

# Pathophysiology of Autoimmune Thrombocytopenia: Current Insight with a Focus on Thrombopoiesis

Irene Marini<sup>1</sup> Tamam Bakchoul<sup>1</sup>

<sup>1</sup> Department of Transfusion Medicine, Medical Faculty of Tübingen, University of Tübingen, Tübingen, Germany

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**Address for correspondence** Tamam Bakchoul, MD, Department of Transfusion Medicine, Medical Faculty of Tübingen, University Hospital of Tübingen, Otfried-Müller-Straße 4/1, 72076 Tübingen, Germany (e-mail: tamam.bakchoul@med.uni-tuebingen.de).

## Abstract

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by a significant reduction in the number of circulating platelets which is frequently associated with bleeding. The total count of platelets in the body is finely regulated by the balance between platelet production and destruction. Although the pathogenesis of ITP is still not completely elucidated, it is largely recognized that the low platelet count observed in ITP patients is due to alterations of both mechanisms. An abnormal proliferation of autoreactive T cells leading to the breakdown of immune tolerance to platelet antigens is suggested to be responsible for the up-regulated proliferation of autoantibody producing B cells. Consequently, the immune response induces enhanced T cell-mediated cytotoxicity and antibody-mediated platelet destruction through phagocytosis, complement activation and apoptosis. An additional contribution to the pathophysiology of ITP is given by alterations of thrombopoiesis caused by platelet-reactive autoantibodies or cytotoxic T cells leading to impaired megakaryocyte differentiation and platelet production. All these processes involved in ITP pathophysiology account for the complexity and heterogeneity in the clinical manifestation and therapy responsiveness of this disorder. For this reason, a better understanding of the different underlying mechanisms in ITP is necessary to develop more efficient therapeutic treatments in the future. In this review, we will provide an update on the pathophysiology of ITP with a particular focus on the impact of impaired thrombopoiesis.

## Keywords

- ▶ autoantibodies
- ▶ platelet
- ▶ megakaryocytes
- ▶ autoimmune disorders
- ▶ acquired thrombocytopenia

## Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by bleeding due to isolated thrombocytopenia with platelet count less than  $100 \times 10^9/L$ .<sup>1–3</sup> The incidence of ITP ranges between 3.3 and 3.9 per 100,000 per year in adults, and between 1.9 and 6.4 per 100,000 per year in children.<sup>3,4</sup> While a brief course with spontaneous remission is frequently observed in the majority of children with ITP, most adult patients display chronic ITP which can be associated with clinically significant bleeding including haemorrhages in skin or mucous membranes such as petechiae, purpura and rarely intracranial manifestations.<sup>5,6</sup> Based on

these clinical symptoms the primary therapeutic aim in ITP is to reduce the risk of severe bleeding.

The exact mechanism of autoimmunity leading to ITP is still unclear, but includes an alteration of the balance between effectors and regulatory cells.<sup>7</sup> This imbalance results in a breakdown of the immune tolerance causing increased platelet clearance and impaired thrombopoiesis. Similarly to other autoimmune disorders, molecular mimicry with bacterial or viral proteins might be one reason for pathogenesis of ITP. In fact, it was reported a cross-reactivity of anti-platelet autoantibodies with human immunodeficiency virus, hepatitis C virus and *Helicobacter pylori* in secondary ITP.<sup>8,9</sup> Under non-pathological conditions the immune system is finely regulated

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by humoral and cellular components, including primarily regulatory T and B cells (T regs and B regs). Functional alterations in these regulatory immune cells in ITP patients have been observed and are thought to contribute to ITP pathogenesis. For a long time, it was thought that the low platelet count is solely caused by enhanced destruction of platelets opsonized by anti-platelet antibodies.<sup>10–12</sup> However, recent studies have shown that T cell cytotoxicity and impaired megakaryopoiesis are additional pathomechanisms in ITP. In this review, we will focus on the mechanisms leading to platelet destruction in ITP with a particular focus on current findings concerning alterations of thrombopoiesis.

