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## A distinct plasmablast and naive B-cell phenotype in primary immune thrombocytopenia

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## **Title Page**

### **Article Title:**

A distinct plasmablast and naive B-cell phenotype in primary immune thrombocytopenia.

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Immunophenotype of primary ITP

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## **Abstract**

Primary immune thrombocytopenia is an autoimmune disorder in which platelet destruction is a consequence of both B- and T-cell dysregulation. Flow cytometry was used to further characterize the B- and T-cell compartments in a cross-sectional cohort of 26 immune thrombocytopenia patients including antiplatelet antibody positive (n=14) and negative (n=12) patients exposed to a range of therapies, and a cohort of matched healthy volunteers. Markers for B-cell activating factor and its receptors, relevant B-cell activation markers (CD95 and CD21) and markers for CD4+ T-cell subsets, including circulating T follicular helper-like cells were included. Our results indicate that an expanded population of CD95+ naive B-cells correlated with disease activity in immune thrombocytopenia patients regardless of treatment status. A population of CD21-naive B-cells was specifically expanded in autoantibody-positive immune thrombocytopenia patients. Furthermore, the B-cell maturation antigen, a receptor for B-cell activating factor, was consistently and strongly upregulated on plasmablasts from immune thrombocytopenia patients. These observations have parallels in other autoantibody-mediated diseases and suggest that loss of peripheral tolerance in naive B-cells may be an important component of immune thrombocytopenia pathogenesis. Moreover, the B-cell maturation antigen represents a potential target for plasma cell directed therapies in immune thrombocytopenia.

## Introduction

Primary immune thrombocytopenia (ITP) is a clinical diagnosis given to patients with an unexplained, prolonged isolated thrombocytopenia. ITP is a rare but chronic condition in adults and is associated with significant bleeding-related morbidity and mortality.<sup>1</sup> The condition is characterized by both platelet destruction and impaired platelet production. A role for platelet-directed antibodies was established in the 1960s with transfer experiments showing that thrombocytopenia could be induced by transfer of the gamma-globulin fraction of ITP patient serum.<sup>2</sup> Using the most sensitive assays, antibodies binding platelet membrane glycoproteins are present in approximately 50% of patients.<sup>3</sup> The mechanism by which B-cell tolerance is lost is debated, but an elevated serum level of B-cell Activating Factor (BAFF) is likely to be an important contributing factor.<sup>4</sup> BAFF drives B-cell maturation, promotes B-cell survival and augments immunoglobulin production by binding three surface B-cell receptors: BAFF receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA).<sup>5</sup> An expanded CD95 (Fas receptor) positive population of B-cells has also been described in ITP and there are reports of fewer regulatory B-cells, defined both as CD24<sup>hi</sup>CD38<sup>hi</sup> B-cells and by IL-10 production.<sup>6,7</sup>

A modern view of ITP pathogenesis places these B-cell abnormalities within a complex network of abnormalities affecting multiple immune cell lineages. T-cells, in particular, contribute to platelet destruction both by facilitating the production of class-switched, high affinity autoantibody and through B-cell

independent mechanisms such as cell-mediated cytotoxicity directed against platelets.<sup>8</sup> The latter may be the primary mechanism of disease in a subset of patients with no detectable anti-platelet antibodies.<sup>9</sup> High-affinity autoantibody production is facilitated by T follicular helper cells (TFH), a subset recently reported to be expanded proportional to germinal centre and plasma cell numbers within the spleens of ITP patients.<sup>10</sup>

This study sought to extend existing knowledge of immune dysregulation in ITP by performing detailed flow cytometry-based immunophenotyping of the B- and T-cell compartments. An interest in the therapeutic potential of belimumab, an anti-BAFF humanized monoclonal antibody, led us to focus on BAFF and its receptors in B-cells. While recent studies of immune populations in splenectomy specimens from patients with ITP have by their nature enrolled patients with refractory disease receiving significant immunodulatory therapy, we chose to enroll a cross-section of ITP patients in order to ensure the broadest possible applicability of our findings. Therefore autoantibody positive and negative ITP patients were recruited across a range of platelet counts and prior treatments including rituximab and splenectomy despite the known effects of these therapies on B-cells with the intention of identifying candidate biomarkers of relevance to future clinical trials. An initial analysis was performed comparing splenectomy- and rituximab-naive ITP patients with healthy volunteers, and significant results were evaluated in the larger cohort.

## **Methods**

### **Patients and healthy volunteers**

A cross-sectional cohort of adult patients with a clinical diagnosis of chronic ITP was recruited from patients in the UK ITP registry visiting the outpatient clinic of the Royal London Hospital Department of Haematology (Table 1 and Table S2). All patients able to give informed consent were considered for inclusion; the only exclusion criterion was ongoing immunosuppressive or cytotoxic therapy for a non-ITP diagnosis (one renal transplant recipient). Recruitment was stratified to give approximately equal numbers of patients by anti-platelet antibody status. All participants provided one venous blood sample; a subset of patients provided a second sample at a later time-point. None of the patients had received a platelet transfusion in the 10 days prior to or intravenous immunoglobulin in the 21 days prior to venesection.

Age- (within 10 years) and sex-matched healthy volunteers (HV) were recruited locally from within the GSK donor pool in parallel with the ITP patients. Ethical approval was obtained from the National Research Ethics Service, London REC, Ref. 07/H0718/57 (ITP patients) and National Research Ethics Service, Hertfordshire REC, Ref. 07/H0311/103 (GSK donor pool). The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents.



### **Anti-platelet autoantibodies.**

EDTA-anticoagulated venous blood was tested within three days of venesection. Direct tests (for platelet bound IgG and IgM) were determined using the platelet immunofluorescence test (PIFT)<sup>11</sup> and by the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay<sup>12</sup> to determine whether there was platelet bound IgG localised to platelet glycoproteins (GP)IIb/IIIa, GPIa/IIa or GPIb/IX. A patient was considered to have platelet autoantibodies if the results of the direct PIFT or the direct MAIPA assay were greater than the mean +3 standard deviations of the values obtained for platelets obtained from at least three normal blood donors on the same day. These investigations were performed at the Histocompatibility and Immunogenetics Laboratory, NHS Blood & Transplant – Filton.

