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Review article

CD4$^+$ T cell phenotypes in the pathogenesis of immune thrombocytopenia

Milos Kostic[a,*], Nikola Zivkovic[b], Ana Cvetanovic[c], Goran Marjanović[a,d]

[a] University of Nis, Medical Faculty of Nis, Department of Immunology, Blvd. dr Zorana Djindjica 81, 18000 Nis, Serbia
[b] University of Nis, Medical Faculty of Nis, Department of Pathology, Blvd. dr Zorana Djindjica 81, 18000 Nis, Serbia
[c] University of Nis, Medical Faculty of Nis, Department of Oncology, Blvd. dr Zorana Djindjica 81, 18000 Nis, Serbia
[d] Clinical Centre Nis, Department of Hematology and Clinical Immunology, Vojislava Ilića 1, 18000 Nis, Serbia

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ABSTRACT

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by low platelet counts due to enhanced platelet clearance and compromised production. Traditionally, ITP was regarded a B cell mediated disorder as anti-platelet antibodies are detected in most patients. The very nature of self-antigens, evident processes of isotype switching and the affinity maturation of anti-platelet antibodies indicate that B cells in order to mount anti-platelet immune response require assistance of auto-reactive CD4$^+$ T cells. For a long time, ITP pathogenesis has been exclusively reviewed through the prism of the disturbed balance between Th1 and Th2 subsets of CD4$^+$ T cells, however, more recently new subsets of these cells have been described including Th17, Th9, Th22, T follicular helper and regulatory T cells. In this paper, we review the current understanding of the role and immunological mechanisms by which CD4$^+$ T cells contribute to the pathogenesis of ITP.

1. Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a low platelet count ($< 100 \times 10^9$/L) due to enhanced platelet clearance and compromised production, typically manifesting with bleeding [1]. Traditionally, the acronym ITP stood for "idiopathic thrombocytopenic purpura", however in 2009, The International Working Group (IWG) introduced instead the term "immune thrombocytopenia" to emphasize two facts: immune mechanisms that mediate the pathogenesis of ITP and a significant number of patients who do not clinically present with purpura [2]. According to the IWG, ITP is classified as primary disease, when it presents as an isolated clinical syndrome (the diagnosis of exclusion) or secondary, when it is associated with other disorders including autoimmune and lymphoproliferative diseases, infection or medication [2–4]. Based on the disease natural history, three distinctive phases of ITP are introduced: i) newly diagnosed ITP (up to 3 months from diagnosis); ii) persistent ITP (between 3 and 12 months); iii) chronic ITP (more than 1 year from diagnosis). The distinction between the three is of relevance for the choice of treatment [2,5]. ITP affects both children and adults, however, children are more prone to developing newly diagnosed and persistent forms of ITP which have a tendency to resolve spontaneously; on the other hand, the chronic form is more prevalent in adults and is most often not self-limiting [6,7].

Historically, ITP was regarded a B cell mediated disorder considering that multiple antibodies against a range of platelet antigens are identified in the majority of patients. Most of these autoantibodies are of the IgG class, directed against platelet membrane glycoprotein complexes – GPIIb-IIIa and GPIb-IX and undergoing affinity maturation through somatic hypermutation of the variable regions. [8–10]. The very nature of self-antigens, evident processes of isotype switching and the affinity maturation of anti-platelet antibodies suggest that B cells in order to mount deleterious immune response in ITP require assistance of autoreactive, platelet specific CD4$^+$ T cells, implicating the importance of these cells in disease pathogenesis. In concordance to this, after *in vitro* GPIIIa stimulation, a significant CD4$^+$ T cell proliferative response and IL-2 production was observed in peripheral blood mononuclear cell (PBMC) cultures obtained from ITP patients in contrast to healthy donors [11,12]. Furthermore, accumulation of oligoclonal T cells was also documented in the peripheral blood of ITP patients [13]. In the experimental model of ITP, depletion of CD4$^+$ T cells completely abolished mice suitability to the disease [14]. Depending on the microenvironment stimuli, activated CD4$^+$ T cells may differentiate to diverse polarization states, defined as Th1, Th2, Th17, Th22, Th9 and regulatory T cells, all of which have distinguished master transcription factors, unique cytokine profiles and functions. In the recent years, CD4$^+$ T cells responsible for mounting antibody dependant anti-platelet immune response have been intensively studied however, similar to

* Corresponding author at: University of Nis, Medical Faculty of Nis, Department of Immunology, Blvd. dr Zorana Djindjica 81, 18000 Nis, Serbia.
E-mail address: milos.kostic@medfak.ni.ac.rs (M. Kostic).

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other autoimmune disorders, exact phenotype profile of these cells in ITP still remain elusive. Besides $CD4^+$ T cells, recent studies also suggested involvement of $CD8^+$ T cell in the depletion of platelets but also disruption of normal thrombocytopoiesis in the bone marrow, especially in ITP patients without detectable anti-platelet antibodies [15–17].

In this review we have summarized the current data supporting T cell relevance in ITP pathophysiology from the aspects of both functional and phenotypic heterogeneity of this cell lineage.

2. Th1 and Th2 immune response in ITP

Th1/Th2 cell dichotomy has been considered the main feature that defines the cellular immune response, and the pathogenesis of many immune mediated disorders was exclusively viewed from the aspect of the altered balance between those two T cell subsets. Th1 cells are induced by IL-12 and IFN-γ, characterized by the expression of *T*-box expressed in T cells (*T*-bet) as a master transcription factor and predominant production of IFN-γ and IL-2. Th2 cells are induced by IL-4, express GATA binding protein 3 (GATA-3) as a master regulator and produce a broader spectrum of cytokines including IL-4, IL-5, IL-9, IL-10 and IL-13 [18].

