Pediatric immune thrombocytopenia: Catching platelets

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Children with acute immune thrombocytopenia have normal numbers of FOXP3 regulatory T cells with enhanced function

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Abstract: Pediatric Immune Thrombocytopenia (ITP) is an autoimmune mediated bleeding disorder associated with loss of immune tolerance. A number of recent studies implicated impaired FOXP3 regulatory T cells (Treg) as a key element in the development of ITP in adults, and Treg as a promising target for immune therapy. In pediatric ITP, functional data are lacking, especially in non-treated individuals. Here we studied the function of Treg in newly diagnosed pediatric ITP patients, who did not receive treatment and were followed for over one year to investigate various Treg parameters from diagnosis to recovery. In contrast with what has been described for adults, the children with acute ITP had normal Treg numbers, phenotype and function, comparable to healthy controls. In addition, no impairment of Treg function was found in patients with chronic ITP. Cross-over suppression assays using patient PBMC collected at diagnosis co-cultured with Treg collected at recovery and vice versa, showed that effector T cells (Teff) from newly diagnosed ITP patients were resistant to suppression by Treg sampled at recovery. However, these effector cells were suppressed normally by Treg from diagnosis. Our findings demonstrate that Treg function is enhanced in an inflammatory environment in which activated Teff show increased resistance to suppression. (TIKI-study NTR1563)
1. Introduction

Pediatric Immune Thrombocytopenia (ITP) is a bleeding disorder with an incidence of about 5 in 100,000 children.\(^1\) The disease pathophysiology is not fully understood, heterogeneous, and has been suggested to differ from disease onset at adult age.\(^{1-4}\) Three types of the disease are recognized; newly diagnosed (previously known as acute), persistent, and chronic ITP.\(^5\) Newly diagnosed pediatric ITP is characterized by a sudden onset of bleeding and low platelet counts as the only abnormality. Disease onset is often associated with a viral illness a few weeks prior to appearance of symptoms.\(^1\) A slight increased incidence seems to be associated with Measles-mumps-rubella (MMR) vaccination in early childhood.\(^6\) ITP becomes persistent once the disease is not resolved after three months. Approximately 25% of pediatric ITP patients develop chronic ITP, which has recently been defined as a platelet count below 100*10^9/L for over one year.\(^5\) In contrast, adult ITP usually has a subtle onset, is not associated with preceding infections or vaccinations, and results in approximately 70-90% of the cases in chronicity.\(^2-4\)

ITP is an autoimmune disease in which loss of tolerance plays a critical role in disease onset. Autoreactive antibodies, mainly directed against the platelet and megakaryocyte glycoprotein IIb/IIIa complex, are held responsible for both accelerated platelet destruction as well as reduced platelet production.\(^4,7,8\) In addition, it has been shown that ITP patients possess activated platelet specific autoreactive T cells \(^9-11\), whereas these cells are tolerogenic if present in healthy individuals, suggesting loss of tolerance in ITP patients.\(^7\) Since FOXP3 regulatory T cells (Treg) play a crucial role in maintenance of peripheral tolerance, they have been the focus of research in autoimmune diseases for the last decade.\(^12\) Several studies have suggested that Treg defects may underlie ITP pathology in mice and humans.\(^13-15\) Initially, Treg were defined as CD25\(^{\text{high}}\) expressing CD4\(^+\) T cells; however this phenotype is shared with activated effector T cells (Teff). By discovery of the Forkhead box P3 (FOXP3) transcription factor as the master regulator of Treg development and function, it became possible to identify Treg more accurately.\(^16,17\) Nevertheless, even FOXP3 can be upregulated by Teff and therefore phenotypic findings should ideally be confirmed by functional assays.\(^18,19\) Although current literature is contradicting, the majority of studies show diminished Treg numbers and function in ITP
patients, concluding that Treg are indeed affected in ITP and proposing Treg as the future target for immunomodulating therapies.\textsuperscript{14,20,21} This is supported by data showing that intensive treatment with corticosteroids and 2\textsuperscript{nd} line therapy with thrombopoietin (TPO) agonists seems to overcome the defects observed in these Treg.\textsuperscript{22-24}