## Pathophysiology of ITP

### T Cell in ITP; Dysfunction in Regulation and Effector Function

The loss of immunological tolerance to autoantigens expressed on patients' own platelets is one of the critical issues in the pathophysiology of ITP. In this context, several studies reported T cell abnormalities with an imbalance in T helper (Th)1/Th2 ratio in ITP patients.<sup>13,14</sup> T regs are another subgroup of immune cells that play a critical role in maintaining self-tolerance under physiological conditions. These cells suppress cell- and antibody-mediated autoimmune responses.<sup>15</sup> Dysfunction of these cells is thought to be involved in pathogenesis of ITP.<sup>16,17</sup> For instance, T regs were found to have reduced *in vitro* immunosuppressive activity.<sup>18</sup> Moreover, an association was demonstrated between the severity of the disease (bleeding and thrombocytopenia) and the reduction of T regs.<sup>19</sup> In fact, using a murine model, an increased severity of thrombocytopenia after depletion of CD8 T cell has been reported. While upon CD8 T regs re-transfusion correction in platelet counts was observed, indicating that CD8+ T regs are required to maintain the immunological tolerance.<sup>20</sup> These findings were supported by an additional study performed using an animal model. Nishimoto et al showed that approximately 40% of T reg-deficient mice developed thrombocytopenia which lasted for up to 5 weeks. Thrombocytopenic mice produced immunoglobulin G (IgG) anti-platelet antibodies which mainly target the glycoprotein (GP) Ib/IX. Of note, transfer of purified T regs into the deficient mice restored platelet count.<sup>21</sup> Taking into consideration that up to 50% of ITP patients have no detectable autoantibodies in their blood, an alternative mechanism for platelet destruction in ITP is very obvious.<sup>22,23</sup> In fact, an increased number of T cells and higher cytotoxic activity have been reported in ITP patients with no detectable anti-platelet antibodies.<sup>24</sup> In the same line, increased levels of splenic T cells were detected in ITP patients who did not respond to the anti-B cell depleting treatment. These results support the hypothesis that T cells are involved in the pathophysiology of ITP in a B cell-independent manner. So far, the target peptides bound by platelet-specific T cells expressed on major histocompatibility complex (MHC) class I have not been identified. Interestingly, a study proposed that platelets preferentially activate naïve T cells and that they can express the pathogen-derived

peptides in the context of MHC class I.<sup>25</sup> Although the role of antigen presentation on MHC class I in ITP development is still unclear, this represents a promising area for further investigations.

Accumulating evidence suggests that cytotoxic T lymphocytes contribute directly to the increased platelet destruction.<sup>24,26</sup> It was shown that ITP patients have autoreactive T cell clones against cryptic GPIIb/IIIa epitopes<sup>27</sup> and enhancement of oligoclonal T cells.<sup>28</sup> In addition, T cells from ITP patients were found to harbour increased cytotoxicity against autologous platelets.<sup>29</sup> In active ITP patients, lysis of radiolabelled autologous platelets by purified CD3 + CD8+ T cells as effector cells was observed, but not in ITP patients in remission.<sup>26</sup> Apoptosis has also been suggested as another mechanism of platelet destruction caused by activated autoreactive T cells. This hypothesis is supported by the observation that purified CD8+ T cells from ITP patients overexpress molecules involved in cytotoxicity and induce upregulation of the apoptotic marker annexin V in autologous platelets.<sup>26</sup> Based on these findings, the concept that apoptosis and perforin/granzyme-mediated cytotoxicity represent an important pathway through which cytotoxic T cells destroy autologous platelets in ITP patients is corroborated.

### B Cell in ITP; Activated Humoral Autoimmune Response

It is generally accepted that ITP is associated with dysfunctions of B cells. The contribution of B cells to the pathogenesis of ITP is not restricted to the production of autoantibodies. Enhanced numbers of B cells have been observed in the red pulp spleen sections of ITP patients, suggesting that these cells contribute to the autoantigen stimulation in ITP.<sup>30,31</sup> The development and survival of B cells is regulated by the B cell activating factor of the TNF family (BAFF).<sup>32</sup> Several studies showed higher serum levels of BAFF in untreated ITP patients, which were significantly reduced upon successful immunosuppressive therapy.<sup>33</sup> Moreover, the up-regulation of THR7 in mice with ITP led to an enhancement in BAFF, with a subsequent decrease in platelet count.<sup>34,35</sup> These findings indicate that survival of autoreactive B cells is enhanced by BAFF in ITP patients.