### **Flow cytometry**

Venous blood was collected into lithium heparin tubes (Fisher Scientific) and arrived at the laboratory within 3 hours. Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). For transitional B-cells, PBMC were pre-incubated with MitoTracker green (Invitrogen) at 10nM concentration for 20 minutes at 37°C, then washed before adding antibodies.<sup>13</sup> For B-cell immunophenotyping, a common antibody panel consisting of anti-CD38 (PE-Cy7, eBioscience 25-0389), anti-CD27 (APC, eBioscience 17-0279), anti-CD19 (APC-Cy7, BD Biosciences 557791), anti-IgD (biotin, BD Biosciences cat 555777) and anti-CD3 (Pac Orange, Invitrogen CD0330) was used, with the following antibodies added to individual tubes as required: anti-CD10 (PE, BD Bioscience 555375), anti-IgG (FITC, BD

Biosciences 555786), anti-IgM (PerCP Cy5.5, BioLegend 314512), anti-CD95 (PE, BioLegend 305608), anti-CD21 (PE, BD Biosciences 555422), anti-CD24 (PerCP eFluor710, eBioscience 46-0247), anti-BCMA (PerCP eFluor710, custom GSK conjugate), anti-BAFFR (FITC, BioLegend 316904), and anti-TACI (PE, BioLegend 311906).

For T-cell immunophenotyping, a common skeleton consisting of anti-CD3 (APC-Cy7, BioLegend 300318), anti-CD4 (Pacific Blue, BioLegend 317429), anti-CD45RA (PerCP Cy5.5, eBioscience 45-0458), and anti-CXCR5 (PE, R&D Systems FAB190P) was supplemented with anti-CCR6 (PE-Cy7, BioLegend 353418), anti-PD1 (APC, BD Biosciences 558694) and anti-CXCR3 (FITC, R&D Systems FAB160F) for general T-cell subsets/T-follicular helper cells and with anti-CD25 (PE-Cy7, BD Biosciences 557741) and anti-CD127 (AF647, BD Biosciences 558598) for regulatory T-cells. Normal rat serum and normal mouse serum was added to all tubes to minimize non-specific binding (Table S1).

In each case  $1 \times 10^6$  cells were stained in 100uL at room temperature in the dark for 20 minutes. Tubes containing anti-IgD were then stained with streptavidin eFluor 450 (eBiosciences 48-4317) for a further 20 minutes.

Cells were resuspended in 200uL FACS buffer and acquired immediately on a BD Canto II (BD Biosciences). A compensation matrix was calculated using BD CompBeads for all stains except Mitotracker, where positive and negative live cells were used. Gating was performed in FlowJo vX (Miltenyi). B-cell gating is shown (Figure 1A). CD4+ T-cells are gated as CD4+CD3+ cells in the lymphocyte

gate. Memory CD4+ T-cells are gated as CD45RA-. Tregs are gated as a discrete CD25 high, CD127 low population. For quality assurance, each ITP sample was run in parallel with a HV sample using the same antibody mixes; only technically adequate samples were included in the analysis.

### **BAFF Enzyme Linked Immunosorbent Assay (ELISA)**

Serum was extracted from venous blood collected into serum tubes and centrifuged at 2000rpm for 15 minutes. This was stored at -80°C until the ELISA could be performed, in three overlapping batches. The Quantikine Human BAFF/BLyS/TNFSF13B Immunoassay (R&D Systems, cat # DBLYS0) was used according to the manufacturer's instructions.

### **Data analysis and statistical methods.**

The main analysis was performed using a 'patient-level' cohort, comprising one sample per ITP patient and a matched HV sample, stratified by prior splenectomy or rituximab use. Where patients provided multiple samples, the time-point with the lowest platelet count was chosen. Twenty-six ITP patients fulfilled these criteria for the B-cell analysis and 18 patients for the T-cell analysis with healthy volunteers matched to each analysis. Two patients had FACS data without a platelet count; these were only excluded from analyses requiring a platelet count. Four patients in the ITP cohort had platelet count and FACS data from multiple time-points, allowing a repeated measures analysis.

All data analysis was performed using R ([www.r-project.org](http://www.r-project.org)). Between group comparisons were performed using pairwise Wilcoxon signed-rank tests, except for a repeated measures analysis of the association between platelet count and CD95+ naive B-cells where a linear mixed effects model was implemented using the nlme package in R. An alpha level of 0.05 was considered significant.

## Results

### Major B-cell populations.

A cross-sectional cohort of 26 ITP patients, 12 of whom had active disease (i.e. platelet count  $< 50 \times 10^9/L$ ) was matched to 26 HV (Table 1). Nine ITP patients were on no current treatment and had not received prior B-cell modulating therapies; 12 patients had previously received rituximab or a splenectomy and a number were also receiving other agents including mycophenolate, azathioprine and romiplostim (Table S2). Fourteen ITP patients had detectable platelet-bound IgG or IgM antibodies, and seven of these had antibodies with the following specificities: GPIIb/IIIa (4), GPIb/IX (1) or both GPIIb/IIIa and GPIb/IX (2).

The major B-cell subsets (i.e. B-cells overall, naive (CD27-IgD+) B-cells, CD27+IgD+ memory B-cells, CD27+IgD- memory B-cells, 'double-negative' (CD27-IgD-) B-cells and circulating plasmablasts gated as shown in Figure 1A) were compared between splenectomy- and rituximab-naive ('untreated') ITP patients (n=14) and HV. No differences were observed in these headline populations between ITP patients and HV (Figure 1B). There were also no differences between untreated ITP patients and HV in the most immature

population of peripheral blood B-cells, transitional B-cells, despite phenotyping these in detail (Figures 1C-D).

### **B-cell expression of CD95 (Fas receptor) and CD21 (Complement receptor 2).**

There were, however, differences in the B-cell surface expression of CD95 and CD21, both linked previously to autoimmune disease.<sup>14,15</sup> Overall, CD95 was expressed in a bimodal fashion on the B-cell surface (Figure 2A) with the proportion of CD95+ cells increasing stepwise along the B-cell differentiation pathway (i.e. median proportion in HV of CD95+ naive B-cells = 1%, IgD+CD27+ memory B-cells = 16%, IgG+IgD-CD27+ memory B-cells = 43% and circulating plasmablasts = 98%, Figure 2B). In naive and IgD+CD27+ memory B-cells, there was a small but highly statistically significant expansion of the CD95+ population in untreated ITP patients compared to HV (Figure 2B). ITP patients with prior splenectomy or rituximab also had a higher median proportion of CD95+ IgD+CD27+ memory B-cells than their untreated counterparts and a positive association was observed with time since rituximab therapy (Figure 2C-D), suggesting an additional effect of B-cell depletion. This is in contrast to the proportion of CD95+ naive B-cells, which was increased similarly in all ITP patients regardless of prior rituximab and splenectomy exposure and which was not associated with time post-rituximab.

The size of the CD95+ naive population was correlated with disease activity, with the largest expansion observed in those patients with platelet counts  $< 50 \times 10^9/L$  (Figure 2E). Using the four ITP patients who had been bled at a second time-

point (ranging from 56 to 455 days between samples), including one who had previously had a splenectomy and one who had received rituximab 2.6 years prior to the first blood draw, we found that that within individual patients, an improving platelet count was associated with a reduction in the proportion of CD95+ naive B-cells ( $p=0.05$ , repeated measures using a linear mixed effects model, Figure 2F). Prior rituximab or splenectomy was also associated with an expansion of the proportion of CD95+ cells compared to HV in IgD-CD27+ memory B-cells (data not shown).

