Individualized prognosis in childhood immune thrombocytopenia

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ITGB1-expressing CD4$^+$ T Effector Cell Response Associates with Chronic Childhood Immune Thrombocytopenia

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ABSTRACT

The current paradigm of the pathophysiology in the autoimmune bleeding disorder childhood immune thrombocytopenia (ITP) includes a humoral a cellular immune response against platelet self-antigens. Multiple perturbations of lymphocyte subsets have been associated with ITP and proposed as markers associated with a chronic or transient ITP prognosis. Here we aimed to identify immune markers of recovery from newly diagnosed ITP, using our recent TIKI randomized controlled trial (N=158). Participants were randomized to observation or IVIg treatment, recovery was monitored for one year. Immune cells from diagnosis were analyzed in a centralized laboratory by flow cytometry, single-cell RNA sequencing (scRNA-seq) and T- and B- cell receptor sequencing (scTCR-seq and scBCR-seq). We found that chronic *vs* transient ITP patients show increased levels of an activated CD4 T effector memory cell population, that is characterized by *ITGB1* and co-expression of the pro-inflammatory genes include *GNLY, GZMB, GZMA, PRF1,* and the activation markers *AHNAK, ANXA2, KLF6, KLRB1,* and *IL32.* Moreover, CD8 T cells were found to be increased relative to CD4 cells in chronic compared to transient ITP. CD4 T effector cell frequency and CD4/CD8 ratio were predictive of complete recovery from newly diagnosed ITP, both after IVIg treatment and during observation. Conceptually, the identified *ITGB1*-expressing CD4 T cells may explain previously identified enhanced immune activation with increased IL-2 production in platelet-stimulated PBMC of chronic ITP patients. Other previously suggested immune markers show limited promise when validated in our large prospective study.

INTRODUCTION

Childhood immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterized clinically by a primary hemostasis defect due to thrombocytopenia, with platelets below 100 x 10^9/L (1). ITP can be clinically distinguished into different phases from newly diagnosed to persistent and chronic ITP(1). Patients with transient ITP recover within three months of the diagnosis. The morbidity of ITP is most severe in patients who exhibit persistent thrombocytopenia and chronic disease courses (2,3). With presently available clinical characteristics, ITP disease courses are predicted with a moderate accuracy (4-6), and further predictive biomarkers are needed to explain diverging disease courses and to better target clinical management. The current paradigm of the immune dysregulation in ITP pathophysiology features a humoral antibody-mediated and a cellular CD8 T cell-mediated immune response against platelet self-antigens (7,8). At the same time, it is also assumed that a functional deficiency or reduced number of T regulatory cells may contribute to the self-directed immune response (8,9). These immune responses are the biological hallmarks that result in platelet clearance (10-13), impairment of platelet function (14-16), platelet apoptosis(17), and the impairment of megakaryocyte differentiation and platelet production (18-22). In childhood ITP specifically, it has been described that newly diagnosed ITP patients show a reduced number of CD4 T cells and a decreased CD4/CD8 ratio (23). This includes a reduction in CD4+ CD25+, and potentially regulatory FOXP3+ T cells (23-25). CD19+ B cells were found to be increased in newly diagnosed ITP (23). Finally, Natural Killer (NK) CD16+ CD56+ cells were found to be decreased in ITP (25). To differentiate between patients who recovery from ITP vs those who show chronic disease, two studies found that the CD4+ CD25hi cells were lower in chronic vs transient ITP (23,25). Moreover, platelet-stimulated PBMC were found to produce more IL-2 in chronic ITP patients (26), indicating a relatively higher in vivo T cell pre-activation in these patients. Given the key pathophysiological role of the immune system in ITP, the immune markers represent major candidates to improve prediction of recovery, or to anticipate resistance to immune-directed therapy (6).

In our recent TIKI randomized controlled trial, 200 children with newly diagnosed ITP were randomized to careful observation or IVIg treatment, and were followed for one year (27). Using data from this trial, we aimed to identify immune markers that can differentiate transient ITP *vs* persistent or chronic ITP. To this end, the immune repertoire was evaluated at the time of diagnosis by flow cytometry, and gene expression and clonality were evaluated

by single-cell RNA sequencing (scRNA-seq) paired with T- and B- cell receptor sequencing (scTCR-seq and scBCR-seq).

METHODS

Study participants and sample processing

Samples were obtained from the *Therapy With or Without Intravenous Immunoglobulins in Kids with ITP* (TIKI) randomized controlled multicenter trial (27). Briefly, children with newly diagnosed ITP (within 72h of diagnosis) below 16 years of age with a diagnosis platelet count ≤20 x 10^9/L were randomized to careful observation or single-dose intravenous immunoglobulin (IVIg) treatment. Patients were not eligible for participation in case of major or life-threatening bleeding that required medical intervention (Buchanan bleeding score 4 or 5). Parents and children aged ≥12 years gave written informed consent for participation. The study was approved by the Ethical Review Board of University Medical Center Utrecht and conducted in accordance with the Declaration of Helsinki. Blood samples were obtained by venipuncture. EDTA-anticoagulated whole blood obtained at the study recruitment was transported to our national reference laboratory for immune phenotyping by flow cytometry, as detailed below. Of a subset of patients, heparin-anticoagulated whole blood at the diagnosis and during five follow-up visits was immediately transported by courier to a central laboratory facility (Sanquin, Amsterdam) for isolation of fresh peripheral blood mononuclear cells (PBMC) from whole blood, using Ficoll. Trained laboratory personnel performed the isolation at the institute's biobank (Cryobiology, Sanquin) according to a standard operating procedure. The cells were aliquoted and cryopreserved at -80°C. A complete recovery from ITP was defined as a platelet count ≥ 100 x 10^9/L (1). Bleeding symptoms were assessed using the modified Buchanan bleeding score (28,29).

Immune phenotyping by flow cytometry

Immune cells were enumerated and phenotyped by flow cytometry using three separate tubes in a routine diagnostic workflow (Sanquin Diagnostics). The CD3, CD4, CD8, B and NK cells were identified with CD3 FITC, CD16 PE, CD56 PE, CD45 PerCP-Cy5.5, CD4 PE-Cy7, CD19 APC and CD8 APC-Cy7 (BD Biosciences; Vianen, The Netherlands; #644611). Staining of 25µL EDTA-anticoagulated whole blood was performed in TruCount tubes (BD Biosciences #340334), allowing absolute and relative cell counts. Regulatory T cells (CD4 T, $CD4^+$ $CD25^+$ $CD127^{lo}$) were identified by CD4 FITC, CD25 PE-Cy7, CD127-Alexa Fluor 647 (BD 560249) and CD3 PerCP (BD Biosciences #345766). In a second tube, the CD4 naive ($CD4^+$ $CD45RO^-$ $CD27^+$),

effector memory (CD4 T, $CD4^+$ $CD45RO^+$ $CD27^-$) and central memory ($CD4^+$ $CD45RO^+$ $CD27^+$) subsets were identified by staining with CD3 PerCP (BD Biosciences #345766), CD4 APC (BD Biosciences #345771), CD45RO PE (BD Biosciences #347967) and CD27 FITC (Sanquin Reagents; Amsterdam, The Netherlands; M1764). Data analysis was performed with a standardized workflow in flowJo or BD FACSDiva. Each sample was separately visualized and inspected.