Recently, several studies provided robust evidence concerning the involvement of B regs in chronic ITP patients.<sup>36,37</sup> B regs (CD19 + CD24hiCD38hi) maintain peripheral tolerance by secretion of IL-10 leading to T regs recruitment and/or differentiation, and reduction of CD4+ T cell functionality.<sup>38,39</sup> Notably, functional impairment of B regs was observed in non-splenectomized ITP patients with chronic ITP.<sup>36</sup>

The presence of autoantibodies produced by B cells in ITP is fully established.<sup>40</sup> The first demonstration of the pathogenic effect of these antibodies took place in the early 1950s. Harrington and Hollingsworth demonstrated, for the first time, the existence of a 'thrombocytopenic factor' in the blood of ITP patients. They induced transient thrombocytopenia in healthy recipients who received 500 mL of whole blood from ITP patients. In these subjects a significant reduction in platelet count after 2 hours and a complete absence of platelets after 24 hours, with a gradual recovery after a few days,<sup>41</sup> were observed. Years later, Shulman et al revealed that the

'thrombocytopenic factor' was present in the IgG fraction of plasma leading to the hypothesis that the anti-platelet factor was an antibody.<sup>10</sup> Finally, at the end of the 1990s, it was found that the antibodies are mainly directed against platelet surface proteins.<sup>42,43</sup> Based on these findings, intensive efforts have been taking place for more than two decades to identify the antigen specificity of antibodies in ITP. A deeper understanding was achieved with the introduction of immunocapture assays, in particular the monoclonal antibody-specific immobilization of platelet antigens (MAIPA).<sup>44</sup> Using this assay, it has been demonstrated that autoantibodies in ITP are mainly directed against GPIIb/IIIa and GPIb/IX, but also GPIV, GPVI, GPIa/IIa and GPV.<sup>45</sup> More recent research revealed that autoantibodies recognize several epitopes and require different conformations. Anti-GPIIb/IIIa antibodies, for example, frequently bind to cation-dependent conformational antigens on  $\alpha$ IIb $\beta$ 3, but not to another  $\beta$ 3-containing integrin,  $\alpha$ v $\beta$ 3.<sup>46,47</sup> These autoantibodies bind specifically to the  $\beta$ -propeller domain of  $\alpha$ IIb, predominantly from the N-terminus to the W4:4–1 loop of the  $\beta$ -propeller domain. Interestingly, it was observed that in the presence of a single amino acid substitution in  $\alpha$ IIb, the reactivity of some autoantibodies is completely inhibited, suggesting that target epitopes are extremely restricted to conformational epitopes.<sup>48</sup> A similar effect was observed by He et al for antibodies targeting the GPIb/IX, which mainly recognize a short amino acid sequence (333–341) on GPIb $\alpha$ .<sup>43</sup> Although most antibodies bind the GPIb component of the receptor complex GPIb/IX/V, little is known about relevant autoepitopes on the entire complex.<sup>49–51</sup> Given the variety of the target antigens, we have summarized the characteristics of GPs targeted by ITP autoantibodies in **Table 1**.

Despite this heterogeneity in target antigens, anti-GPIIb/IIIa antibodies have been shown to have restricted  $\kappa/\lambda$ -chain usage.<sup>48,52</sup> Roark and colleagues demonstrated by sequencing analysis of the Fab region of immunoglobulin arrangements using phage display libraries constructed from splenocytes of ITP patients that anti-platelet autoantibodies in ITP use highly limited immunoglobulin variable regions.<sup>53</sup> Moreover, they have noticed a selective incorporation of the VH3–30 variable heavy chain gene segment suggesting that the antigenic repertoire in ITP is fairly limited and that anti-platelet antibodies are generated from a restricted number of B cell clones.

Taken together, immune dysregulation in ITP is not limited to T cell subsets. B cells play a central role as a regulatory cell as well as effector cells in a complex molecular network that has not been elucidated so far.

