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ORIGINAL ARTICLE

Increased effector memory CD4$^+$ T cells are associated with chronic childhood immune thrombocytopenia

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Abstract

Background: Antibody- and T-cell immune responses against platelet self-antigens are key features of childhood immune thrombocytopenia (ITP). Reliable diagnostic and prognostic immune markers remain underdeveloped and are not currently used in clinical practice.

Objectives: To validate previously suggested biomarkers and identify novel predictors of chronic childhood ITP.

Methods: We analyzed immune profiles in 158 children with newly diagnosed ITP at the time of diagnosis prior to treatment ("Treatment With or Without IVIg for Kids with ITP" randomized controlled trial). Spontaneous platelet recovery and response to intravenous immunoglobulin were evaluated during 1 year.

Results: Neither CD4$^+$, CD8$^+$, CD19$^+$, nor natural killer cell populations predicted the development of chronic ITP or recovery during follow-up. However, we observed a significant age-adjusted increase in effector memory CD4$^+$ T cells by 1.4% (95% CI, 0.4-2.4) in chronic ITP. A frequency of effector memory CD4$^+$ T cells above the median was associated with a reduced likelihood of recovery, with an age- and treatment-adjusted

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hazard ratio of 0.55 (95% CI, 0.35-0.85). Stratification by effector memory CD4$^+$ T-cell levels demonstrated additional prognostic value over the Childhood ITP Recovery Score, a clinical prediction model. Single-cell RNA-sequencing of peripheral blood mononuclear cells of 6 children confirmed an expansion of effector memory CD4$^+$ T cells in chronic ITP, characterized by high expression of *ITGB1* (integrin β1, CD29).

Conclusions: Increased integrin β1-positive effector memory CD4$^+$ T cells are associated with chronic ITP and improve the prognostic value of a clinical prediction model. T-cell phenotyping could be valuable for prognosis and personalized treatment in newly diagnosed childhood ITP.

KEYWORDS
immune system, immune thrombocytopenia, IVIg, pediatrics, prognosis

1 | INTRODUCTION

Childhood immune thrombocytopenia (ITP) is an acquired autoimmune bleeding disorder characterized by thrombocytopenia, defined as a platelet count $< 100 \times 10^9$/L [1]. Most children experience a transient disease course, with recovery typically occurring within 3 months of diagnosis [1]. However, the morbidity is significantly higher in those with persistent (>3 months) and chronic (>12 months) ITP [2,3]. The underlying pathophysiology of ITP remains incompletely understood, and it remains unclear why some patients recover while others develop chronic disease. Current clinical prediction models for ITP prognosis have only moderate accuracy [4–6], highlighting the need for biomarkers that can better predict disease progression and guide clinical management.

The prevailing understanding of ITP pathophysiology involves both humoral (antibody-mediated) and cellular (CD8$^+$ T-cell-mediated) immune responses against platelet self-antigens [7,8]. A functional deficiency or reduced number of regulatory T cells is also thought to contribute to inadequate suppression of the autoimmune response (loss of immune tolerance) [7,9]. These immune dysfunctions lead to increased platelet clearance [10–13], impaired platelet function [14–16], platelet apoptosis [17], and impaired megakaryocyte differentiation and platelet production [18–22].

Childhood ITP differs from adult ITP due to the unique factors of a developing immune system [23], frequent onset after infections or vaccinations, and overall prognosis [24]. In pediatric ITP, many divergent findings on immune characteristics have been reported, often stemming from small, heterogeneous studies with varying methodologies. Newly diagnosed children showed reduced numbers of CD4$^+$ T cells and a lower CD4$^+$/CD8$^+$ ratio [25]. This included a decrease in CD4$^+$ CD25$^+$ (which represent activated cells, including regulatory), and potentially FOXP3$^+$ T regulatory cells [25–27]. Additionally, CD19$^+$ B cells were elevated in newly diagnosed ITP [25], while CD16$^+$ CD56$^+$ natural killer (NK) cells were decreased [27]. Concerning prognosis, two studies found that CD4$^+$ CD25^{hi} cells were lower in chronic ITP when compared with transient ITP [25,27], suggesting a role in disease persistence. Moreover, patients with chronic ITP show higher levels of interleukin-2 (IL-2) production by platelet-stimulated peripheral blood mononuclear cells (PBMCs) [28], indicating increased T-cell preactivation. These immune markers are candidates for predicting disease course and guiding immune-targeted therapies [6].

Our randomized controlled trial, "Treatment With or Without IVIg for Kids with ITP" (TIKI), involved 200 children with newly diagnosed ITP who were randomly assigned to either careful observation or intravenous immunoglobulin (IVIg) treatment, and followed for 1 year [29]. This trial provides a valuable platform to evaluate predictive markers for spontaneous recovery, IVIg response, and the development of chronic ITP. In this preplanned secondary study, we aimed to validate previously suggested immune predictors and identify novel markers for disease recovery and chronicity. Immune phenotyping was performed at diagnosis by flow cytometry, with stored samples further analyzed for gene expression and clonality using single-cell RNA-sequencing (scRNA-seq), including T- and B-cell receptor sequencing (scTCR-seq and scBCR-seq).

2 | METHODS

2.1 | Study participants and sample processing

Samples were obtained from the TIKI randomized controlled multicenter trial [29]. Written informed consent for participation was given by parents and children aged ≥12 years. The study was approved by the ethical review board of the University Medical Center Utrecht and conducted in accordance with the Declaration of Helsinki. Briefly, children with newly diagnosed ITP (within 72 hours of diagnosis) <16 years of age with a platelet count $\leq 20 \times 10^9$/L were randomly assigned to undergo careful observation or single-dose IVIg treatment (Supplementary Methods). Patients were not eligible for participation in case of major or life-threatening bleeding that required medical intervention (Buchanan bleeding score 4 or 5). Blood samples were

obtained by venipuncture after inclusion and randomization, within 72 hours of diagnosis, as IVIg had to be administered by that time. EDTA-anticoagulated whole blood was transported to our national reference laboratory for immune phenotyping by flow cytometry, as detailed below. Of 40 patients, heparin-anticoagulated whole blood at diagnosis and during 5 follow-up visits was immediately transported by courier to a central laboratory facility (Sanquin) for isolation of fresh PBMCs from whole blood, using Ficoll. Trained laboratory personnel performed the isolation at the institute's biobank (Cryobiology) according to a standard operating procedure. The cells were aliquoted and cryopreserved at −80 °C.

2.2 | Clinical outcomes

A complete recovery from ITP was defined by International Working Group criteria as a platelet count $\geq 100 \times 10^9$/L [1]. Absence of a complete recovery 3 and 12 months after the diagnosis were defined as persistent and chronic ITP, respectively. Bleeding symptoms were assessed using the modified Buchanan bleeding score [30,31]. The primary clinical outcome was longitudinal complete recovery. Secondary outcomes were association with clinical outcome by 12 months (complete recovery vs chronic ITP), or 3 to 6 months (transient/persistent ITP), as indicated.

