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# Increased percentages of circulating T follicular helper cells associate with disease subtype and activity in pediatric immune cytopenias

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**Abstract**

Immune thrombocytopenia (ITP), warm autoimmune hemolytic anemia (wAIHA), and Evans Syndrome (ES) have unpredictable disease activity and are sometimes associated with monogenetic disorders and/or extra-hematologic autoimmunity. ITP and immune neutropenia remain diagnoses of exclusion, hindering the diagnosis of ES in patients with multiple cytopenias. As there are no predictors of immunologic comorbidities in these patients, it is difficult to determine who would benefit from genetic testing and/or evaluations for extra-hematologic autoimmunity. As circulating $CD4^+$ T follicular helper (cTfh) cells promote autoimmunity, we quantified cTfh cells, associated clinical characteristics, and transcriptional signatures in a cohort of 153 pediatric patients with immune cytopenias (85 with ITP, 26 with wAIHA, and 42 with ES). cTfh cell percentages exceeding 9.5% had 76% sensitivity and 86% specificity for distinguishing ES from ITP or wAIHA, irrespective of disease activity, with positive predictive value of 0.68 and negative predictive value of 0.91. Increased percentages of cTfh cells were associated with active cytopenia and decreased with treatment in patients with improving cytopenias over time, suggesting the utility of cTfh measurement for clinical monitoring. Increased percentages of cTfh cells were also associated with underlying immune disorders and extra-hematologic autoimmunity, thus identifying patients who would benefit from more extensive immunologic evaluation. Single cell RNA-sequencing of cTfh cells and plasma cytokines revealed increased IFN-α/β and IFN-γ signaling in patients with active wAIHA and ES, respectively. These results not only uncover immunologic pathways differentiating subtypes of immune cytopenias, but also demonstrate applications of existing clinical tests in diagnosing immune cytopenias and in identifying patients who require more extensive evaluation for immunologic comorbidities.

## Introduction

Pediatric immune cytopenias are challenging to diagnose and manage because they range from self-resolving, single lineage cytopenias to life-threatening immune dysfunction affecting multiple lineages and organs. These disorders include immune thrombocytopenia (ITP), warm autoimmune hemolytic anemia (wAIHA), immune neutropenia, and Evans Syndrome (ES) affecting multiple lineages. While patients with immune cytopenias can achieve sustained remission, a relapsing/remitting or chronic course is common, particularly for those with ES. Pediatric mortality due to ES ranges from 7-35%.[3,4] The diagnosis of ES is challenging due to the lack of established diagnostic tests for ITP or immune neutropenia, leading to a high rate of misdiagnosis.[5,6] There are no known markers of disease activity, immunologic comorbidities, or treatment response.

T cells, including T regulatory and T follicular helper cells, have been implicated in the pathogenesis of ITP in adults but have not been as robustly studied in pediatric immune cytopenias.[7–9] T follicular helper (Tfh) cells are a subset of $CD4^+$ T cells that reside in lymphoid organs, where they activate B cells.[10] They are essential for germinal center formation and antibody production.[10] Circulating T follicular helper (cTfh) cells are $CD4^+CXCR5^+PD1^+$ T cells that share T cell receptor clonotypes and functions with germinal center Tfh cells.[10] Tfh cells are known to drive B cell expansion, activation, and the production of protective antibodies as well as autoantibodies in patients with autoimmunity.[11–13] Measurement of cTfh cells has been studied for the diagnosis and management of many different diseases, including sickle cell disease,[14] primary antibody deficiency,[15] stem cell transplant,[16] and cancer.[17] cTfh cell expansion has been described in pediatric patients with active untreated ES[18,19] and chronic ITP,[20–23] but the clinical features and transcriptional changes associated with cTfh expansion in patients with immune cytopenias remain incompletely understood.

To address these questions, this study evaluated the clinical utility of quantifying cTfh cells in a cohort of 153 pediatric patients with ITP, wAIHA, and ES with different disease activity, treatments, and comorbidities, reflecting the heterogeneity seen in clinical practice.

## Methods

### *Study Cohort*

This single-center prospective cohort study enrolled 153 pediatric patients with immune cytopenias who had research samples drawn at the time of routine clinical care and 78 healthy controls. This study was approved by the Boston Children's Hospital Institutional Review Board and was conducted in accordance with the *Helsinki Declaration* of 1975, as revised in 2013. Patients and/or their legal guardians provided informed consent and assent when appropriate. The inclusion criteria were a diagnosis of ITP, wAIHA, or ES as determined by the treating hematologist, including patients with and without immune disorders and extra-hematologic autoimmunity. Patients were included regardless of cytopenia or treatment status at time of enrollment. Clinical and laboratory data were collected via review of the medical record. Primary or secondary immune disorder was defined as IEI (**Supplementary Data 1**), common variable immunodeficiency[24], or prior hematopoietic stem cell transplant. Extra-hematologic autoimmunity was defined as autoimmunity affecting another organ system other than blood (erythrocytes, platelets, or leukocytes) (**Table 1**).

Patients who were receiving immune cytopenia-directed medication at time of cTfh measurement were considered on treatment. Intravenous immunoglobulin (IVIG) within 4 weeks and rituximab within 6 months before cTfh measurement were considered on treatment. Active cytopenia was defined as cytopenia presence on clinical laboratory results at time of cTfh measurement. Inactive cytopenia was defined as no cytopenia at time of cTfh measurement. Improving disease was defined as ability to discontinue treatment and/or cytopenia resolution. Worsening disease was defined as recurrence of a previously resolved cytopenia. No status

change was defined as no change in whether cytopenia was present and no change in whether a patient was receiving medication compared to the previous timepoint.

*Flow cytometry*

cTfh cells were measured by flow cytometry as a percentage of $CD4^+$ T cells expressing CXCR5 and PD-1 (**Supplementary Figure 1, Supplementary Data 1**).

*Cytokine measurement*

Cytokine levels in plasma were measured with LEGENDplex multiplex bead-based assay panels (**Supplementary Data 1**).

*Single cell RNA sequencing*

Peripheral blood mononuclear cells (PBMCs) were freshly isolated by Ficoll-Paque density gradient centrifugation. $CD4^+$ T cells were isolated from PBMCs from controls and patients using magnetic separation (Miltenyi Biotec, 130-096-533). Single cell RNA sequencing (ScRNA-seq) was performed on $CD4^+$ cells from controls and patients with a total of 10,000 cells per library. Sequencing data have been deposited to National Center for Biotechnology Information Sequence Read Archive (SRA) accession number PRJNA1233813.

*Statistical Analysis*

Data distribution was tested for normality using D'Agostino & Pearson, Anderson-Darling, Shapiro-Wilk, and Kolmogrov-Smirnov tests. The Mann-Whitney test was used for comparisons between two groups. Kruskal-Wallis and Dunn's multiple comparisons tests were used for comparisons of three or more groups. Spearman's correlation was used to evaluate associations between two variables. Receiver operating characteristic (ROC) curve was used to determine sensitivity and specificity. Logistic regression was performed using the mctest package[25] with and without Firth correction. Statistical tests were performed using Graphpad Prism v10.0.0 or R version 4.3.1. Graphs were created with Graphpad Prism v10.0.0.

## Results

*Clinical characteristics*

This study enrolled 78 controls and 153 pediatric patients with immune cytopenias: 85 patients with ITP, 26 with wAIHA, and 42 with ES, with their clinical and demographic characteristics as outlined in **Table 1** and **Supplementary Table 1**. There were 32 patients with immune disorders, including 29 with genetically defined disorders, one with common variable immunodeficiency lacking a genetic cause, and two with a history of hematopoietic stem cell transplantation (**Table 2**). All 42 patients with ES had genetic testing performed and 17 (40.5%) had a genetic diagnosis. Of the 26 patients with wAIHA, 22 (84.6%) had genetic testing performed and 5 (22.7%) of those tested had a positive result. Of the 85 patients with ITP, 36 (42.4%) had genetic testing performed and 8 (22.2%) of those tested had a positive result (**Figure 1A, B** and **Table 2**).

*Clinical features associated with increased percentages of cTfh cells*

Percentages of cTfh cells were significantly higher in patients with ES than in those with ITP ($p<0.0001$), wAIHA ($p=0.0007$), or controls ($p<0.0001$) (median 13.05% in those with ES, 4.82% in those with ITP, 6.11% in those with wAIHA, and 5.16% in controls) (**Figure 2A**). Patients with active or inactive ES had higher percentages of cTfh cells than controls (**Figure 2B**). In contrast, patients with active or inactive ITP or wAIHA had comparable percentages of cTfh cells to controls, irrespective of disease activity (**Figure 2B**). Receiver operating characteristic curve analysis revealed that cTfh exceeding 9.5% had 76% sensitivity and 86% specificity for distinguishing ES from ITP or wAIHA (**Figure 2C**). cTfh measurements greater than 9.5% had a positive predictive value of 0.68 (95% CI 0.54-0.80) and negative predictive value of 0.91 (95% CI 0.84-0.95) for ES as compared to ITP or wAIHA alone. cTfh percentages were highest in patients with active ES off treatment (**Figure 2D**). Among patients with ES, only those with normal blood cell counts without treatment had cTfh percentages comparable to controls (**Figure 2D**).

We next studied cTfh percentages in patients with longitudinal measurements over time. Of the 51 patients with more than one measurement of cTfh cell percentages, 27 had ITP, 6 had AIHA, and 18 had ES. Most patients with ES with improving disease had normalization of cTfh percentages over time (**Figure 2E** and **Supplementary Figure 2**). Among those with persistently active ES, most had cTfh percentages that remained above 11.3%, which we previously published as the 97.5$^{th}$ percentile of cTfh percentages in 210 controls.[26] In contrast, most patients with ITP (88.5%, n=23) and wAIHA (87.5%, n=7) had cTfh values that remained <11.3% (**Figure 2E** and **Supplementary Figure 2**). There was no significant correlation between disease duration and cTfh percentages in patients with ITP, wAIHA, or ES (**Supplementary Figure 3**).

To identify specific immune features associated with cTfh cell expansion, we substratified cTfh percentages by clinical features: (1) primary or secondary immune disorder, (2) extra-hematologic autoimmunity, or (3) elevated anti-nuclear antibody (ANA) titer. Patients with a primary or secondary immune disorder or extra-hematologic autoimmunity accompanying their immune cytopenia had significantly higher cTfh percentages than those lacking these features (**Figure 2F**). We next investigated these associations within each subtype of immune cytopenia. Among patients with ITP and ES, those with an associated primary or secondary immune disorder had increased percentages of cTfh cells compared to those with a history of immune cytopenias alone (**Supplementary Figure 4**). Extra-hematologic autoimmunity was also associated with increased cTfh cell percentages in patients with ITP but not ES or wAIHA, suggesting that extra-hematologic autoimmunity denotes a clinically distinct subgroup of patients with ITP (**Supplementary Figure 4**). As immune cytopenias can be a presenting feature of systemic lupus erythematosus (SLE), ANA is often measured as part of the diagnostic evaluation for these disorders. Using an ANA threshold associated with SLE[27], we found no difference in cTfh between patients who had an ANA ≥1:160 vs. <1:160 in the total cohort (**Figure 2F**) or in patients with ITP or ES. Among patients with wAIHA, cTfh percentages were

increased in patients with ANA ≥1:160 (**Supplementary Figure 4**). We next assessed percentages of regulatory T cells (Tregs), which have been implicated in the pathogenesis of immune cytopenias.[28] $CD4^{+}CD25^{hi}CD127^{low}$ Tregs were measured by a CLIA-approved clinical flow cytometry test in 42 study participants (15 with ITP, 8 with wAIHA, and 19 with ES). Percentages of Tregs were not different among these patients and did not correlate with cTfh percentages (**Supplementary Figure 5**).