### Mechanisms Leading to Autoantibody-Mediated Platelet Clearance

Autoantibody-mediated thrombocytopenia can be induced by different mechanisms such as phagocytosis of platelets upon antibody binding, apoptosis, desialylation, complement activation and impairment of platelet production (**Fig. 1**). Interestingly, in addition to the fast clearance of platelets, ITP-autoantibodies are able to induce functional alterations of the circulating cells. In fact, although it is extremely difficult to investigate platelet functions in subjects with low platelet counts, functional defects in patients characterized by severe bleeding have been reported.<sup>54</sup> In particular, a decrease of platelet degranulation after ADP stimulation as well as a reduced ability to form microaggregates upon stimulation have been shown in ITP patients. In accordance with these findings, another group observed decreased levels of P-selectin- and activated GPIIb/IIIa-positive platelets upon adenosine diphosphate (ADP) or thrombin receptor-activating peptide (TRAP) activation in paediatric ITP patients with severe bleeding.<sup>55</sup>

### Fc-Dependent Platelet Phagocytosis

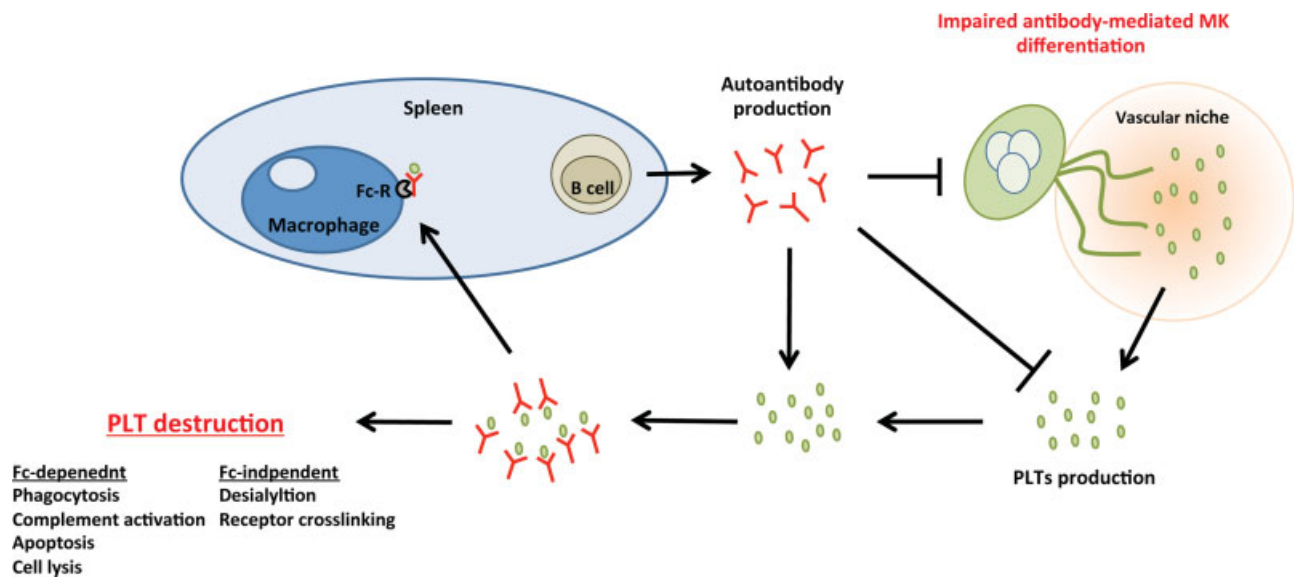
Opsonized platelets are primarily destroyed by macrophages in the spleen. This process is mediated by low affinity Fc gamma receptors (FcγRs) IIA and FcγRIIA which are linked to immune receptor tyrosine-based activation motifs (ITAMs) in the intracellular domain. Upon autoantibody binding, ITAMs are phosphorylated by the tyrosine kinase Syk, triggering the phagocytosis.<sup>56,57</sup> Although all FcγRs are involved in activating the immune system, FcγRIIB is the only inhibitory Fc receptor. This is due to its immune receptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail which inhibits phagocytosis and the release of pro-inflammatory cytokines by macrophages and dendritic cells. In this context, the ratio between different FcγRs is critical to correctly regulate the immune response. In fact, in patients responding to *H. pylori* eradication, a shift in monocyte FcγRs expression toward FcγRIIB has been reported.<sup>58</sup> The relevant