2.3 | Immune phenotyping by flow cytometry

Immune cells were enumerated and phenotyped by flow cytometry using 3 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 #644611), respectively. Staining of 25µL EDTA-anticoagulated whole blood was performed in TruCount tubes (BD Biosciences #340334). Regulatory $CD4^+$ T cells ($CD25^+$ $CD127^{lo}$; Supplementary Figure S1) were identified by CD4 FITC, CD25 PE-Cy7, CD127-Alexa Fluor 647 (BD #560249), and CD3 PerCP (BD Biosciences #345766). The $CD4^+$ CD45RO-negative ($CD4^+$ $CD45RO^-$, Supplementary Figure S1), effector memory ($CD4^+$ $CD45RO^+$ $CD27^-$) 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 M1764).

Where indicated, cryopreserved PBMCs were thawed, washed, and stained with CD29 APC (Biolegend #303007), CCR7 FITC (Biolegend #353215), CD45RO PE (BD Biosciences #347967), CD3 PerCP (BD Biosciences #345766), CD4 Pacific Blue (Biolegend #300521) and CD27 BV510 (Biolegend #302836). Alternatively, for T-helper subsets, staining was performed with CXCR5 BB515 (CD185, BD #564625), CD8 APC (BD #345775), CD14 AF700 (BD #561029), CD29 BV421 (BD #568994), CCR6 BV605 (CD196, BD #562724), CD45RA BV786 (BD #563870), CD4 BUV395 (BD #563550), CD27 BUV568 (BD #748705), CD3 BUV805 (BD #612893), CCR4 PE-Dazzle (Biolegend #359420), CXCR3 PE-Cy7 (BD #560831), and APC-H7 viability stain, with samples analyzed on a FACSymphony (BD Biosciences). The Th1 and Th1-like CD4 cells were defined as $CXCR5^-$, $CXCR3^+$ and $CCR6^{+/-}$, and Th2 CD4 cells as $CXCR5^-$, $CXCR3^-$, $CCR6^-$, and $CCR4^+$. Data analysis was performed with a standardized workflow in flowJo or BD FACSDiva. Each sample was separately visualized and inspected.

2.4 | 10X genomics scRNA-seq

Cryopreserved PBMCs from 6 children with newly diagnosed ITP were analyzed by scRNA-seq. Two of these children later developed chronic ITP. The remaining 4 patients displayed transient ITP (resolution within 3-12 months) and were used as age-matched controls at a 2:1 ratio for the chronic ITP cases. Samples were selected based on availability at the time of the experiment. The median difference in age between the transient and chronic patients was 1 year (Supplementary Table S1; immune phenotyping and platelet autoantibody data, Supplementary Table S2). Detailed methods are available in the Supplementary Methods. Briefly, single-cell sequencing was performed using gel bead emulsions [32]. Samples were processed according to 10X recommendations for the v3.1 5′ V(D)J kit. For analysis, data were loaded in Seurat version 3 [33]. The consensus dataset of the Blood Atlas (Human Protein Atlas [34]) was used for interpretation of differential gene expression per blood cell type.

2.5 | 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. Age-adjusted mean differences for groups were calculated by linear regression (in R notation, *cells* ∼ *age* + *group*). Survival curves (longitudinal complete recovery) were visualized by Kaplan–Meier plots. The effect sizes (hazard ratios [HRs]) and 95% CIs were obtained using Cox-proportional hazard models. A P value of $< .05$ was considered statistically significant.

3 | RESULTS

3.1 | $CD4^+$, $CD8^+$, B, and NK cell counts are not associated with ITP prognosis

The absolute counts of $CD3^+$, $CD4^+$, $CD8^+$ T cells, $CD19^+$ B cells, and $CD56^+$ $CD16^+$ NK cells of newly diagnosed ITP patients were within the age-appropriate respective reference interval (N = 158; Figure 1 [23,35,36]). Moreover, the absolute $CD3^+$, $CD4^+$, $CD8^+$ T cells, $CD19^+$ B cells, and $CD56^+$ $CD16^+$ NK cell counts at the time of diagnosis were similar between patients who recovered and those