However, the assumption that Treg are affected in pediatric ITP is based on research performed in chronic adult ITP patients. Adult ITP differs from pediatric ITP in onset and course of disease, and might therefore have different disease pathology.\textsuperscript{1-4} To our knowledge, only two phenotypic studies have been performed in pediatric ITP patients, describing lower Treg numbers compared to healthy controls.\textsuperscript{25,26} Second, chronic adult ITP patients form a rather heterogeneous study population, varying in disease course and treatment regimens. In studies showing Treg impairment in adult ITP, the majority of patients received treatment.\textsuperscript{14,20,22,23,27-31} Since the available therapeutic agents are mainly immunomodulating in nature, such as Intravenous Immunoglobulin (IVIg) and corticosteroids, they may have affected study outcomes. Third, the published studies are hard to value since they are often limited to phenotypic findings, using a variety of Treg definitions, often identifying them as CD4\textsuperscript{+}CD25\textsuperscript{high} cells, and lack a follow up. Therefore, our goal was to investigate the role of Treg in pediatric ITP in a homogeneous population; children with newly diagnosed ITP, who did not receive treatment and were followed for one year. Besides Treg numbers and phenotype, we assessed Treg function at diagnosis and upon recovery, making use of a cross-over assay. With this approach we demonstrate that newly diagnosed pediatric ITP patients express normal Treg numbers and function. Moreover, in our cross-over suppression assay we found indications for increased suppressive capacity of Treg during active disease. Together, Treg defects do not seem to play a key role in the onset of pediatric ITP.

2. Methods

2.1. Patients

Thirteen children aged 3 months to 16 years with newly diagnosed ITP (<20\textsuperscript{th}10\textsuperscript{9}/L) were included in a larger multicenter randomized clinical trial (TIKI-
study NTR1563). Exclusion criteria are described in supplemental methods. For this study, only patient receiving no treatment were included. Peripheral blood was collected at fixed time points; ranging from diagnosis for one year. In addition 3 children with chronic ITP, defined as a platelet count below 100*10^9/L for over a year, were tested in the suppression assay. All chronic patients had a mild or moderate bleeding tendency (score 2-3 on the scale of Buchanan), and received no therapy for at least 3 months prior to inclusion. Blood obtained from random donors (aged 18-30) was used as a healthy control (HC). Parents and patients aged 12 years and older gave written informed consent. The study was approved by the Institutional Review Board of the University Medical Center Utrecht and performed in accordance with the Declaration of Helsinki

2.2. Cell isolation

Blood was collected in a vacutainer with 17 IU sodium heparin and a coagulation tube PBMC were isolated according to the manufacturers’ protocol using Ficoll Isopaque density gradient centrifugation and cryopreserved in FCS containing 10% DMSO. Plasma and serum were frozen within 24 hrs at -80°C.

2.3. Cell culture

Cells were cultured in RPMI-1640 medium, supplemented with 2mM L-glutamine, 100U/mL penicillin streptomycin, and human 10% AB serum, in 96-well plates. Cells were cultured at 37°C and 5% CO². Plates were used directly or pre-coated with 50ul 1.5μg/mL α-CD3 for stimulation.

2.4. Flow cytometry

2.5*10⁴ Cells were washed twice in PBS with 2%FCS, and subsequently stained for CD3, CD4, CD25, CD45RA, CD45RO, GITR, CTLA4 and FOXP3. Teff were identified as CD3⁺CD4⁺, Treg were defined as CD3⁺CD4⁺FOXP3⁺ in phenotyping assays and as CD4⁺CD25^high^CD127^low^ in functional assays. Samples were assessed on FACSCanto II, sorting was done on a FACS Aria and analyzed using FACS Diva or FlowJo. Antibodies used for flow cytometry are listed in supplemental methods.
2.5. Suppression assay

To investigate suppressive capacity of Treg, 1*10^4 PBMC were co-cultured with FACS-sorted Treg (CD4^+CD25^{high}CD127^{low}) in different ratios; Treg:PBMC 1:10, 1:2 and 1:1, in presence of α-CD3 as a stimulus. Cells were cultured in RPMI medium enriched with 10% AB medium, as previously described. At day 5 supernatant was carefully removed for cytokine analysis and 1μCi ^3H-thymidine added, which was measured after 16-18 hours.

2.6. Cytokine detection in supernatant

Cytokine levels of IFNγ, TNFα and IL17 were measured in supernatant retrieved from suppression assay cell cultures at day five. Cytokine concentrations were measured with standardized Immune Multiplex technology as previously described.

2.7. Statistical analysis

Statistical analyses were performed using SPSS. Data are shown as median with interquartile range (IQR) or as mean ± standard error of the mean (SEM). To compare 2 groups the Mann-Whitney U test was applied, and paired patient samples were tested with a paired t-test or the Wilcoxon matched pairs test. For statistical analysis of multiple groups the Kruskall-Wallis test was performed, together with a Bonferroni correction. P-values <.05 were considered statistically significant.