Having identified several clinical features associated with elevated cTfh, we used logistic regression to model these relationships. A diagnosis of ES (odds ratio (OR): 13.25, 95% confidence interval (CI): 4.43-44.92, $q$=0.0001), immune disorder, defined as a primary or secondary immune disorder and/or extra-hematologic autoimmunity (OR: 9.88, 95% CI: 3.42-32.22, $q$=0.0001), and active cytopenia (OR: 4.90, 95% CI: 1.73-15.79, $q$=0.0015) were associated with increased percentages of cTfh cells (**Figure 2G, Supplementary Table 2**). Treatment with cytopenia-directed medications at the time of sampling was inversely associated with cTfh percentages (OR: 0.30, 95% CI: 0.09-0.90, $q$=0.0165) (**Figure 2G**).

*Transcriptional profiles of cTfh cells in patients with immune cytopenias*

We hypothesized that the transcriptional profiles of cTfh cells from patients with immune cytopenias differed from controls, even in those with normal percentages. We used single-cell RNA-sequencing (scRNAseq) to analyze cTfh cells from patients with ES (subdivided into those with active or inactive ES and those on or off treatment), active or inactive wAIHA on treatment, and active ITP off treatment (**Figure 3A, B**). Pathway analysis was performed on differentially expressed genes (fold change of at least $\pm$1.5, adjusted $p$<0.05) in each group compared to controls. cTfh cells from patients with active ES off treatment exhibited transcriptional profiles indicative of increased interferon (IFN) signaling, which encompassed targets downstream of IFN-$\alpha/\beta$ (*OASL*, *IFITM3*) and IFN-$\gamma$ (*IFNG*, *CXCR3*, *IL21*) signaling, activation of the T cell receptor and effector kinases (PI3K/AKT) that support the metabolic demands of cell activation,

and apoptosis (**Figure 3C, D**). Additionally, these cells also had decreased IL-7 receptor signaling, a pathway important for T cell survival.[29] In contrast, patients with active or inactive ES on treatment had reduced signaling downstream of T cell activation, the Th1 pathway, and IFN-γ (**Figure 3C, D**), reflecting treatment effects rather than disease activity. In patients with active ES on treatment, cTfh cells exhibited upregulation of the vitamin D receptor/retinoic acid receptor axis known to suppress genes important for the differentiation of Tfh and cTfh cells.[30] Patients with inactive ES on treatment had upregulated signaling in the PD-1 pathway, which inhibits T cell activation and mediates T cell exhaustion. Compared to controls, cTfh cells from patients with inactive ES off treatment had no pathways enriched for differentially expressed genes relevant to T cell development and/or function, consistent with their clinical status. Furthermore, all patients except for those with inactive ES off treatment had transcriptional evidence of increased apoptosis, indicating persistent differences in cTfh transcriptional profiles even in the absence of active cytopenias.

Among patients with wAIHA, there was increased expression of genes indicative of IFN-α/β signaling in those with and without active cytopenias, even though all patients in these cohorts were receiving immunomodulatory treatments. Patients with active wAIHA had increased inflammatory signaling compared to controls, evidenced by increased expression of targets downstream of IFN-γ, the pathway that fuels T cell activation, and IL-8. In contrast, those with inactive wAIHA had reduced antigen presentation and less Th1 compared to controls. Lastly, the transcriptional profiles of cTfh cells from patients with active ITP off treatment exhibited a lack of inflammatory signaling. Compared to controls, patients with active ITP off treatment had decreased expression of genes important for T cell activation (Notch1, STAT3, and CD28 costimulation), IL-3, a cytokine produced by activated T cells, and TGF- β, a regulator of Tfh cell development (**Figure 3C**). Despite the normal percentages of cTfh cells in patients with wAIHA and ITP, scRNA-seq analysis shows that increased IFN signaling distinguishes the cTfh cells in those with wAIHA.

We next used unsupervised hierarchical clustering of differentially expressed genes in the IFN-α/β and IFN-γ pathways to compare the disease groups (**Figure 4**). Patients with active ES off treatment and active AIHA on treatment clustered together with the most robust IFN signatures. Those with active ES off treatment had the highest expression of genes indicative of IFN-γ signaling (*IFNG*, *CXCR3*, *IL21*), while patients with active wAIHA on treatment had the highest expression of genes downstream of IFN- α/β signaling (*IFIT1*, *MX1*, *OAS1/2/3*, *ISG15/18*, among others). Patients with inactive wAIHA on treatment and active ES on treatment formed the next cluster (**Figure 4**). cTfh cells from patients with inactive wAIHA on treatment exhibited increased expression of IFN-stimulated genes compared to those with active ES on treatment, underscoring the robust IFN signature in patients with wAIHA regardless of cytopenia presence. Patients with inactive ES on or off treatment, active ITP, and controls formed the last cluster; among these, patients with ITP clustered most closely with controls. Collectively, these findings identify Type I and Type II IFN signaling as immunologic mechanisms distinguishing subgroups of patients with immune cytopenias.

*Cytokine profiles*

As the circulating cytokine milieu is known to affect the transcriptional profiles of cTfh cells, we measured 23 cytokines in the plasma of patients with ES, wAIHA, and ITP and in controls. These cytokines included those induced by IFN-α/β, IFN-γ, NF-κB, and Th1/Th2/Th17 effector T cells (**Figure 5** and **Supplementary Figure 6**). We found that CXCL9, a cytokine induced by IFN-γ, was significantly higher in patients with ES (p=0.0008) than controls (**Figure 5A**), consistent with the increased IFN-γ signaling seen in cTfh cells of patients with active ES. Plasma CXCL9 levels were higher in patients with wAIHA than controls (p=0.0357) (**Figure 5A**), also concordant with the pattern of increased IFN-γ signaling observed in cTfh cells from patients with active wAIHA on treatment. In our total cohort, there was a modest but statistically significant correlation between CXCL9 levels and cTfh percentages (p=0.0003, r=0.3267)

(**Figure 5B**). Additionally, levels of the chemokine ligand CCL2 were significantly higher in patients with wAIHA than controls (p=0.0170) (**Figure 5C**). IFN-α/β upregulates the expression of CCL2, which activates and recruits T cells and macrophages to sites of inflammation.[31–33] The increased circulating CCL2 levels were concordant with the transcriptional signature of IFN-α/β signaling in cTfh cells from patients with wAIHA. We identified no correlation between CCL2 levels and cTfh percentages (**Figure 5D**), suggesting that cTfh expansion is associated with Type II, rather than Type I, IFN signaling. There were no significant elevations in the levels of any other cytokines evaluated in patients with ITP, wAIHA, or ES compared to controls (**Supplementary Figure 6**).

**Discussion**

Quantification of cTfh cells is a clinically available test that can aid in diagnosing Evans syndrome, identifying patients with immune disorders and extra-hematologic autoimmunity, and monitoring immune cytopenia activity. Patients with ES had higher cTfh percentages than those with ITP or wAIHA, and those with active ES had the highest percentages. We found that cTfh cell expansion was also associated with presence of extra-hematologic autoimmunity and underlying immune disorders, identifying patients with immune cytopenias who would benefit from immunologic evaluations. cTfh values decreased with treatment and in patients with improving cytopenias over time, suggesting that cTfh measurement may be useful for clinical monitoring. Transcriptional studies of cTfh cells and circulating cytokine profiles revealed IFN-γ and IFN-α/β as key pathways associated with active ES and wAIHA, respectively, which could be potential therapeutic targets.

Quantification of cTfh percentages can aid in the diagnosis of ES, as cTfh exceeding 9.5% had 76% sensitivity and 86% specificity for distinguishing ES from ITP or wAIHA. Kumar et al. have previously shown that 24 pediatric patients with active ES had increased cTfh percentages compared to 22 patients with chronic ITP and 24 healthy individuals, none of whom

were receiving treatment.[18] Our study shows that cTfh percentages distinguish ES from ITP or wAIHA in patients with a spectrum of disease activity and cytopenia-directed treatments. This is clinically relevant because the lack of diagnostic tests for ITP or immune neutropenia, which remain diagnoses of exclusion, often make it difficult to determine whether a patient with multiple cytopenias has ES or cytopenias of other etiologies.[6,34,35] Given the finding of cTfh expansion in patients with ES, cTfh measurement may facilitate the diagnosis of ES in a patient with cytopenias of unclear etiology. Future prospective validation studies using samples collected at the time of diagnosis will be essential to establish the diagnostic utility of cTfh.

Presence of high cTfh could be integrated into clinical practice as a marker of immune dysregulation. Among patients with ES, cTfh percentages were comparable to controls only in patients without active cytopenias off treatment. Longitudinal cTfh measurements normalized in patients with ES with improving disease, suggestive that cTfh normalization may reflect treatment efficacy. Larger longitudinal cohorts are needed to establish the clinical utility of cTfh as a biomarker for patient monitoring. cTfh cell percentages can be measured using equipment and techniques readily available in most clinical laboratories with a low reagent cost.[26]

Logistic regression analysis showed that elevated cTfh percentages were also associated with immune disorders, including inborn errors of immunity, secondary immune disorders, and extra-hematologic autoimmunity. It is often not apparent at diagnosis which patients with immune cytopenias have immune disorders, as predictors of underlying immune disorders in pediatric immune cytopenias are lacking.[36] It is consequently difficult to determine which patients require functional immunologic testing, genetic testing, and multidisciplinary evaluation for extra-hematologic autoimmunity, which is not currently standard of care for all patients with immune cytopenias. We found that increased percentages of cTfh cells were associated with underlying immune disorders in patients with ITP as well as ES (**Supplementary Figure 2**). Therefore, cTfh cell measurements may also identify patients with immune cytopenias who require functional immunologic and genetic testing as well as

multidisciplinary evaluation for extra-hematologic autoimmunity. Measurement of cTfh cells helps support additional diagnostic testing, including genetic testing, for underlying immune disorders.

There are numerous studies evaluating T cells patients with ITP that are complementary to the current study (**Supplementary Table 3**). Some of these studies identified increased percentages of Tfh and decreased Treg cells in patients with ITP, while others showed no increase in cTfh in pediatric patients with ITP.[10,18,21,22,37–40] It is important to note that there are key methodological differences across studies in how cTfh and Treg cells are measured and in the ages of the patient populations studied.[41] The variability in markers used to define cTfh and Treg cells complicates comparisons across studies.[41–44] Furthermore, most prior studies of cTfh cells in immune cytopenias focused on adults with ITP, who have distinct clinical phenotypes from children with ITP[45] and differences in baseline immunity.[46,47] Even within a given age group, there are additional clinical features, such as the presence of anti-platelet antibodies, that may be associated with increased cTfh cells in patients with ITP.[22] In our cohort, patients with ITP who had elevated cTfh cells were those patients who had underlying genetic causes of autoimmunity and/or multisystem autoimmunity.

In addition to differences in cTfh percentages among patients with ES, wAIHA, and ITP, we showed that these disorders can be distinguished by the transcriptional changes in cTfh cells. cTfh cells from patients with active ES off treatment exhibited a gene signature indicative of IFN-γ and IFN-α/β, consistent with increased circulating levels of CXCL9, a chemokine ligand induced by IFN-γ. These findings are concordant with those of Kumar et al. in which targeted transcriptomics of flow-sorted $CD4^{+}CD127^{+}CD25^{low}CXCR5^{+}$ T cells from patients with active untreated ES showed increased expression of *IFNG* and *CXCL9*, with the latter declining after sirolimus treatment.[18,19] We also found that multiple types of immunomodulatory treatment reduced expression of IFN-γ gene targets, which further declines with improving disease. We note that the correlation between CXCL9 and cTfh percentages was modest, suggesting that

IFN- γ may affect other aspects of the cTfh population, including function. However, patients on treatment without active cytopenias still had increased expression of genes indicative of apoptosis and PD-1 signaling, demonstrating that normalization of cTfh percentages does not necessarily indicate normalization of gene expression. Only patients off treatment and without cytopenias had cTfh cell transcriptional profiles comparable to controls.