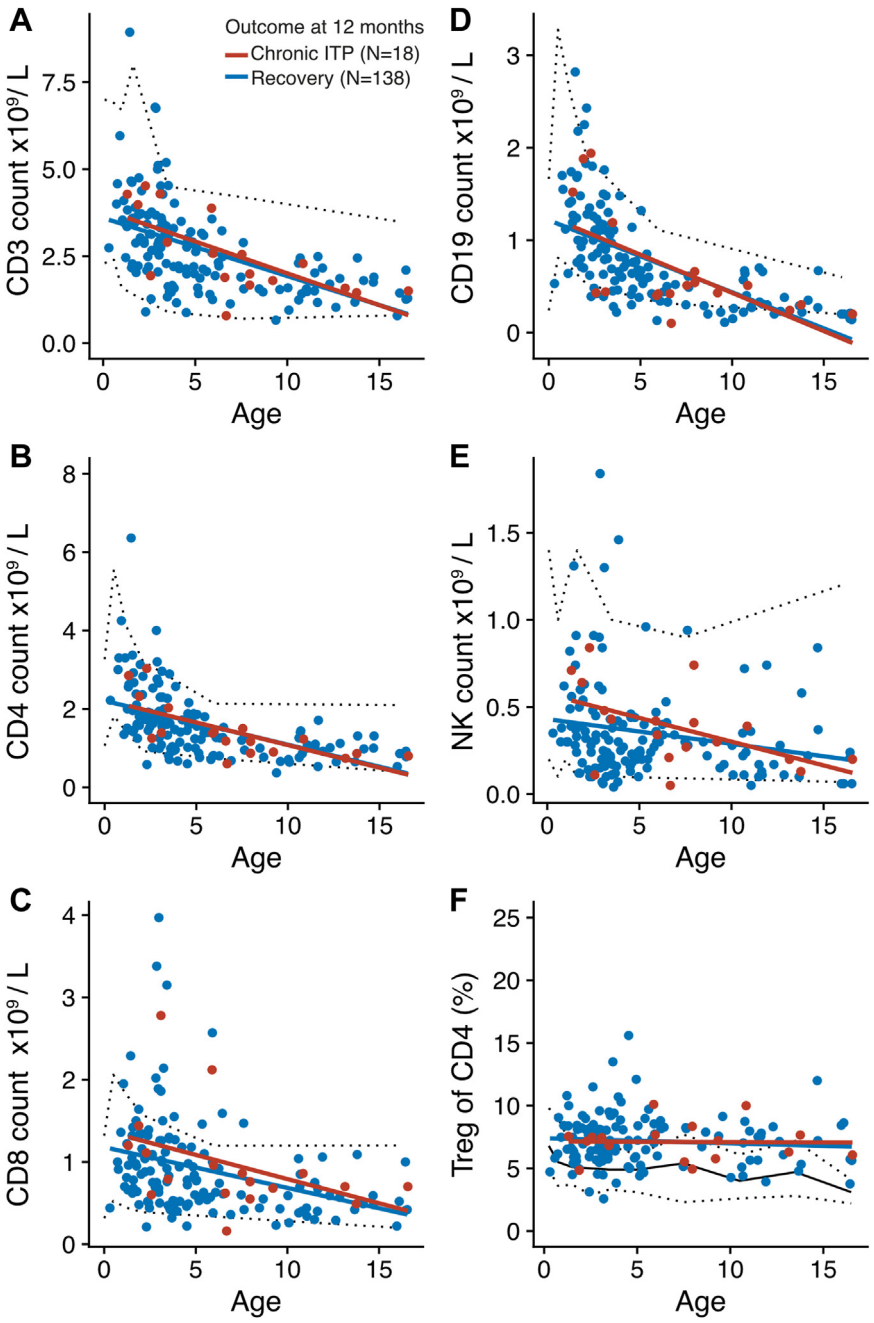

FIGURE 1 Immune phenotyping by flow cytometry in children with newly diagnosed immune thrombocytopenia (ITP, *N* = 156). Reference data of healthy children from the Netherlands for absolute cell counts (dotted lines, 2.5% and 97.5%)[23,36]. Regulatory $CD4^+$ T-cell reference data of healthy children from the Netherlands, with the same gating, shown as median (black line) and range (dotted lines)[35]. NK, natural killer cells; Treg, regulatory $CD4^+$ T cells.

with chronic ITP at 12 months follow-up (Figure 1A–E); they also did not differentiate the time of recovery during 1 year after ITP diagnosis (Cox-proportional hazard model, adjusted for age and stratified by treatment). Relative cell counts are displayed in Supplementary Figure S2. While regulatory $CD4^+$ T cells (Treg; $CD25^+$ $CD127^{low}$) were suggested to be lower in ITP, in this dataset, the frequency was within the age-appropriate reference range, or slightly above (Figure 1F). No differences in Treg frequency were observed between children who recovered and those with chronic ITP (Figure 1F).

In sum, our study could not validate any of the previously suggested $CD4^+$, regulatory $CD4^+$, $CD8^+$, $CD19^+$, or NK cell counts as a suitable predictor of ITP prognosis. Thus, we pursued the identification of novel markers of prognosis.

3.2 | Increased effector memory $CD4^+$ T cells are associated with chronic ITP

The evaluation of effector memory ($CD45RO^+$ $CD27^-$) $CD4^+$ T cells revealed an age-adjusted increase of 1.4% (95% CI, 0.4-2.4; *P* = .005; Figure 2A) in patients with chronic vs recovered ITP. This was consistent with a significant increase in the absolute effector memory

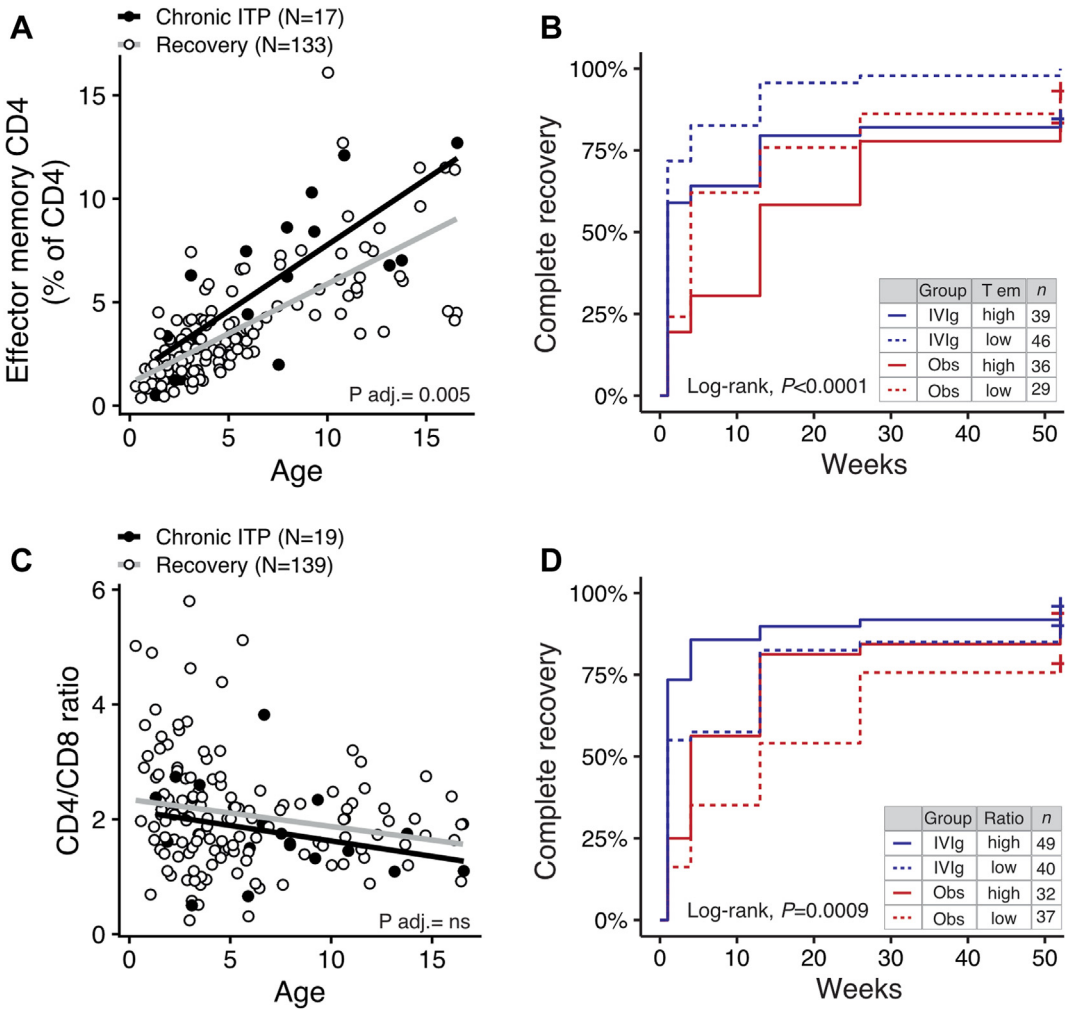

FIGURE 2 Prognostic significance of effector memory $CD4^+$ T cells and $CD8^+$ cells in newly diagnosed childhood immune thrombocytopenia (ITP). Data for spontaneous recovery as well as response to intravenous immunoglobulins. (A) Frequency of effector memory $CD4^+$ T cells ($CD27^-$ $CD45RO^+$ of $CD3^+$ $CD4^+$ lymphocytes) for patients who developed chronic ITP as compared with those who recovered. While direct reference data was not available, the age trend was as expected from previous data [23,35]. (B) Longitudinal recovery dependent on effector memory $CD4^+$ T frequency at, above, or below the median ("high" and "low"). (C) $CD4^+/CD8^+$ ratio for children with chronic immune thrombocytopenia compared with those who recovered. Response to intravenous immunoglobulins is seen as difference compared with patients randomized to careful observation. (D) Longitudinal recovery is dependent on a "high" $CD4^+/CD8^+$ ratio (at/above the median) or "low" ratio (below the median). IVIg, intravenous immunoglobulins; TEM, effector memory $CD4^+$ T cells; Obs, careful observation group.