3. Results

3.1. Baseline characteristics

A total of 13 newly diagnosed pediatric ITP patients were included in this study. According to the International Working Group criteria, 9 patients were classified as early recovered ITP patients, indicating that they recovered fully within 3 months. Four patients were classified as persistent ITP patients, implying a late recovery between 3 and 12 months after initial diagnosis. Two patients, 1 early recovered and 1 persistent, received immunomodulating therapy (prednisone and IVIg) during study participation. From those patients, only samples obtained before therapy (at diagnosis and one week later) were
tested, to eliminate the possibility of drug interference in our experiment outcomes. One early recovered patient dropped out, thus from this patient also only early time point samples were tested (Table 1). Three chronic pediatric ITP patients were included in this study. All chronic patients expressed moderate to severe bleeding at the time of inclusion, although platelet counts varied highly (Table 2).

Table 1 Baseline characteristics of newly diagnosed pediatric ITP patients

<table>
<thead>
<tr>
<th>Newly Diagnosed Pediatric ITP patients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male)</td>
<td>69*</td>
</tr>
<tr>
<td>Age at inclusion (years)</td>
<td>3 [1-14]</td>
</tr>
<tr>
<td>Platelet count at diagnosis (*109/L)</td>
<td>5 [1-18]</td>
</tr>
<tr>
<td>Platelet count at recovery (*109/L)</td>
<td>250 [171-377]</td>
</tr>
<tr>
<td>Duration ITP (months)#</td>
<td>&lt;3 [1-12]</td>
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</tbody>
</table>

*data are represented as a percentage or as median [range] 
#measured at fixed timepoints indicating recovery before 1, 3, 6 or 12 months.

Table 2 Baseline characteristics of chronic pediatric ITP patients

<table>
<thead>
<tr>
<th>sex</th>
<th>age at inclusion (year)</th>
<th>Duration ITP (year)</th>
<th>Bleeding tendency (score according to Buchanan)</th>
<th>Treatment past 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td>M</td>
<td>17</td>
<td>9</td>
<td>2</td>
<td>none</td>
</tr>
<tr>
<td>F</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>none</td>
</tr>
</tbody>
</table>

3.2. Treg numbers during acute illness and after recovery in newly diagnosed pediatric ITP do not differ from healthy controls.

A decrease in Treg percentage, defined as the percentage of CD4+FOXP3+ cells of the total CD3·CD4+ T lymphocyte population in peripheral blood, is considered an important indicator of possible Treg failure in a broad range of autoimmune diseases, including ITP. To determine Treg levels during the natural course of ITP in children, samples obtained at diagnosis (referred to as acute illness) as well as, after recovery were analyzed for %FOXP3+ within CD3·CD4+ T lymphocytes, and compared to HC (Figure 1A). FACS analysis showed similar Treg numbers during acute illness and recovery, which equaled HC (Figure 1B). In addition, paired analysis of acute illness and recovery of the same patient did not reveal a difference in Treg percentage either (Figure 1C). Analysis of FOXP3 Mean Fluorescence Intensity (MFI) also showed no differences (Figure 1D).
Figure 1 Treg numbers during onset (acute illness) and recovery of pediatric ITP do not differ from HC, whereas CTLA4-expression is reduced at disease onset. PBMC were stained for CD3, CD4 and FOXP3 expression and analyzed by flow cytometry (A-D). Representative dotplot showing the FOXP3+ cells within CD3+CD4+ T cells (A). Accumulative data of the percentage of Treg during acute illness (AI), upon recovery (R), and in healthy controls (HC), presented in groups (B) and pair-wise within patients (C). FOXP3 MFI in AI, R and HC (D). Representative dot plot showing CTLA4 expression within CD3+CD4+ T cells (grey) and within CD3+CD4+FOXP3+ Treg (black) (E). Accumulative data of the percentage CTLA4+ Treg during AI, R and in HC presented in groups (F) and pair-wise within patients (**P=.0018) (G). Representative histogram showing the MFI of CTLA4 expression during acute illness and upon recovery in a patient (H).
3.3. Treg express reduced CTLA4 levels during acute illness that restore upon patient recovery