The transcriptional signature of cTfh cells in wAIHA was previously unknown. We identified a robust Type I IFN signature in cTfh cells from patients with wAIHA in tandem with increased expression of CCL2, a target of IFN-α. The causal link between Type I IFNs and AIHA has been previously established by reversible AIHA caused by pegylated IFN-α and by the development of AIHA in patients with Type I interferonopathy.[48–51] These findings highlight Type I and Type II IFN pathways as potential therapeutic targets for treatment of AIHA and ES, respectively. Future studies are needed to functionally validate the IFN pathway findings and to elucidate the mechanistic relationship between CXCL9 levels and cTfh percentages. Future studies are also needed to determine if blocking Type I or II IFNs with JAK inhibitors may be an effective treatment option for patients with wAIHA, regardless of a genetically defined defect impairing regulation of JAK/STAT signaling. In contrast, cTfh cells from patients with ITP had no transcriptional evidence of activation, so additional investigations are needed to identify markers of active ITP beyond thrombocytopenia. We acknowledge that Th1 skewing is has been identified in many adult cohorts with ITP.[52–55] However, studies have shown that Th1 skewing in children with ITP is much more variable, with some studies showing that only a minority of children with ITP have Th1 skewing.[56,57]

Monogenic immune disorders, known as inborn errors of immunity (IEI), are present in at least 65% of pediatric patients with ES,[58,59] but data regarding IEI frequency in children with ITP and wAIHA remains sparse.[60,61] In this cohort, 40.5% of patients with ES, 22.7% of patients with wAIHA and 22.2% of patients with ITP who underwent genetic testing had a positive result. However, these estimates are limited by the lack of uniform genetic testing across all patient

groups. Given the high prevalence of IEI in patients with ES[58,59], standard of care includes genetic and functional immunologic testing.[62] In contrast, genetic testing is not routinely offered to patients with wAIHA and ITP, even though both are increasingly recognized in immune disorders.[60,61,63] The relatively high rate of genetic findings in patients with ITP and wAIHA in this cohort suggests that genetic evaluation should also be considered in children with single lineage immune cytopenias, especially those with increased cTfh cells. Identification of a genetic disorder informs treatment with targeted or curative therapies, screening for extra-hematologic autoimmunity, and risk of malignancy.[64,65]

This study has several limitations. Due to the study design, samples were not all collected at the time of diagnosis. Prospective validation studies at the time of diagnosis are needed to determine whether increased percentages of cTfh cells in a patient with a single lineage immune cytopenia predicts an increased risk of future development of ES and/or extra-hematologic autoimmunity. Additionally, the number of longitudinal samples was limited, so the effect of specific treatments on cTfh over time could not be evaluated. As ES is a rare disease, future multi-center studies are needed for validation of these findings. As we have shown an expansion of cTfh cell percentages and increased IFN-γ signaling in patients with ES, future studies are needed to elucidate the mechanism by which these immunologic features contribute to disease activity. Many studies have shown that cTfh cells induce B cell activation and differentiation into antibody-secreting cells in disorders characterized by a loss of tolerance, including systemic lupus erythematosus, chronic graft versus host disease, and primary immune regulatory disorders.[66–69] We thus hypothesize that cTfh cells have a similar role in promoting ES disease activity, although future studies are needed to identify the epitopes recognized by autoreactive T and B cells in ES and wAIHA.

In conclusion, this study shows how quantification of cTfh cells can advance clinical characterization of immune cytopenias by identifying disease subtypes as well as patients with genetic and immunologic co-morbidities and by indicating changes in disease activity. Although

cTfh cells are known to drive B cell proliferation and lead to autoantibody production, further study is needed to understand why cTfh cell expansion occurs. Our results highlight CCL2 and CXCL9 as two chemokine ligands that can be used as indicators of Type I and Type II IFN signaling, respectively. We have identified Type I and II IFN signaling as inflammatory pathways upregulated in wAIHA and active ES, respectively, thereby setting the foundation for future studies investigating these pathways as therapeutic targets. As the measurement of cTfh, CXCL9, and CCL2 are relatively inexpensive clinically available tests, these results address the urgent need for better diagnostic tools for patients with immune cytopenias.

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## Tables

**Table 1.** Demographic and clinical features at time of sampling

| **Clinical feature** | **Patients N=153** | | | **Controls N=78** |
|---|---|---|---|---|
| **Disease type** | ITP (n=85) | wAIHA (n=26) | ES (n=42) | |
| **Age,** years, median (range) | 10.6 (1-22) | 13.3 (0.5-27) | 16.9 (0.5-33) | 12.0 (1.3-47.1) |
| **Sex,** female, n (%) | 45 (53%) | 17 (65%) | 15 (36%) | 44 (56.4%) |
| **Race,** n (%) | | | | |
| White (%) | 46 (54%) | 19 (73.1%) | 27 (64.3%) | 48 (61.5%) |
| Asian (%) | 2 (2.4%) | 0 (0%) | 1 (2.4%) | 3 (3.8%) |
| Black (%) | 0 (0%) | 1 (3.8%) | 1 (2.4%) | 5 (6.4%) |
| Other (%) | 13 (15.3%) | 3 (11.5%) | 6 (14.3%) | 11 (14.1%) |
| Unknown (%) | 24 (28.2%) | 3 (11.5%) | 7 (16.7%) | 11 (14.1%) |
| **Ethnicity,** n (%) | | | | |
| Hispanic or Latino (%) | 11 (12.9%) | 1 (3.8%) | 4 (9.5%) | 12 (15.4%) |
| Unknown (%) | 23 (27%) | 6 (23.1%) | 10 (23.8%) | 10 (12.8%) |
| **Time from diagnosis,** days, median (range) | 374 (4-5,827) | 372 (2-3,563) | 1420 (38-10,697) | |
| **Active cytopenia present**, n (%) | 53 (62%) | 8 (31%) | 24 (57%) | |
| **On treatment,** n (%) | 31 (36%) | 24 (92%) | 32 (76%) | |
| Immunoglobulin replacement | 6 | 5 | 6 | |
| Corticosteroid | 2 | 15 | 8 | |
| Anti-CD20 therapies | 4 | 7 | 1 | |
| Bortezomib | 0 | 1 | 0 | |
| Sirolimus | 1 | 3 | 5 | |
| Mycophenolate mofetil | 1 | 5 | 11 | |
| Azathioprine | 0 | 0 | 1 | |
| Thrombopoietin receptor agonists | 21 | 0 | 6 | |
| Abatacept | 0 | 0 | 2 | |
| Ruxolitinib | 0 | 1 | 1 | |
| G-CSF | 0 | 0 | 1 | |
| **Immune disorders** | 8 (9%) | 6 (23%) | 18 (43%) | |
| Inborn error of immunity | 8 | 4 | 17 | |
| CVID | 0 | 0 | 1 | |
| Post-HSCT | 0 | 2 | 0 | |
| **Extrahematologic autoimmunity** | 7 (8%) | 8 (31%) | 12 (29%) | |
| Hepatitis | 1 | 4 | 4 | |
| GI tract | 3 | 2 | 3 | |
| Thyroiditis | 2 | 1 | 4 | |
| Skin | 3 | 1 | 1 | |
| Pulmonary | 1 | 0 | 4 | |
| Systemic lupus erythematosus | 1 | 2 | 0 | |
| Primary sclerosing cholangitis | 0 | 2 | 0 | |
| Type 1 diabetes | 0 | 0 | 1 | |
| **ANA titer $\geq$ 1:160** | 10 (20%, n=50) | 3 (20%, n=15) | 7 (21%, n=33) | |

Some patients have more than one type of extrahematologic autoimmunity. Patients post-HSCT had undergone stem cell transplant for RAG1 deficiency (n=1) and Hurler syndrome (n=1). No patients had undergone splenectomy. ITP = immune thrombocytopenia; wAIHA = warm autoimmune hemolytic anemia; ES = Evans syndrome; CVID = common variable immune deficiency; HSCT = hematopoietic stem cell transplant.

**Table 2. Genetically-defined immune disorders in this study cohort.**

| Immune cytopenia | Genetic Disorder | Gene | Variant |
|---|---|---|---|
| ITP | ALPS | *FAS* | c.856G >A, p.G286R |
| | ch19p13.3 duplication* | 19p13.3 | 19p13.3(260911-4342341x3) *de novo* duplication |
| | DiGeorge syndrome | 22q11.2 | 22q11.2 deletion |
| | Kabuki syndrome† | *KMT2D* | c.12415_12416del, p.Val4139Phefs*28 |
| | Kabuki syndrome | *KMT2D* | c.18267G>T, p.V5423F |
| | Kabuki syndrome | *KMT2D* | c.44221G>T, p.C1474F |
| | NFKB1 haploinsufficiency | *NFKB1* | c.1153G>A, p.Gly385Ser |
| | IKZF1 haploinsufficiency | *IKZF1* | c.1537G>C, p.Glu513Gln |
| wAIHA | DOCK11 deficiency | *DOCK11* | c.1162G>A, p.Gly388Arg |
| | Kabuki syndrome | *KMT2D* | c.13151C>G, p.Ala4384Gly |
| | NFKB1 haploinsufficiency | *NFKB1* | c.259-1G>C |
| | RAG1 deficiency, post-HSCT | *RAG1* | c.1210C>T, p.Arg404Trp<br>c.983G>A, p.Cys328Tyr |
| | STAT1 GOF | *STAT1* | c.604A>G, p.Met202Val |
| ES | ALPS | *FAS* | c.185C>G, p.Pro92Arg |
| | A20 haploinsufficiency | *TNFAIP3* | c.2281C>T, p.Arg761Cys |
| | CD25 deficiency | *IL2RA* | c.530G>A, p.Trp177*<br>c.800del, p.Lys267Argfs*95 |
| | CTLA4 haploinsufficiency | *CTLA4* | c.347T>A, p.I116N |
| | DiGeorge syndrome | 22q11.2 | 22q.11.21 (18661724_21661435)x1 dn, *de novo* deletion of at least 2.9 Mb within cytogenic band 22q11.21 |
| | Kabuki syndrome | *KMT2D* | c.6265_6266delinsCAAT mutation |
| | Kabuki syndrome | *KMT2D* | c.13696G>T, p.Glu4566 |
| | Kabuki syndrome | *KMT2D* | c.15731_15732delAA |
| | Kabuki syndrome | | variant ID unavailable |
| | NFKB1 haploinsufficiency | *NFKB1* | *NFKB1* c.884G>A, p.Trp295* |
| | NFKB1 haploinsufficiency | *NFKB1* | *NFKB1* c.1855_1856del, p.Val619PhefsTer10 |
| | NFKB2 haploinsufficiency | *NFKB2* | *NFKB2* c.1354G>A, p.Gly452Ser |
| | PLAID | *PLCG2* | c.2866C>T, p.Arg956Cys |
| | SAMD9L deficiency | *SAMDL9* | c.1549T>C, p.Trp517Arg |
| | SOCS1 haploinsufficiency | *SOCS1* | c.24delA, p.Ala9Profs∗76 |
| | STAT3 GOF | *STAT3* | c.2144 C>T, p.Pro715Leu |
| | STAT3 GOF | *STAT3* | c.1199A>T, p.Asn400Ile |

*This disorder is associated with immune dysregulation and immunodeficiency.[70,71]
†Although the numbers of patients with Kabuki syndrome in our cohort was likely influenced by our center's multidisciplinary Roya Kabuki program, others have similarly identified immune cytopenias as a common feature of Kabuki syndrome.[72,73] ITP = immune thrombocytopenia; wAIHA = warm autoimmune hemolytic anemia; ES = Evans syndrome; ALPS = autoimmune lymphoproliferative syndrome; GOF = gain-of-function; HSCT = hematopoietic stem cell transplant; PLAID = PLCG2-associated antibody deficiency and immune dysregulation.