$CD4^+$ count (chronic ITP increase, 0.016 ± 0.008 × 10^9/L; Supplementary Figure S3). In newly diagnosed childhood ITP, IVIg treatment results in higher recovery rates within the first 6 months compared with observation alone, but it does not influence progression to chronic ITP [29]. Here, we observed that patients with a $CD4^+$ effector memory count above the median displayed a lower proportion of complete recovery over a 1-year follow-up and a higher rate of chronic ITP, both after randomization to IVIg treatment and observation (Figure 2B). The adjusted HR (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).

We also observed that children with a $CD4^+/CD8^+$ ratio below the median exhibited a lower proportion of complete recovery during follow-up, with an aHR of 0.64 (95% CI, 0.46-0.89; adjusted for age and treatment; Figure 2D). This difference was most pronounced during the first 6 months after diagnosis (Figure 2D). When the 12-month outcome was considered, children with chronic ITP showed no significant changes in the $CD8^+$ T-cell frequency vs recovered ITP (age-adjusted mean increase 2.9%; 95% CI, −1.2% to 7.0%; P = .17; Supplementary Figure S2), and no difference in $CD4^+$ cells. Similarly, the $CD4^+/CD8^+$ T-cell ratio showed no significant change in chronic ITP (mean age-adjusted decrease, 0.2 ± 0.2 ratio units; Figure 2C), suggesting limited utility for prediction of chronic ITP (Figure 2D).

In a multivariate model, both the $CD4^+$ effector memory frequency and the $CD4^+/CD8^+$ ratio were independently associated with the ITP prognosis (Supplementary Table S3). Moreover, the predictive ability was independent of a preceding infection, total leukocyte and lymphocyte counts, and the presence of IgG or IgM autoantibodies by

TABLE Proportion of complete response/chronic immune thrombocytopenia stratified at the time of diagnosis by the Childhood Immune Thrombocytopenia Recovery Score and the effector memory CD4$^+$ T-cell frequency.

	High probability of recovery			Intermediate probability			Low probability		
Month	Pre-test	aTEM < median	aTEM ≥ median	Pretest	aTEM < median	aTEM ≥ median	Pretest	aTEM < median	aTEM ≥ median
3	0.80 (99/124)	0.88 (52/59)	0.72 (47/65)	2/5	1/3	1/2	0.47 (8/17)	0.60 (6/10)	0.29 (2/7)
6	0.89 (111/125)	0.88 (53/60)	0.89 (58/65)	2/5	1/3	1/2	0.61 (11/18)	0.82 (9/11)	0.29 (2/7)
12	0.94 (117/125)	0.97 (58/60)	0.91 (59/65)	3/5	2/3	1/2	0.67 (12/18)	0.82 (9/11)	0.43 (3/7)
Chronic ITP	0.06 (8/125)	0.03 (2/60)	0.09 (6/65)	2/5	1/3	1/2	0.33 (6/18)	0.18 (2/11)	0.57 (4/7)

Data are the proportion (n/N) of patients exhibiting complete response/chronic immune thrombocytopenia 3, 6, 12 months after the diagnosis. Pretest probability as observed after stratification by Childhood Immune Thrombocytopenia Recovery Score group.

aTEM, age-adjusted effector memory CD4$^+$ T-cell frequency (residuals of linear model, see Methods); ITP, immune thrombocytopenia.

Monoclonal Antibody-Specific Immobilization of Platelet Antigens assay. (Supplementary Table S3). The age-adjusted effector memory CD4$^+$ T-cell frequency dichotomized at the optimum cutoff (Youden's J value) resulted in a chronic ITP sensitivity of 0.53 (95% CI, 0.28-0.77) and specificity was 0.82 (0.74-0.88), with positive and negative predictive values of 0.27 (0.13-0.46) and 0.93 (0.87-0.97), respectively.

We next assessed whether the effector memory CD4$^+$ T-cell frequency could further stratify prognosis after a clinical prediction model had already been used, ie, after application of the externally validated Childhood ITP Recovery Score [4]. One-percentage point increase in the age-adjusted frequency of effector memory CD4$^+$ T cells had an aHR of 0.88 for complete recovery (95% CI, 0.80-0.97; P = .008; Supplementary Table S4), adjusted for treatment and the Childhood ITP Recovery Score. Improved risk stratification by effector memory CD4$^+$ was shown for patients at high, intermediate, or low probability of recovery by the Childhood ITP Recovery Score between 3 and 12 months of follow-up, including chronic ITP (Table). Thus, the frequency of effector memory CD4$^+$ T cells was a prognostically useful addition to the clinical prediction model, independent of age.

Functionally, the effector memory CD4$^+$ T cells of newly diagnosed ITP patients showed enrichment for circulating Th1/Th1-like and Th2 cells (median 41% and 26%, respectively; N = 9), as compared with naive T cells (0% and 3%, respectively). The CD29$^+$ effector memory CD4$^+$ T cells showed similar proportion of Th1/Th1-like cells and Th2 cells as compared with all CD4 effector memory T cells (median difference −0.1% for Th1/Th1-like and +0.6% for Th2).

3.3 | scRNA-seq identifies an *ITGB1*-expressing CD4$^+$ cluster associated with chronic ITP

For further identification of potential markers of prognosis, scRNA-seq paired with TCR- and BCR-seq was performed on PBMCs of 6 newly diagnosed ITP patients, two of whom displayed persistent thrombocytopenia and were classified as chronic ITP during the study (Figure 3A). Baseline characteristics and flow cytometry data of these patients are shown in Supplementary Tables S1 and S2. 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 S4. 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 between clusters are displayed in Figure 3D and Supplementary Figure S5. Comparing the frequency of clusters between the transient and chronic IT patients, we observed a higher frequency of a cluster of CD4$^+$ T cells expressing fibronectin receptor integrin β1 (*ITGB1*), IL-32 (*IL32*), IL-7 receptor (*IL7R*), and lymphotoxin β (*LTB*) in chronic ITP (mean ± sem, 8.1% ± 1.4% transient vs 14.8% ± 1.2% chronic ITP; Figure 3E and F). Consistent with the relatively increased CD8 frequency in flow cytometry (Supplementary Figure S2), we also observed a minor increase in Hobit (*ZNF683*)-expressing CD8$^+$ T cells (transient 4.0% ± 0.2% *vs* 4.6% ± 0.2% chronic ITP; Figure 3E; individual patient data in Figure 3F). Both findings were consistent with the prognostic markers identified by flow cytometry in the whole blood (see above). The proportion of cells from the other clusters was not different between patients with transient and chronic ITP and was therefore not further considered for this study. The BCR-seq on 739 B cells, limited by the low relative frequency of B cells in PBMC, showed no signs of clonal expansion.