CD21, on the other hand, was present in HV on a median 98% of naive B-cells, 87% of IgG+IgD-CD27+ memory B-cells and 36% of plasmablasts, confirming other reports that this marker is lost stepwise during B-cell terminal differentiation (Figures 3A-B). There was a significant reduction in the proportion of naive B-cells expressing CD21 in untreated ITP patients compared with matched HV (Figure 3B). This was unrelated to disease activity (platelets  $< 50 \times 10^9/L$  versus platelets  $\geq 50 \times 10^9/L$ ,  $p=0.79$ ). Instead, this loss of CD21 on naive B-cells was restricted to patients with detectable platelet autoantibodies and appeared to be ameliorated by treatment with rituximab or splenectomy (Figure 3C). Median platelet counts were similar between antibody positive and negative patients ( $51.5 \times 10^9/L$  versus  $56.5 \times 10^9/L$ ,  $p=0.93$ ).

#### **BAFF and its receptors.**

Serum BAFF levels were increased in our cohort of ITP patients overall, consistent with other studies. Moreover, in untreated patients there was a trend

toward higher serum BAFF levels in active disease (i.e. platelet count <  $50 \times 10^9/L$ ) compared with patients in remission (Figure 4A). This trend was not seen in the treated group, most likely due to increases in serum BAFF in the setting of rituximab and splenectomy. This is well-described and a consequence of the effect of B-cell depletion on BAFF release and synthesis.<sup>16</sup> There were no differences in serum BAFF levels by autoantibody status (data not shown).

In the absence of published data on the expression of BAFF receptors following rituximab, we analysed both treated and untreated ITP patients. BAFFR was detectable on all populations of B-cells except for plasmablasts (Figure 4B). TACI was broadly expressed on memory B-cells and plasmablasts but present on only a very small population of naive B-cells (Figure 4C). There were no significant differences in the B-cell expression of either of these markers between HV and untreated ITP patients, but we observed a significant decrease in BAFF receptor expression on a number of B-cell populations and increase in TACI expression on naive B-cells in samples from ITP patients after rituximab or splenectomy (Figure 4C). Neither of these showed a correlation with time since last rituximab dose (data not shown).

BCMA was primarily present on plasmablasts, but was also detected on a small number of double-negative (CD27-IgD-) B-cells. BCMA was markedly and consistently upregulated on plasmablasts in ITP (Figure 4D), irrespective of platelet count, prior treatment or autoantibody status (Figures 4D-F).

### **T-cell phenotype.**

Data on T-cell populations were also available for 18 ITP patients. We found a trend to proportionally fewer CD4+ T-cells in splenectomy- and rituximab-naive ITP patients compared to HV, consistent with previous reports of a reduced CD4:CD8 ratio in ITP (Wilcoxon p-value = 0.053, **Figure 5A**).<sup>17</sup> We found few other differences within the memory T-cell, CXCR5+ memory T-cell and Treg populations when these were analysed as a proportion of their parent populations. Reduced numbers of CD4+ T-cells and memory CD4+ T-cells overall in our cohort of splenectomy- and rituximab-naive ITP patients resulted in a number of differences in subpopulations in absolute terms (Figure 5B).

### **Discussion**

Reduced CD21 and increased CD95 expression on naive B-cells and marked BCMA upregulation on plasmablasts are identified as important components of the immune phenotype of ITP. CD95 is upregulated rapidly upon B-cell activation,<sup>18</sup> and the ITP-specific expansion of CD95+ and of CD21- naive B-cells observed in this study may represent a population of autoreactive B-cells activated in the presence of circulating platelet autoantigens. It is known that self-reactive B-cells emerge from the bone marrow in reasonable quantities,<sup>19</sup> and that peripheral mechanisms of tolerance can be overcome in the presence of high BAFF levels.<sup>20</sup>

Mechanistic data in support of this hypothesis comes from a recent study of B-cells from patients with flaring SLE.<sup>21</sup> Using B-cell receptor repertoire analysis by



next-generation sequencing, the authors demonstrated that a distinct subset of naive B-cells constituted an important source of autoreactive antibody secreting cells. This activated subset was characterized by increased CD95 expression and reduced expression of CD21. Although our flow panels were not designed to study the coexpression of these markers, these findings mirror the expanded CD95+ and CD21- naive B-cell populations we describe here. In SLE, this subset was expanded further during flaring disease, consistent with our observation that expansion of CD95+ naive B-cells in ITP correlated with disease activity.

The loss of CD21, a receptor for C3d that interacts with CD19 to lower the threshold for signaling through the B-cell receptor,<sup>22</sup> has been linked to a subset of naive B-cells in which autoreactive cells are overrepresented. Expansion of this CD21- naive B-cell population has been described in other immune-mediated conditions as well as ITP and SLE including Sjogren's syndrome, common variable immunodeficiency and rheumatoid arthritis.<sup>23-25</sup> It is of interest that in our study CD21- naive B-cells were found predominantly in antiplatelet antibody positive patients, regardless of platelet count. This would be consistent with the hypothesis that even though the size of the CD95+ CD21- naive B-cell population was small, it plays an important role in the generation of autoantibody.

Given this hypothesis, it would have been informative to study the expression of CD95 and CD21 in a cohort with non-immune thrombocytopenia. This population was not available to this study, but is a potential avenue of future research. The lack of differences in the transitional B-cell compartment is in

apparent contradiction to an earlier report of fewer CD24<sup>hi</sup>CD38<sup>hi</sup> B-cells in non-splenectomized patients with active ITP.<sup>6</sup> However, the changes reported were subtle and only found in patients with a platelet count < 50x10<sup>9</sup>/L.

Comparatively small numbers restricted our ability to perform detailed sub-analyses and as such, it is possible that our cross-sectional cohort was not powered to replicate this observation. Alternatively, this may be because we used the gating strategy of Palanichamy *et al*,<sup>13</sup> to distinguish three populations of transitional B-cells. The CD24<sup>hi</sup>CD38<sup>hi</sup> population of the earlier report would represent the more immature T1-2 populations in our study. However, we were able to demonstrate a trend toward expansion of these early B-cell populations in patients post-rituximab, consistent with a previous report.<sup>26</sup>

The dominant finding of our T-cell analysis was a CD4+ T-cell lymphopenia, most prominent in the memory compartment. Such a lymphopenia has not been described previously in ITP and should be interpreted with caution.

Lymphopenia is a recognized consequence of therapy and four of the 16 ITP patients analyzed were receiving antiproliferative agents (i.e. azathioprine, mycophenolate).<sup>27</sup> The lack of observed differences in other subsets, especially Tregs, may reflect the cross-sectional nature of the patient cohort, which was recruited across a range of disease activity.