## Figures

**Figure 1. Genetic testing results.** (A) Frequency of genetic testing in patients with immune thrombocytopenia (ITP), warm autoimmune hemolytic anemia (wAIHA), and Evans syndrome (ES). (B) Frequency of genetic diagnosis in patients with ITP, wAIHA, and ES who had genetic testing performed.

**Figure 2. Quantification of cTfh cells and associated clinical characteristic in patients with immune cytopenias**. (A) Circulating T follicular helper cells (cTfh), measured as % of $CD4^+$ cells, in peripheral blood samples from patients with immune thrombocytopenia (ITP), warm autoimmune hemolytic anemia (wAIHA), Evans syndrome (ES), and controls (Ctrls); ns=p>0.05, ***p<0.001, ****p<0.0001 by Kruskal-Wallis test (B) cTfh, measured as % of $CD4^+$ cells, by presence (+) or absence (-) of cytopenia at time of sample collection in patients with ITP, wAIHA, and ES as compared to controls (Ctrls) (C) ROC curve comparing cTfh values in patients with ES as compared to non-ES immune cytopenias (isolated ITP or wAIHA) (D) cTfh, measured as % of $CD4^+$ cells, by active cytopenia presence (+) or absence (-) at time of sample collection and whether patient was receiving (+) or not receiving (-) medication treatment at the time of sample collection, in patients with ES and controls (Ctrls); ns=p>0.05, **p<0.01, ****p<0.0001 by Kruskal-Wallis test (E) Longitudinal evaluation of cTfh in patients with improving disease and persistently active disease showing number (No.) of patients with ITP (n=26), wAIHA (n=8), and ES (n=18) who had cTfh that remained <11.3% (white), declined from high to <11.3% (green), increased from normal to >11.3% (grey), and remained >11.3% (black) over time (F) cTfh, measured as % of $CD4^+$ cells, in patients with immune cytopenias with (+) and without (-) extra-hematologic autoimmunity, primary or secondary immune disorder, and anti-nuclear antibody (ANA) ≥1:160. Primary immune disorder refers to genetically defined inborn

error of immunity (IEI) (n= 29). Secondary immune disorder refers to patient who developed immune cytopenia post-stem cell transplant or met clinical criteria for Common Variable Immune Deficiency (CVID) without a genetic diagnosis (n=2). ANA was measured in a subset of participants: 58.8% (n=50) of patients with ITP, 57.7% (n=15) of patients with wAIHA, and 76.2% (n=32) of patients with ES. ns=p>0.05, **p<0.01, ****p<0.0001 by two-tailed Mann-Whitney test (G) Logistic regression analysis of clinical factors associated with high percentages of cTfh cells (>11.3%). Immune disorders include primary immune disorders (inborn errors of immunity), secondary immune disorders, and extra-hematologic autoimmunity. Each patient was included only once for each variable. FDR-adjusted q values are shown.

**Figure 3. Analysis of cTfh transcriptional profiles obtained by single cell RNA-sequencing.** (A) Uniform Manifold Approximation and Projection (UMAP) of circulating T follicular helper (cTfh) cells in patients with the indicated immune cytopenias. ES = Evans syndrome; wAIHA = warm autoimmune hemolytic anemia; ITP = immune thrombocytopenia (B) Clinical characteristics of the patients studied in this analysis. tx = treatment (C) Pathway analysis of differentially expressed genes (-1.5<fold change<1.5, adjusted p value <0.05) in cTfh cells from the indicated patient groups compared to controls. IFN = interferon; IL-1 = interleukin-1; IL-3 = interleukin-3; IL7R = interleukin-7 receptor; IL-8 = interleukin-8; PI3K= Phosphoinositide 3-Kinase; TCR = T cell receptor; Th1 = T helper 1; VDR/RXR = vitamin D receptor / retinoid X receptor

**Figure 4. Unsupervised hierarchical clustering of differentially expressed genes in the IFN-α/β and IFN-γ pathways**. Patients were categorized based on cytopenia type (ES = Evans syndrome, ITP = immune thrombocytopenia, wAIHA = warm autoimmune hemolytic anemia), activity, and treatment (tx). Active indicates patient with active cytopenia present at time of

sample collection. Inactive indicates patient without active cytopenias present at time of sample collection.

**Figure 5. Cytokine levels** (A) C-X-C Motif Chemokine Ligand 9 (CXCL9) levels in plasma samples from patients with immune thrombocytopenia (ITP), warm autoimmune hemolytic anemia (wAIHA), Evans syndrome (ES), and controls (Ctrls); ns=$p>0.05$, *$p<0.05$, ***$p<0.001$ by Kruskal-Wallis test to correct for multiple comparisons. (B) Spearman correlation of CXCL9 levels with circulating T follicular helper cell (cTfh) percentages in all patients and controls. (C) C-C Motif Chemokine Ligand 2 (CCL2) levels in plasma samples from patients with ITP, wAIHA, ES, and controls; *$p<0.05$ by Kruskal-Wallis test to correct for multiple comparisons. (D) Spearman correlation of CCL2 levels with cTfh percentages in all patients and controls.

# Figure 1

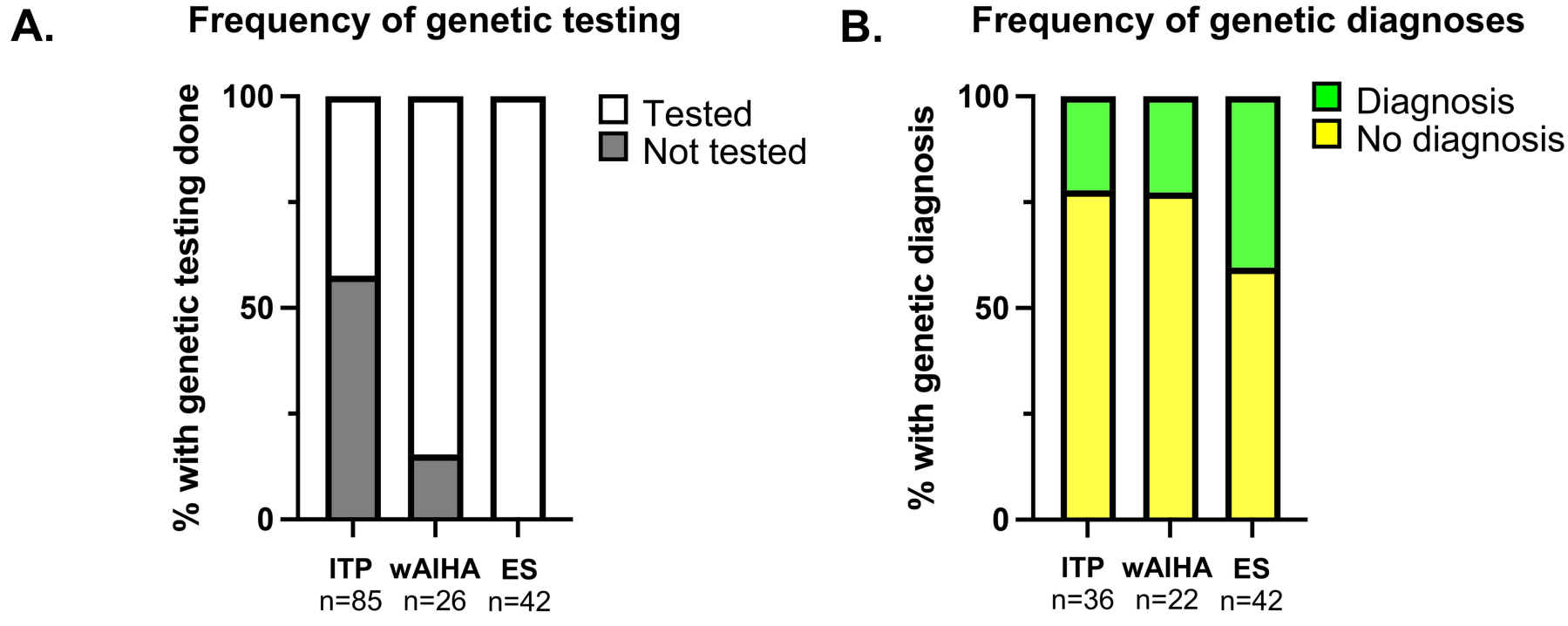

A.
Frequency of genetic testing
% with genetic testing done
100
50
0
Tested
Not tested
ITP
wAIHA
ES
n=85
n=26
n=42
B.
Frequency of genetic diagnoses
% with genetic diagnosis
100
50
0
Diagnosis
No diagnosis
ITP
wAIHA
ES
n=36
n=22
n=42

