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

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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 Δ 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.

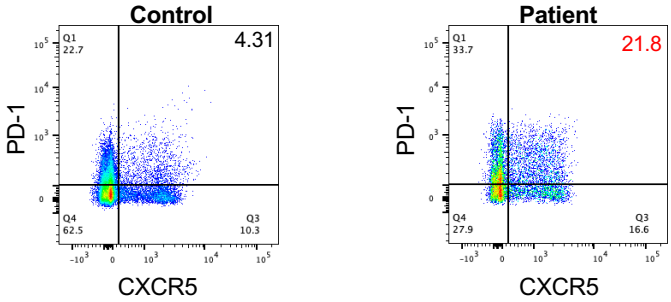
Supplementary Figure 6. Levels of plasma cytokines in patients with immune cytopenias ns=p>0.05, *p<0.05, ***p<0.001 by Kruskal-Wallis test.

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

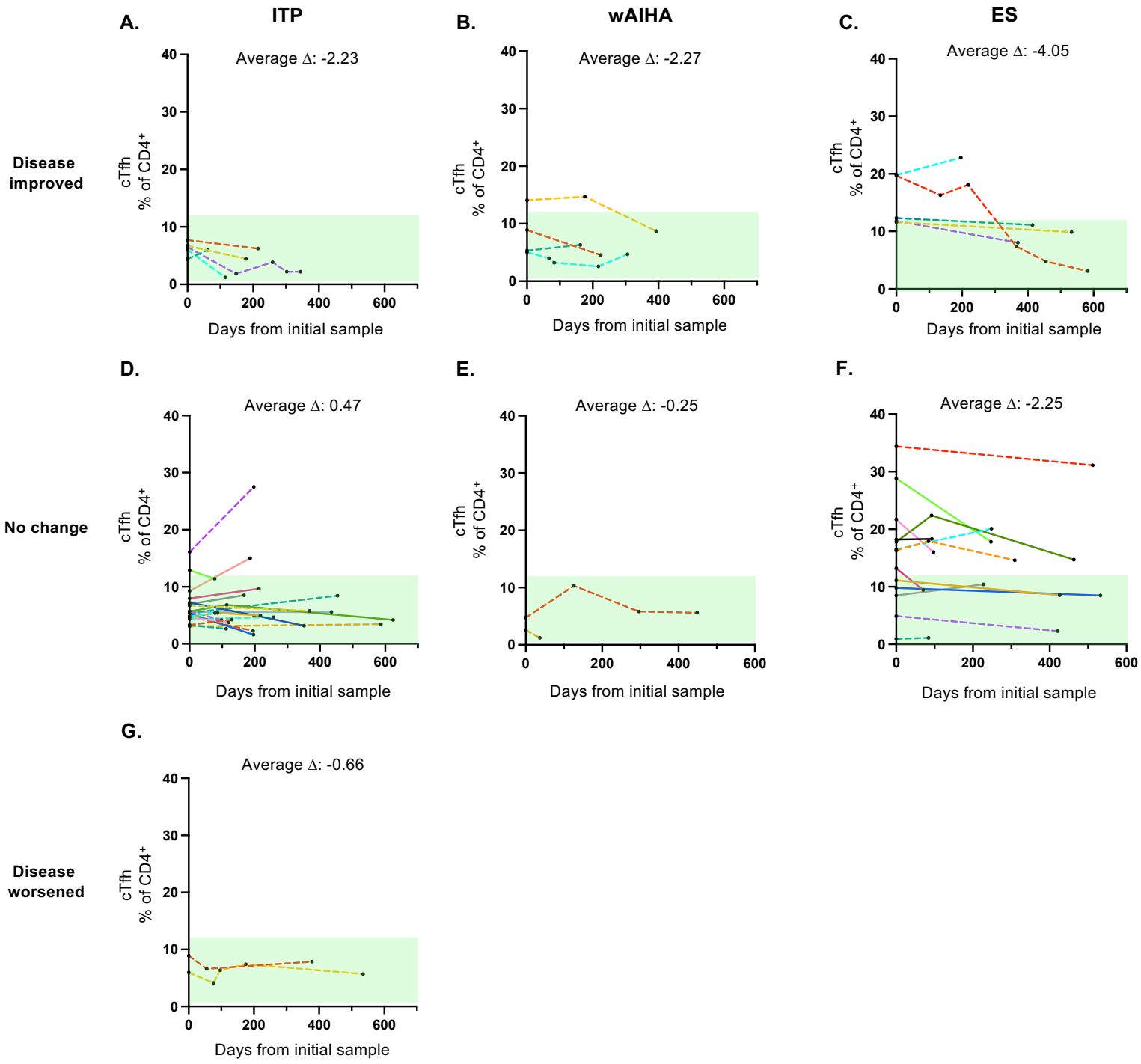
Supplementary Table 2. Logistic regression of factors associated with high circulating T follicular helper cell (cTfh) % of $CD4^{+}$ T cells, using standard methods and Firth's penalized logistic correction.