10X Genomics Single cell RNA-sequencing and alignment

In six patients with newly diagnosed ITP who subsequently developed chronic ITP (N=2) or with transient ITP (N=4), we evaluated PBMC by single cell RNA-sequencing (scRNA-seq). The median difference in age between the transient and chronic patients was 1 year (Supplementary Table 1; cell counts and immune phenotyping, Supplementary Table 2). Single cell sequencing was performed using gel bead emulsions (30). Samples were processed according to 10X recommendations for the v3.1 5' V(D)J kit. Briefly, PBMC samples were thawed in RPMI and 10% FCS and concentration was adjusted according to 10x genomics instructions. Cells were loaded in 5' Chromium Next GEM Single Cell Immune Profiling kits for gene expression v3.1. Human T and B cell Chromium Single Cell V(D)J Enrichment kits were used for targeted TCR and Ig enrichment. Steps were performed according to the manufacturer protocol. Sequencing was performed on an Illumina Novaseq S4. The mean coverage was at least 40 000 reads per cell. Raw sequencing data were mapped to the human transcriptome GRCh38 release 3.0.0. with 10X Genomics CellRanger version 3.0.

Pre-processing, normalization and scaling

Data were loaded in Seurat version 3 (31). scTCR-seq and scIg-seq derived clonotype data was stored as metadata in the Seurat object, and visualized using the *scRepertoire* package. Cells with low or high number of unique features (<200, >2000) or ≥10% mitochondrial reads were excluded. Samples were log-normalized and the reads were scaled. Data were integrated using anchoring by CCA in Seurat with the functions *FindAnchors*.

Clustering

Principal component analysis was used for dimensionality reduction, the 13 first principal components were used for UMAP. Canonical marker expression and the differentially expressed genes of each cluster (Seurat function *FindAllMarkers*) were used to annotate clusters. The consensus dataset of the Blood Atlas (32) was used for interpretation of differential expression of genes per blood cell type.

Differential gene expression

Non-integrated data were used for differential expression (Seurat slot `RNA`). Differential marker expression between groups was tested with the function *FindMarkers*, using standard parameters with adjustment of p-values for multiple testing.

Statistical analysis

All statistical analyses were performed in R version 3.6.0 and 4.0.2 (R Core Team). Continuous data were compared using a Welch's t-test or a Mann-Whitney U test, as appropriate. Survival curves (longitudinal complete recovery) were visualized by Kaplan-Meier plots. The effect sizes (hazard ratios) and 95% confidence intervals were obtained by Cox-proportional hazard models.

RESULTS

Age-adjusted T, B and NK cells numbers and association with ITP prognosis

Using flow cytometry for immune cell phenotyping and enumeration at the time of diagnosis (available for N=158/200 patients in the trial), we analyzed all data as a function of age, thus taking into account the normal immune development during childhood. The comparison of the absolute cell counts with healthy children showed that the lymphocyte subsets of ITP patients were within the respective reference interval for their age (Schmidt et al, medRxiv, *manuscript submitted*). For prognosis, we observed no differences in absolute counts of CD3, CD4 or CD8 T cells, CD19 B cells, or NK cells between patients with transient or chronic ITP (Figure 1A-E). The mean of relative CD8 T counts was slightly higher in chronic ITP *vs* transient ITP, with a mean age-adjusted increase of 2.9 % (95% CI, -1.2 - 7.0 %; P=0.17; Figure 1G). None of the of the CD4, CD19 or NK cell counts showed differences (Figure 1F, 1H-I).

Effector memory CD4 T cells and CD8 T cells associate with chronic ITP

The evaluation of activated CD4 effector memory ($CD45RO^+$ $CD27^-$) T cells by flow cytometry revealed a higher frequency in chronic ITP, with a mean age-adjusted increase of 1.4 % (95% CI, 0.4 – 2.4; P= 0.005; Figure 2A). Patients with a high CD4 effector memory count (≥ median) displayed a lower proportion of complete recovery over a one-year follow-up in our randomized controlled trial, irrespective of whether patients were allocated to treatment with IVIg, or observation (Figure 2B). The adjusted hazard ratio (aHR) of a CD4 effector memory count above the median for complete recovery was 0.55 (95% CI, 0.35 - 0.85; adjusted for age and treatment).

In line with the relatively increased CD8 cells observed in chronic ITP patients (Figure 1G), a reduced CD4/CD8 T cell ratio was associated with chronic ITP (mean age-adjusted decrease, 0.2 ± 0.2 ratio units; Figure 2C). Similarly, the patients with a low CD4/CD8 ratio (< median) exhibited a worse prognosis during follow-up, both for IVIg-treated patients and patients that were observed (Figure 2D). The aHR for complete recovery for patients with a low CD4/CD8 ratio was 0.64 (95% CI, 0.46 – 0.89; adjusted for age and treatment). In a multivariate model, both the CD4 effector memory frequency and the CD4/CD8 ratio were independently associated with the ITP prognosis (Supplementary Table 3). Moreover, the predictive ability

was independent of a preceding infection, total leukocyte and lymphocyte counts, and the presence of IgG or IgM autoantibodies by MAIPA (Supplementary Table 3).
In summary, chronic ITP patients seemed to show an increase in CD4 T effector memory cells and CD8 T cells at the time of diagnosis.

Similar number of regulatory T cells in chronic and transient ITP

The analysis of CD4 regulatory T cell counts by flow cytometry revealed no differences between transient and chronic ITP patients (Supplementary Figure 2). Comparing the regulatory T cell frequencies with reference data from 145 healthy controls of the same age (33), noting minor possible differences in staining and gaiting, the regulatory T cells in ITP patients were not reduced (not tested; Supplementary Figure 2).

Single cell RNA-seq identifies an ITGB1-expressing CD4 cluster associated with chronic ITP

Single cell RNA-seq paired with TCR- and BCR-seq was carried out on PBMC of six newly diagnosed ITP patients, two of which became chronic during the course of the study. (longitudinal platelet counts and the scRNA-seq workflow in Figure 3A). Baseline characteristics and flow cytometry data of these patients are shown in Supplementary Tables 1-2. After filtering, a total of 7965 cells were classified into 17 clusters by their gene expression (Figure 3B). Gene expression per cluster is shown in Supplementary Figure 3. Annotating the 17 clusters based on canonical markers and expressed genes, they encompassed T cells (*CD3G*), NK cells (*KLRD1*), B cells (*CD79A*), monocytes (*LYZ*), and plasmacytoid dendritic cells (*LILRA4*). Of note, all clusters were present in both transient and chronic ITP patients (Figure 3C). The key canonical and differentially expressed genes are displayed in Figure 3D and Supplementary Figure 4. Comparing the frequency of clusters between the transient and chronic IT patients, we observed a higher frequency of a cluster of CD4 fibronectin receptor integrin β1 (*ITGB1),* interleukin 32 *(IL32),* interleukin 7 receptor *(IL7R),* lymphotoxin beta *(LTB)*-expressing T cells in chronic ITP (mean ± sem, 8.1 ± 1.4 % transient *vs* 14.8 ± 1.2 % chronic ITP; Figure 3E-F). We also observed a minor increase in CD8 Hobit (*ZNF683)*-expressing T cells (transient 4.0 ± 0.2 % *vs* 4.6 ± 0.2 % chronic ITP; Figure 3E-F). The proportion of cells from the other clusters were not different between patients with transient and chronic ITP (Figure 3E).