Figure 2

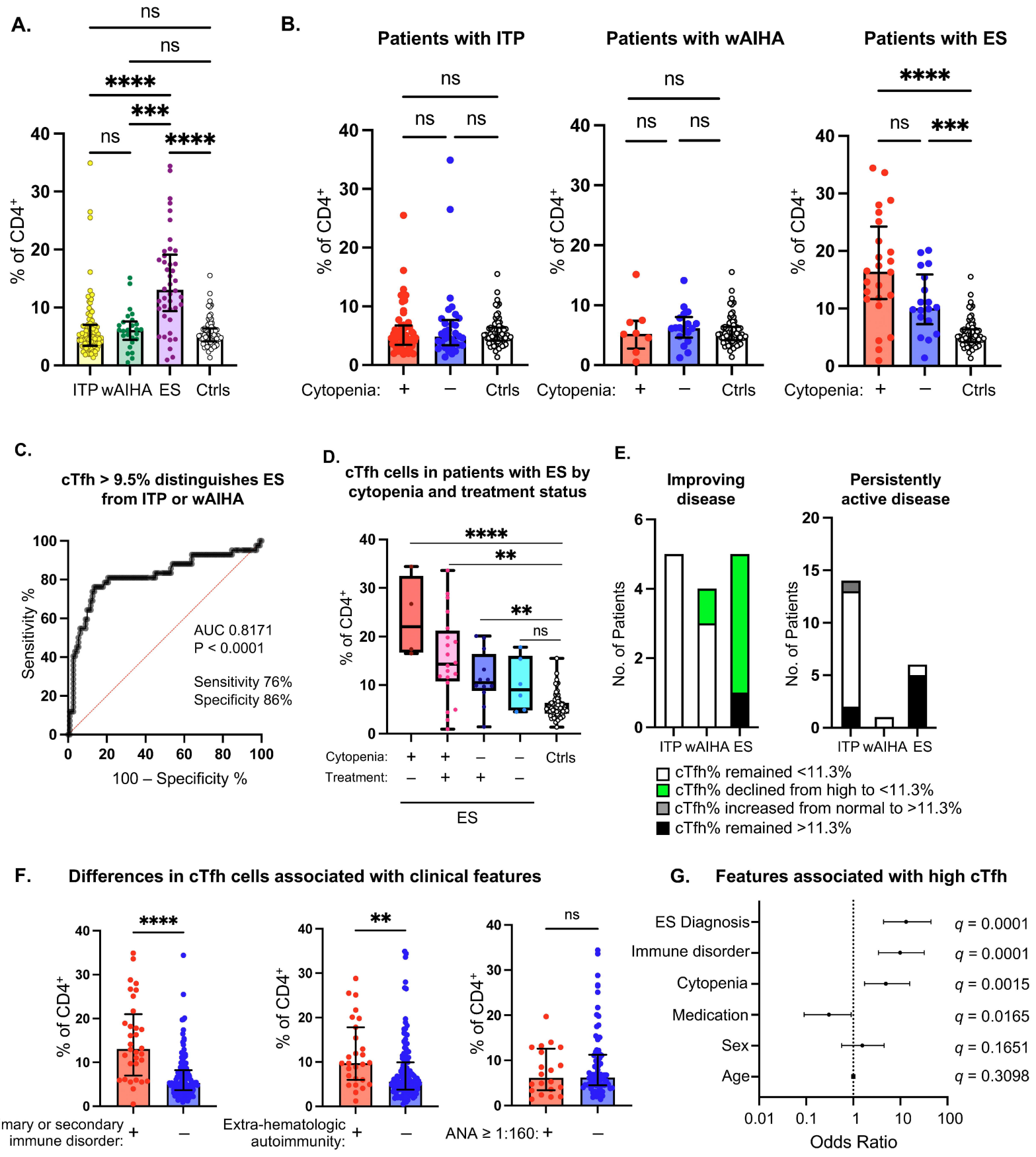
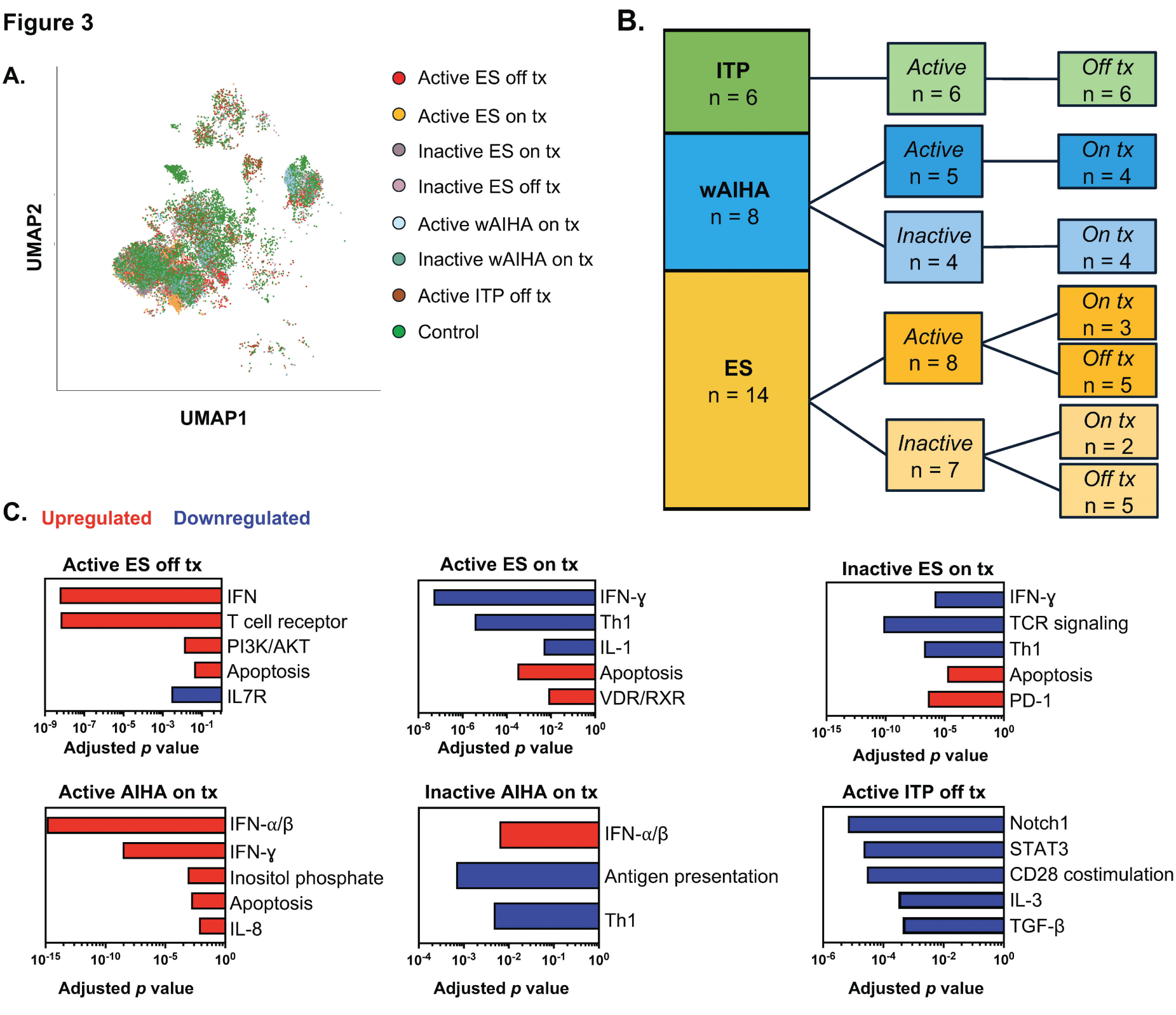

A.
B.
Patients with ITP
Patients with wAIHA
Patients with ES
% of CD4+
ITP wAIHA ES Ctrls
Cytopenia: + − Ctrls
C.
cTfh > 9.5% distinguishes ES from ITP or wAIHA
Sensitivity %
100 – Specificity %
AUC 0.8171
P < 0.0001
Sensitivity 76%
Specificity 86%
D.
cTfh cells in patients with ES by cytopenia and treatment status
Cytopenia:
Treatment:
ES
E.
Improving disease
Persistently active disease
No. of Patients
cTfh% remained <11.3%
cTfh% declined from high to <11.3%
cTfh% increased from normal to >11.3%
cTfh% remained >11.3%
F.
Differences in cTfh cells associated with clinical features
Primary or secondary immune disorder:
Extra-hematologic autoimmunity:
ANA ≥ 1:160:
G.
Features associated with high cTfh
ES Diagnosis
Immune disorder
Cytopenia
Medication
Sex
Age
q = 0.0001
q = 0.0001
q = 0.0015
q = 0.0165
q = 0.1651
q = 0.3098
Odds Ratio

Figure 3


A.
UMAP2
UMAP1
Active ES off tx
Active ES on tx
Inactive ES on tx
Inactive ES off tx
Active wAIHA on tx
Inactive wAIHA on tx
Active ITP off tx
Control
B.
ITP
n = 6
wAIHA
n = 8
ES
n = 14
Active
n = 6
Off tx
n = 6
Active
n = 5
On tx
n = 4
Inactive
n = 4
On tx
n = 4
Active
n = 8
On tx
n = 3
Off tx
n = 5
Inactive
n = 7
On tx
n = 2
Off tx
n = 5
C.
Upregulated
Downregulated
Active ES off tx
IFN
T cell receptor
PI3K/AKT
Apoptosis
IL7R
Adjusted p value
Active ES on tx
IFN-γ
Th1
IL-1
Apoptosis
VDR/RXR
Adjusted p value
Inactive ES on tx
IFN-γ
TCR signaling
Th1
Apoptosis
PD-1
Adjusted p value
Active AIHA on tx
IFN-α/β
IFN-γ
Inositol phosphate
Apoptosis
IL-8
Adjusted p value
Inactive AIHA on tx
IFN-α/β
Antigen presentation
Th1
Adjusted p value
Active ITP off tx
Notch1
STAT3
CD28 costimulation
IL-3
TGF-β
Adjusted p value

# Figure 4

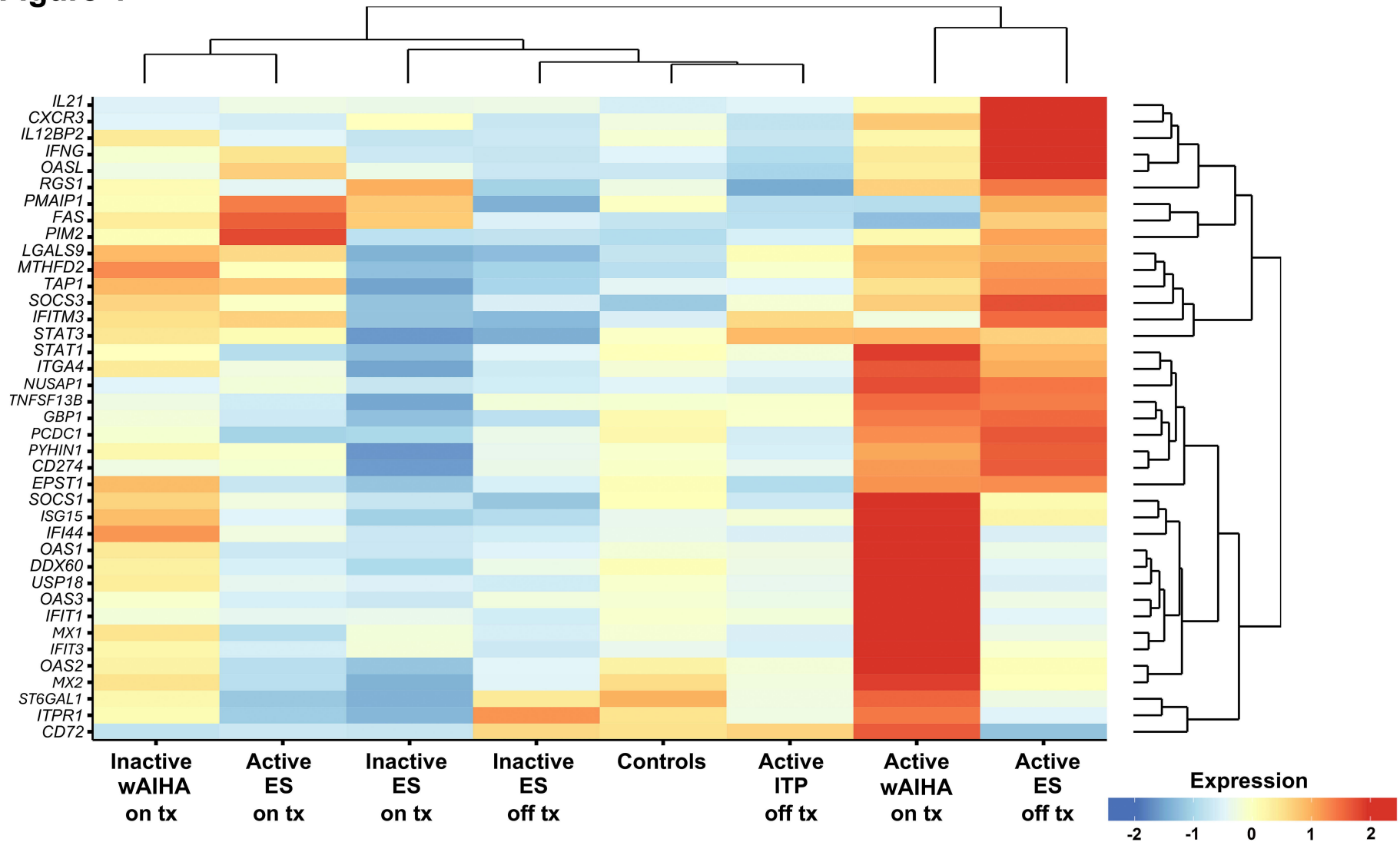
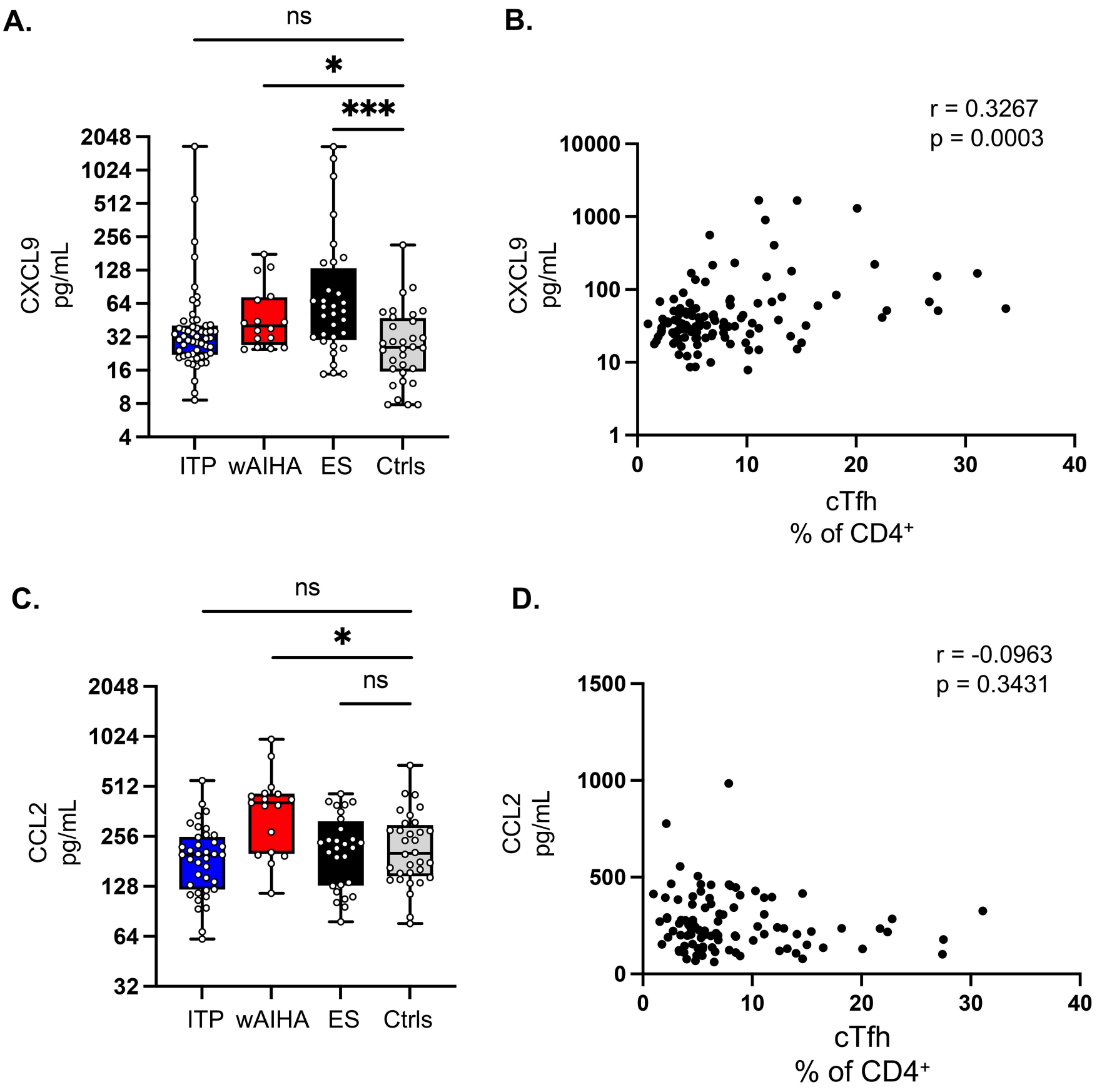