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

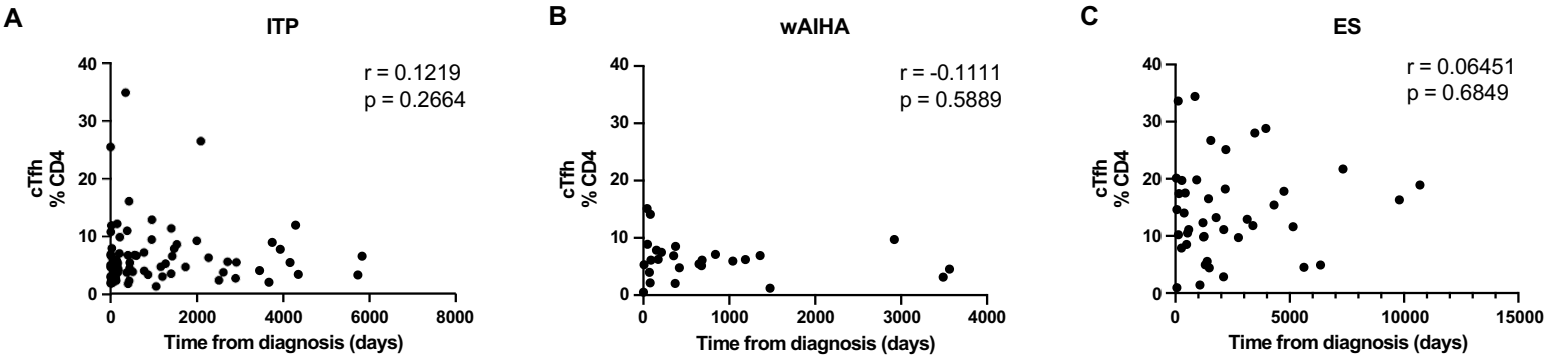
Supplementary Figure 1



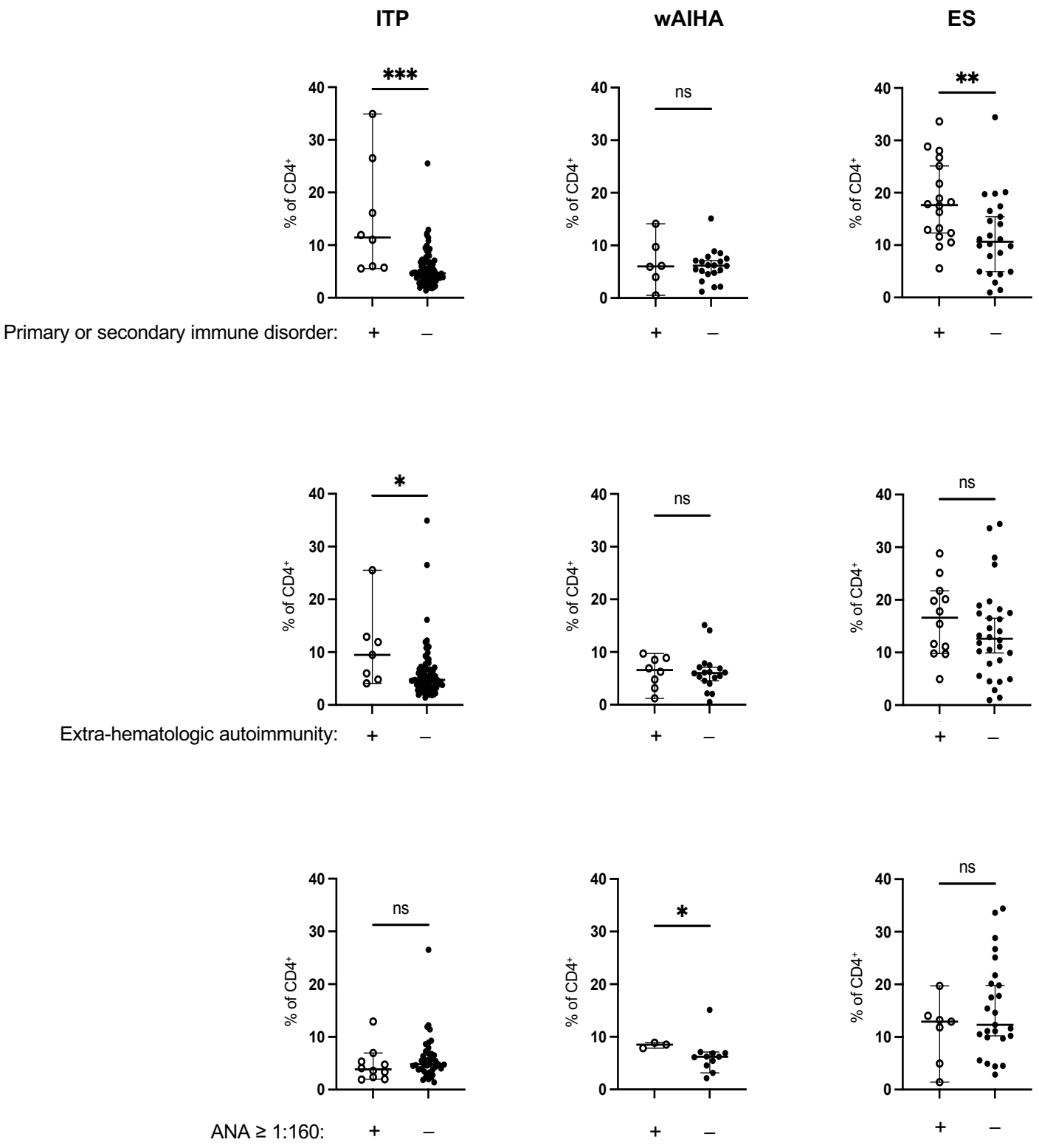