Next we tested a panel of Treg associated phenotypic markers. We investigated the expression of glucocorticoid induced tumor necrosis factor receptor family related gene (GITR) and Cytotoxic T lymphocyte Antigen 4 (CTLA4). GITR is associated with the activation status of T lymphocytes and Treg suppressive function.\textsuperscript{35} No differences in expression of GITR were observed in Treg and CD4\textsuperscript{+} T lymphocytes between acute illness, upon recovery and in comparison with HC (data not shown). CTLA4 is a protein receptor essential for the suppressive function of Treg and associated with a variety of autoimmune diseases.\textsuperscript{36-39} Interestingly, we observed that during acute illness patient Treg displayed a decrease in total CTLA4 expression, stained as percentage of CTLA4\textsuperscript{+} within CD3\textsuperscript{+}CD4\textsuperscript{+}FOXP3\textsuperscript{+} cells (\textbf{Figure 1E}). Upon recovery CTLA4 expression normalized and reached similar levels as the HC (\textbf{Figure 1F}). Paired analysis revealed a significant increase in CTLA4 (*P=.0018) on Treg upon recovery (\textbf{Figure 1G}). Also, the level CTLA4 expression (fluorescence intensity) was increased upon recovery (\textbf{Figure 1H}). In contrast, CTLA4 expression by non-Treg CD4\textsuperscript{+} lymphocytes was consistently low and similar to HC, both during acute illness and upon recovery (data not shown). Similar results were obtained in paired analysis.

3.4. Treg exhibit suppressive capacity, both during acute illness and upon recovery, similar to HC

To investigate actual Treg suppressive function we performed autologous suppression assays. For this purpose we used a customized Treg suppression assay taking total PBMC as effector cells as published previously.\textsuperscript{33,40} In this assay the \textit{in vivo} situation is reflected as closely as possible by not sorting out one single effector population, thereby also taking activation status of other cells (i.e. myeloid cells) into account. In addition, it makes it possible to perform functional assays with the small cell numbers obtained from juvenile patient samples. Paired patient samples were tested at acute illness and after recovery and compared to HC. CD4\textsuperscript{+}CD25\textsuperscript{high}CD127\textsuperscript{low} Treg, (\textbf{Supplementary Figure 1}), were sorted and co-cultured in different ratios with autologous PBMC for 5 days, followed by analysis of \textsuperscript{3}H-thymidine incorporation after anti-CD3 stimulation. To determine maximal proliferation capacity, 1*10\textsuperscript{4} PBMC were
cultured and proliferation was set to 100%. 3H-thymidine counts did not differ between acute illness and recovery (1851 ± 805, and 2458 ±1202, P=.65), and depended on the CD3 stimulus. Furthermore, we showed that Treg alone did not proliferate by themselves in presence of a CD3 stimulus. Furthermore, when the number of input PBMC was doubled, the proliferation also doubled, confirming that there was no expansion limitation due to volume restriction in our assay (Figure 2A). FACS analysis of sorted Treg revealed a high percentage of FOXP3+ cells in the sorted CD4+CD25highCD127low T cells (74.6% ± 2.7) that did not differ between groups. When PBMC were cultured in presence of an increasing ratio of Treg, proliferation levels declined accordingly from 100% to approximately 30% in the 1:1 ratio. Results obtained were similar in all groups; acute illness (36.3% ±4.5%), recovered (21.3% ±5.3) and HC (29.3% ± 14.6) (Figure 2B). In conclusion; Treg from newly diagnosed ITP patients exhibited equal suppressive capacity to that of healthy controls, both when sampled during the acute phase and after recovery.

3.5. Treg express normal suppressive capacity in chronic pediatric ITP patients

Next, we investigated whether Treg suppressive capacity is affected in chronic pediatric ITP patients in line with findings in adult chronic ITP patients.22 Autologous suppression assays were performed and compared to a HC. Proliferation of PBMC without addition of Treg was set at 100%. Again, no volume restriction was observed when the number of input PBMC was doubled and no proliferation occurred in absence of plate-bound α-CD3 nor when Treg were cultured alone (Supplementary Figure 3). When PBMC were cultured in presence of an increasing ratio of Treg, proliferation levels declined down to 30% when cultured in a 1:1 ratio, both for patients (25.8% ± 3.26) and HC (34.7%) (Figure 2C). Thus, Treg show normal suppressive function in chronic pediatric ITP patients.
Figure 2 Treg from newly diagnosed and chronic ITP patients normally suppress Teff proliferation. CD4+CD25+CD127low cells were sorted by flow cytometry and co-cultured with PBMC in the presence of plate-bound anti-CD3. At day 5, 3H-thymidine was added, followed by readout after 16-18hrs culture. Proliferation of 1*10⁴ PBMC without Treg was set at 100%, displayed by the dotted line. Results show percentage of proliferation relative to 1*10⁴ PBMC alone. Bars represent mean +/- SEM. Samples from diagnosis (Acute illness, AI), recovery (R) and healthy controls (HC) All conditions were performed in triplicates (A-B). Teff proliferation with 1*10⁴ PBMC without Treg was set at 100%. Culturing 2*10⁴ PBMC revealed no volume restriction. No proliferation was seen in absence of a T cell stimulus, and Treg were anergic when sorted without Teff (n=6) (A). To assess suppressive capacity of Treg, PBMC were co-cultured with an increasing number of Treg ranging from 1:10, 1:2 and 1:1 Treg:PBMC. (n=6) (B). Chronic pediatric ITP patients (PT) were tested in the autologous suppression assay in a similar fashion. PBMC were co-cultured with an increasing number of Treg ranging from 1:10, 1:2 and 1:1 Treg:PBMC. (n=3). Conditions were performed in triplicates (C).
3.6. At acute onset of ITP, highly activated Teff are suppressed only by Treg from the same timepoint.