**Table 1** Characteristics of glycoproteins (GPs) targeted by ITP autoantibodies

Targets of AAbs	Copies/PLT	Ligand	Functions	Related disorders
GPIIb/IIIa ( $\alpha$ IIb $\beta$ 3)	50,000–80,000	Fibrinogen	Platelet adhesion and aggregation	Glanzmann's syndrome
GPIb/IX/V	~50,000	von Willebrand factor	Platelet adhesion and aggregation	Bernard–Soulier syndrome
GPIV	12,000–25,000	Collagen	Platelet activation and aggregation	–
GPIa/IIa	2,000–4,000	Collagen	Platelet activation and aggregation	–
$\alpha$ v $\beta$ 3	300–400	Vitronectin	Platelet adhesion and aggregation	–

Abbreviations: AAbs, autoantibodies; ITP, immune thrombocytopenia; PLT, platelet.

Source: Clemetson KJ, Clemetson JM. Platelet receptors. In: Michelson AD, ed. Platelets. 3rd ed. San Diego, CA: Elsevier/Academic Press; 2013; 169–194.



**Fig. 1** Mechanisms causing platelet destruction in immune thrombocytopenia. Platelets (PLTs) are destroyed in the circulation through different mechanisms. Autoantibodies, produced by B cells, can induce phagocytosis, complement activation, apoptosis and cell lysis via their Fc domain. In addition, Fc-independent platelet destruction mechanisms have been suggested recently including desialylation and activation. (Adapted from Kashiwagi et al 2013, International Journal of Hematology.)

role of the FcγRIIB was also demonstrated in a study, using animal model, which showed that its presence on splenic macrophages is necessary for the protective effects of intravenous immunoglobulin (IVIG).<sup>59</sup>

### Complement Activation

Complement activation represents an additional important mechanism of platelet destruction. Several *in vitro* studies<sup>60,61</sup> documented antibody-mediated complement fixation to platelets from ITP patients. GPIIb/IIIa and GPIb/IX are the major targets of autoantibodies with complement fixation capability.<sup>62</sup> The biological significance of this observation was demonstrated using an established mouse model of ITP.<sup>63</sup> The authors showed that platelet destruction by complement-fixing autoantibodies is significantly increased in the presence of complement.<sup>64</sup> Taking these findings into account, novel therapies for ITP could target complement factors. For instance, eculizumab was shown to rescue thrombocytopenia in patients with anti-phospholipid syndrome by inhibition of the downstream components of complement.

### Fc-Independent Platelet Destruction

The Fc-mediated platelet phagocytosis is a well-known mechanism in ITP pathogenesis. However, it has been estimated that 20% of ITP patients are refractory to the standard therapies that target the Fc receptor signalling,<sup>65</sup> including splenectomy and IVIG. This indicates that, at least for this subgroup of patients, the antibody-mediated platelet destruction involves different pathways and site of clearance. Recent studies proposed two Fc-independent mechanisms.<sup>66,67</sup> Using a murine model, Li and coworkers showed that ITP autoantibodies induce glycan modification of platelet surface GPs, which are recognized by Ashwell–Morell receptors, expressed on hepatocytes, leading to accelerated platelet clearance in the liver.<sup>68</sup> In some patients, this may explain the ineffectiveness of

splenectomy which represents the ultimate ITP therapeutic option for refractory subjects. Interestingly, 2 years later a retrospective study involving 61 ITP patients reported a correlation between platelets desialylation and a reduced response to first-line treatments corroborating the hypothesized Fc-independent mechanism.<sup>69,70</sup> Another Fc-independent mechanism has been suggested by Quach et al who showed that non-responding ITP patients often produce autoantibodies targeting the ligand binding domain of GPIIb/IX. This specific binding can activate GPIIb/IX by platelet receptor-crosslinking, inducing unfolding of its mechanosensory domain and the consequent platelet destruction.<sup>71</sup> However, it is currently unclear whether the unfolding of GPIIb mechanosensory domain is the earlier event required for sialidase neuraminidase-1 translocation and platelet desialylation.