Pioneering studies of Th1 cell role in the pathogenesis of ITP were performed by determining the levels of Th1 signature cytokines in patients' serum or plasma; however this approach prove to be unreliable, considering many inconsistencies in reported results. Specifically, elevated serum levels of IFN-γ, IL-10 and IL-2 were found in both pediatric and adult ITP patients that might suggest Th0/Th1 polarization; but that was not consistent across all studies [19–25]. The other approach was to investigate mRNA levels of Th1 cytokines and master transcription factors in patients' PBMCs; and similar to previous studies, both higher [25–27] and unaltered [28,29] expression levels of IFN-γ, IL-2, as well as *T*-bet were reported. Direct Th1 cell detection by flow cytometric analysis also provided opposing results [28–31]. On the other hand, in the spleen, which is the primary site for the activation of platelet-reactive T and B cells [32], higher frequency of Th1 cells was detected, especially in rituximab (RTX) non-responder patients [33].

Similar to Th1 cells, conflicting data on aberrant Th2 cell mediated immune response in ITP are present. Several studies have shown lower, unaltered or even higher Th2 associated cytokine serum levels in both pediatric and adult ITP patients [19–23,28]. In contrast to serum level, gene expression analyses generally show lower expression levels of Th2 cytokines and GATA-3 in PBMCs and splenocytes isolated from ITP patients [26,28,34], accordingly, lower percentage of Th2 cells is found in peripheral blood of ITP patients compared to healthy controls [28,29,35].

In addition to the differences in study designs and methodology, many discrepancies in the reported results could be partially explained by the fact that primary ITP is a diagnosis of exclusion. Therefore, especially in the early, yet unrecognized stages, various other disorders could be classified as primary ITP. Another feasible explanation may be substantial individual variations in the immune system constitutional characteristics influenced by genetic features, environment and age [36,37]. In order to overcome these individual variations, in many studies, Th1 and Th2 immune profiles were not considered separately yet in the contexts of their mutual relationship. Both *T*-bet/GATA-3 ratio in PBMCs and splenocytes [28,38] and Th1/Th2 cell ratio in the peripheral blood of patients with ITP was shown to be increased [28,30,35] and inversely correlated with platelet counts [26]. The conventional ITP therapies such as high-dose dexamethasone (HD-DXM) or RTX [23,29,39] as well as experimental treatments, including IL-11 and GM-CSF [38,40], restore the Th1/Th2 balance to the control levels, resulting in clinical improvement. Upon relapse, the cytokine profile tends to revert to the pretreatment levels [23]. Taken together these data indicate that ITP is a Th1 dominant disease, a view currently widely accepted.

2.1. Th1/Th2 dysregulation and platelet destruction

Th1/Th2 immune imbalance observed in patients with ITP is directly involved in the pathogenesis and progression of the disease, including platelet destruction mediated by both autoantibodies and $CD8^+$ T cells. Splenic monocytes and macrophages play a major role in the phagocytosis of autoantibody opsonized platelets via the Fcγ receptor (FcγR) engagement, thus stimulating both platelet autoantigen presentation and clearance [32,41]. Fcγ receptors are heterogeneous family of membrane glycoproteins widely distributed on different immune cells, capable of binding the Fc fragment of IgG antibody. Both monocytes and macrophages express activating (FcγRI, FcγRIIA/C, FcγRIIIA) but also inhibitory (FcγRIIB) FcγRs the simultaneous stimulation of which establishes a threshold needed for cell activation that consequentially regulates their functional response [42]. Although FcγR polymorphisms that increase binding affinity to IgG were documented in ITP patients [43–45], the Th1/Th2 cytokine axis could induce quantitative and qualitative alterations in phagocyte FcγR expression pattern thus influencing platelet clearance. In monocyte/macrophage cell cultures, IFN-γ was able to up-regulate expression of activating FcγRs and down-regulate expression of inhibitory FcγRIIB, whereas IL-4 produced completely opposite effects [46]. Accordingly, the higher FcγRI expression and increased FcγRIIA/IIB ratio was detected on monocytes isolated from ITP patients at both mRNA and protein level which was normalized following HD-DXM or thrombopoietin receptor agonist therapy [47,48]. While not consistently, in the spleen, reduced FcRIIB expression was reported on macrophages [44,49]; furthermore, their phagocytic capacity was significantly increased and there was a positive correlation with the Th1/Th2 ratio [50]. In addition to effects on FcγR expression, IFN-γ primed macrophages also enhance the expression of costimulatory molecules CD80 and CD86. They have a higher capacity to produce proinflammatory cytokines including IL-12, which in turn favors Th1 polarization and additional IFN-γ production, thus forming a positive feedback loop [51,52]. In ITP patients, higher expression levels of CD80 and CD86 were indeed found on splenic macrophages [44] together with higher plasma level of IL-12 [53]. Interestingly, a specific subset of IL-12 producing $CD16^+$ monocytes was shown to be expanded in patients with active ITP. *In vitro* these cells were able to promote T cell differentiation into Th1 cells [54]. Taken together, these results suggest that strong Th1 biased environment may promote alterations in FcγR expression on monocyte-macrophage cell lineage toward a proinflammatory profile, thus establishing a lower threshold for cellular activation and increase in their phagocytic capabilities. Consequently, FcγR mediated internalization of antibody opsonized platelets by macrophages induces enhanced processing and subsequent presentation of cryptic platelet peptides (e.g. GPIIb/IIIa) to $CD4^+$ T cells which, upon antigen recognition and costimulation, facilitate B cell activation and anti-platelet antibody production. In support of this theory, macrophage cell cultures pulsed with opsonized platelets were shown to promote GPIIb/IIIa specific antibody production when cocultured with autologous GPIIb/IIIa reactive T cells and B cells [41].