Enhanced activation of ITGB1-expressing CD4 memory T cells in chronic ITP

In line with these observed differences in CD4 and CD8 clusters, we further evaluated T cell subtypes in the ITP patients. Sub-clustering revealed 11 distinct T cell clusters (Figure 4A). All T cell clusters were present in both transient and chronic ITP patients. The clusters encompassed four naive C-C motif chemokine receptor 7 (*CCR7)*-expressing CD4 T cell populations (Figure 4B), two *IL7R*-expressing activated CD4 T cell clusters, two *CCR7*-expressing naive CD8 T cell clusters, and three CD8 effector T cell clusters that showed expression of *ZNF683*, cathepsin W (*CTSW)* and C-C motif chemokine ligand 5 (*CCL5*; Figure 4B). Using TCR-seq, we solely observed clonotype expansion in the activated memory CD8 T cells (Figure 4B-C), whereas the cells in other clusters represented unique clonotypes. There was no evidence of differences in clonal expansion of T cells from patients with transient or chronic ITP (Figure 4D). The CD4 cells with differences between transient and chronic ITP identified in global clustering showed as two populations of *CCR7* negative, *ITGB1* expressing CD4 cells (Figure 4E). By co-expression analysis, these two clusters were functionally synonymous with the CD4 T effector memory cells that are identified by flow cytometry, showing low levels of *CCR7, CD27,* L-selectin/CD62L (*SELL)* and activation of cytokine production (Figure 4F). Moreover, re-grouping of CD4 and CD8 cells by *CCR7* expression (independent of scRNA-seq clustering) confirmed the increase of CD4 memory cells in chronic ITP (Supplementary Figure 5).

Compared to other T cells, the identified *CCR7* and *ITGB1* expressing clusters have an activated memory pro-inflammatory phenotype, expressing high levels of the *ITGB1,* desmoyokin *(AHNAK),* annexin A2 *(ANXA2), KLF6,* CD161 *(KLRB1)* and *IL32* (Figure 4B). We observed no expression of the cytotoxic markers *GNLY, GZMB* or *PRF1* in these cells (Nicolet 2021 bioArxiv; doi: 10.1101/2021.02.10.430576), also not if we grouped cells by positive or negative *ITGB1* expression (Supplementary Figure 6).

By differential expression analysis in the *ITGB1*-expressing CD4 T cell clusters, compared to transient ITP cells in the same cluster, the cells of chronic ITP patients displayed upregulation of *IL32,* thymosin beta 4, X-linked *(TMSB4X),* S 100 calcium binding protein A11 *(S100A11),* myosin light chain 12A *(MYL12A),* and interferon induced transmembrane protein 1 (*IFITM1;* Figure 5A-B)*,* indicating enhanced activation of these memory T cells.

In summary, scRNA-seq suggested effector memory CD4 cells expressing *ITGB1* as a potential marker for chronic ITP. Furthermore, a minor increase in CD8 T cells might be characteristic

of chronic ITP. No other immune cell subcluster showed expansion between transient and chronic ITP patients.

Discussion

The main result of our study is that chronic *vs* transient ITP patients show increased levels of an activated CD4 T effector memory cell population, that is characterized by *ITGB1* and co-expression of the pro-inflammatory genes *GNLY, GZMB, GZMA, PRF1,* and activation markers *AHNAK, ANXA2, KLF6, KLRB1,* and *IL32.* Moreover, CD8 T cells were found to be increased relative to CD4 cells in chronic compared to transient ITP. The strengths of our study include the uniform sampling time at the diagnosis (before treatment), centralized laboratory analysis, prospective follow-up of all patients, the chance to assess effect of IVIg and spontaneous recovery by randomization, and most importantly, the large number of patients available for immune evaluation, allowing for adjustment for age effects.

Conceptually, Semple and colleagues showed 25 years ago that T cells displayed more enhanced in-vitro stimulated IL-2 production in chronic *vs* transient ITP patients, and this correlated with IL-2, IFN-y and IL-10 serum cytokine levels (26). Our study may explain this phenomenon by showing an expanded subcluster of *ITGB1*-expressing CD4 T cells that express enhanced levels of pro-inflammatory markers in chronic ITP. We further extend this finding to clinical prognosis by showing that it may be useful to distinguish chronic and transient ITP disease courses independent of age, a history of infection, or platelet autoantibodies. Recent data show that ITP patients showing spontaneous disease resolution or good response to IVIg can be predicted at the time of the diagnosis by clinical and molecular characteristics (6,34). Given the non-specific diagnosis of ITP by thrombocytopenia without secondary laboratory criteria, presumably chronic ITP represents a different underlying disease pathology. Thus, the identified immune markers in this study further explain some of the underlying differences in the pathology and clinical outcome of childhood ITP.

Previous research

In contrast to prior hypotheses, based on previous literature, most T cell subsets showed no differences between transient and chronic patients by scRNA-seq and flow cytometry. Partially this may be explained by a lack of adjustment for the normal development of the immune system during childhood in prior studies (see also Schmidt; medRxiv). Moreover, in a systematic review, we found that many immune studies in childhood ITP used varying sampling times from the diagnosis for transient vs chronic patients, and include a mix of patients before/after treatment (35). We observed no differences in Treg frequencies

between transient and chronic ITP by flow cytometry. Rather we found potentially a slightly increased number compared to healthy children, which could be physiological during immune activation and T cell expansion (36). Of note, we did not assess the functional capacity of Treg cells. For the scRNA-seq experiment, no Treg cluster was identified by unsupervised analysis, and supervised analysis (PCA on key expressed T reg genes (37)) identified < 100 cells), in agreement with the ~5-10% expected rate of CD4 cells; a number too low for functional profiling assessment.

Limitations

A limitation of our study was that we did not have age-matched healthy children available for comparison; however, comparison to healthy children was not a primary outcome and we were able to use indirect reference data from previous studies. Moreover, only few chronic ITP patients were included in this study – in agreement with the low prevalence of chronic childhood ITP - but we could utilize our full cohort to analyze longitudinal follow-up of patients over a full year, effectively showing even early differences in recovery by the identified immune subsets. The expression analysis of *IL32, S100A11* or *TMSB4X* expression along with the frequency of CD4 effector memory cells might have allowed improved discrimination of chronic vs transient ITP in flow cytometry. Furthermore, at this point, we could not analyze the longitudinal changes in T cells subsets as well as effects of (successful) treatment. In addition, deep immune phenotyping by high-dimensional flow cytometry may allow for further identification of differences in immune cell subsets that our present methods were not able to detect.

Conclusions

Our study shows CD4 effector T cells are independently associated with prognosis in a large dataset of childhood ITP patients. This marker could be used in addition to other clinical and molecular markers to predict disease outcomes in childhood ITP. Other previously suggested immune markers show limited promise when validated in our large prospective study.

Figure Legends

Figure 1. Immune phenotyping by flow cytometry in children with newly diagnosed ITP (N=146). (A-E) Absolute and (F-I) relative counts of CD3 T cells, CD4 T helper cells, CD19 B cells and NK subsets, showing no differences between patients with transient (grey line, open circles) or chronic ITP (black line, black dots). (G) The relative CD8 cytotoxic T cell counts were slightly elevated in chronic ITP.