3.3 | Enhanced activation of *ITGB1*-expressing CD4$^+$ memory T cells in chronic ITP

Given these differences in CD4$^+$ and CD8$^+$ clusters found by scRNA-seq, we further evaluated T-cell subtypes in the ITP patients. After filtering out other cell types, sub-clustering revealed 11 distinct T-cell subclusters (Figure 4A). All T-cell subclusters were present in both

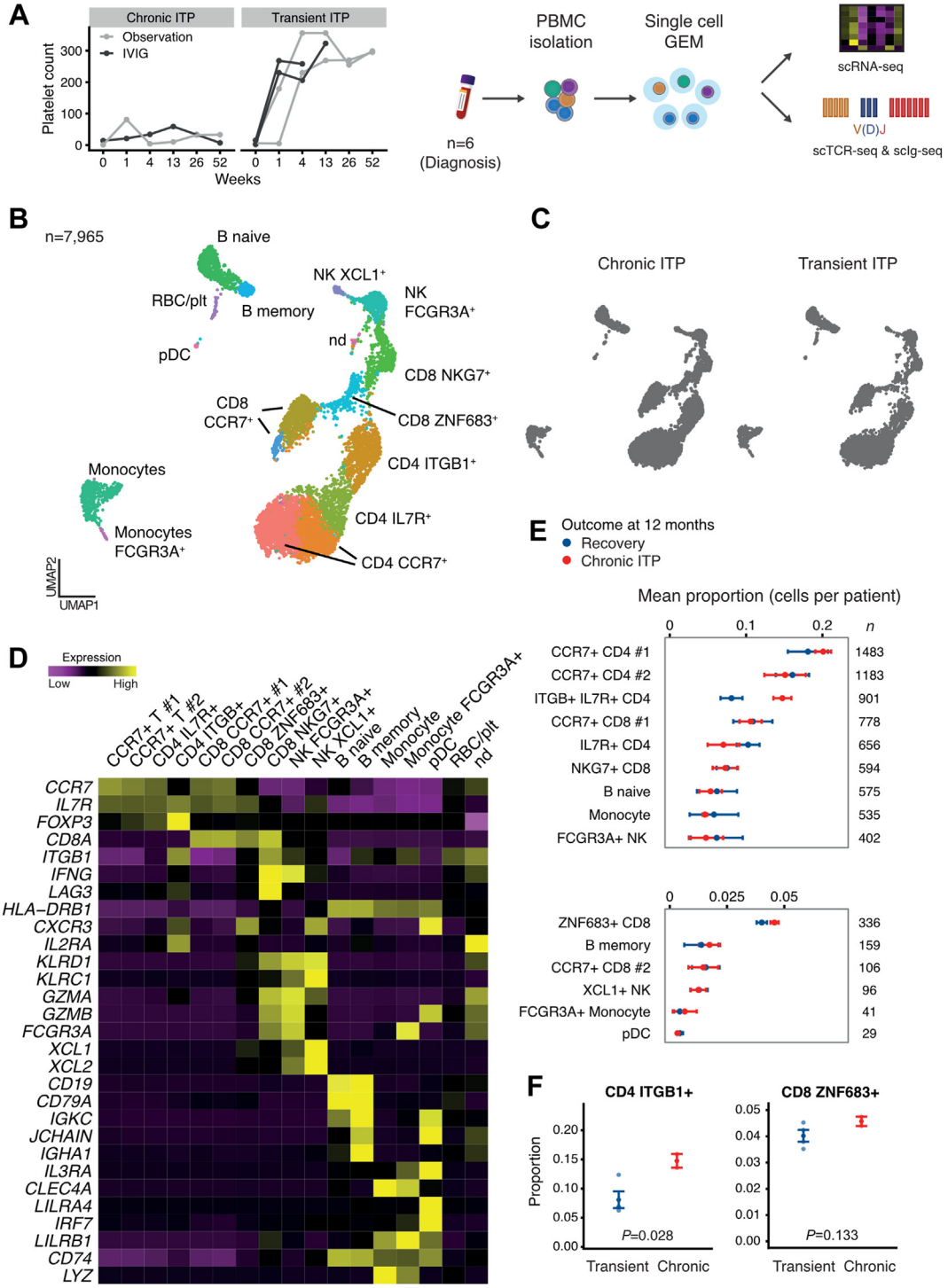

FIGURE 3 Immune characteristics of childhood immune thrombocytopenia (ITP) peripheral blood mononuclear cells analyzed by single-cell RNA-sequencing (scRNA-seq). (A) Left panel, longitudinal platelet counts of patients with chronic or transient ITP during 1-year follow-up. Right panel, 10X Genomics workflow. Libraries were prepared for scRNA-seq, as well as scRNA-seq for T- and B-cell receptors. (B) UMAP projection of identified peripheral blood mononuclear cells, using unsupervised clustering on RNA gene expression. (C) All UMAP clusters were present in both 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 *ITGB1*+ *IL7R*+ CD4$^+$ T effector memory cells and *ZNF683*+ CD8$^+$ T cells. PMBC, peripheral blood mononuclear cells. GEM, gel beads-in-emulsion; UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction.

transient and chronic ITP patients. The subclusters encompassed 4 naive C-C motif chemokine receptor 7 (*CCR7*)-expressing CD4$^+$ T-cell populations (Figure 4B) and 2 *IL7R*-expressing activated CD4$^+$ T-cell clusters. In addition, 2 *CCR7*-expressing naive CD8$^+$ T-cell clusters were identified, as well as 3 CD8$^+$ effector T-cell clusters that showed expression of *ZNF683*, cathepsin W *(CTSW)*, and C-C motif chemokine