In addition to antibody mediated platelet destruction, Th1/Th2 dysregulation could also affect platelet clearance by modifying $CD8^+$ T cell activity. Significant role of $CD8^+$ T cells in ITP pathogenesis has been indicated in many different experimental studies performed on murine models of ITP [55,56] but also in the clinical studies [15,16]. Direct lysis is suggested to be the main pathogenic mechanism by which $CD8^+$ T cells promote platelet depletion, especially in patients without detectable anti-platelet antibodies [16,57]. However, these cells are also shown to mediate platelet apoptosis [15] and compromise normal thrombocytopoiesis by inhibiting megakaryocyte apoptosis and platelet production [17]. Furthermore, both megakaryocytes and platelets could elicit platelet specific $CD8^+$ T cell response considering that in ITP these cells express costimulatory molecules (CD80 and CD86) and present self-antigens in conjunction with MHC class I molecules. This

could be one of the potential mechanisms involved in ITP progression [19,58,59].

Analogous to Th cells, $CD8^+$ T cells are also classified in various subsets, including Tc1, Tc2, Tc17, Tc22, Tc9 and $CD8^+$ T regulatory cells, based on their cytokine profile and specific functions. It is believed that $CD8^+$ T cell differentiation is directly influenced by the polarization state of $CD4^+$ T cells that facilitate their activation and promote their functions [60]. In the peripheral blood and the spleen of ITP patients, higher Tc1/Tc2 ratio as well as relative number of Tc1 cells was documented and, interestingly, there was a significantly positive correlation with the frequency of Th1 cells [29,31,39]. Currently, we do not have a thorough understanding of how precisely Tc1/Tc2 deviation in $CD8^+$ T cell populations promotes ITP development. However, two potential scenarios are postulated. Firstly, by producing IFN-γ, these cells could further promote Th1 biased microenvironment [61], stimulating platelet sequestration and destruction by macrophages and antibody production by B cells. Secondly, both autocrine and paracrine IFN-γ signaling, alone or in synergy with other Th1 polarizing cytokines such as IL-12, can substantially enhance $CD8^+$ T cell cytolytic potential [62,63] and thus promote platelet lysis. Accordingly, Tc1 cells are shown to have higher lytic capacity then Tc2 cells, and their signature cytokines (IFN-γ and IL-4) antagonistically modulate $CD8^+$ T cell cytolytic activity *in vivo* [64]. Compared to healthy controls, in spleens of RTX non-responder ITP patients, parallel to increased number of Tc1 cells, $CD8^+$ T cells indeed showed higher cytotoxic activity based on granzyme B expression [33].

2.2. The origin of Th1/Th2 dysregulation

The very origin of Th1/Th2 dysregulation in ITP still remains unclear. Constitutional alterations could be one of potential risk factors, considering that some IFN-γ and IL-4 gene polymorphisms were associated with ITP susceptibility. IFN-γ +874 TT genotype, linked to higher expression of IFN-γ, was found more frequently in chronic ITP patients in contrast to low-expression IFN-γ +874 AA genotype which was significantly lower [65]. In addition, higher-expression-IFN-γ genotype, as well as higher-function-IFN-γ-receptor genotype (IFN-γR -611GA), correspond to higher Th1/Th2 ratio and more severe thrombocytopenia [66]. Similarly, IL-4 (IL-4 VNTR intron 3) and IL-4 receptor (IL-4Rα Q576R) polymorphisms were detected in ITP patients and associated with a higher number of required treatment regimens [65,67], however, that was not seen in all studies [65,68], probably due to small sample sizes and ethnic homogeneity of the investigated subjects.

In addition to genetic factors directly involved in defining the characteristics of Th1 and Th2 immune response, Th1/Th2 dysregulation is also associated with altered innate immune functioning of antigen presenting cells (APCs) that are crucial for T cell activation and subsequent polarization. Professional APCs, such as dendritic cells (DCs), macrophages and B cells, express numerous pattern-recognition receptors (PPRs), including toll-like receptors (TLRs), the stimulation of which leads to the secretion of different cytokines directly involved in the polarization process of activated $CD4^+$ T cells. Both TLR7 and TLR4 expression was found increased in ITP patients [69,70]. In macrophages obtained from ITP patients, TLR7 stimulation induced significantly higher IL-12 production compared to the control group, and in a murine model of ITP promoted Th1 polarization which corresponded to decreased platelet count [71]. The increased expression of TLR4 was found on monocytes, but also on $CD4^+$ T cells where TLR4 expression level corresponded to the higher IFN-γ/IL-4 ratio [70,72,73]. Considering that TLRs recognize pathogen associated molecular patterns (PAMPs), the alterations in TLR expression may suggest that ITP develops as a reflection of aberrant immune response to infectious agents. Accordingly, association between infection and ITP onset, as well as disease exacerbation, is well established especially in children but also adults [4,74,75]. Platelets themselves also express TLR4 which upon stimulation with lipopolysaccharide (LPS) in the presence of anti-platelet antibodies leads to increased platelet phagocytosis. This may explain why Gram-negative infections result in worsening of thrombocytopenia in some ITP patients [76].