Finally, there was a strong upregulation of BCMA on plasmablasts in ITP. The role of BCMA in the pathogenesis of autoimmune disease is complex and incompletely understood. While the sole B-cell phenotype of BCMA<sup>-/-</sup> mice appears to be impaired survival of long-lasting plasma cells,<sup>28</sup> when crossed onto

a lupus prone background BCMA<sup>-/-</sup> mice exhibited a range of pathologies including increased plasma cell number, elevated systemic BAFF and an increased titre of anti-nuclear antibody than their BCMA sufficient counterparts.<sup>29</sup> Similarly, little is known about the regulation of BCMA transcription and membrane expression. Certainly Blimp-1 and IRF4 are important positive regulators,<sup>30</sup> consistent with BCMA's predominant expression on plasma cells and plasmablasts, however whether BCMA regulates, or is regulated by, its ligands BAFF and APRIL is unknown. Additional weight for a role for BCMA in autoimmune disease comes from studies in SLE, where B-cell expression of BCMA has been shown to be elevated.<sup>31</sup> Current therapies, including rituximab, do not target plasma cells well. The restricted tissue expression pattern of BCMA, its important role in plasma cell survival and its increased expression in ITP and other autoimmune disease together make it an attractive target for novel plasma cell directed therapies.

Our study adds significant detail to an emerging B-cell phenotype that is shared between a number of antibody-mediated autoimmune diseases. This phenotype is characterized by expanded populations of CD95<sup>+</sup> and CD21<sup>-</sup> naive B-cells, elevated levels of serum BAFF and elevated BCMA expression on plasmablasts. As well as ITP, aspects of this phenotype have also been observed in SLE, common variable immunodeficiency and rheumatoid arthritis.<sup>23,32</sup> Individually, these autoimmune diseases are rare, but a common immune phenotype may streamline the development of effective therapies targeting multiple diseases. The CD95<sup>+</sup> population of naive B-cells in particular is identified as warranting further study as a potential biomarker for this phenotype, being both correlated

with disease activity within and between individuals, and robust to commonly used B-cell modulating therapies.

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## Tables

**Table 1. Baseline demographics, treatment received and autoantibody status for ITP patients and HV used in the B-cell analysis.**

	ITP	HV
Demographics		
Patients in cohort, number	26	26
Median age, years (range)	36 (22-63)	37 (19-62)
Gender, number (%)		
Female	16 (62%)	16 (62%)
Male	10 (38%)	10 (38%)
ITP duration, years (range)	7.3 (1.1-37.4)	
Prior rituximab or splenectomy		
Rituximab alone, number (%)	7 (27%)	
Splenectomy alone, number (%)	1 (3.8%)	
Both, number (%)	4 (15%)	
Current thrombopoetin receptor agonist, number (%)	6 (24%)	
Anti-platelet antibodies, number (%)		
Positive by direct MAIPA (IgG)	7 (28%)	
Positive by direct PIFT (IgG & IgM)	13 (50%)	
Positive by direct PIFT & direct MAIPA	14 (54%)	

Median platelet count,  $\times 10^9/L$  (IQR)\* 52 (36-119)

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\*missing=2. MAIPA (monoclonal antibody immobilization of platelet antigens); PIFT (platelet immunofluorescence test); IQR (interquartile range); HV (healthy volunteer); ITP (immune thrombocytopenia).



## Figure Legends

**Figure 1. Analysis of major B-cell subsets: differences by diagnosis.** (A) Gating strategy for B-cell subsets. (B) Major B-cell subsets expressed as a percentage of CD19+ cells and stratified by diagnosis (i.e. healthy volunteers or splenectomy- and rituximab- naive ITP patients). (C) Gating strategy for transitional B-cells. Mitotracker positive gate defined using expression on switched memory B-cells. Transitional B-cells are Mitotracker+ IgD+CD27-. T1 transitional population is CD10+; T2 & 3 populations are distinguished by CD38 expression. (D) Transitional B-cell populations, expressed as a percentage of B-cells and stratified by diagnosis as in B. Significant pairwise p-values (Wilcoxon signed-rank test) are shown. ritux/splen (rituximab or splenectomy); mem (memory). Plasmablasts are gated out of other B-cell subsets.

**Figure 2. CD95+ (Fas receptor+) naive B-cells are more frequent in ITP patients.** (A) Representative gating, showing distribution of CD95+ cells across the major B-cell subsets. (B) Proportions of CD95+ cells across B-cell subsets, stratified by diagnosis (i.e. healthy volunteers or splenectomy- and rituximab-naive ITP patients). (C) Proportions of CD95+ naive and IgD+CD27+ B-cells stratified by diagnosis and prior rituximab or splenectomy. (D) Proportions of CD95+ naive and IgD+CD27+ B-cells by timing of rituximab, for those ITP patients who had received prior rituximab. (E) CD95+ cells as a proportion of naive B-cells, stratified by platelet count. (F) CD95+ naive B-cells by platelet

count for ITP patients where a second time-point is available ( $\Delta$  Splenectomy;  $\square$  Rituximab;  $\circ$  Neither). Significant pairwise p-values (Wilcoxon signed-rank test) are shown in B-C & E. P-value in F is generated using a repeated measures linear mixed effects model. PB (plasmablast); DN (IgD-CD27- B-cells); plt = platelet count; ritux/splen (rituximab or splenectomy). Plasmablasts are gated out of other B-cell subsets.

**Figure 3. CD21- (complement receptor 2-) naive B-cells are more frequent**

**in ITP patients.** (A) Representative gating, showing distribution of CD21- cells across the major B-cell subsets. (B) Proportions of CD21- cells across B-cell subsets, stratified by diagnosis (i.e. healthy volunteers or splenectomy- and rituximab- naive ITP patients). (C) CD21- cells as a proportion of naive B-cells, stratified by anti-platelet antibody status. Significant pairwise p-values (Wilcoxon signed-rank test) are shown. sw mem + PB (switched memory B-cells and plasmablasts i.e. CD27+IgD- B-cells); DN (double-negative B-cells i.e. CD27- IgD- B-cells); nonsw memory (non-switched memory i.e. CD27+IgD+ B-cells); Ab (antiplatelet antibody); ritux/splen (rituximab or splenectomy).