Since Treg mediated suppression depends both on Treg functionality and the receptiveness of Teff to suppression, we performed cross-over suppression experiments, enabling us to determine if their relative activity changed during disease course in the patients. PBMC obtained at acute illness, were co-cultured with sorted CD4⁺CD25\textsuperscript{high}CD127\textsuperscript{low} Treg obtained at recovery, and vice versa. Due to limitations defined by the number of cells available, only the 1:2 Treg:PBMC ratio was tested. Strikingly, during acute disease, only Treg obtained from the same time point were able to suppress proliferation, but not Treg obtained at recovery. Teff obtained at recovery were suppressed well by both Treg populations (obtained at acute illness or recovery). In line with these observations, we found that Teff obtained after recovery to be suppressed even stronger by Treg from acute illness, albeit not significantly (Figure 3A).

3.7. Pro-inflammatory cytokine production is also suppressed by Treg from ITP patients with acute disease

While proliferation of Teff was effectively suppressed by Treg, it is possible that these Teff still produce cytokines driving pro-inflammatory responses, for example at the onset of ITP. Therefore, we tested TNF\(\alpha\) and IFN\(\gamma\) cytokine suppression at a Treg:PBMC ratio of 1:2 in cultures from both the regular autologous suppression assays and the cross-over settings, which was compared to the production of these cytokines by 1*10\(^4\) PBMC alone. The average cytokine production by 1*10\(^4\) PBMC alone of acute illness and recovery was set at 100%. Production of TNF\(\alpha\) declined in the presence of Treg in all groups, both autologous suppression assays and cross-over tests (Figure 3B). IFN\(\gamma\) production also declined or remained constant in the presence of Treg compared to 1*10\(^4\) PBMC alone. The cross-over test in which Teff obtained during acute illness were co-cultured with Treg after recovery displayed an increase in IFN\(\gamma\) production (Figure 3C). This might suggest that Teff are more active during acute disease which is compensated by increased activity of Treg in the same time phase, but this activity pattern reverts to less activity of both Treg and Teff after recovery.
Figure 3 Teff cells from timepoint acute illness fail to respond to suppression by Treg from timepoint recovery in newly diagnosed pediatric ITP patients. To distinguish between Teff and Treg function a cross-over suppression assay was performed in which PBMC sampled at diagnosis were co-cultured with Treg harvested after recovery and vice versa, in a 1:2 ratio (white- and black tiled column). To compare these data with the regular autologous suppression assays, as described in Figure 2, the proliferation percentage of acute illness (AI) as well as, recovery (R) in the 1:2 ratio are depicted on each side (light gray and black column). Bars represent mean ±SEM (n=6). PBMC from timepoint acute illness co-cultured with Treg obtained at recovery (PBMC AI-Treg R), could not be suppressed, (white tiled column) whereas PBMC from timepoint recovery were suppressed strongly by Treg from timepoint acute illness (*P=.03) (PBMC R-Treg AI, black tiled column) All conditions were performed in triplicates. (A). To investigate whether inflammatory cytokine production is also suppressed adequately in a suppressive environment the amount of TNFα (B) and IFNγ (C) present in the supernatant of the suppression assays was tested. Supernatants were removed at day five and cytokine concentration was measured by luminex. Results show the 1:2 ratio in autologous suppression assays (light gray and black columns) as well as, in the cross-over suppression assays (white tile and black tile columns) and are compared to the average cytokine production by 1*10⁴ PBMC alone of bot acute illness and recovery (dotted line). Bars show cytokine level in pg/ml as mean ±SEM (n=3).
CHAPTER 2

Since human Treg have been shown to be able to produce cytokines under inflammatory conditions, we assessed whether Treg produced pro-inflammatory cytokines TNFα, IFNγ, and IL17 following PMA/Ionomycin stimulation. However, this Treg obtained at acute illness or after recovery did not differ in this respect between each other or HC group, indicating that patient Treg do not express an increased pro-inflammatory phenotype. However, CD4+ T cells produced significantly more TNFα during active disease (*P=.0027) (Supplementary Figure 2). In summary, cytokine analyses of culture supernatants demonstrated that Treg not only suppress Teff proliferation adequately but also suppress inflammatory cytokine production.