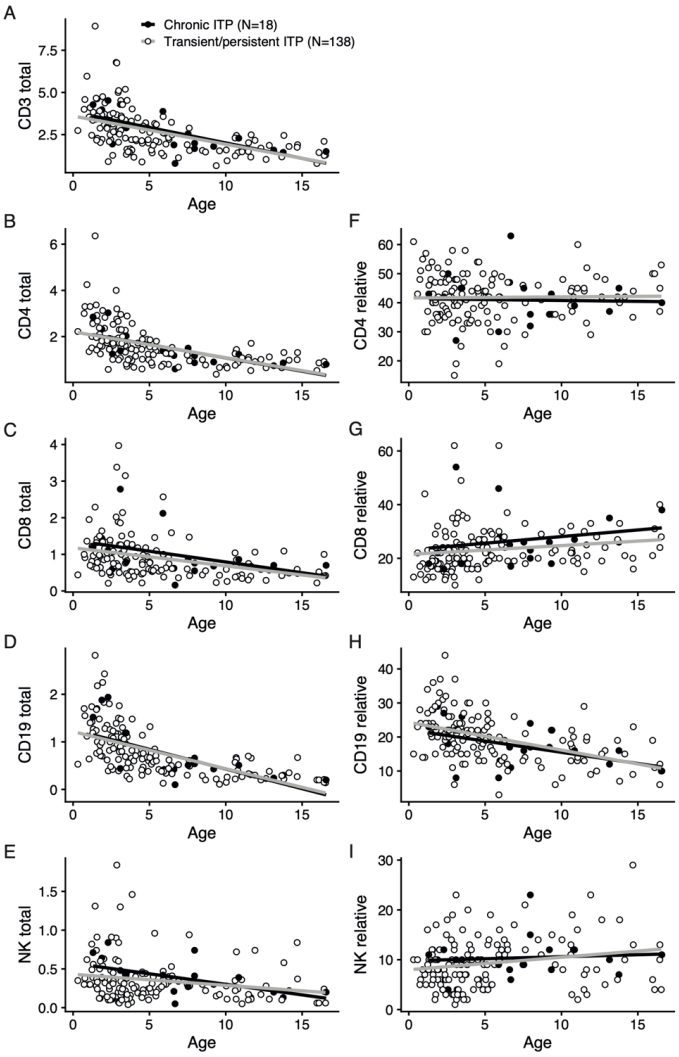


Figure 2. Prognostic significance of CD4 T effector memory cells and CD8 cells in newly diagnosed childhood ITP. Shown are data for spontaneous recovery as well as response to intravenous immunoglobulins (IVIg). (A) Over all age groups, T effector cells, defined as CD4+ CD27lo CD45RO+ cells lymphocytes, were found to be increased in patients who developed chronic ITP as compared to those who had transient or persistent ITP. (B) Patients with T effector frequency at or above, or below the median ("high" and "low") showed, skewed a prognosis irrespective of treatment. (C) Over the various ages, the CD4/CD8 ratio was decreased in patients with worse chronic ITP compared to no chronic ITP. (D) Patients with a "high" CD4/CD8 ratio (at or above the median) displayed favorable recovery, as compared to patients with a "low" ratio (below the median).

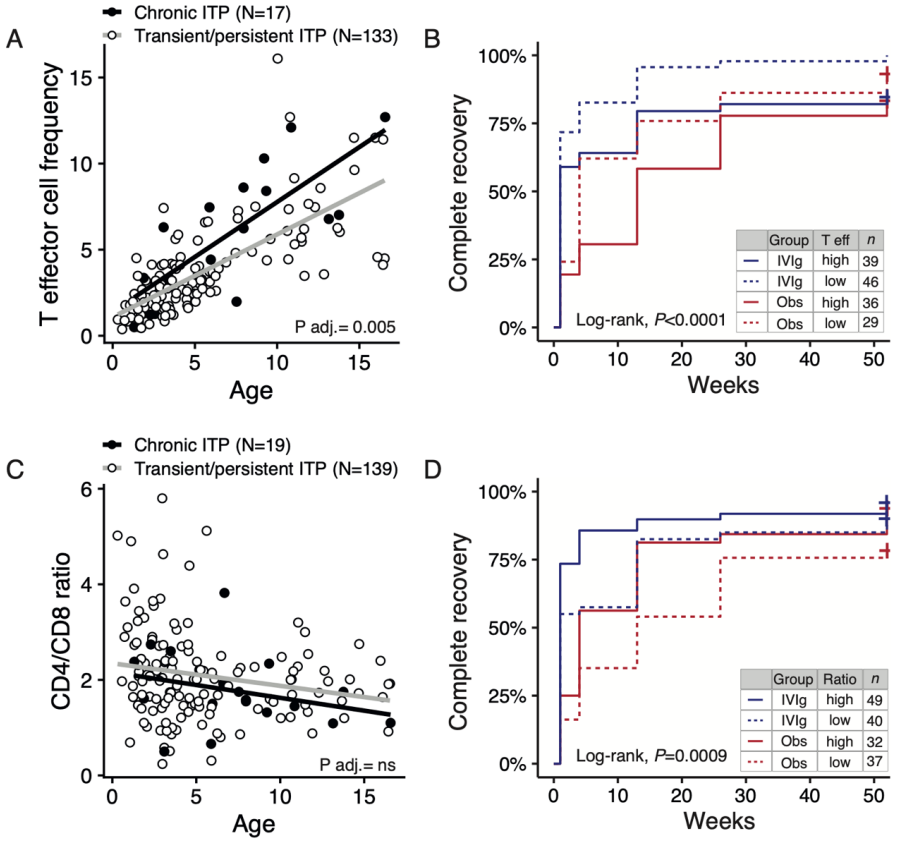
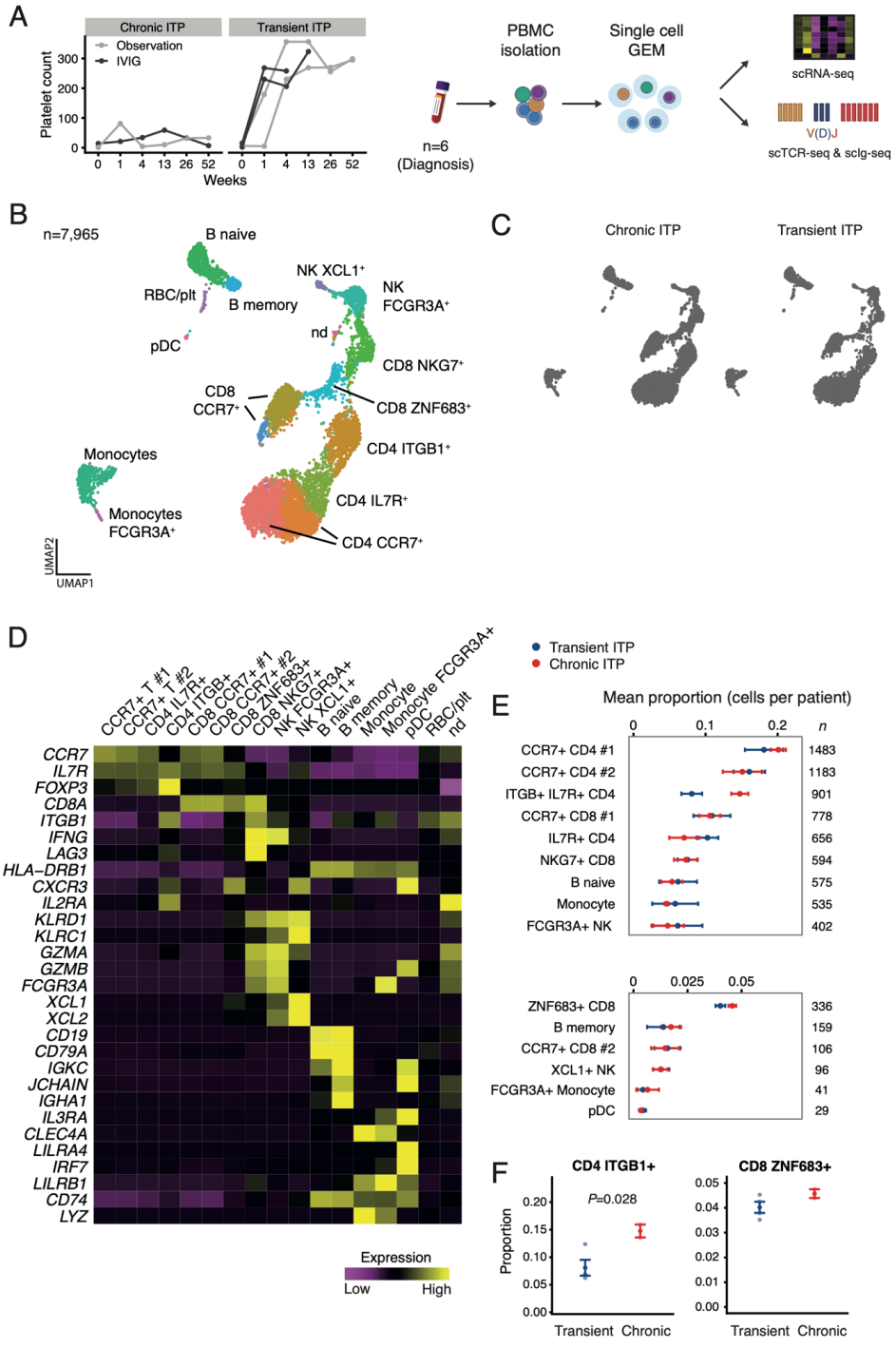