IL21
CXCR3
IL12BP2
IFNG
OASL
RGS1
PMAIP1
FAS
PIM2
LGALS9
MTHFD2
TAP1
SOCS3
IFITM3
STAT3
STAT1
ITGA4
NUSAP1
TNFSF13B
GBP1
PCDC1
PYHIN1
CD274
EPST1
SOCS1
ISG15
IFI44
OAS1
DDX60
USP18
OAS3
IFIT1
MX1
IFIT3
OAS2
MX2
ST6GAL1
ITPR1
CD72
Inactive wAIHA on tx
Active ES on tx
Inactive ES on tx
Inactive ES off tx
Controls
Active ITP off tx
Active wAIHA on tx
Active ES off tx
Expression
-2
-1
0
1
2

**Figure 5**


A.
ns
*
***
2048
1024
512
256
128
64
32
16
8
4
CXCL9
pg/mL
ITP
wAIHA
ES
Ctrls
B.
r = 0.3267
p = 0.0003
10000
1000
100
10
1
CXCL9
pg/mL
0
10
20
30
40
cTfh
% of CD4+
C.
ns
*
ns
2048
1024
512
256
128
64
32
CCL2
pg/mL
ITP
wAIHA
ES
Ctrls
D.
r = -0.0963
p = 0.3431
1500
1000
500
0
CCL2
pg/mL
0
10
20
30
40
cTfh
% of CD4+

**Supplementary methods**

*Study cohort*

Controls included pediatric patients enrolled from outpatient hematology and immunology clinics. The patients included as controls had mild food or environmental allergies not requiring treatment or resolving iron deficiency with either normal or mildly low hemoglobin. These patients were evaluated in a clinical setting where personal and family history was evaluated. Control patients were excluded if they had a history of autoimmunity, immune dysfunction, malignancy, or solid organ or stem cell transplantation or had symptoms of an active infection.

Genetic testing included clinical next generation sequencing panels for inborn error of immunity (IEI) and whole exome sequencing through the Children's Rare Disease Collaborative.[1,2] As previously described,[73] Emedgene software (Illumina), a machine learning program that utilizes explainable artificial intelligence, was used to assist the identification of putative variants. However, we supplemented Emedgene analysis with manual curation and analysis of all variants with a minor allelic frequency of less than 0.001 in gnomAD v4.1.0, regardless of Emedgene classification. Positive genetic results were defined as either a pathogenic variant diagnostic of an immune disorder or a variant of uncertain significance and a clinical and laboratory phenotype consistent with the genetic disorder.

Thrombocytopenia was defined as platelet count $<150*10^6$ cells/μL, neutropenia was defined as absolute neutrophil count <1500 cells/μL, anemia was defined as hemoglobin below lower limit of normal for age and sex.

*Flow cytometry*

Flow cytometry was performed on 100 μL of whole blood collected in sodium heparin anticoagulant tubes. Anti-human monoclonal antibodies (mAbs) were used for staining. Data were acquired on LSRFortessa (BD Life Sciences) cell analyzer and were analyzed using FlowJo™

v10.8 Software (BD Life Sciences). Reagents and mAbs for flow cytometry included: CD4 (BioLegend #317420), PD-1 (BioLegend, #329907), CXCR5 (BioLegend, #356904), CXCR3 (BioLegend, #353716), and CCR6 (BioLegend, #353434).

T regulatory cells (Tregs) were measured on a clinical basis in a Clinical Laboratory Improvement Amendments-certified laboratory that specifies Treg as $CD4^{+}CD25^{hi}CD127^{low}$ T cells.

*Cytokine measurements*

Cytokines were measured using LEGENDplex multiplex bead-based assay panels including Human Inflammation Panel 1 (BioLegend, #740809): IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, CXCL8 (IL-8), IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33 and Human Proinflammatory Chemokine Panel 1 (BioLegend, #741081): CXCL8 (IL-8), CXCL10 (IP-10), CCL11 (Eotaxin), CCL17 (TARC), CCL2 (MCP-1), CCL5 (RANTES), CCL3 (MIP-1α), CXCL9 (MIG), CXCL5 (ENA-78), CCL20 (MIP-3α), CXCL1 (GROα), CXCL11 (I-TAC), CCL4 (MIP-1β). Values that resulted below lower limit of detection were assigned the lowest detectable value for that cytokine.

*Single cell RNA sequencing*

Single cell gene expression libraries from multiplexed samples were prepared using GEM-X Single Cell 5' Reagent Kits v3 (10x Genomics) and sequenced on an Illumina NovaSeq 6000 S4 system with 150-bp paired-end sequencing. Libraries were processed by using 10X Genomics' CellRanger version 9.0.0 and GRCh38 as the reference. Downstream analysis was performed using nf-core/scrnaseq version 3.0.0[74] and nf-core/scdownstream version 0.0.1dev[75] using the default parameters. Both filtered and unfiltered counts were passed to the scdownstream pipeline. Subsequent processing followed the canonical Seurat-based analyses[76], where we chose maximum dimension of 50, cluster resolution of 0.8, and the MAST test in FindMarkers. Cell type annotation was performed using ScType[77] with $CD4^{+}$ T cell gene markers

from Azimuth (celltype.l2)[78] and *CXCR5*, *PDCD1*, and *ICOS* for identifying cTfh cells. Library demultiplexing was performed using Cellsnp-lite[79] followed by Vireo[80]. Pathway analysis of differentially expressed genes (-1.5 < fold change < 1.5, false discovery rate <0.05) between groups was performed with Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood City, Calif).

*Statistical Analyses*

To complement the Dunn's multiple comparisons tests with Bonferroni corrections performed for comparisons of three or more groups, we calculated the false discovery rate (FDR) using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli using a Q value of 5% to calculate FDR adjusted q values.

**Supplementary Figure 1**. **Gating Strategy** to identify $CXCR5^{+}PD1^{+}$ cells for quantification of circulating T follicular helper cells (cTfh) as % of $CD4^{+}$ T cells in a control sample and a patient sample

**Supplementary Figure 2. Longitudinal evaluation of cTfh in patients with ITP, wAIHA, and ES** (A) Patients with immune thrombocytopenia (ITP) with improving disease over time (B) Patients with warm autoimmune hemolytic anemia (wAIHA) with improving disease over time (C) Patients with Evans syndrome (ES) with improving disease over time (D) Patients with ITP with no change in disease status over time (E) Patients with wAIHA with no change in disease status over time (F) Patients with ES with no change in disease status over time (G) Patients with ITP who had worsening disease over time. Each differently colored line represents a unique patient. Dashed line indicates off medication treatment, solid line indicates on medication treatment. Green shading indicates normal circulating T follicular helper cell (cTfh) % of $CD4^{+}$ T cells (<11.3%). Average $\Delta$ indicates average change in cTfh from first to last measurement.

**Supplementary Figure 3. cTfh is not associated with disease duration** in patients with (A) immune thrombocytopenia (ITP), (B) warm autoimmune hemolytic anemia (wAIHA), or (C) Evans syndrome (ES) as evaluated by Spearman correlation.

**Supplementary Figure 4. Clinical features associated with cTfh in each immune cytopenia subtype.** Circulating T follicular helper cell (cTfh) % of $CD4^{+}$ T cells in patients with immune thrombocytopenia (ITP), warm autoimmune hemolytic anemia (wAIHA), and Evans syndrome (ES) with (+) or without (-) extra-hematologic autoimmunity, primary or secondary immune disorder, and anti-nuclear antibody (ANA) $\geq$ 1:160; ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001 by two-tailed Mann-Whitney test.

**Supplementary Figure 5. Regulatory T cells as measured by clinical flow cytometry** (A) $CD4^{+}CD25^{hi}CD127^{low}$ regulatory T cells in immune thrombocytopenia (ITP), warm autoimmune hemolytyic anemia (wAIHA), and Evans syndrome (ES); ns=p>0.05 by Kruskal-Wallis test (B) $CD4^{+}CD25^{hi}CD127^{low}$ regulatory T cells are not correlated with circulating T follicular helper cell (cTfh) % of $CD4^{+}$ T cells by Spearman correlation.

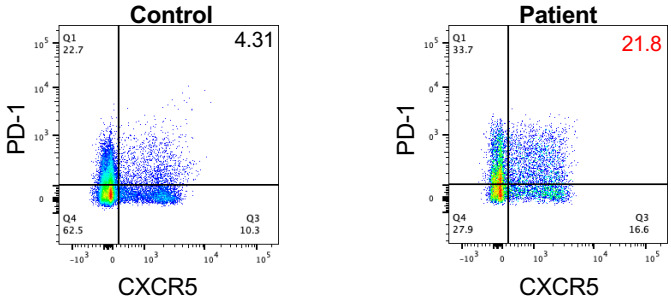
**Supplementary Figure 6. Levels of plasma cytokines in patients with immune cytopenias** ns=p>0.05, *p<0.05, ***p<0.001 by Kruskal-Wallis test.