4. Discussion

During the past decade, research in adult ITP has focused on Treg number and function, and their possible role in the progression of acute into chronic disease. Research in this area faces numerous challenges due to the heterogeneous disease pathology, possible fluctuations in disease activity, and the widespread use of immunomodulating therapies which are likely to affect study outcomes. In addition, there are signs that pediatric ITP has a different underlying disease mechanism than adult ITP. Here we describe normal Treg number and function in a homogeneous population of newly diagnosed pediatric ITP patients. These children did not receive treatment and were followed for one year enabling comparison of various T cell parameters with disease progression in time. Despite the relatively small sample sizes, this setting allowed us to investigate the role of Treg in newly diagnosed pediatric ITP in a way not possible for chronic adult patients. Phenotyping at disease onset and after recovery revealed normal number of Treg in both groups, within patients and in comparison to HC. This is in accordance with Yu et al. and others who studied adult chronic ITP patients by the current gold standard, identifying Tregs as CD4+CD25+FOXP3+ T cells. In contrast, two pediatric ITP studies described low Treg levels compared to HC. Unfortunately both studies lacked a follow-up, as well as functional tests, or omitted FOXP3 staining. In adults, others have also described low Treg numbers. Even though this may indicate a difference between adult and pediatric ITP, it might also be due to the focus on refractory patients, the lack of FOXP3 staining, or influenced by the frequent use of corticosteroids. The last possibility is of particular concern, as the
study by Li et al. revealed that Treg numbers gradually normalized in ITP after stopping intensive treatment with dexamethasone and prednisone.\textsuperscript{23} In addition, we also found a normal Treg suppressive function in newly diagnosed in out chronic pediatric ITP patients. This fits with the normal Treg phenotype we observed, except for the diminished expression of CTLA4 during the disease onset, which restored upon recovery. CTLA4 is constitutively expressed on Treg and essential for their suppressive function. Furthermore, it is up-regulated on Teff after activation, inducing a negative feedback loop.\textsuperscript{37} Together with our results from the functional assays, we have no reason to assume malfunction of Treg in pediatric ITP. Possibly, the lower CTLA4 levels observed in Treg in newly diagnosed ITP reflect their activated status in which CTLA4 might be shedded due to trogocytosis as is suggested by Wing et al.\textsuperscript{37} Importantly, next to normal Treg percentages and phenotype we observed good suppressive capacity of these cells in both stages of disease, acute illness and after recovery. It has to be noted that we used a customized Treg suppression assay taking total PBMC as effector cells as published previously.\textsuperscript{33,40} In this assay the \textit{in vivo} situation is reflected as closely as possible by not sorting out one effector population and also taking APC activation status into account. Furthermore it makes suppression assays with small cell numbers obtained from juvenile patient samples possible.

In the cross-over model, we found that during acute disease, Teff are more difficult to suppress but this is also compensated by enhanced Treg activity. After recovery the activity of both Teff and Tregs lowered. Since Treg mediated suppression at both stages of disease to a similar extent as HC, these findings point at a compensatory mechanism in which Treg adapt to the inflammatory environment during acute illness by becoming more suppressive in this particular environment. Adaptation of Treg to an inflammatory environment, the so-called functional specialization of Treg, has previously been described in mice.\textsuperscript{47} In concordance with our data, Walter et al. showed that human Treg produced more cytokines in an inflammatory environment, including TNFα, IFNγ, IL17 and IL10, while their Treg phenotype was preserved and even exhibited increased suppressive capacity.\textsuperscript{43} However, although we did not observe increased pro-inflammatory cytokine production by Treg during acute disease, their suppressive capacity was increased compared to Treg from after recovery by a yet unknown mechanism. Evidence is accumulating that increased resistance of Teff to suppression rather than malfunctioning Treg play a role in a variety of autoimmune diseases. This is supported by previous studies with
similar findings in other immune disorders, i.e. Juvenile Idiopathic Arthritis (JIA), Reumatoid Arthritis (RA), Systemic Lupus Erythematosous (SLE), Multiple Sclerosis (MS) and Diabetus Mellitus Type 1 (DMT1) patients and mice. However, to our knowledge, this is the first study to demonstrate increased Teff resistance in a within-patient follow-up model combining acute illness with recovery status in ITP.