Figure 3. Immune characteristics of childhood ITP PBMC analyzed by scRNA-seq. (A) Left panel, longitudinal platelet counts of patients with chronic or transient ITP during one-year follow-up. Right panel, 10X Genomics workflow. Libraries were prepared for scRNA-seq, as well as scTCR-seq and scIg-seq for T and B cell receptors. (B) UMAP projection of identified PBMC, using unsupervised clustering on RNA gene expression. (C) All UMAP clusters were present both in chronic and transient ITP. (D) Canonical and differentially expressed markers between clusters. (E-F) Frequency of cells by clusters. Most clusters showed similar frequencies, except for ITGB+ IL7R+ CD4 T effector memory cells and ZNF683+ CD8 T cells.


A
Chronic ITP
Transient ITP
Observation
IVIG
Platelet count
300
200
100
0
0 1 4 13 26 52
0 1 4 13 26 52
Weeks
n=6
(Diagnosis)
PBMC
isolation
Single cell
GEM
scRNA-seq
V(D)J
scTCR-seq & sclg-seq
B
n=7,965
B naive
NK XCL1+
NK
FCGR3A+
RBC/plt
B memory
nd
pDC
CD8 NKG7+
CD8
CCR7+
CD8 ZNF683+
CD4 ITGB1+
Monocytes
CD4 IL7R+
Monocytes
FCGR3A+
CD4 CCR7+
UMAP2
UMAP1
C
Chronic ITP
Transient ITP
D
CCR7+ T #1
CCR7+ T #2
CD4 IL7R+
CD4 ITGB+
CD8 CCR7+ #1
CD8 CCR7+ #2
CD8 ZNF683+
CD8 NKG7+
NK FCGR3A+
NK XCL1+
B naive
B memory
Monocyte
Monocyte FCGR3A+
pDC
RBC/plt
nd
CCR7
IL7R
FOXP3
CD8A
ITGB1
IFNG
LAG3
HLA–DRB1
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IL2RA
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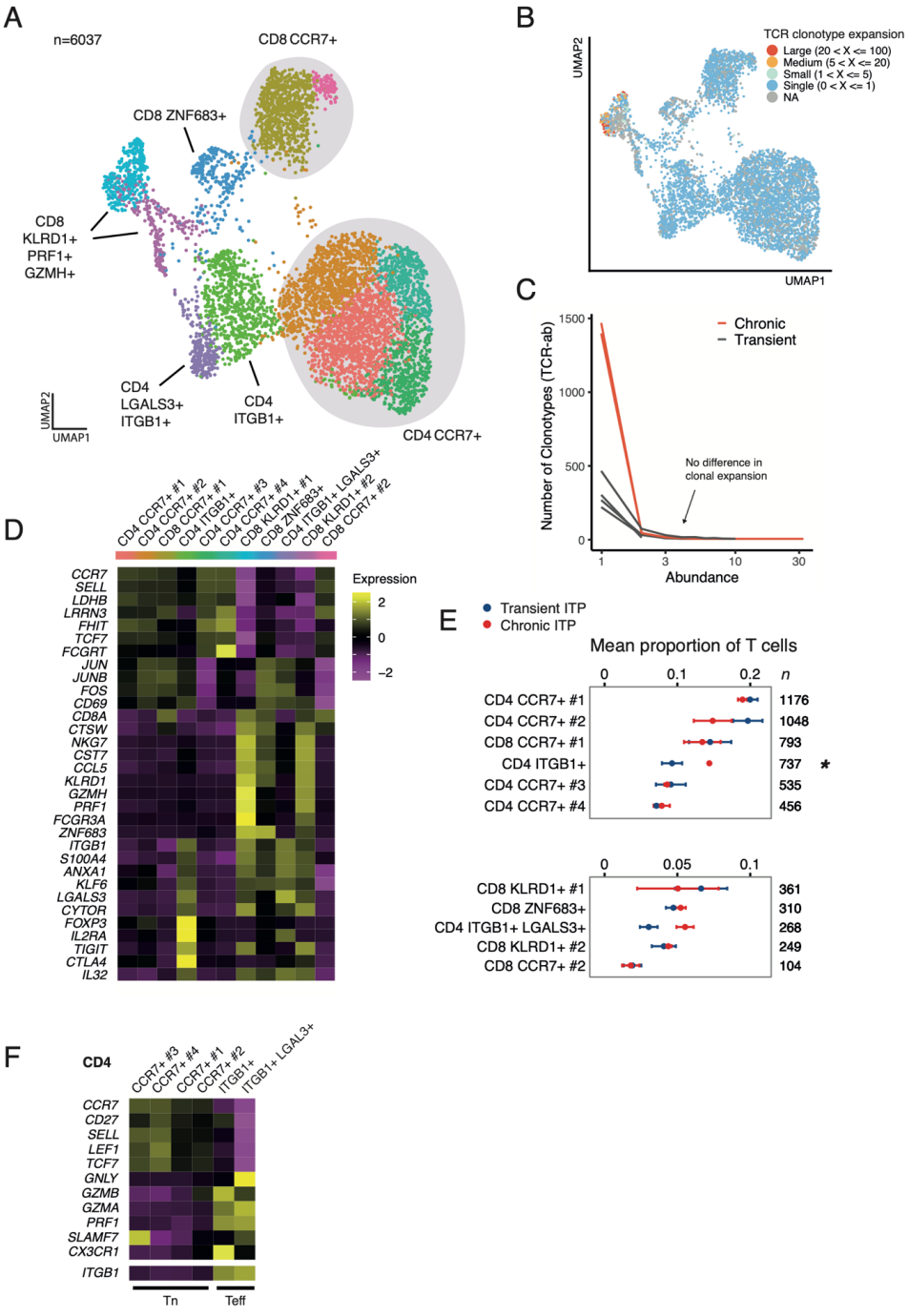
KLRC1
GZMA
GZMB
FCGR3A
XCL1
XCL2
CD19
CD79A
IGKC
JCHAIN
IGHA1
IL3RA
CLEC4A
LILRA4
IRF7
LILRB1
CD74
LYZ
Expression
Low
High
E
Transient ITP
Chronic ITP
Mean proportion (cells per patient)
0
0.1
0.2
n
CCR7+ CD4 #1 1483
CCR7+ CD4 #2 1183
ITGB+ IL7R+ CD4 901
CCR7+ CD8 #1 778
IL7R+ CD4 656
NKG7+ CD8 594
B naive 575
Monocyte 535
FCGR3A+ NK 402
0
0.025
0.05
ZNF683+ CD8 336
B memory 159
CCR7+ CD8 #2 106
XCL1+ NK 96
FCGR3A+ Monocyte 41
pDC 29
F
CD4 ITGB1+
P=0.028
Proportion
0.20
0.15
0.10
0.05
0.00
Transient Chronic
CD8 ZNF683+
0.05
0.04
0.03
0.02
0.01
0.00
Transient Chronic