### Antibody-Mediated Platelet Apoptosis

The platelet life cycle is regulated by the intrinsic apoptotic pathway similarly to nucleated cells. Considering this, the contribution of ITP autoantibodies in inducing platelet apoptosis was investigated by several groups using well-defined apoptosis markers such as depolarization of the mitochondrial transmembrane potential, Bcl-2 family protein expression, caspase-3 and 9 activation and phosphatidylserine (PS) exposure.<sup>72–74</sup> One of the first studies performed using a mouse model for ITP showed that upon injection of anti-GPIIb antibodies, murine platelets displayed apoptotic features including destruction of the mitochondrial potential, caspase activation and PS expression.<sup>75</sup> In human ITP, platelet apoptosis has been reported in paediatric and adult patients with acute ITP which was ameliorated by immunoglobulin infusion.<sup>76,77</sup> Interestingly, in a recent study apoptotic platelets were found in ITP patients expressing anti-GPIIb/IIIa and anti-GPIb autoantibodies but not in those carrying anti-GPIa/IIa autoantibodies.<sup>78</sup> This suggests a possible preferential specificity of the autoantibodies in inducing platelets



apoptosis. Although, the exact mechanism of autoantibody-mediated platelet apoptosis is not yet completely clear, these findings suggest a relevant contribution of the apoptotic pathway in the ITP pathogenesis, opening novel horizons for deeper investigations.