Despite the small sample sizes we were able to perform functional assays in a well-defined cohort of treatment naïve patients. These data indicate that Treg are well-adapted to the inflammatory environment during the acute state of pediatric ITP, in which they retain their FOXP3 phenotype and may temporarily become more suppressive, compensating for the increased activity of Teff from the same environment.

5. Acknowledgments

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6. Authorship Contributions

AL, KH, YV, CS, MH, MB, BP, GV and FW designed the study. AL, YV and KH performed the experiments. AL, KH, GV, BP and FW carried out the data analysis. All authors contributed to data interpretation. AL, and FW wrote the manuscript. All authors contributed to revising the manuscript for intellectual content and style and all approved the final version.

7. Conflict of Interest Disclosures

The authors declare no competing financial interests.
Reference List


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Supplementary Methods

2. Methods

2.1. Patients

A total of 13 children aged 3 months to 16 years with newly diagnosed ITP were included. These patients participated in a larger multicenter randomized clinical trial (TIKI-study NTR1563). Newly diagnosed ITP was defined as isolated thrombocytopenia with a platelet count below 20*10^9/L at presentation. Patients with severe or life threatening bleeding at presentation, defined as score 4 or higher on the scale of Buchanan, were excluded. Other exclusion criteria consisted of the presence of clinical features that were not compatible with the diagnosis of newly diagnosed ITP and the use of immunomodulating therapy within 4 weeks prior to diagnosis.

During the follow-up of one year the selected patients did not receive treatment, unless clinically required based on bleeding tendency. Samples obtained after therapeutic intervention were excluded for analyses. Peripheral blood was collected at fixed timepoints; at diagnosis, 1 week, 1 month, 3 months and after 1 year. In addition three children aged 5 to 16 years with chronic ITP were tested in the suppression assay. Chronic ITP was defined as isolated thrombocytopenia with a platelet count below 100*10^9/L for over a year. All chronic patients had a mild or moderate bleeding tendency (score 2-3 on the scale of Buchanan). None of them received therapy for at least 3 months prior to inclusion. Blood obtained from healthy individuals (aged 18-30) was used as a healthy control (HC). All patients were subjected to history, physical examination and a full blood analysis at the time of inclusion.

Parents and patients aged 12 years and older gave written informed consent. The study was approved by the Institutional Review Board of the University Medical Center Utrecht and performed in accordance with the Declaration of Helsinki.

2.2. Cell isolation

Blood was collected in a 10 mL BD Vacutainer with 17 IU sodium heparin (Becton Dickinson and Company, Plymouth, United Kingdom) and in a 4mL BD coagulation tube (Becton Dickinson and Company, Plymouth, United
Kingdom). Plasma was obtained after 10 min centrifugation at 1000RPM, and frozen within 24 hrs at -80°C. PBMC were isolated according to the manufacturers’ protocol using Ficoll Isopaque density gradient centrifugation (GE Healthcare BioSciences AB) and cryopreserved in FCS (Invitrogen) containing 10% DMSO (Sigma-Aldrich). Serum was obtained after 10 min centrifugation at 3000RPM and frozen within 24 hrs at -80°C.

2.3. Cell culture

Cells were cultured in RPMI 1640 medium (Invitrogen), supplemented with 2mM L-glutamine (Invitrogen), 100U/mL penicillin streptomycin (Invitrogen), and 10% human AB serum, in round bottom 96-well plates (Nunc). Cells were cultured at 37°C and 5% CO2. Plates were used directly or pre-coated with 50μl 1.5μg/mL α-CD3 (OKT-3, eBioscience) for stimulation.

2.4. Flow cytometry

2.5*10^4 Cells were washed twice in FACS buffer, consisting of PBS supplemented with 2% FCS (Invitrogen) and 0.1% sodium azide (Sigma-Aldrich), and subsequently stained for CD3, CD4, CD25, CD45RA, CD45RO, GITR, CTLA4 and FOXP3. Samples were assessed on FACSCanto II (BD Biosciences) and analyzed using FACS Diva Version 6.13 (BD Biosciences) or FlowJo Version 7.6.5 (Tree Star inc.). All antibodies used for flow cytometry are listed in supplemental methods.

2.5. Cytokine stimulation

To assess cytokine production 2*10^5 cells were cultured for 5 hours in presence of 20ng/mL PMA (MP Biomedicals) and 1 μg/mL Ionomycin (Calbiochem). As a negative control cells were cultured without stimulus. After 30 minutes 1:1500 Golgistop (BD Biosciences) was added to all culture conditions. Cells were washed twice in FACS buffer and stained for surface markers and intracellular cytokines, according to the manufacturers’ protocol.