**Figure 4. Analysis of serum BAFF and its receptors: BCMA is markedly**

**upregulated on the surface of plasmablasts in ITP, regardless of activity or**

**autoantibody status.** (A) Serum BAFF, stratified by past treatment and diagnosis. BAFFR (B) and TACI (C) median fluorescence intensity (MFI) across the major B-cell subpopulations, stratified by diagnosis and prior treatment. (D) Plasmablast BCMA MFI, stratified by diagnosis and prior treatment. (E-F)

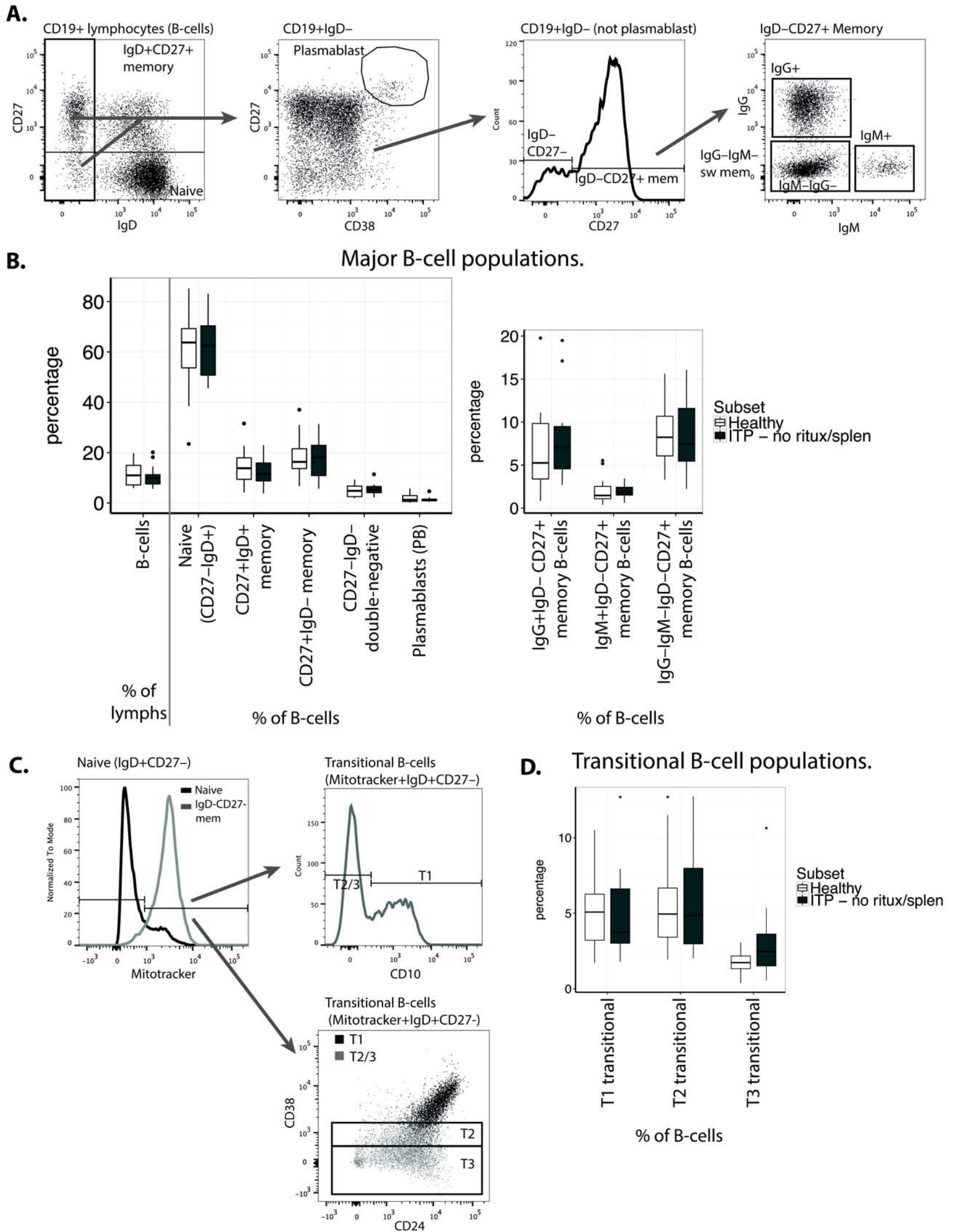
Plasmablast BCMA MFI, stratified by platelet count and autoantibody status.

Significant pairwise p-values (Wilcoxon signed-rank test) are shown. Plt (platelet count); ritux/splen (rituximab or splenectomy).

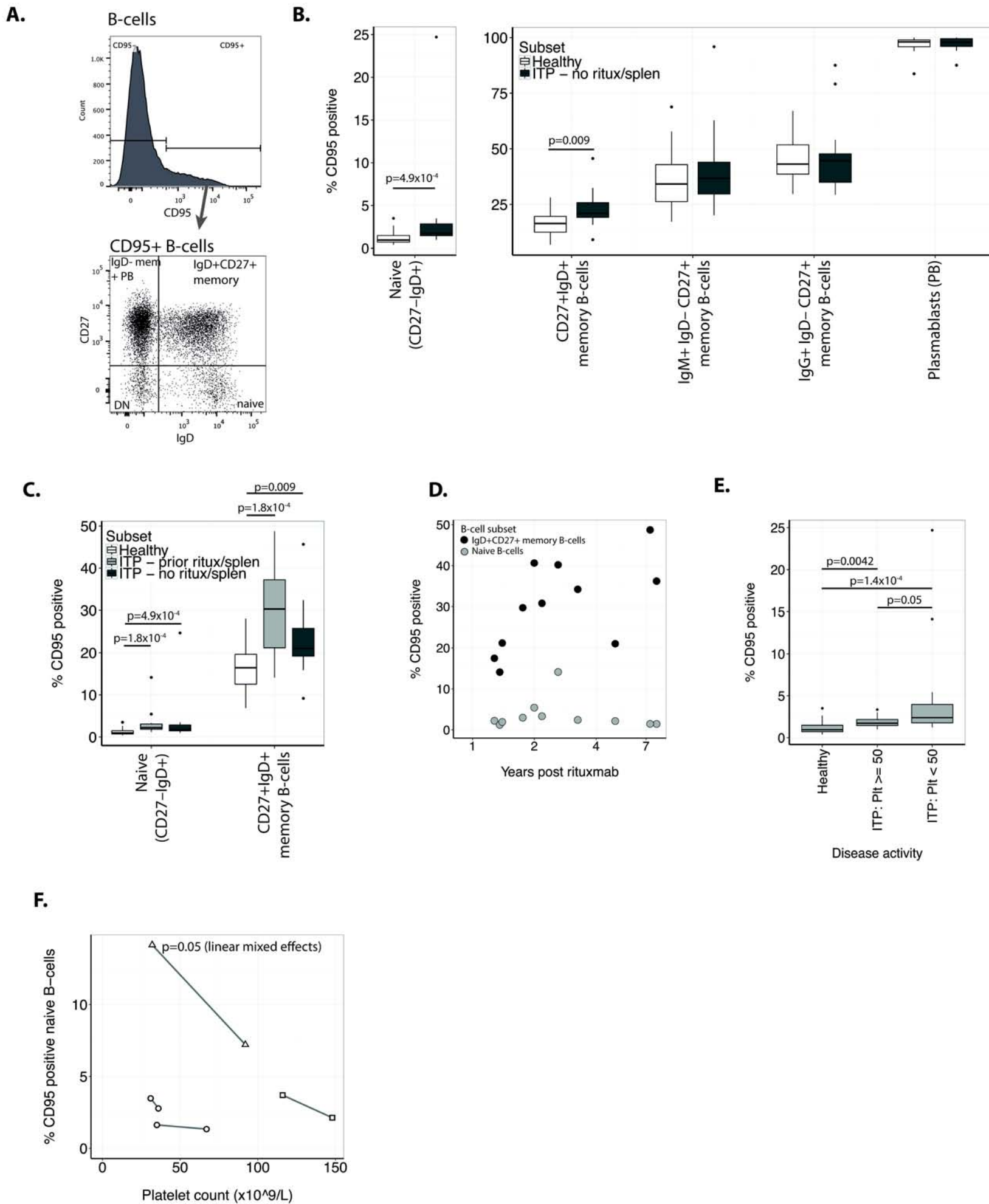
**Figure 5. CD4+ T-cells in ITP and healthy volunteer samples by diagnosis.**