Supplementary Figure 2



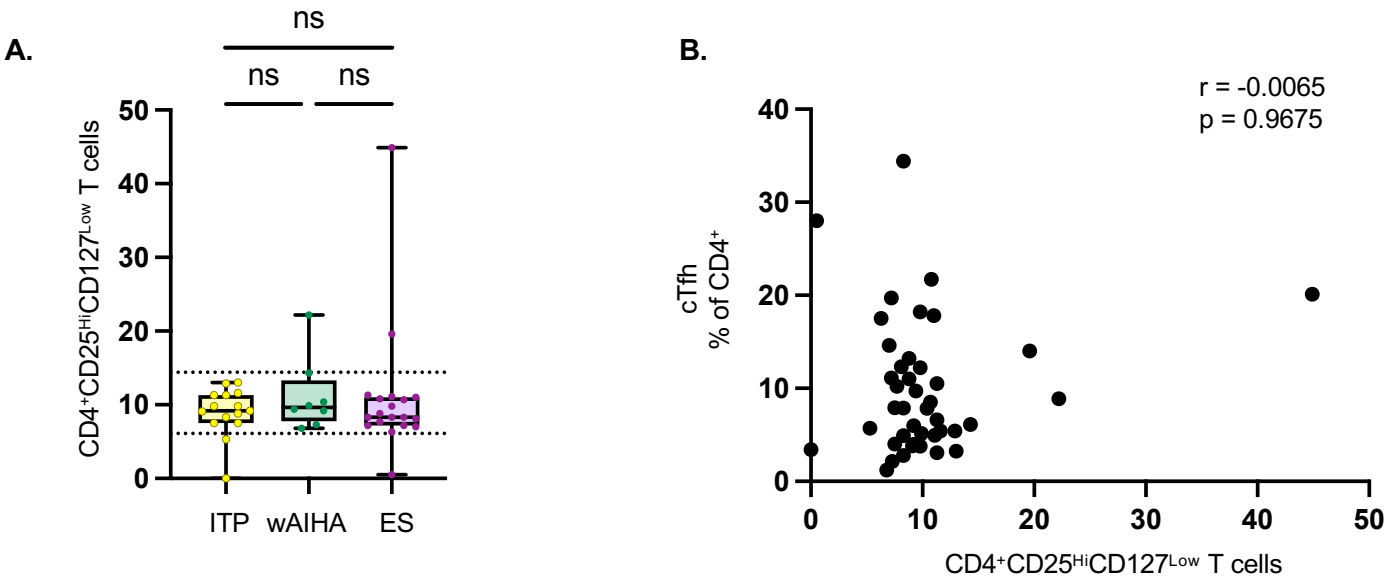
Supplementary Figure 3



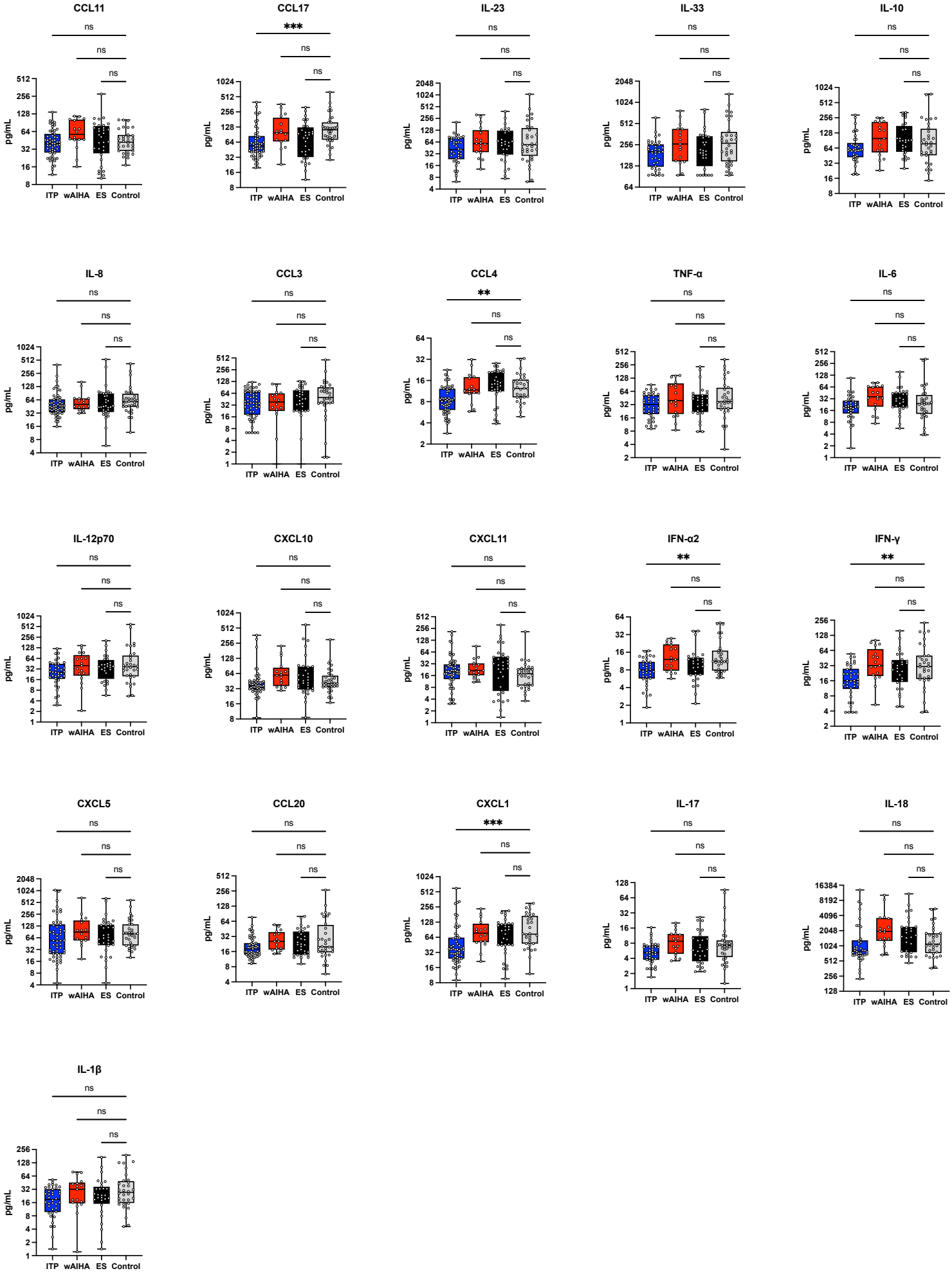
Supplementary Figure 4



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. 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' 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.