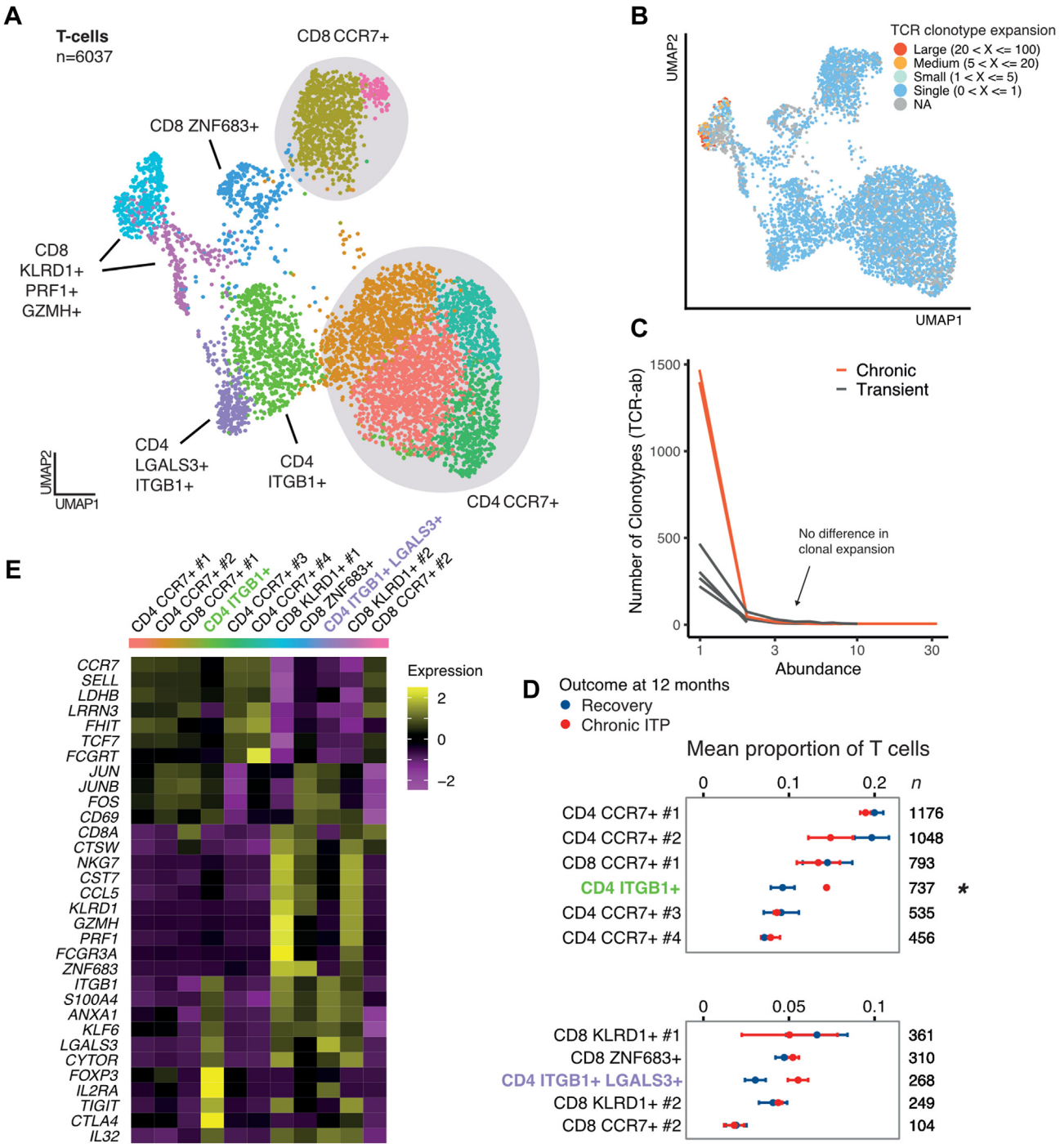

FIGURE 4 T-cell subclusters in childhood immune thrombocytopenia (ITP). (A) Single-cell RNA-sequencing UMAP projection of T-cell subclusters, using unsupervised clustering on RNA expression. (B) TCR clonotypes (paired scTCR-seq) showed expansion in $CD8^+$ *KLRD1*+ effector cells. Other clusters showed mostly single unique clones. (C) No differences were observed in clonal expansion of T cells between chronic and transient ITP patients. The higher absolute number of single clones in chronic ITP patients was due to more sequenced cells. (D) Frequency of cells by cluster. The 2 *ITGB1*+ and *ITGB1*+ *LGALS3*+ $CD4^+$ T-cell clusters showed higher frequencies in chronic ITP. $^*P < .05$. Error bars show standard error of the mean for 2 to 4 patients. (E) 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. NA, not available; TCR, T-cell receptor; TCR-ab, T-cell receptor antibody; scTCR, T-cell receptor sequencing; UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction.

ligand 5 (*CCL5*; Figure 4B). Using TCR-seq, we solely observed clonotype expansion of few cells in the activated memory $CD8^+$ T-cell subclusters (Figure 4B), whereas cells in other subclusters represented unique clonotypes. There was no evidence of clonal expansion from patients with transient or chronic ITP, ie, T-cell clonotypes with >1 cell (Figure 4C); chronic ITP patients had more sequenced cells and