While, splenic macrophages are recognized as key triggers of anti-platelet immune response in ITP [41], altered DC function can also affect Th1/Th2 balance. Compared with healthy donors, monocyte derived DCs from ITP patients were shown to have higher expression level of costimulatory molecules and efficiently promote Th1 polarization via enhanced IL-12 production [77]. In the physiological context, monocyte derived DCs *per se* are prone to induce Th1 cell polarization [78]. However, it appears that this feature is more pronounced in ITP patients. In addition to phenotype alterations, changes in the distribution pattern of specific DC subsets could contribute to the disease development and progression. In a murine model of ITP, a significant deficiency of tolerogenic and an increase of activating DCs were reported in animals' spleens during active phase of the disease. It has also been shown that intravenous immunoglobulin treatment could raise platelet counts by stimulating thymic release of tolerogenic DC and their re-distribution in the spleen [79].

Plasmacytoid DCs (pDCs) are another unique subset of DCs producing IFN-α and β but also other proinflammatory cytokines, which are of particular importance for antiviral protection [80]. The involvement of pDCs in ITP pathogenesis is still controversial. Increased number of pDCs and higher plasma level of IFN-α were reported in patients with ITP. Moreover, patients' plasma was able to increase T cell stimulatory capacity of monocytes and myeloid DCs. This effect was suppressed by blocking IFN-α receptor, suggesting the important role of this IFN in the pathogenesis of ITP [81]. In addition to stimulatory effects on T cell activation, IFN-α is well known to be potent Th1 promoting factor [82,83]. Interestingly, IFN-α/β production in pDCs is multifold increased following TLR7 stimulation [84], and its expression, as previously mention, was found to be increased in ITP [69]. However, in the study of *Saito et al.* reduced frequency of pDCs was found in the blood of both primary and *Helicobacter pylori* associated ITP patients. Even more, the numbers of circulating pDCs highly correlated with the platelet counts [85]. In some of the studies no statistically significant differences in the numbers of circulating pDCs were observed between ITP patients and healthy controls [86]. Thereby, further research is warranted to establish the precise role of pDCs in ITP pathogenesis.

3. Th17 immune response in ITP

Significant advances in the research of $CD4^+$ T cell biology made clear that established Th1/Th2 paradigm cannot completely explain the vast heterogeneity of this cell population, which led to the definition of new, unique Th subsets. Accordingly, more than a decade ago, Th17 cells were described as a T cell subset that expresses retinoic acid-related orphan receptor γT (RORγT) as a master regulator and predominantly produces IL-17, IL-21 and IL-22. Interleukin-6, IL-1, IL-21, IL-23, and TGF-β were identified as the major signaling cytokines involved in Th17 cell commitment [18].

First evidence to suggest the pathogenetic role of Th17 cells in ITP was based on elevated plasma levels of IL-17 and other Th17 associated cytokines IL-1, IL-6 and IL-23 [87–89]. Moreover, IL-23 was found to positively correlate with IL-17 plasma levels and the number of Th17 cells, while it was negatively correlated with the platelet counts, suggesting important role of this cytokine in promoting Th17 immune response and disease pathogenesis [89,90]. At the mRNA level, the expression of IL-17 and IL-23, their relevant receptors as well as RORγT was found increased in PBMCs isolated from ITP patients [89,91]. In concordance, during the active phase of the disease, patients had higher frequency of Th17 cells, which were alongside neutrophils identified as the main IL-17 producers [31,87–89,91]. The percentage of Th17 cells in the blood was also proposed to be a positive indicator of sensitivity to corticosteroid treatment, considering that corticosteroid non-responding newly diagnosed ITP patients before the treatment had

significantly lower numbers of Th17 cells than responders [92]. In addition to Th17 cells, Tc17 subset of $CD8^+$ T cells was found to be expanded in ITP patients, which could be another significant cellular source of IL-17 [93]. In several studies, however, the frequency of circulating Th17 cells did not differ significantly between patients and healthy controls [35,94]. Analysis of IL-17 expression in the bone marrow of ITP patients revealed that macrophages and/or monocytes, but not lymphocytes, had increased IL-17 expression. Interestingly, better clinical responses to thrombopoietin receptor agonist therapy were achieved in the IL-17 low expression patients than in those with a high expression profile of IL-17, indicating that IL-17 could be also involved in disturbed thrombocytopoesis [95].

3.1. Th17 cells and platelets

It appears that Th17 cells and platelets have multifaceted and proactive mutual relationship. Upon activation, multiple mediators with pronounced immunomodulatory properties are rapidly released from platelet storage granules. Specifically, platelet factor 4 (PF4), also known as CXCL4, stored in α-granules, has been found to directly stimulate Th17 differentiation program in naïve T cells [96], and, additionally, it is a well-known inhibitor of megakaryopoiesis and platelet production [97]. In ITP, platelets are constantly activated [98,99], thus, higher level of CXCL4 could promote the continuation and amplification of Th17 mediated autoimmune process. Although, CXCL4 is the most abundant mediator of platelet granules, other products of activated platelets such as platelet-derived serotonin, CXCL1, CCL5, and platelet-activating factor (PAF) were also shown to promote Th17 cell differentiation [100]. On the other hand, platelets express functional IL-17 receptor and its stimulation facilitates their activation [101], indicating that Th17 cells and platelets might form a self-perpetuating cycle. Interestingly, human megakaryocyte progenitors, before complete maturation, may act as professional APCs and augment Th17 immune response by secreting Th17 inducing cytokines including IL-1, IL-6, IL-18, TGF-β, and IL-23 [102]. Direct IL-17 effect on megakaryopoiesis is still unknown. However, IL-21 as an important cytokine of the Th17 spectrum, promoted megakaryocyte differentiation, but did not have any effects on mature megakaryocytes and platelet production process itself. Curiously, in contrast to enhanced megakaryopoiesis, IL-21 decreased total platelet counts due to higher platelet consumption in the spleen and in the liver [103].