(A) T-cell populations expressed as a proportion of their parent population, stratified by diagnosis (i.e. healthy volunteers (white, n=18) or splenectomy- and rituximab- naive ITP patients (dark grey, n=9)). (B) The same T-cell populations, expressed in absolute numbers (healthy volunteers (white, n=9); splenectomy- and rituximab- naive ITP patients (dark grey, n=8)). Significant pairwise p-values (Wilcoxon signed-rank test) are shown. ritux/splen (rituximab or splenectomy); mem (memory i.e. CD45RA-).

**Figure 1.**

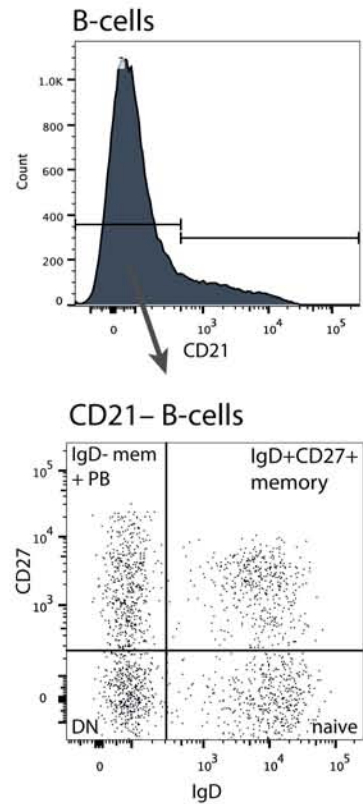


**Figure 2.**

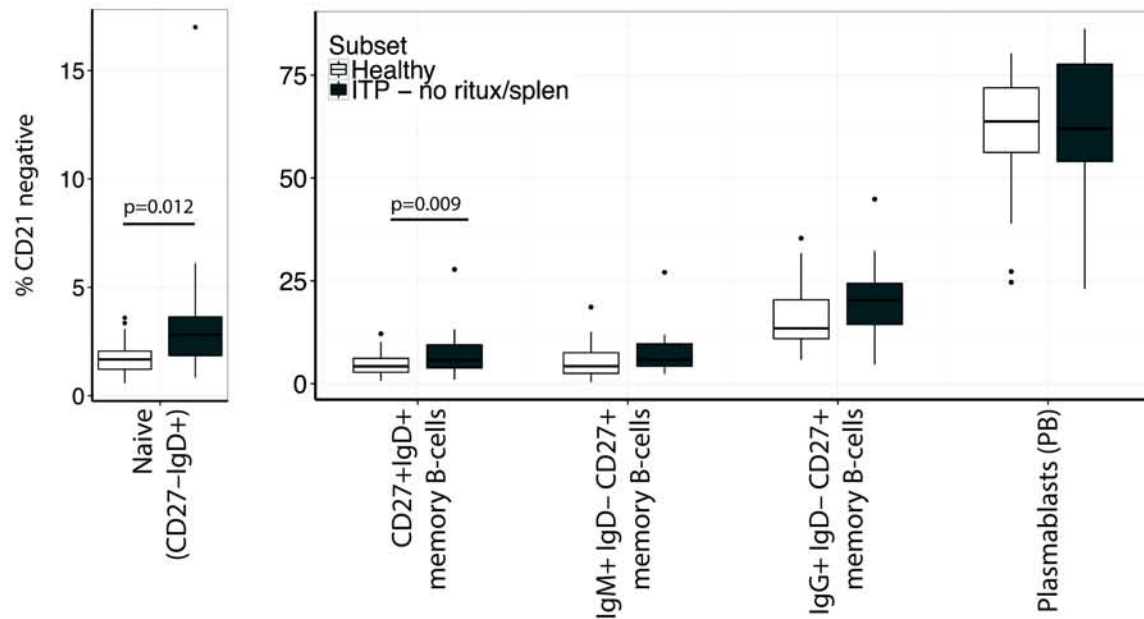


**Figure 3.**

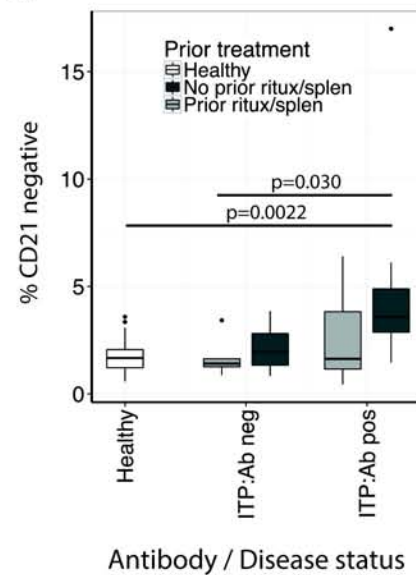
**A.**

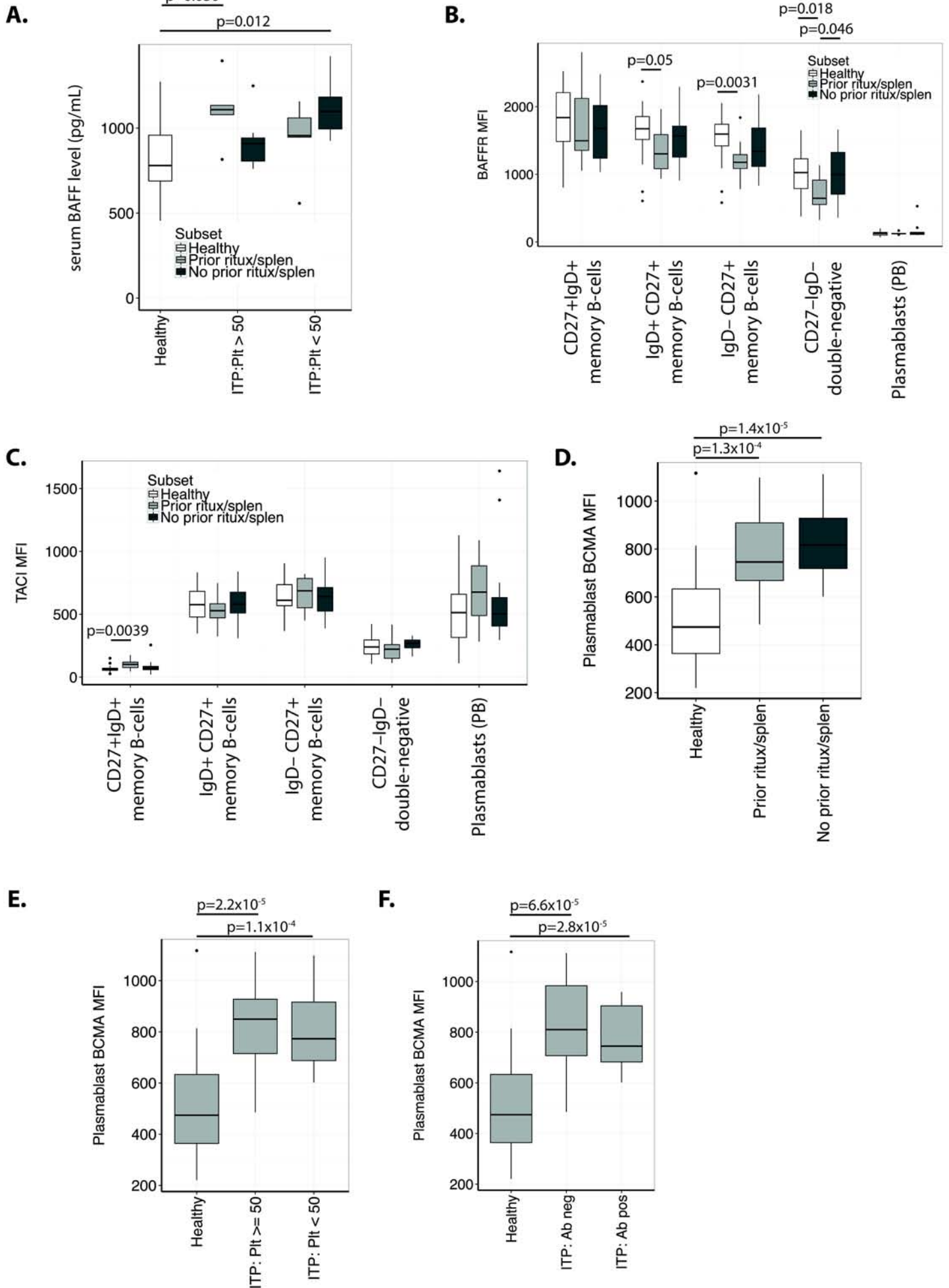


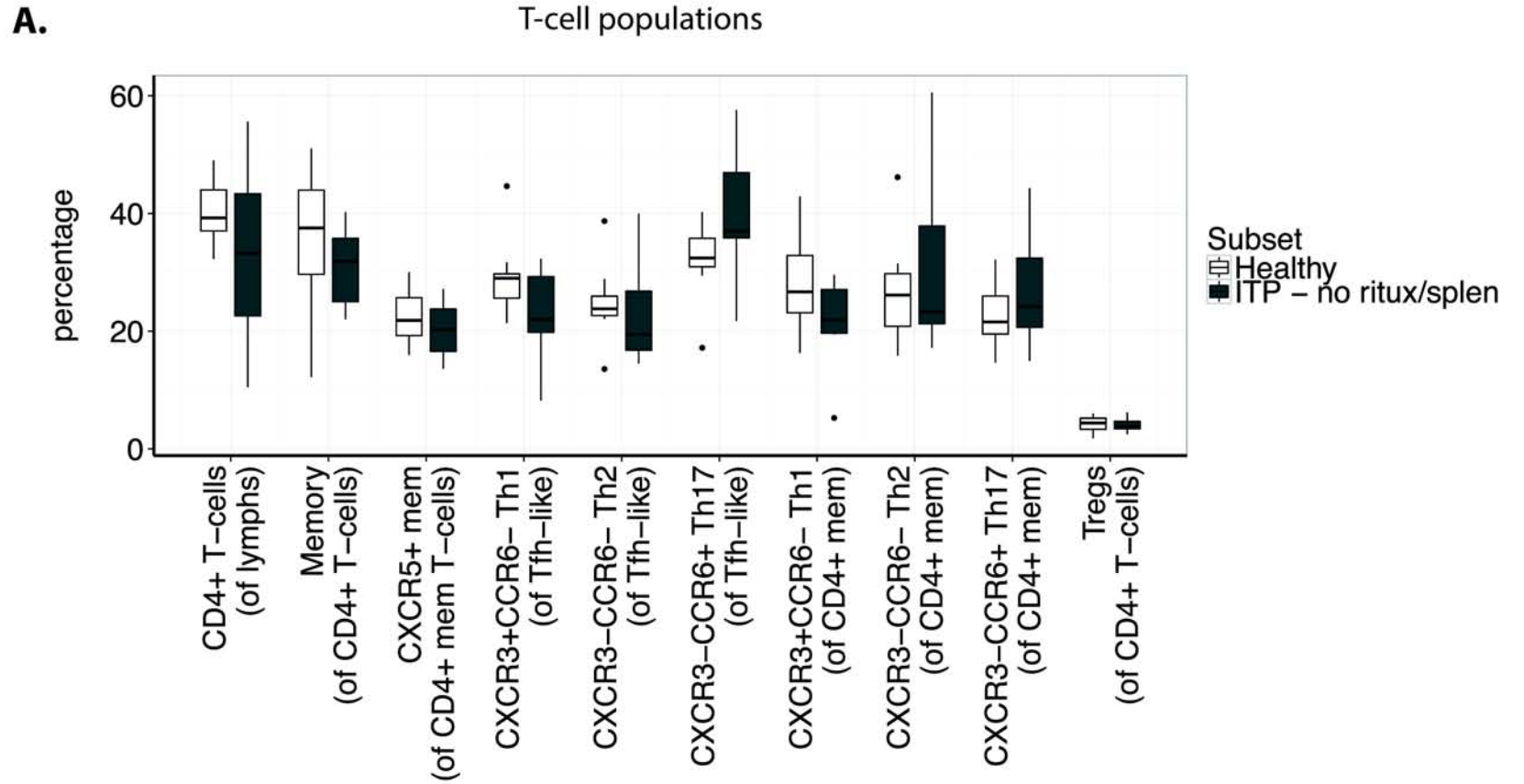
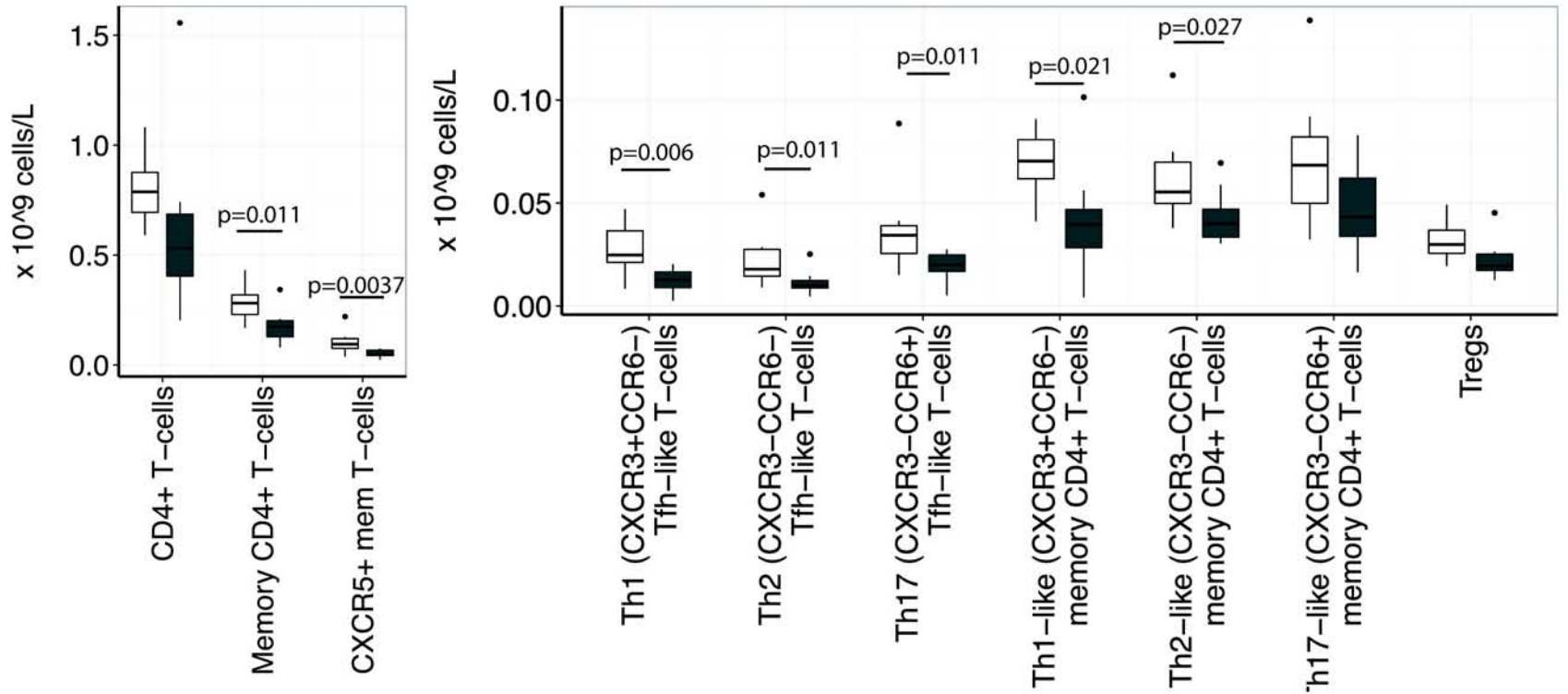
