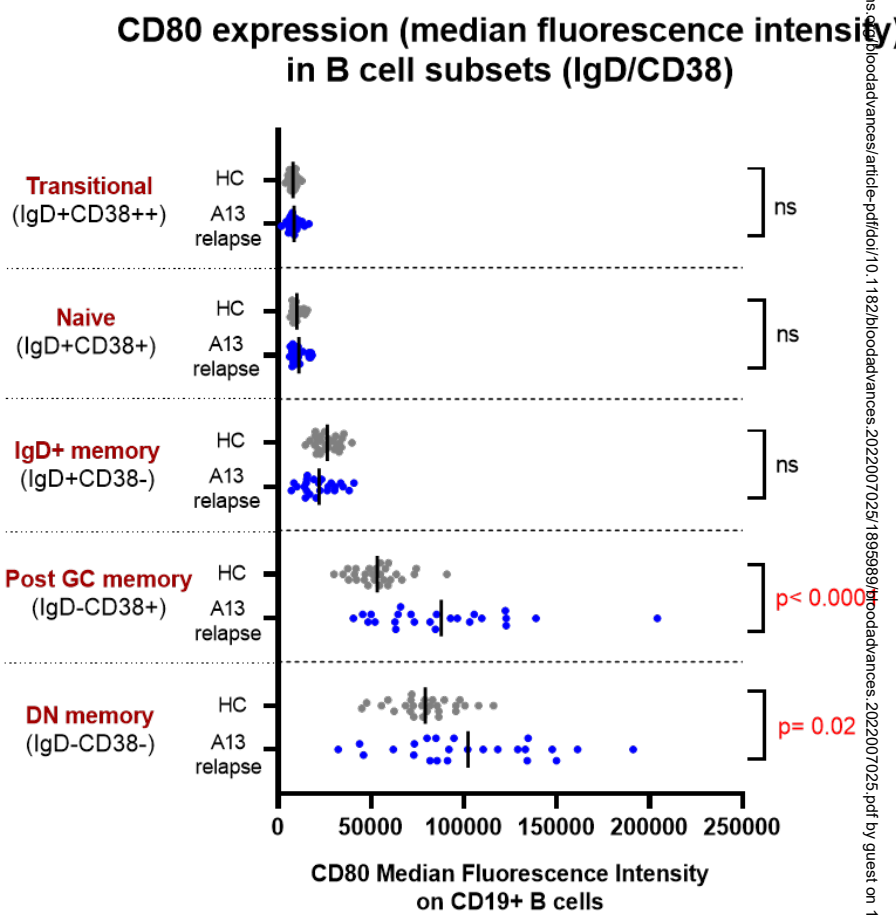


Figure 5 a)

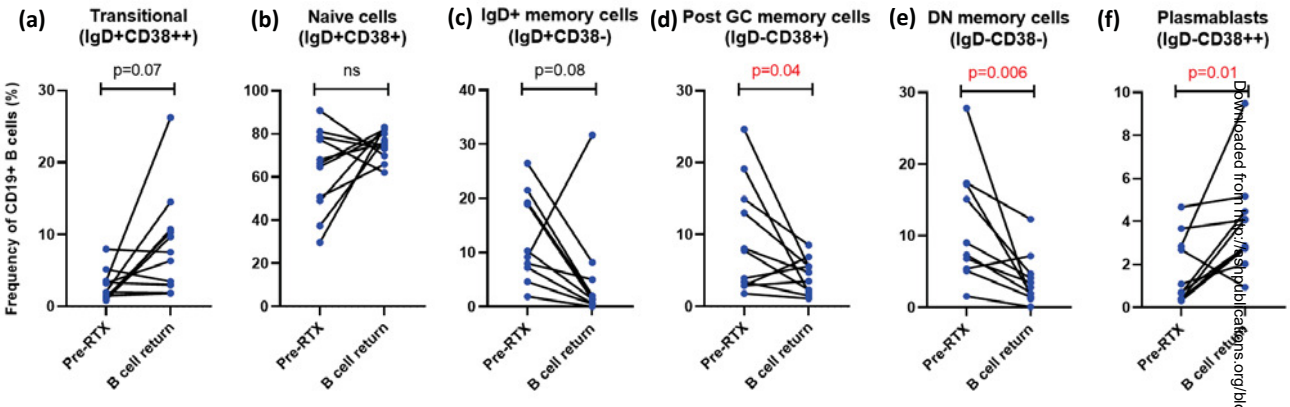


Figure 5 b)

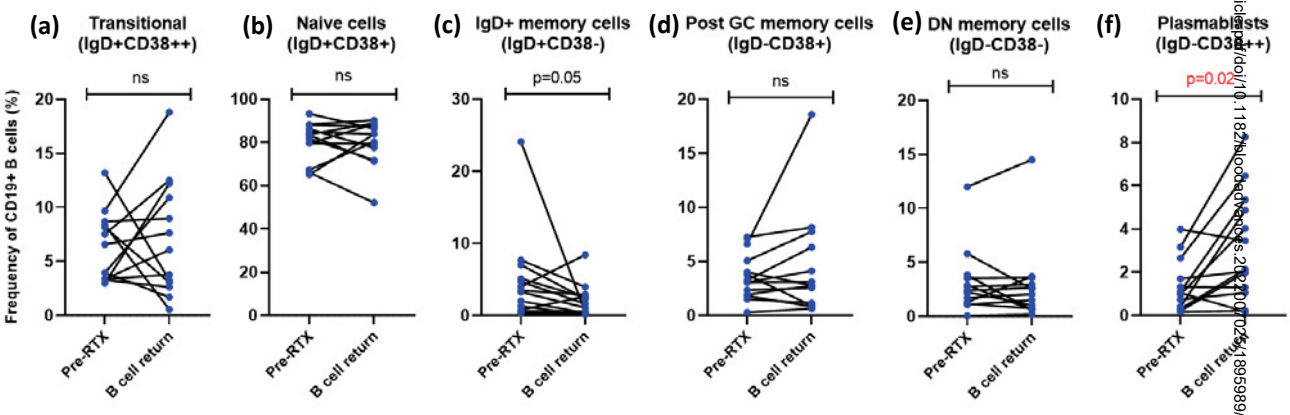


Figure 6