Figure 4. T cells in childhood ITP analyzed by scRNA-seq paired with scTCR-seq. (A) UMAP projection of T cell subclusters, using unsupervised clustering on RNA expression. (B) TCR clonotypes showed expansion in CD8 KLRD1+ effector cells. Other clusters showed mostly single unique clones. (C) No differences were observed in clonal expansion of T cells between chronic and transient ITP patients. (D) Canonical and differentially expressed markers between T cell clusters. Expression is shown per gene and cluster, with yellow indicating higher expression in the cluster, and purple lower expression. (E) Frequency of cells by cluster. CD4 ITGB1+ and CD4 IGTB1+ LGALS3+ T cell clusters showed higher frequencies in chronic ITP. (F) Co-expression of naive and activation markers in CD4 T cell subclusters.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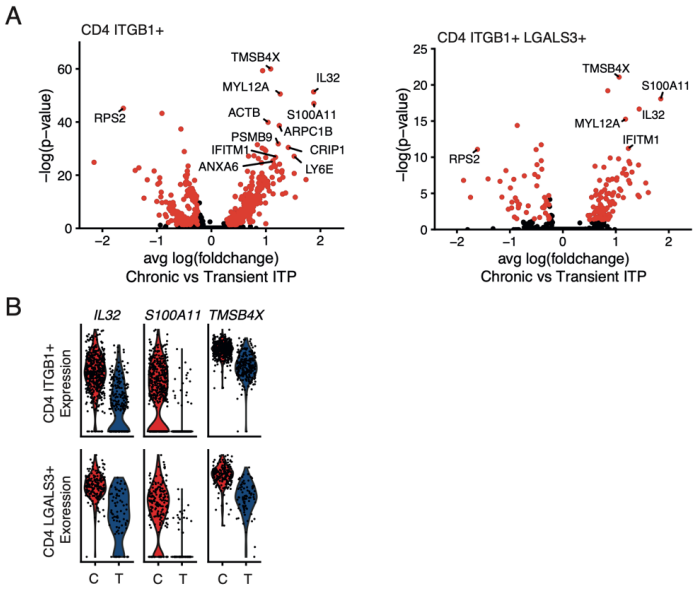
CD8 CCR7+
CD8 ZNF683+
CD8 KLRD1+ PRF1+ GZMH+
CD4 LGALS3+ ITGB1+
CD4 ITGB1+
CD4 CCR7+
UMAP2
UMAP1
B
TCR clonotype expansion
Large (20 < X <= 100)
Medium (5 < X <= 20)
Small (1 < X <= 5)
Single (0 < X <= 1)
NA
UMAP2
UMAP1
C
Chronic
Transient
Number of Clonotypes (TCR-ab)
1500
1000
500
0
No difference in clonal expansion
1
3
10
30
Abundance
D
CD4 CCR7+ #1
CD4 CCR7+ #2
CD8 CCR7+ #1
CD4 ITGB1+
CD4 CCR7+ #3
CD4 CCR7+ #4
CD8 KLRD1+ #1
CD8 ZNF683+
CD4 ITGB1+ LGALS3+
CD8 KLRD1+ #2
CD8 CCR7+ #2
CCR7
SELL
LDHB
LRRN3
FHIT
TCF7
FCGRT
JUN
JUNB
FOS
CD69
CD8A
CTSW
NKG7
CST7
CCL5
KLRD1
GZMH
PRF1
FCGR3A
ZNF683
ITGB1
S100A4
ANXA1
KLF6
LGALS3
CYTOR
FOXP3
IL2RA
TIGIT
CTLA4
IL32
Expression
2
0
−2
E
Transient ITP
Chronic ITP
Mean proportion of T cells
0
0.1
0.2
n
CD4 CCR7+ #1
CD4 CCR7+ #2
CD8 CCR7+ #1
CD4 ITGB1+
CD4 CCR7+ #3
CD4 CCR7+ #4
1176
1048
793
737
535
456
*
0
0.05
0.1
CD8 KLRD1+ #1
CD8 ZNF683+
CD4 ITGB1+ LGALS3+
CD8 KLRD1+ #2
CD8 CCR7+ #2
361
310
268
249
104
F
CD4
CCR7+ #3
CCR7+ #4
CCR7+ #1
CCR7+ #2
ITGB1+
ITGB1+ LGAL3+
CCR7
CD27
SELL
LEF1
TCF7
GNLY
GZMB
GZMA
PRF1
SLAMF7
CX3CR1
ITGB1
Tn
Teff

Figure 5. Differential expression in CD4 ITGB1+ T cells between transient and chronic ITP. (A) Volcano plots showing upregulation of activation markers IL32, S100A11, ANXA6, TMSB4X, MYL12A, IFITM1, with similar fold-changes in both T cell subclusters, indicating a more pronounced activation of cells in this cluster in chronic ITP patients. (B) Violin plots showing individual cell expression of IL32, S100A11 and TMSB4X for patients with transient [T] or chronic [C] ITP.



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Supplementary Tables

Supplementary Table 1. Clinical characteristics of patients with transient *vs.* chronic ITP analyzed by single cell-RNAseq.

	Group	Age	Sex	Platelets	Buchanan Score	Preceding Infection	Days with symptoms	NOPHO recovery chance	*FCGR2C*-ORF	Randomization	More samples available?
T244 G001	Chronic	8.0	F	14	2	No	7	Intermediate	No	IVIg	Longitudinal
T316 G005	Chronic	6.7	F	1	2	Yes	5	High	NA	Observation	Longitudinal
T263 G002	Transient	6.4	M	16	3	Yes	1	High	No	Observation	-
T269 G006	Transient	6.0	F	6	3	No	1	High	No	Observation	-
T281 G004	Transient	4.4	M	15	3	No	7	High	No	IVIg	-
T440 G008	Transient	4.5	F	2	3	No	1	High	No	IVIg	Diagnosis and longitudinal

Modified Buchanan Score by Bennett Blood 2008. History of preceding infection during 28 days before diagnosis. NOPHO recovery, score group by Edslev BJH 2008. FCGR2C-ORF as in Schmidt Blood Advances 2019.
NA, not available.