Genetic background for Th17 cell abnormalities in ITP was mainly associated with IL-17F gene polymorphisms. IL-17F is the most recently discovered cytokine of the IL-17 family, mainly produced by Th17 cells, but also activated monocytes, basophils and mast cells [104]. The frequency of G allele of IL-17F rs763780 polymorphism was found to be significantly decreased in ITP patients, suggesting its protective features [105]. On the other hand, IL-17F rs763780 AA genotype was associated with a more severe thrombocytopenia at the time of the diagnosis [106]. The exact mechanisms by which IL-17F gene polymorphisms, as well as IL-17F itself, are implicated in ITP development are still unknown; however in the animal model of ITP, elevated IL-17F expression was observed in splenocytes [107]. These polymorphism association studies should be viewed with caution, considering that they were mainly performed on a single ethnic population, and the results were not reconfirmed in other ethnic groups [108].

4. Regulatory T cells in ITP

Regulatory T cells (Tregs) are a specific subset of $CD4^+$ T cells actively engaged in establishing immune homeostasis by maintaining self-tolerance and contracting the immune response. These physiological properties of Tregs are achieved by different means, including direct inhibition of potentially autoreactive T cells by producing immunosuppressive mediators (TGF-β and IL-10) or by direct cell-to-cell contact via inhibitory molecules such as the programmed death ligand 1 (PD-L1) and the cytotoxic T lymphocyte associated antigen 4 (CTLA-4). Immunosuppressive effects are also achieved indirectly by promoting tolerogenic APC phenotypes. Forkhead box P3 (FoxP3) is the major lineage-specific transcription factor which is induced by TGF-β signaling and amplified by IL-2, after antigen recognition [18].

4.1. Numerical and functional abnormalities of Tregs

Both numerical and functional abnormalities of Tregs have been implicated in the pathogenesis of various autoimmune disorders [109]. In concordance, decreased Treg frequency was reported in the peripheral blood of ITP patients during the active phase of the disease when compared with the patients in remission and control subjects, in both adults and children [35,110–113]. Tregs were also found to be reduced in number, in the bone marrow and the spleen of ITP patients [114,115]. In contrast, some studies show no significant difference in Treg frequency, especially when Tregs were identified as $CD4^+$ $CD25^+$ $FoxP3^+$ cells [115–118]. While those markers are most commonly used for Treg detection, other $CD4^+$ $FoxP3^-$ T cell subsets with regulatory functions have been firmly established including IL-10 producing T regulatory-1 cells (Tr1) and TGF-β producing T helper 3 cells (Th3) [119]. Thereby, in the evaluation of ITP immune profile, functional assessment of $CD4^+$ T cell immunosuppressive effects could be more interpretive than their phenotypic characteristics. In this regard, $CD4^+$ $CD25^+$ T cells isolated from ITP patients with active disease had significantly diminished suppressive effect on cell proliferation when co-cultured with auto or allologous $CD4^+$ $CD25^-$ lymphocytes compared with the control subjects [116,117,120]. Functional defects of Tregs were associated with decreased production of immunosuppressive cytokines TGF-β and IL-10, which serum/plasma levels were found to be lower in ITP patients and positively correlate with the platelet counts [117,121–123]. Treg mediated suppression is also achieved by direct cell to cell contact via inhibitory molecules PD-L1 and CTLA-4. PD-L1 is a principal ligand of the coinhibitory receptor - programmed death 1 (PD-1), preferentially expressed on effector T cells where stimulation initiates intracellular signaling pathways that inhibit cell activation and function. Currently, PD-1/PD-L1 signaling is a well-established physiological mechanism in down-regulating self-reactive T cells, thus, it is considered to be an important factor in promoting both central and peripheral T cell autotolerance. Interestingly, Tregs also express PD-1, however PD-1 signaling pathway in Tregs has significant role in their development, survival and sustaining of their suppressive capacity [124]. In PBMCs isolated from ITP patients, reduced expression of both PD-1 and PD-1L was detected. Unfortunately, further phenotype analyses were not performed in order to specifically identify the cells expressing those molecules [125]. CTLA-4 is another inhibitory molecule expressed by Tregs with high binding affinity for costimulatory molecules - CD80 and CD86, required for complete T cell activation. By employing CTLA-4, Tregs deprive other T cells of costimulatory molecules, thus inhibiting their activation. PBMCs of the patients with acute ITP showed decreased expression of CTLA-4 at both protein and mRNA level and, interestingly, HD-DXM treatment enhanced CTLA-4 expression in responding but not in non-responding patients [126,127]. In addition to PD-1/PD-1L and CTLA-4, other recently identified molecules expressed by Tregs are also involved in contracting the immune response and maintaining self-tolerance. CD39 is a membrane protein with enzymatic activity that degrades extracellular adenosine triphosphate (ATP) to adenosine monophosphate (AMP), which is then dephosphorylated into adenosine by CD73 molecule (ecto-5′-nucleotidase). Adenosine signaling in lymphocytes via adenosine A_{2A} receptor can elicit diverse functions in the immune system, including suppression of proinflammatory cytokine production by effector T cells but also activation of Tregs [128]. The expression of CD39 on $CD4^+$ $CD25^+$ Tregs as well as its enzymatic activity was found to be decreased in ITP patients and it could be restored by HD-DXM therapy. Those abnormalities could cause a reduced adenosine production leading to