2.6. Suppression assay

To investigate suppressive capacity of Treg, 1*10^4 PBMC were co-cultured with sorted Treg (CD4^+CD25^highCD127^low) in different ratios; Treg:PBMC 1:10, 1:2 and 1:1. Cells were cultured in 100μl RPMI medium enriched with 10% AB medium,
as previously described. At day 5 25 μl supernatant was carefully removed for cytokine analysis and 1 μCi ³H-thymidine was added. ³H-thymidine incorporation was measured after 16-18 hours. As controls 1*10⁴ and 2*10⁴ PBMC were cultured without Treg, 1*10⁴ Treg were cultured without PBMC and 1*10⁴ PBMC were cultured without α-CD3 stimulus. All conditions were performed in triplicates.

2.7. Cytokine detection in supernatant

Cytokine levels of IFNγ, TNFα and IL17 were measured in supernatant retrieved from suppression assay cell cultures at day five. Cytokine concentrations were measured with standardized Immune Multiplex technology as previously described.

2.8 Antibodies used for flow cytometry.

For phenotyping PBMC were stained with the following antibodies: anti-CD3-PE/Cy7 (UCHT1, Biolegend), anti-CD4-APC/CY7 (RPA-T4, Biolegend), anti-FOXP3-eFluor 450 (eBioscience), anti-CTLA4-APC (BD biosciences), anti-GITR-FITC (110416, R&D Systems), anti-CD45RA-FITC (JS-83, eBioscience), anti-CD45RO-PE (UCHL1, Biolegend). To stain cells for sorting of Treg the following monoclonal antibodies were used: anti-CD4-FITC (RPA-T4, eBiosciences), anti-CD25-APC (M-A251, BD biosciences), and anti-CD127-PB (A019D5, BD biosciences). For PMA-Ionomycin stimulation PBMC were stained with the following antibodies: anti-CD3-PE/Cy7 (UCHT1, Biolegend), anti-CD4-APC (RPA-T4, eBioscience), anti-FOXP3-eFluor 450 (PCH101, eBioscience), anti-IFNγ-PE (25723.11, BD biosciences), anti-IL17-FITC (eBio64DEC17, eBioscience) and anti-TNFα-FITC (MAb11, Biolegend).

2.9. Statistical analysis

Statistical analyses were performed using SPSS (IBM SPSS Statistics 20). Data are shown as median with interquartile range (IQR) or as mean ± standard error of the mean (SEM). To compare 2 groups the Mann-Whitney U test was applied, and paired patient samples were tested with a paired t-test or the Wilcoxon matched pairs test. For statistical analysis of multiple groups the Kruskall-Wallis test was performed, together with a Bonferroni correction. P-values <.05 were considered statistically significant.
**Supplementary Figures**

**Supplementary Figure 1** Sorting strategy applied to isolate CD4\(^+\)CD25\(^-\)CD127\(^{low}\) Treg. PBMC were stained for CD4, CD25 and CD127. The left dotplot shows the live gate, the middle one the CD4\(^+\) population and the right the final sort gate for CD4\(^+\)CD25\(^-\)CD127\(^{low}\) Treg.
Supplementary Figure 2 Cytokine production following PMA and Ionomycin stimulation in paired patient samples and HC. $2 \times 10^5$ PBMC were cultured for 5 hours in presence of 20 ng/mL PMA and 1 μg/mL Ionomycin. After stimulation Cells were stained for surface markers and pro-inflammatory intracellular cytokines; IFNγ, TNFα and IL17. The left panel shows the percentage of cytokine production within Treg, the right panel in CD4$^+$ T lymphocytes. Results are depicted as dot-plots representing paired patients samples of acute illness and recovery and single HC samples. (A). TNFα production by CD4$^+$ T lymphocytes is reduced in recovered patients compared to acute illness (B). No differences are observed in IL17 production within patients or compared to HC (C).
Supplementary Figure 3 Control settings of the suppression assay of chronic pediatric ITP patients. CD4+CD25+CD127low cells were sorted by flow cytometry and co-cultured with PBMC. At day 5, 3H-thymidine was added, followed by readout after 16-18 hrs culture. 1*10^4 PBMC were cultured in absence of Treg to assess normal proliferation and set to 100% (dotted line). Bars represent mean +/- SEM of n=3. No significant differences were observed in control settings between patients and HC. Culturing 2*10^4 PBMC revealed no volume restriction. No proliferation was seen in absence of a T cell stimulus, and Treg were anerg and sorted without Teff.