**B.**



**C.**



**Figure 4.**

**Figure 5.****B.** T-cell populations – absolute counts



## A distinct plasmablast and naive B-cell phenotype in immune thrombocytopenia.

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### Supplementary Information

**Supplementary Table 1.** Flow cytometry staining panels.

Channel	FITC	PE	PerCP Cy5.5 PerCP-eF710	PE-Cy7	APC AF647	APC- Cy7	Pac Blue V450	Am Cyan Pac Oran
<b>Transit 1</b>	<i>Mito tracker</i>	CD10	CD24	CD38	CD27	CD19	IgD	CD3
<b>Transit 2</b>	<i>Mito tracker</i>	Iso type	CD24	CD38	CD27	CD19	IgD	CD3
<b>Activated 1</b>	IgG	CD95	IgM	CD38	CD27	CD19	IgD	CD3
<b>Activated 2</b>	IgG	CD21	IgM	CD38	CD27	CD19	IgD	CD3
<b>BAFFR/TACI</b>	BAFF R	TACI	BCMA	CD38	CD27	CD19	IgD	CD3
<b>BAFFR/TACI Iso</b>	BAFF R ( <i>FMO</i> )	TACI	BCMA	CD38	CD27	CD19	IgD	CD3
<b>BAFF R Iso/TACI</b>	( <i>FMO</i> )	TACI	BCMA	CD38	CD27	CD19	IgD	CD3
<b>T reg</b>	CXCR3	CXCR5	CD45RA	CD25	CD127	CD3	CD4	
<b>TFH 1</b>	CXCR3	CXCR5	CD45RA	CCR6	PD-1	CD3	CD4	