Supplementary Table 2. Immune phenotyping and clinical chemistry results**.** Top panel, absolute cell counts. Bottom panel, relative cell frequencies.

pid	Leukocytes	Neutrophils	Lymphos	CD3	CD4	CD8	CD4/CD8 ratio	CD19	NK
T244	5.1	2.3	3.28	1.99	1.17	0.76	1.55	0.54	0.74
T316	4	1.6	0.95	0.79	0.6	0.16	3.82	0.1	0.05
T263	10.3	5.6	NA	NA	NA	NA	NA	NA	NA
T269	9.8	3.7	NA	NA	NA	NA	NA	NA	NA
T281	10.6	4.4	NA	NA	NA	NA	NA	NA	NA
T440	11.6	5.6	4.34	2.76	1.88	0.72	2.6	1.14	0.44

Counts are x 10^9/L.
NA, not available.

pid	% CD3	% CD4	% CD8	% CD19	% NK	% T reg	% T naive	% T cm	% T eff
T244	61	36	23	17	23	4.94	63.9	26.7	6.24
T316	83	63	17	11	6	NA	NA	NA	NA
T263	NA	NA	NA	NA	NA	NA	NA	NA	NA
T269	NA	NA	NA	NA	NA	NA	NA	NA	NA
T281	NA	NA	NA	NA	NA	NA	NA	NA	NA
T440	64	44	16	26	10	15.6	82	11.6	1.75

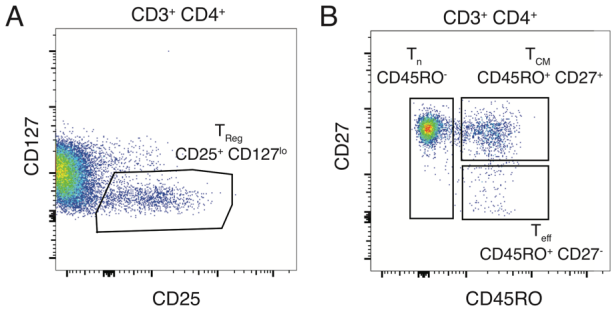
T reg, frequency of CD3+ CD4+ CD25+ $CD127^{lo}$ cells.
T naive, frequency of CD3+ CD4+ CD45RO- cells.
T central memory, frequency of CD3+ CD4+ CD45RO+ CD27+ cells.
T effector, frequency of CD3+ CD4+ CD45RO+ CD27- cells.
NA, not available.

Supplementary Table 3. Multivariate effect of CD4 T effector cell frequency and CD4/CD8 ratio on complete recovery from newly diagnosed ITP. Two Cox-proportional hazards model are presented.

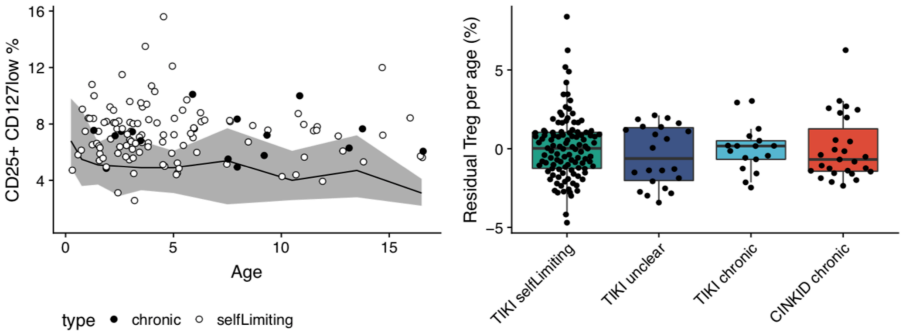
Variable	Adjusted Hazard Ratio (95% CI)	P-value
Model 1: adjusted for age in years, IVIg treatment		
CD4 T effector cell frequency, below median	1.78 (1.13; 2.82)	0.014
CD4/CD8 ratio, below median	0.64 (0.45; 0.92)	0.015
Model 2: adjusted for preceding infection, leukocyte count, lymphocyte count, positive IgM MAIPA (GPIIb/IIIa), positive IgG MAIPA (GP IIb/IIIa), age in years, IVIg treatment		
CD4 T effector cell frequency, below median	1.81 (1.11; 2.96)	0.017
CD4/CD8 ratio, below median	0.56 (0.37; 0.83)	0.004

Supplementary Figures

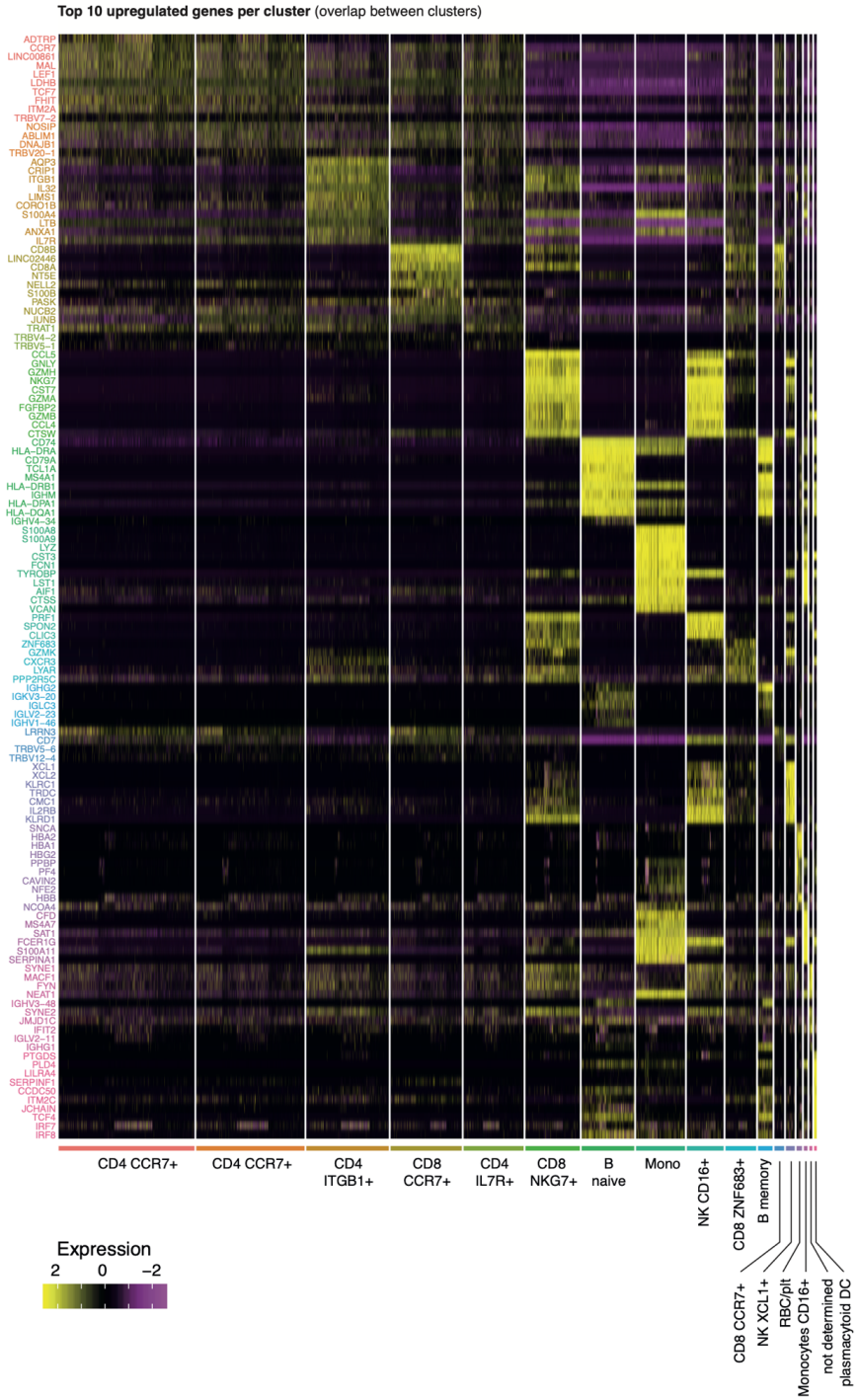
Supplementary Figure 1. Flow cytometry gating. Separate tubes were stained for surface markers of regulatory T cell (A) and naive, central memory and effector T cells (B). A preselection was made on CD3+ CD4+ cells and lymphocytes in FSC/SSC. Gates were set manually and inspected visually for each sample. Previously shown in Supplementary Data Schmidt et al. JTH (2021).