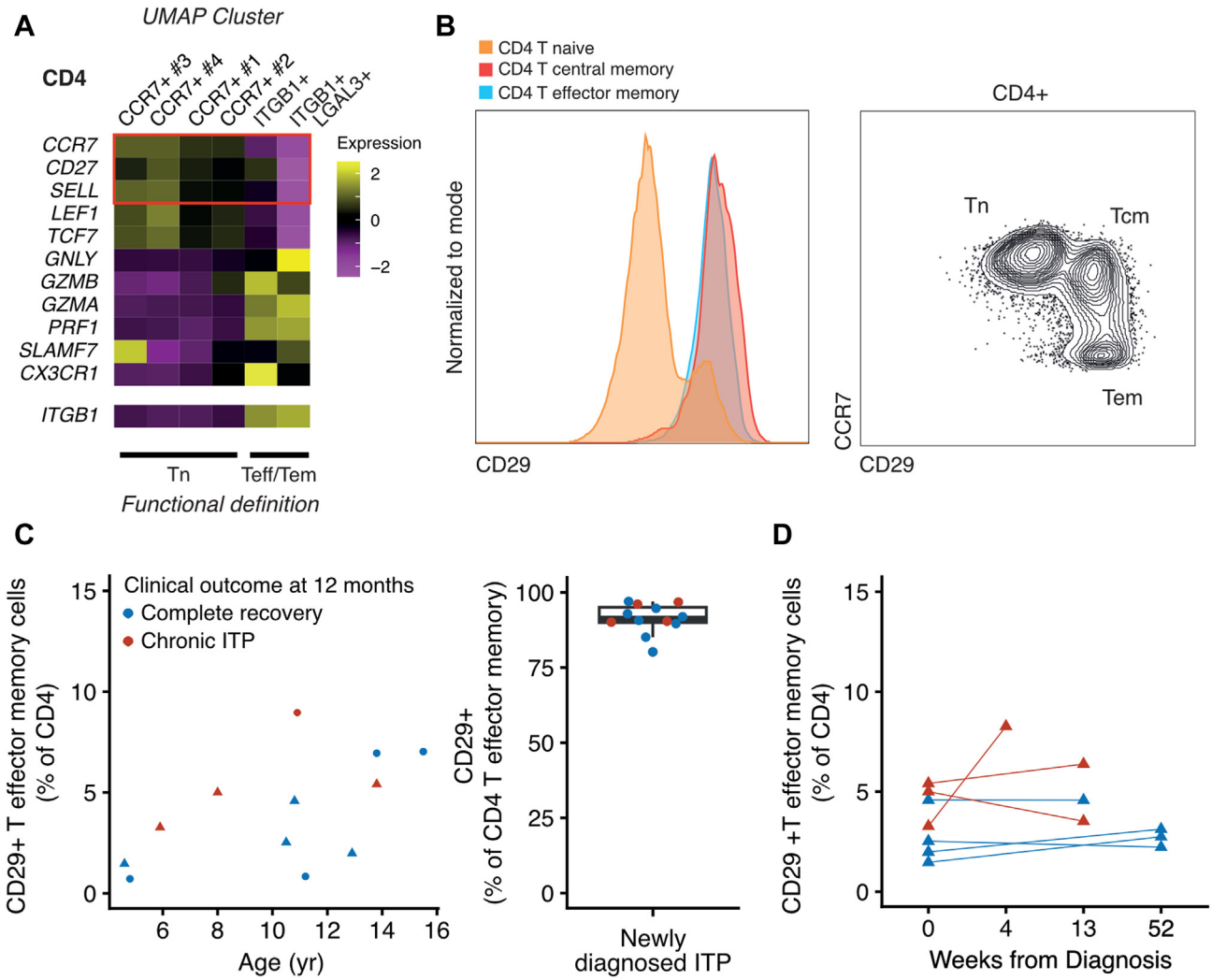

FIGURE 5 *ITGB1* expression and CD29 protein levels in CD4$^+$ effector memory T cells in newly diagnosed immune thrombocytopenia (ITP). (A) RNA coexpression of naive and activation markers in CD4$^+$ T-cell single-cell RNA-sequencing T-cell subclusters (see Figure 4). The *ITGB1*+ and *ITGB1*+ *LGAL3*+ T-cell subclusters were negative for CCR7 and coexpressed markers of CD4$^+$ effector memory cells, while *ITGB1*-negative clusters showed expression consistent with naive CD4$^+$ T cells. The red box indicates markers commonly used in flow cytometry for identification of Tn/Teff/Tem. (B) Surface staining of CD29 (*ITGB1*) by flow cytometry among naive, central memory, and effector memory CD4$^+$ T cells (cryopreserved peripheral blood mononuclear cells of newly diagnosed ITP; representative sample, quantification in panel C). (C) Left panel, CD29 effector memory CD4$^+$ T cells of cryopreserved peripheral blood mononuclear cells at the time of diagnosis, showing patients with chronic ITP vs complete recovery at 12 months follow-up (N = 12). Triangles indicate patients with available longitudinal sample, as shown in panel D. Right panel, percentage of CD29-positive cells among effector memory CD4$^+$ T cells in newly diagnosed ITP. (D) Longitudinal changes in CD29-positive T effector memory cells. One patient with the sample available 4 weeks after diagnosis was treated with intravenous immunoglobulin. Tn, naive T cells; Tcm, central memory CD4$^+$ T cells; Tem, effector memory CD4$^+$ T cells; UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction.

therefore showed a higher absolute number of single clones. By non-supervised clustering of expressed genes, the CD4$^+$ cells with differences between transient and chronic ITP identified in global clustering showed as 2 subclusters of *CCR7*-negative, *ITGB1*-expressing CD4$^+$ cells, one of which also expressed *LGALS3* (Figure 4D; *dark-violet* and *light-green* colored subclusters). Compared with other T-cell subclusters, these 2 identified *ITGB1*-expressing subclusters have an activated memory phenotype, expressing high levels of *ITGB1*, desmoyokin *(AHNAK)*, annexin A2 *(ANXA2)*, *KLF6*, CD161 *(KLRB1)*, and *IL32* (Figure 4E). In these *ITGB1*-expressing CD4$^+$ T-cell subclusters, compared with transient ITP, the cells of chronic ITP patients displayed upregulation of *IL32*, thymosin β4, X-linked *(TMSB4X)*, S 100 calcium binding protein A11 *(S100A11)*, myosin light chain 12A *(MYL12A)*, and interferon induced transmembrane protein 1 (*IFITM1*), indicating T-cells activation (Supplementary Figure S6; differential expression).

3.4 | Consistent increase in CD29$^+$ effector memory CD4$^+$ T cells in chronic ITP by flow cytometry

We next examined the relationship between the scRNA-seq CD4$^+$ clusters that were associated with chronic ITP (Figures 3 and 4) and

the effector memory CD4$^+$ T cells identified by flow cytometry (Figure 2). First, the 2 *ITGB1*-expressing CD4$^+$ subclusters showed low levels of *CCR7*, *CD27*, L-selectin/CD62L (*SELL*), and activation of cytokine production (Figure 5A; RNA expression for all cells in subclusters, all individuals). This was consistent with an increase of effector memory CD4$^+$ T cells in chronic ITP as identified by flow cytometry. Additionally, 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 S7). Moreover, by flow cytometry, CD29 (*ITGB1*) was present on the surface of central memory and effector memory CD4$^+$ T cells, but not naive CD4$^+$ (Figure 5B), a finding that is also observed in healthy adult controls. Central memory CD4$^+$ cells are positive for CCR7, differentiating these cells from the scRNA-seq subclusters (Figure 5A, B).

Cryopreserved PBMC from newly diagnosed children of the TIKI study at the time of diagnosis displayed a mean age-adjusted 2.8% ± 1.2% increase of CD29$^+$ effector memory CD4$^+$ T cells in chronic ITP (N = 12; Figure 5C, left panel). This was in full agreement with the results from scRNA-seq and flow cytometry in the whole study cohort. Furthermore, a median 91% (IQR, 90%-95%) of effector memory CD4$^+$ T cells were positive for CD29 (Figure 5C, right panel), suggesting a strong overlap of the scRNA-seq cluster with the effector memory CD4$^+$ population identified by immune phenotyping (see above). Among children with available follow-up samples (n/N = 7/12), CD29$^+$ CD4$^+$ effector memory T cells were relatively stable 3 and 12 months after diagnosis (Figure 5D). In summary, these data suggest higher integrin β1-expressing effector memory CD4$^+$ T cells and a minor increase in CD8$^+$ T cells as characteristics of chronic ITP at the time of diagnosis.

4 | DISCUSSION

The primary result of our study is that children with newly diagnosed ITP who later developed chronic ITP had a higher frequency of effector memory CD4$^+$ T cells at diagnosis than those who recovered. These cells were characterized by the expression of *ITGB1* (integrin β1, CD29). Notably, effector memory CD4$^+$ T cells emerged as independent predictors of chronic ITP and could further stratify patient prognosis when combined with the externally validated Childhood ITP Recovery Score (http://itprecoveryscore.org/) [4]. While there is some overlap between chronic ITP and patients who recovered, no single biomarker can be expected to perfectly predict outcomes in complex diseases like ITP. However, the T-cell markers can enhance risk stratification when combined with other predictive factors, as we show with the Childhood ITP Recovery Score.

The study design allowed for the assessment of IVIg effects and spontaneous recovery through randomization. In the TIKI trial, children randomly assigned to IVIg showed higher recovery rates up to 6 months after diagnosis when compared with observation, but there was no effect on the development of chronic ITP [29]. Importantly, the relatively large sample size enabled adjustment for age-related effects, considering the evolving immune system in children, a factor that may have been inadequately accounted for in previous studies.