<b>TFH 2</b>	CXCR3	CXCR5	CD45RA	( <i>FMO</i> )	PD-1	CD3	CD4	

FMO ('fluorescence minus one' control)

**Supplementary Table 2.** Immunosuppression received by patients at time of first bleed.

	Prior rituximab	Time since last Rituximab (years)	Splenectomy	Romiplostim at bleed	Other immunosuppression
1	Yes	7.88	No	Yes	No
2	Yes	7.33	No	No	MMF 500mg bd
3	Yes	2.61	No	No	No
4	Yes	2.18	No	Yes	No
5	Yes	1.76	No	Yes	No
6	Yes	1.40	No	No	No
7	Yes	1.28	No	No	No
8	Yes	4.96	Yes	Yes	No
9	Yes	3.26	Yes	No	Aza 100mg d
10	Yes	2.00	Yes	Yes	No
11	Yes	1.36	Yes	Yes	No
12	No		No	No	No
13	No		No	No	MMF 1g bd
14	No		No	No	No
15	No		No	No	MMF 1g bd
16	No		No	No	MMF 250mg bd
17	No		No	No	No
18	No		No	No	No
19	No		No	No	MMF 250mg d
20	No		No	NA	No
21	No		No	No	No
22	No		No	No	MMF 500mg bd
23	No		No	No	No
24	No		No	No	No
25	No		No	No	No
26	No		Yes	No	Aza 75mg d

MMF (mycophenolate mofetil); Aza (azathioprine); NA (not available).