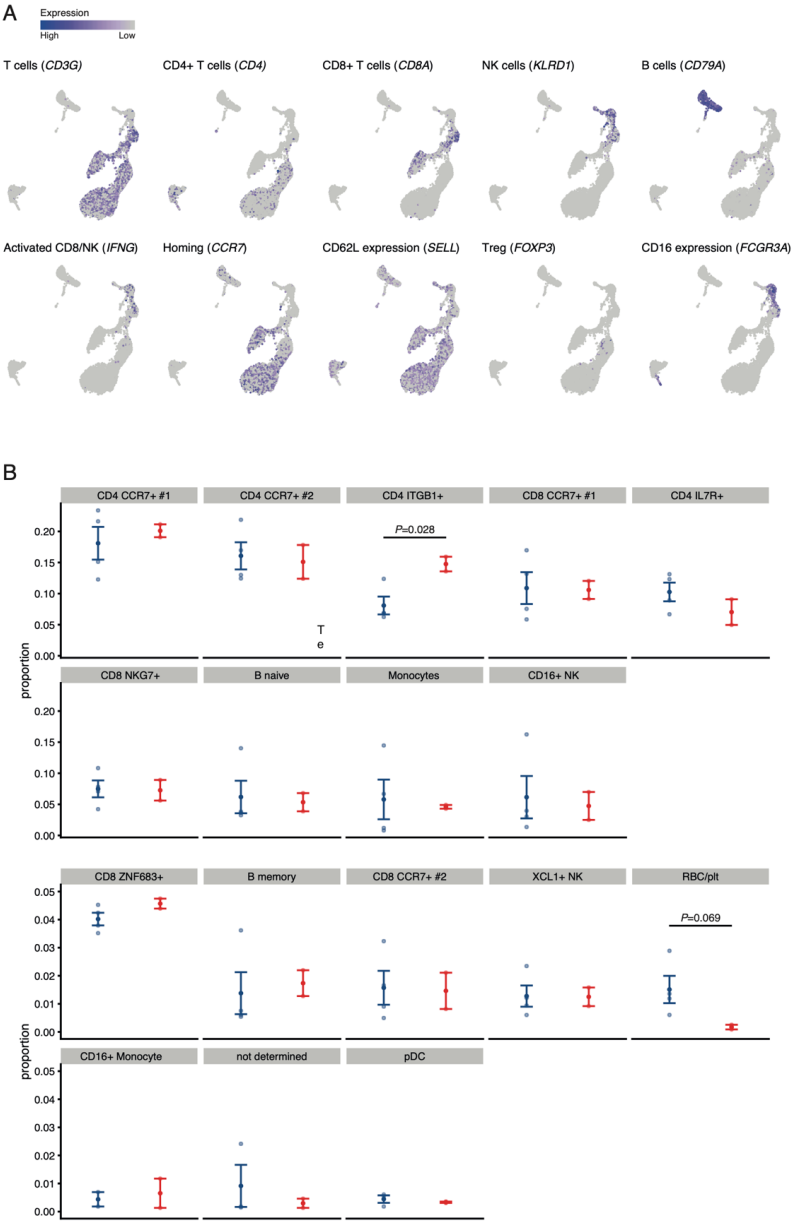
Supplementary Figure 2. T regulatory cells (CD4 CD25+ CD127lo) are not different between chronic and transient ITP. (A) Comparison of levels in chronic ITP (black dots) and transient ITP (white circles). Reference data from 145 healthy children from the Netherlands, stained by the same markers, are shown in the shaded grey area and the median per age as black line {vanGent:2009kq}. (B) T regulatory cell levels, corrected for age (residual), showed no difference between patients with different prognosis.



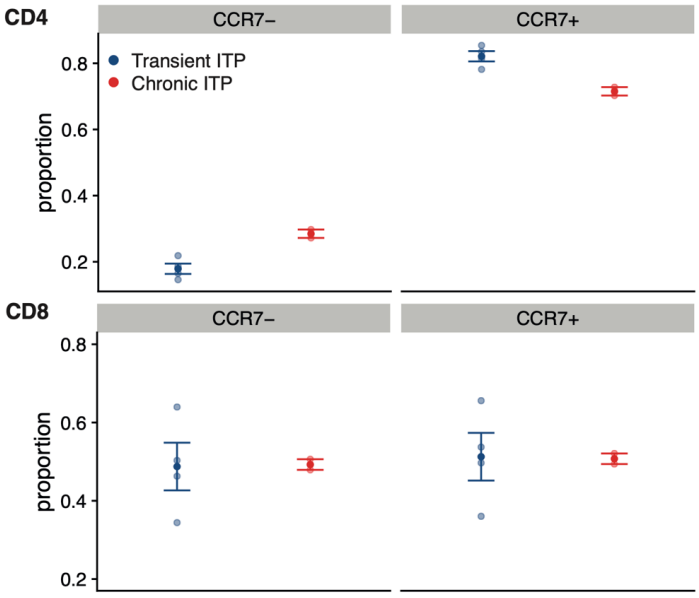
Supplementary Figure 3. Gene expression per scRNA-seq cluster.



Supplementary Figure 4. Canonical gene expression per cluster (A) and frequencies between chronic and transient ITP (B).



Supplementary Figure 5. CCR7-positive (naïve) and negative (effector) CD4 and CD8 cells in transient and chronic ITP.



Supplementary Figure 6. Differential expression of *ITGB1*-positive vs *ITGB1*-negative CD4 T cells. These cells did not exhibit a CD4+ cytotoxic T cells gene expression profile, i.e., showing no upregulation of granulysin (*GNLY),* granzyme B (*GZMB),* granzyme A *(GZMA),* or perforin (*PRF1)*. Compare also Nicolet 2021 bioArxiv; doi: 10.1101/2021.02.10.430576.