**Supplementary Table 1.** Patient and control ages at time of sample collection

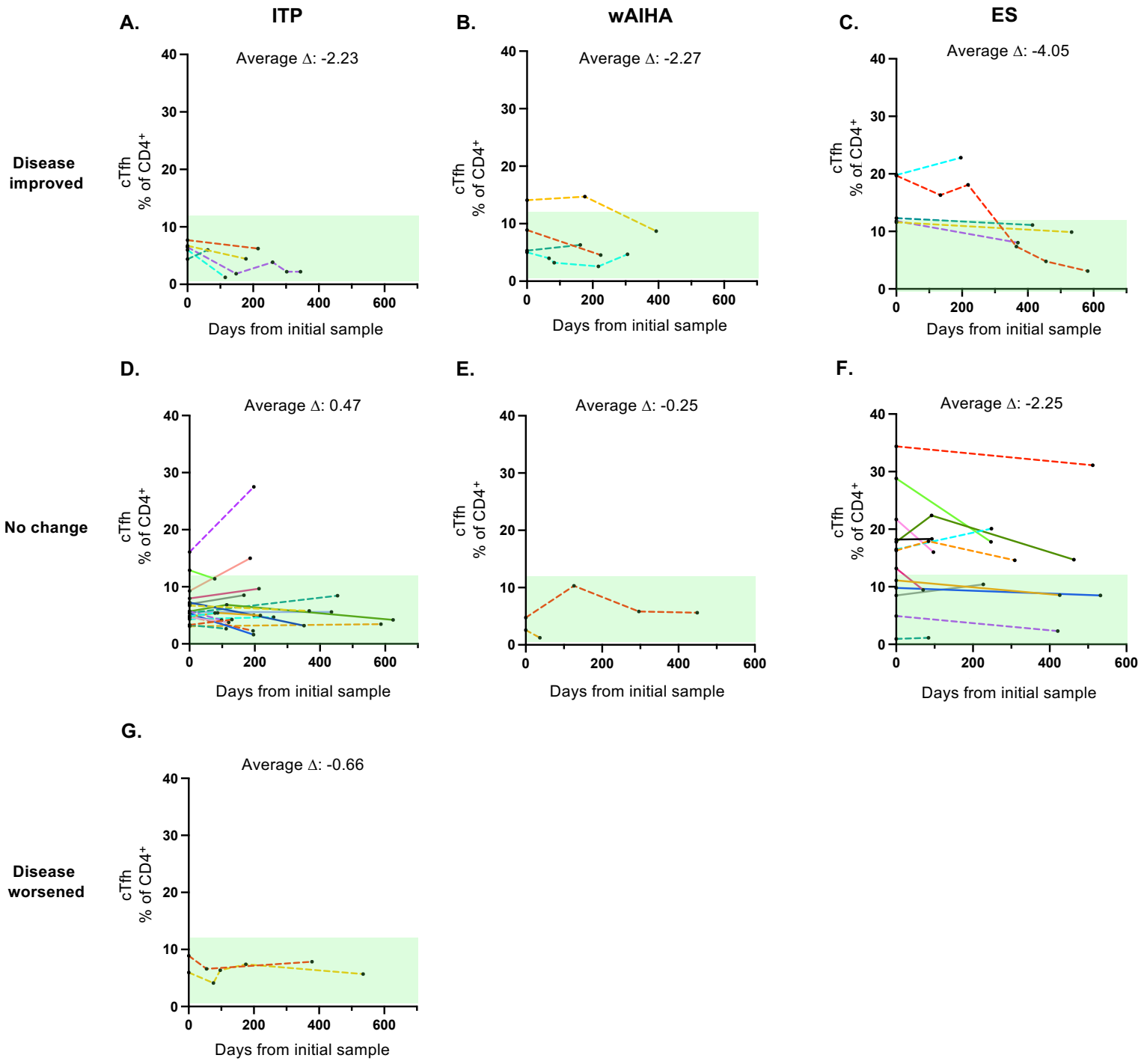
**Supplementary Table 2.** Logistic regression of factors associated with high circulating T follicular helper cell (cTfh) % of $CD4^{+}$ T cells, using standard methods and Firth's penalized logistic correction.