compromised immunosuppressive function of Tregs, enhanced production of proinflammatory cytokines thus contributing to the perpetuation of autoimmune process. Additionally, the expression of adenosine A_{2A} receptor in $CD4^+$ cells was decreased in ITP patients, suggesting that those cells are less sensitive to adenosine stimulation [129]. Considering that adenosine A_{2A} receptor activation suppresses the production of IL-2, IL-17, TNF-α and IFN-γ, but up-regulates the expression of PD-1, CTLA-4 and FoxP3 [130], adenosine A_{2A} receptor agonists could be a promising strategy for ITP treatment.

Additional evidence supporting the important role of Tregs in ITP development was obtained by analyzing the effects of different therapeutic strategies used in ITP treatment on Treg compartment. In different studies, in addition to the aforementioned HD-DXM treatment, thrombopoietin receptor agonists, RTX and intravenous immunoglobulins were also shown to normalize both numerical and functional Treg aberrations [117,120,131–133]. Moreover, platelet specific Tregs could be successfully generated *de novo* from non-regulatory $CD4^+$ $CD25^-$ $CD45RA^+$ cells isolated from ITP patients, which makes them very attractive for antigen-specific cellular immunotherapy. Tregs generated in such a way could suppress proliferation of anti-platelet $CD4^+$ T cells by modulating the stimulatory capacity of DCs *in vitro*, however their clinical safety and efficacy still remains to be investigated [134].

4.2. Treg/Th17 dysregulation

Tregs and Th17 cells serve different functions but share a common signaling pathway induced by TGF-β. The definite fate of activated $CD4^+$ T cell is defined by additional microenvironmental stimuli, specifically TGF-β alone drives $CD4^+$ T cell differentiation into Treg but in the presence of IL-6, IL-1, IL-21 or other proinflammatory cytokines gives rise to Th17 cell subset [18]. Therefore, factors affecting Treg/Th17 balance could be regarded as a corner stone of the immunological tolerance, considering anti- and pro-inflammatory nature of these cells. In ITP, Treg/Th17 balance is skewed toward Th17 cells, and long term correlates with disease activity [135,136]. The precise immunological mechanisms that underline the Th17/Treg imbalances in ITP are still largely unknown. Considering the differentiation patterns of Tregs and Th17 cells, it is plausible that immune deviation towards Th17 profile is a secondary event that reflects previously established proinflammatory environment. However, in the study of *Catani et al.* it was shown that circulating DCs isolated from ITP patients as well as DCs generated *ex vivo* from PBMCs had reduced ability to up-regulate the expression of immunomodulatory enzyme indoleamine 2,3-dioxygenase 1 (IDO1) which is crucial in promoting Treg development and the maintenance of their suppressive function [137]. Simultaneously, IDO1 activity in DCs blocked IL-6 expression thus inhibited Th17 cell differentiation but also Treg re-programming to IL-17 producing T cells (Th17 like T cells) [138]. These results suggest that decreased IDO1 expression in DCs may initially skew immune response toward proinflammatory Th17 phenotype and consequently disturb delicate balance between Tregs and Th17 cells. Additionally, CTLA-4-Ig fusion protein was able to successfully enhance IDO1 expression in DCs isolated from ITP patients and restore their ability to suppress T cell proliferation and promote Treg development, suggesting its therapeutic potential [139]. Disturbed Treg/Th17 balance in ITP was also associated with aberrant expression of microRNAs (miRs). By modulating post-transcriptional gene expression, miRs influence T cell activation and differentiation but also provide stability to the specific T cell subsets [140]. In ITP, miR-146a, commonly associated with various immune mediated disorders, was found to be decreased in PBMCs [141]. MiR-146a deficiency favors Th17 differentiation by stimulated IL-6 and IL-21 production and simultaneously inhibits the differentiation of Tregs [142]. Accordingly, in ITP patients, miR-146a levels negatively correlated with the number of Th17 cells and positively with the Treg number as well as platelet counts [141]. In addition to miR-146a, other miRs including miR-99a, miR-182-5p, miR-183-5p and miR-125a-5p were also suggested to contribute to Treg/Th17 imbalance in ITP patients [143,144].

5. Th22 immune response in ITP

Th22 cells were originally described in 2009 as a unique subset of $CD4^+$ T cells characterized by the production of IL-22, IL-13, TNF-α and the expression of aryl hydrocarbon receptor (AhR) as an important transcription regulator [145]. Lack of the expression of the signature cytokines (IFN-γ, IL-4, IL-17) and master transcription factors (*T*-bet, GATA-3, RORγT) of Th1, Th2 and Th17 cells, respectively, as well as a specific chemokine receptor pattern (CCR4, CCR6, CCR10) distinguish this cell lineage from other Th profiles [145–147]. Proinflammatory cytokines IL-6 and TNF-α were firstly identified to drive Th22 cell differentiation program; whereas TGF-β appeared to exert the repressive effects [146]. Further, *in vitro* studies revealed that the optimal Th22 differentiation conditions could be achieved with the combination of four factors IL-6, IL-23, IL-1β and the tryptophan derivative 6-formylindolo[3,2-b]carbazole (FICZ) in the presence of TGF-β receptor inhibitor [148]. Plasmacytoid DCs in proinflammatory microenvironment were accredited to have the highest potential in inducing Th22 polarization of naïve $CD4^+$ T cells [146].