Conceptually, Semple et al. [28] demonstrated 25 years ago that T cells in patients with chronic ITP produced more IL-2 following *in vitro* stimulation, correlating with elevated serum levels of IL-2, IFNγ, and IL-10 [28]. Our study provides a potential explanation for these findings by identifying an expansion of *ITGB1*-expressing effector memory CD4$^+$ T cells in children with chronic ITP. These cells exhibited higher levels of proinflammatory and activation markers. Furthermore, we extend this observation by showing that effector memory CD4$^+$ T cells may serve as useful markers for distinguishing chronic ITP at the time of diagnosis, independent of factors such as age, infection history, platelet autoantibodies, or clinical prediction scores. We suggest that the elevated levels of CD29$^+$ effector memory CD4$^+$ T cells in chronic ITP may contribute to persistent inflammation and autoimmune responses, as observed in other autoimmune diseases such as ulcerative colitis[37], multiple sclerosis[38,39], and Guillain–Barré syndrome [40]. Expression of integrin β1 associates with potent CD4$^+$ and CD8$^+$ effector cells [41,42]. The elevated presence of these cells could also indirectly reflect immune dysregulation in ITP. Future studies should investigate if the CD29$^+$ CD4$^+$ T cells react directly to autologous platelets or megakaryocytes in co-culture, and if specific targeting of these T cells may be an effective treatment strategy.

Given the nonspecific diagnosis of ITP, characterized solely by thrombocytopenia without definitive secondary laboratory criteria, chronic ITP arguably represents a collection of distinct diseases with varying underlying pathophysiologies, which are currently grouped together under a single diagnosis due to shared clinical features.

Notably, we observed that patients with higher effector memory CD4$^+$ T cells also had a reduced response to IVIg treatment if they were randomized to this (Figure 2B). Thus, integration of this marker with other validated predictors of response (eg, Childhood ITP Recovery Score) may allow to predict if the child will respond in the case of bleeding. Further ITP management may also be affected, such as consideration of the frequency of clinical monitoring, early additional diagnostic evaluations for autoimmunity or hereditary thrombocytopenias, administration of a TPO receptor agonist, and family counseling.

4.1 | Previous research

In contrast to prior literature, external validation of previously suggested immune cell subsets showed no differences between chronic ITP and patients who recovered by flow cytometry, and this was consistent with scRNA-seq. A partial explanation may be a lack of adjustment for the normal development of the immune system during childhood in prior studies, thereby suggesting differences between chronic and recovering ITP patients that are attributable to age. Age plays a significant role in the development of chronic ITP, with infants showing low rates and adolescents experiencing higher rates [24]. Additionally, the frequency of many immune cells changes with age

due to the natural development of the immune system during childhood [23]. Therefore, there is a correlation between chronic ITP, age, and immune variables, which necessitates considering and adjusting for age in related analyses.

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 included a mix of patients before/after treatment [43]. This may further explain differences in contrast to our study, where only treatment-naive patients were assessed at the time of diagnosis. Our findings help reconcile inconsistencies in the pediatric ITP literature, which often arise from small, heterogeneous studies that sample patients at varying times from disease onset, sometimes after immune treatment has begun. In contrast, our study leveraged trial inclusion across 60 hospitals in the Netherlands, where high population density and short laboratory transport times (<2 hours to most locations) preserved sample integrity and consistency. By standardizing patient inclusion at the same diagnostic timeframe (≤72 hours) and conducting diagnostic-grade laboratory assessments over 5 years, we minimized variability and provided robust data that clarify previously conflicting results.

We observed no differences in regulatory $CD4^+$ T-cell frequencies between recovered and chronic ITP, contrasting with several smaller pediatric studies suggesting lower Treg levels in chronic ITP. Our study's strength lies in its larger cohort and high quality of clinical data, as well as standardized sampling time from diagnosis. Previous studies, including markers such as FOXP3 or CD127, had limitations: Zahran et al. [25] included only 8 patients with persistent ITP and none with chronic ITP, while Talaat et al. [27] assessed patients during a later disease phase after steroid or immune therapy, which may have influenced Treg levels. Other studies identified Treg using $CD25^{hi}$ expression alone [26,44], potentially misclassifying activated $CD4^+$ T cells, or comparing ITP patients to healthy controls rather than distinguishing disease courses. Overall, while previous studies suggested a role for Treg in disease persistence, our data indicate that Treg may not be a reliable prognostic marker in pediatric ITP and highlight potential differences between pediatric and adult disease mechanisms.

For our flow cytometry data, CD45RA was not included, and thus it was not possible to identify the re-expression of CD45RA in the terminally differentiated effector memory cells (TEMRA) as was recently described in adult ITP [45]. Thus, whether the elevated CD8 T cells were related to TEMRA cells has to be answered in future studies.

4.2 | 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. Only few ITP patients developed chronic ITP, and given the fixed opportunistic sample size for this study, we were not powered to detect very small differences (magnitude < 1.5% ± 1.0%) in immune cell subsets for validation. To obtain adequate power for such small differences, a significantly larger patient population would need to be recruited. Patients with major organ or life-threatening bleeding at diagnosis were excluded from the study for ethical reasons; these cases are rare and marginally affect the generalizability of our data. Our observation of stable cell numbers during follow-up was based on a small number of individuals with available samples; thus, whether the cells remain stable across a longer period and within a larger population remains to be investigated in future studies. Th1/Th2 profiling was not performed as part of the routine flow cytometry panel. Although post hoc analyses in a subset of patients did not suggest skewing of the Th1/Th2 ratio between $CD29^+$ and all effector memory $CD4^+$ T cells (Results), definitive evidence will require further studies. Children were not eligible for the trial if hereditary thrombocytopenia was expected at the time of diagnosis, but no specific gene panel testing or family investigations were performed in chronic ITP patients. Finally, for the cellular characteristics identified by scRNA-seq and confirmed by flow cytometry, only few patients were available, and the results should be validated further in future studies.

4.3 | Conclusions

Previously suggested immune markers had limited promise when validated in our large prospective study. Our data show that increased effector memory $CD4^+$ cells are independently associated with chronic ITP. Immune phenotyping at the time of diagnosis could be used in addition to other clinical and molecular markers to predict disease outcomes in childhood ITP.

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AUTHOR CONTRIBUTIONS

D.E.S. designed the study, performed laboratory experiments, analyzed and interpreted data and wrote the manuscript; K.M.J.H.-P. designed and performed the clinical studies; B.P.N. designed laboratory experiments, analyzed and interpreted data; M.C.A.B. designed the clinical studies and contributed to the design of the study; N.W. performed laboratory experiments; L.P., R.K., and E.v.d.S. discussed data; G.V. designed and supervised the study and wrote the manuscript; M.d.H. designed and supervised the study, designed the clinical studies, and wrote the manuscript. All authors reviewed and approved the manuscript.

DECLARATION OF COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY

Raw and processed sequencing data were deposited at EMBL-EBI ArrayExpress with accession number E-MTAB-14364

(https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-14364). Code is available from https://github.com/schmidtdav/TIKI-PBMC-scSeq. Further clinical and biological data are available from the corresponding author upon reasonable request. Formal data use, data transfer agreements, and ethical approval may be required depending on the request.

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SUPPLEMENTARY MATERIAL

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