**Supplementary Table 3.** Prior studies of circulating T follicular helper cells (cTfh) in immune cytopenias

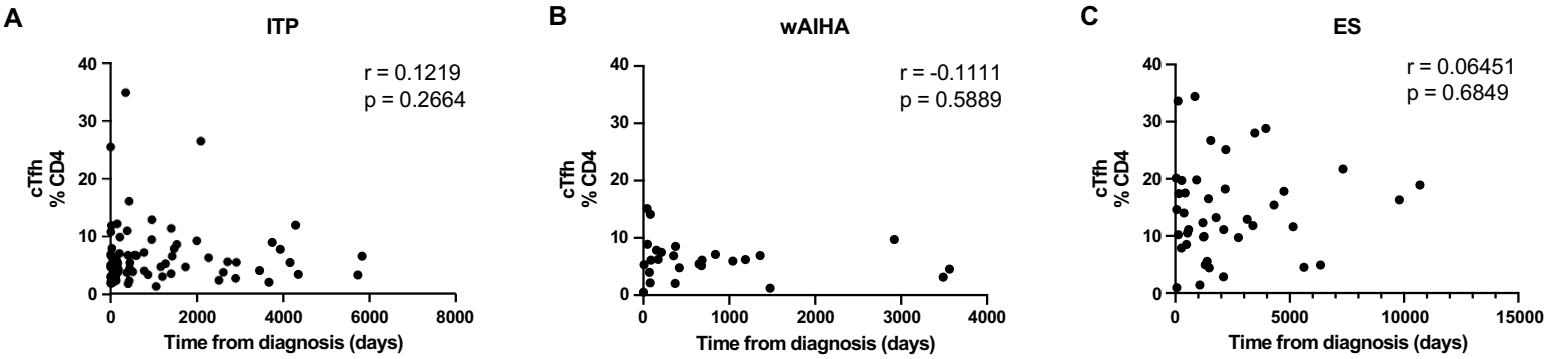
**Supplementary Figure 1**



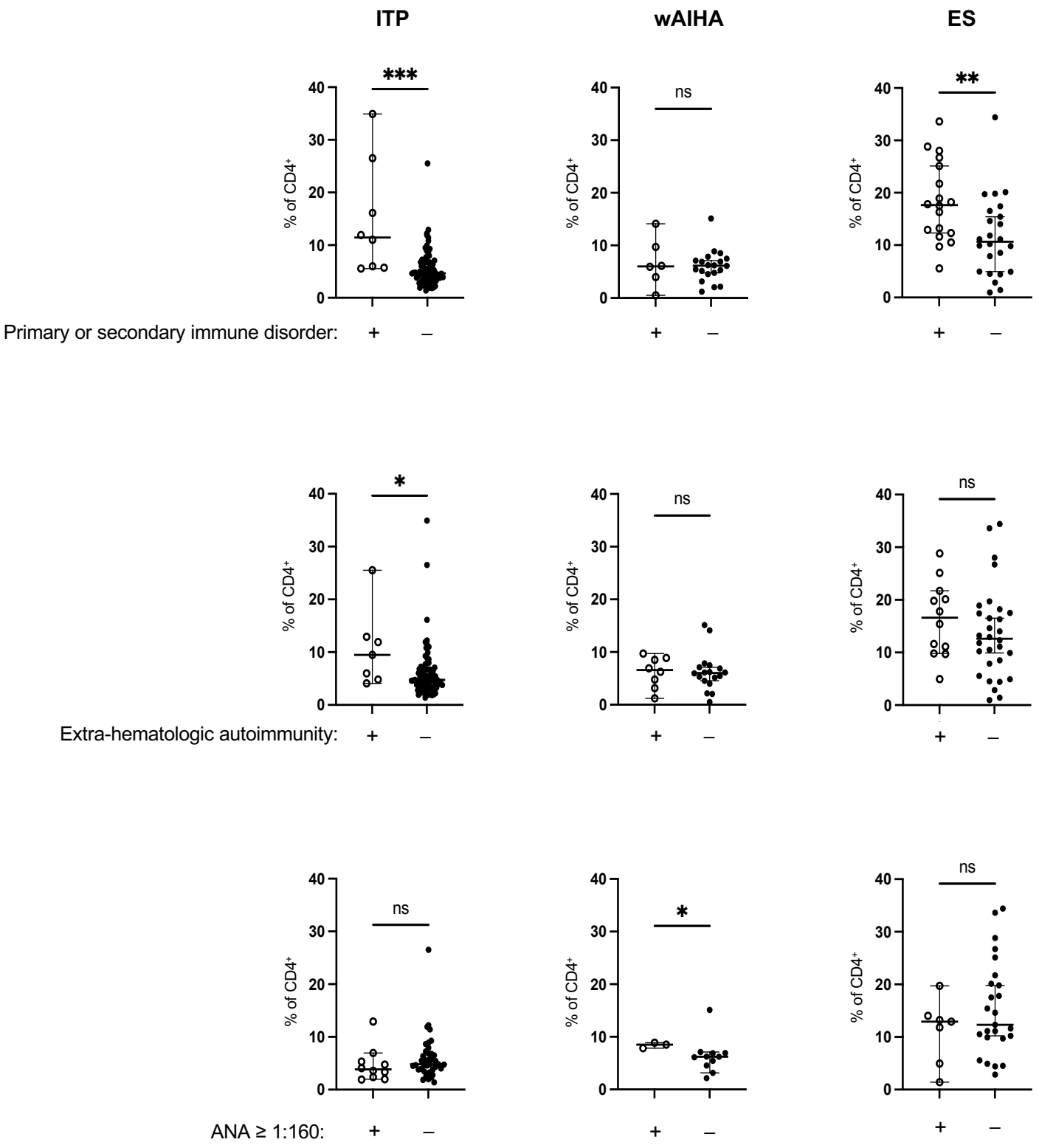
**Supplementary Figure 2**



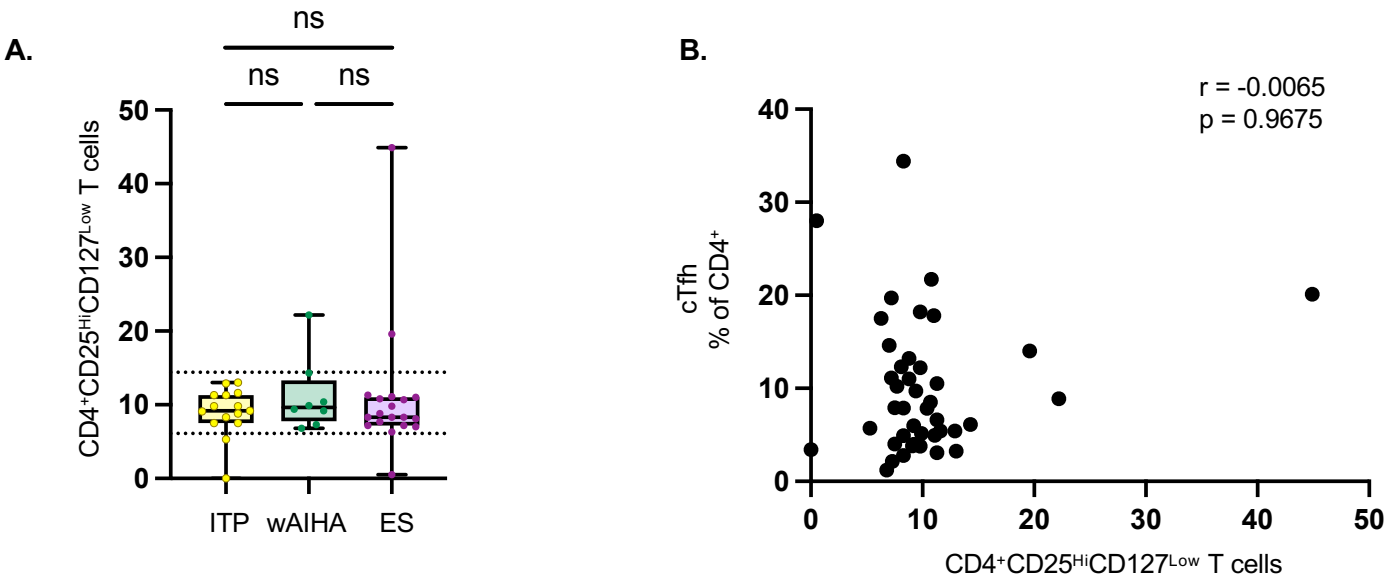
**Supplementary Figure 3**



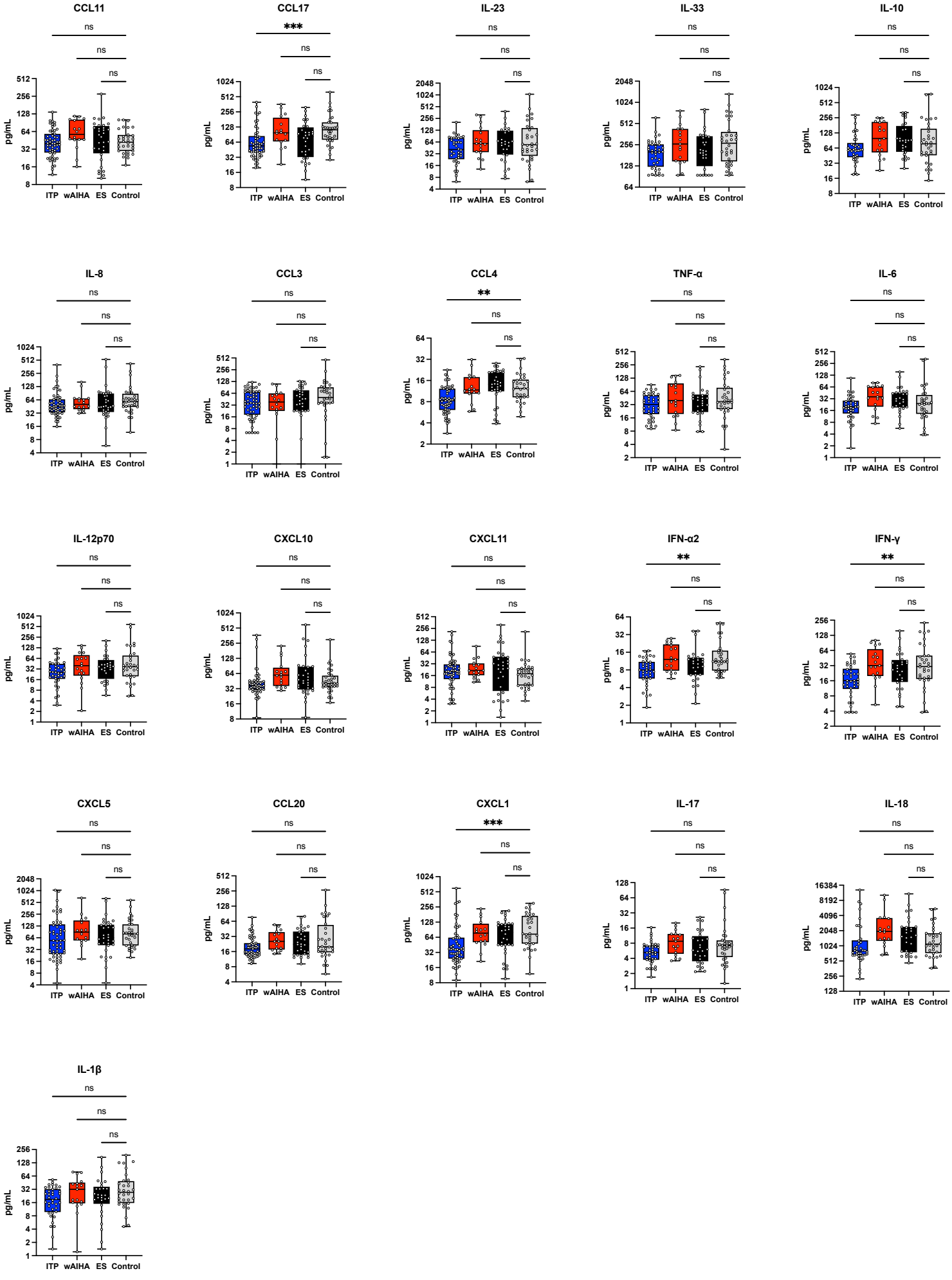
**Supplementary Figure 4**



**Supplementary Figure 5**



# Supplementary Figure 6



**Supplementary Table 1**. Patient and control ages at time of sample collection

| | **<2 years** | **2-<5 years** | **5-<10 years** | **10-<15 years** | **15-<20 years** | **≥20 years** |
|---|---|---|---|---|---|---|
| **ITP** (n=85) n (%) | 8 (9.4%) | 13 (15.3%) | 20 (23.5%) | 23 (27.1%) | 18 (21.2%) | 4 (4.7%) |
| **wAIHA** (n=26) n (%) | 4 (15.4%) | 3 (11.5%) | 2 (7.7%) | 5 (19.2%) | 9 (34.6%) | 3 (11.5%) |
| **ES** (n=42) n (%) | 2 (4.8%) | 2 (4.8%) | 5 (11.9%) | 8 (19.0%) | 13 (31.0%) | 12 (28.6%) |
| **Controls** (n=77) n (%) | 6 (7.8%) | 15 (19.5%) | 13 (16.9%) | 11 (14.3%) | 9 (11.7%) | 12 (29.9%) |

## Supplementary Table 2

| **Standard machine learning regression** | **coef** | **se(coef)** | **lower 0.95** | **upper 0.95** | ***p*** |
|---|---|---|---|---|---|
| (Intercept) | 0.017967169 | 2.23532455 | 0.003115503 | 0.075301344 | 8.23E-11 |
| Age | 1.006714351 | 1.041463171 | 0.929631033 | 1.092067578 | 8.69E-01 |
| Sex | 1.6312913 | 1.732952255 | 0.564470966 | 5.003388636 | 3.69E-01 |
| Diagnosis (ES) | 16.58051366 | 1.865684444 | 5.17728056 | 61.37654814 | 9.40E-07 |
| Medication | 0.26697792 | 1.846825021 | 0.0739913 | 0.843829837 | 2.38E-02 |
| Cytopenia | 5.76168041 | 1.805389251 | 1.921969555 | 19.96702434 | 1.37E-03 |
| Immune disorder (primary or secondary immune disorder or extrahematologic autoimmunity) | 12.07776755 | 1.825580265 | 3.923960165 | 42.61844795 | 7.19E-06 |
| | | | | | |
| **Regression with Firth correction** | **coef** | **se(coef)** | **lower 0.95** | **upper 0.95** | ***p*** |
| (Intercept) | 0.024948222 | 2.072274839 | 0.004854999 | 0.095334865 | 3.10E-10 |
| Age | 1.005608289 | 1.038047744 | 0.932114945 | 1.086708323 | 8.85E-01 |
| Sex | 1.55653512 | 1.662061385 | 0.566662823 | 4.514087399 | 3.93E-01 |
| Diagnosis (ES) | 13.24616457 | 1.769707467 | 4.427580623 | 44.91683595 | 2.00E-06 |
| Medication | 0.3029038 | 1.753345347 | 0.090212256 | 0.901897583 | 3.14E-02 |
| Cytopenia | 4.900996594 | 1.717344084 | 1.733525992 | 15.79496866 | 2.24E-03 |
| Immune disorder (primary or secondary immune disorder or extrahematologic autoimmunity) | 9.882007803 | 1.737294677 | 3.416596568 | 32.21906828 | 1.42E-05 |

**Supplementary Table 3**: Prior studies of cTfh in immune cytopenias

| Study | Summary/Key differences |
|---|---|
| Imbalance of follicular regulatory T (Tfr) cells/follicular helper T (Tfh) cells in adult patients with primary immune thrombocytopenia (PMID 37208911) | This is a study of ITP in adults. The cell markers for cTfh were CD4 and CXCR5 without PD1. This includes cTfh cells and all $CD4^+$ in the early phase of T cell activation, many of which will downregulate CXCR5 to become memory $CD4^+$ T cells. |
| Alterations in B- and circulating T-follicular helper cell subsets in immune thrombotic thrombocytopenic purpura (PMID 35507753) | This is a study of adults with immune TTP (iTTP), which is a different disease from ITP. |
| The Role of Follicular Regulatory T Cells/Follicular Helper T Cells in Primary Immune Thrombocytopenia (PMID 36917965) | This is a study of primary ITP in adults. The cell markers used to for cTfh were CD4 and CXCR5, without evaluation of PD1. This will include cTfh cells and all $CD4^+$ in the early phase of T cell activation, many of which will downregulate CXCR5 to become memory $CD4^+$ T cells. The median CD4+CXCR5+ levels in patients with ITP were reported as 0.04 vs 0.025, p=0.66. |
| CXCL13/CXCR5 axis facilitates TFH expansion and correlates with disease severity in adults with immune thrombocytopenia (PMID 39454362) | This a study of adults with ITP. In this study, the cells were isolated by Ficoll before staining, which changes the frequency of $CD4^+$ T cells. |
| Changes in follicular helper T cells in idiopathic thrombocytopenic purpura patients (PMID 25561904) | This is a study of adults in which peripheral white blood cell, red blood cell, and platelet counts of the ITP patients were all significantly lower than those of the healthy controls. In contrast, our patients with ITP have isolated thrombocytopenia, meaning only their platelet counts were lower than those of healthy individuals.<br>This study used a range of different cell markers: $CD4^+CXCR5^+$ vs. $CD4^+CXCR5^+ ICOS^{high}$ (measured as percentage of $CD4^+CXCR5^+$ that are $ICOS^+$), $CD4^+CXCR5^+PD\text{-}1^{high}$ (measured as percentage of $CD4^+CXCR5^+PD1^+$ cells). A fluorescence intensity of $>10^2$ was the threshold for 'positive' or 'high'<br><br>While Tfh subtypes were significantly higher in patients with platelet antibody positive ITP compared to those lacking platelet antibodies, there were no significant differences in Tfh types between antibody negative ITP and controls. This indicates that adults with ITP do not uniformly have higher Tfh subtypes. |
| Splenic TFH expansion participates in B-cell differentiation and antiplatelet-antibody production during immune thrombocytopenia (PMID 25232056) | This study of adults with ITP measured splenic Tfh cells, which are different from the peripheral blood cTfh population |
| B cell depleting therapy regulates splenic and circulating T follicular helper cells in immune thrombocytopenia (PMID 27863820) | This study of adults with ITP measured splenic Tfh cells, which are different from the peripheral blood cTfh cells. In this study, cells were isolated by Ficoll prior to staining. |
| Differences in frequency and regulation of T follicular helper cells between newly diagnosed and chronic pediatric immune thrombocytopenia (PMID 27667163) | In this pediatric study the media age was 28.8 months $\pm$ 19 months, indicating that many patients were <1 year of age. This study defined cTfh cells as CD4+ $CXCR5^{hi}ICOS^{hi}$ and specified ICOS high as "between $10^2 - 10^3$". |
| Altered circulating T follicular helper cells in patients with chronic immune thrombocytopenia (ref; Exp Ther Med, 2018, vol 16(3); 2471-2477) | This study of adults with chronic ITP defined cTfh cells as $CD4^+CXCR5^+ICOS^+$. Figure 1 shows that a fluorescence intensity of 10 is used as the threshold for $ICOS^+$, which is not the standard used in clinical flow cytometry laboratories. |
| T-follicular helper cell expansion and chronic T-cell activation are characteristic immune anomalies in Evans syndrome (PMID 34424963) | This study included 24 pediatric patients with Evans syndrome and compared them with 22 patients with chronic ITP and 24 healthy controls, finding higher cTfh in patients with Evans syndrome but no association with genetic diagnosis. This study did not evaluate disease activity. |