In the initial study of *Cao et al.* elevated plasma levels of IL-22 were detected in patients with ITP, which correlated with the number of Th1 and Th22 cells (identified as $CD4^+$ $IL22^+$ $IL\text{-}17^-$ T cells), but not Th17 cells [149]. Th17 cells in addition to IL-17 can also produce significant amounts of IL-22, however, due to the lack of correlation with IL-22 levels, the authors speculated that Th22 and Th1 cells could be more important cellular source of elevated IL-22 in ITP [149]. Later studies reconfirmed elevated Th22 immune profile of ITP patients, however, in these studies more precise identification markers of Th22 cells were applied ($CD4^+$ $IL\text{-}22^+$ $IL\text{-}17^-$ $IFN\text{-}\gamma^-$ T cells) and a significant positive correlation between all three investigated cell types (Th22, Th1 and Th17) was found, suggesting their cooperative function during disease development [150,151]. Interestingly, in ITP patients, the frequency of Th22 cells was significantly higher in patients without detectable anti-platelet auto-antibodies [150]. The exact mechanism by which Th22 cells might promote platelet destruction in an antibody independent manner still remains to be investigated. However, it has recently been shown that granzyme B, an enzyme with cytolytic function, usually expressed by $CD8^+$ T cells and natural killer (NK) cells, was also highly expressed in Th22 cells [148]. The expression level of AhR, a transcription factor of Th22 cell lineage responsible for IL-22 production, was also found to be significantly up-regulated in $CD4^+$ T cells isolated from ITP patients and AhR antagonist, resveratrol, was able to diminish the production of both IL-22 and IL-17A, whereas it enhanced the secretion of immunosuppressive IL-10 *in vitro*. This effect was achieved by down-regulating RORγT and promoting FoxP3 expression [152]. Interestingly, RORγt expression albeit essential for Th17 differentiation, was also important for IL-22 production and Th22 cell differentiation [148]. In clinical settings, HD-DXM treatment was also able to decrease IL-22 plasma level and the number of Th22 and Th1 cells in responding patients to the levels that were comparable with healthy controls [153]. Elevated plasma IL-22 level was also detected in pediatric ITP patients, suggesting that Th22 cell could be involved in ITP pathogenesis in children [154].

6. Th9 immune response in ITP

Similar to Th22 cells, within the past decade Th9 cells have been suggested to be a distinct $CD4^+$ T cell lineage. These cells produce large amounts of IL-9 as well as IL-10 and IL-21 and their differentiation is initiated in the presence of IL-4 and TGF-β. The transcription factors interferon regulatory factor 4 (IRF4), signal transducer and activator of transcription 6 (STAT6), basic leucine zipper ATF-like transcription factor (BATF) and GATA-3 are required for Th9 differentiation;

however, IL-9 production is exclusively dependent on the expression of transcription factor PU.1, which is often referred to as a master regulator of this cell lineage [155]. The involvement of IL-4 and especially GATA-3 in Th9 differentiation still raises doubts whether Th9 cells represent a distinct Th lineage or a specific transient differentiation state of Th2 cells, characterized by the production of IL-9 but not other Th2 associated cytokines [156].

Currently, there are limited data on the potential Th9 cell contribution to the immunopathogenesis of ITP. In the study of *Qiao et al.* elevated plasma levels of IL-9, higher expression levels of Th9 associated transcription factors (PU.1, BATF and IRF4) in PBMCs as well as higher percentage of circulating Th9 cells were found in active ITP patients when compared with healthy controls and the patients in remission [157]. Other studies reconfirmed these findings and, additionally, showed negative correlation between Th9 cell numbers and platelet counts [113,158]. Plasma levels of IL-9 and Th9 cells counts were also found to correlate with the levels of IL-17 and Th17 cells in both active and remitting ITP patients [157]. Cooperative function of IL-9 and Th17 cells has been previously suggested both *in vitro* and in the setting of different autoimmune disorders. Specifically, Th17 cells stimulated with TGF-β could acquire ability to produce IL-9; and Th17 cell pathogenicity in the animal models of multiple sclerosis and psoriasis was suggested to be at less partially dependent on the production of IL-9 [159,160]. There are also conflicting results demonstrating that IL-9 production in Th17 cells could limit their pathogenic capacity to induce organ-specific autoimmunity [161].

7. T follicular helper cells in ITP

T follicular helper (T_{fh}) cells are also one of the recently discovered distinct subset of $CD4^+$ T cells specialized in promoting humoral immune response by supporting survival, affinity maturation and immunoglobulin isotype switch of B cells in the germinal centre. T_{fh} cells have unique chemokine receptor profile ($CXCR5^{high}$ $CCR7^{low}$), express different co-stimulatory molecules inducible co-stimulator (ICOS) and CD40L but also inhibitory PD-1 and produce large amount of IL-21 which is considered the most important cytokine of this cell lineage. All of these molecules are closely related to the functions performed by T_{fh} cells. Specifically, CXCR5 induces T_{fh} cell migration into B cell-rich follicles within secondary lymphoid organs, whereas ICOS, CD40L, PD-1 and IL-21 regulate differentiation of activated B cells into antibody producing plasma cells and memory B cells. Differentiation of T_{fh} cells is a complex and multi-step process that requires coordinated activity of both DCs and activated B cells. The essential transcription factor of T_{fh} cells is B cell lymphoma 6 (Bcl-6), and its expression in humans is at least partially dependent on specific cytokine milieu including IL-12, and to a lesser extent TGF-β [162].

T_{fh} cells are particularly interesting from the aspect of ITP pathogenesis, considering that the majority of the patients have anti-platelet antibodies of IgG class, which is produced subsequent to immunoglobulin isotype switching supported by T_{fh} cells. Accordingly, in the blood of ITP patients, the population of circulating T_{fh} cells, identified as both $CD4^+$ $CXCR5^+$ $ICOS^+$ or $CD4^+$ $CXCR5^+$ $ICOS^+$ $PD\text{-}1^{high}$ T cells, was found to be expanded and negatively correlated with the platelet count, suggesting pathogenic role of this cell lineage during disease development [163–165]. More importantly, the subgroup of patients with anti-platelet antibodies had a higher frequency of circulating T_{fh} cells then anti-platelet antibody negative patients [164]. Gene expression analyses of PBMCs have also revealed increased expression of the transcription factors Bcl-6 and c-Maf, highly expressed by mature T_{fh} cells, and, interestingly, decreased expression of PD-1 which is generally considered to be a negative regulator of immune response [163,164]. This gene expression pattern might indicate that beside numerical aberrations of circulating T_{fh} cells in ITP, these cells may also be functionally altered. Finally, serum level of IL-21 was found to be increased and positively correlated with the percentages of circulating T_{fh} cells, additionally, this trend was more pronounced in anti-platelet antibody positive subgroup of patients [163,164]. In support of pathogenicity of T_{fh} cells in ITP are also studies performed on patients' spleens. Splenic T_{fh} cells ($CD3^+$ $CD4^+$ $CXCR5^+$ $ICOS^+$ $PD\text{-}1^{high}$ cells) were found to be expanded in ITP patients compared with healthy controls. In parallel, the mRNA levels of CXCL13 and IL-21, which are both known to be produced by T_{fh} cells, were significantly increased in splenic $CD4^+$ T cells [165]. The significance of these findings was evaluated in the context of promoting B cell mediated anti-platelet immune response. Firstly, the number of Tfh cells positively correlated with different subsets of splenic B cells including pre-germinal center B cells, germinal center B cells and plasma cells, which frequencies were also found to be higher in ITP patients. Secondly, splenic $CD19^+$ B cells, isolated from ITP patients, treated with an anti-IgM, anti-CD40L antibody and IL-21, conditions that mimic antigen stimulation and Tfh cell activity, resulted in production of anti-platelet antibodies in contrast to healthy controls [165]. In pediatric patients, similar results supporting T_{fh} cell involvement in the pathogenesis of ITP were also reported [166].

The beneficial effects of different therapeutic strategies used in ITP treatment (intravenous immunoglobulin, corticosteroids or both) seem to be partially dependent on the alterations in T_{fh} cell compartment. Following treatment, in responding patients, the frequency of circulating T_{fh} cells was significantly reduced in addition to decreased level of IL-21 and reduced expression of Bcl-6 and c-Maf. In contrast, no significant differences were identified in the non-responding patients before and after treatment [163]. B cell depleting RTX therapy was also shown to decrease the number of T_{fh} cells which was in line with the observations that B cells are needed for complete T_{fh} differentiation; however, in this study the therapeutic response was not related to the total number of T_{fh} cells in either spleen or circulation [167].

8. Conclusion

It is well established that $CD4^+$ T cells play an essential and indispensable role in orchestrating multiple immunopathological mechanisms that could lead to the development of different immune mediated disorders. Considering the nature of auto-antibodies produced in ITP, it is plausible that the initial event during disease pathogenesis is actually activation of the auto-reactive, platelet-specific $CD4^+$ T cells that echoes through the B cell production of auto-antibodies. While the role of $CD4^+$ T cells in the pathogenesis of ITP is today firmly established, there are many inconsistencies regarding their phenotypic characteristics. In general, the alterations in the composition of the $CD4^+$ T cell compartment are evident, in terms of increased proinflammatory Th1, Th17 and newly identified Th9, Th22 and Tfh phenotypes and reduced number of cells with immunoregulatory properties such as T regulatory cells. The diversity as well as a phenotypic plasticity of Th profiles implicated in ITP development, indicates that pathogenesis could be more dependent on the constitutional characteristics of patient's immune system, thus it is unlikely that a single, universal immunological pattern of disease development would be ever identified. Another issue is the heterogeneous nature of ITP itself, due to its diagnosis of exclusion which allows that various other diseases in their early stages could be misdiagnosed as primary ITP. However, it is certain that proinflammatory polarized $CD4^+$ T cells contribute to the microenvironment conditions that could promote activation of innate immunity, primarily macrophages and APCs, priming them to become more potent inducers of the immune response. In that context, activated macrophages have increased phagocytic capabilities and consequently more efficiently internalize antibody opsonized platelets inducing enhanced processing and subsequent presentation of cryptic platelet peptides (e.g. GPIIb/IIIa) to $CD4^+$ T cells. Activated platelet specific $CD4^+$ T cells then facilitate B cell activity and additional anti-platelet antibody production, forming a positive feedback loop that sustains autoimmune response in ITP.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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