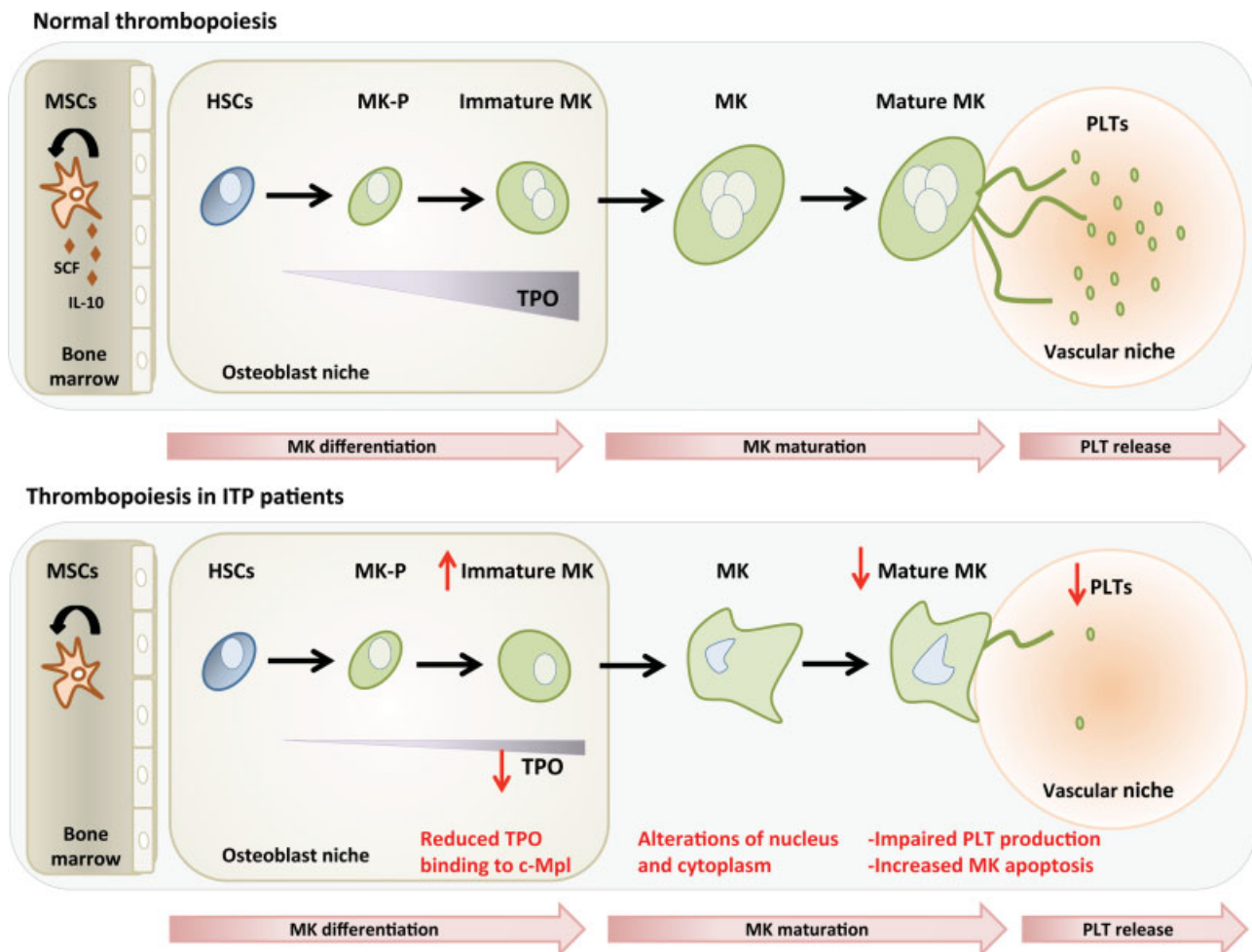
### Physiological Thrombopoiesis

In addition to the established mechanisms leading to platelet destruction, an equivalent role in the pathophysiology of ITP is played by antibody-mediated impaired platelet production. Megakaryopoiesis is a complex process that takes place in the bone marrow involving molecular and cellular changes leading to biogenesis of platelets. The primary activator and regulator of the process is the thrombopoietin (TPO) produced in the liver.<sup>79,80</sup> TPO triggers the differentiation of haematopoietic stem cells (HSCs) into polyploid cells, known as megakaryocytes. Upon complete maturation, megakaryocytes develop proplatelet extensions through the endothelial cells of the vascular niche and finally release platelets into the blood stream (almost 10,000 platelets per megakaryo-

cyte and a total of 100 million each day in healthy subjects).<sup>81</sup> Bone marrow-derived mesenchymal stem cells (MSCs) are essential components in the haematopoietic microenvironment and provide support to megakaryopoiesis. They secrete a wide range of cytokines including interleukin 6 (IL-6), IL-10, IL-11, prostaglandins, stem cell factor (SCF) and leukaemia inhibitory factor which induce megakaryocyte biogenesis and maturation.<sup>82</sup> These cells are capable of self-renewal and differentiation by which they maintain the correct balance between HSC and megakaryocyte differentiation in the bone marrow. Clearly, alterations in any stage of the megakaryopoiesis will affect platelet production (→ Fig. 2).

### Thrombopoiesis in ITP

The first observations of morphological alterations on megakaryocytes from ITP patients were reported in the 1940s. An increase of immature megakaryocytes, characterized by abnormalities in the ploidy state of the nucleus and cytoplasm, was observed in the bone marrow of ITP patients using light microscopy.<sup>83</sup> Interestingly, after transfusion of ITP serum into



**Fig. 2** Altered thrombopoiesis in immune thrombocytopenia (ITP) patients. Upper panel: in physiological condition, thrombopoietin (TPO) induces the differentiation of haematopoietic stem cells (HSCs) into megakaryocyte progenitors (MK-P), immature MKs and finally into the mature MKs characterized by polyploid nucleus. These cells develop proplatelet extensions and release platelets (PLTs) in the blood stream. Mesenchymal stem cells (MSCs), residing in the bone marrow, support the entire process maintaining the balance between self-renewal and differentiation. Lower panel: during thrombopoiesis in ITP patients, different steps of the entire process are altered as shown in the figure (Adapted from Eto K. and Kunishima S., 2016, Blood.)

healthy subjects, the same alterations on megakaryocyte morphology were detected.<sup>84</sup> This finding can be explained by the fact that GPIb and GPIIb/IIIa, the major targets of ITP autoantibodies, are expressed not only on the surface of platelets but also on megakaryocytes during their differentiation. The autoantibody binding results in suppression of megakaryocyte maturation and platelet formation.<sup>85,86</sup> In fact, the antibody-mediated inhibition of platelet production was demonstrated by *in vitro* studies using HSCs that differentiated into megakaryocytes in the presence of ITP autoantibodies targeting GPIb/IX and GPIIb/IIIa. In those studies, impaired megakaryocyte maturation and decreased platelet formation were observed.<sup>87–89</sup> In a more recent study, Zeng et al<sup>90</sup> have investigated the impact of anti- $\alpha v \beta 3$  autoantibody, which is expressed in chronic ITP patients, on megakaryocyte differentiation, survival, migratory and adhesive ability. In fact, the migration and adhesion of megakaryocytes in the vascular niche is an essential process for an efficient thrombopoiesis in the human body. Notably, impaired migration and adhesion of megakaryocytes, but a normal viability and proliferation of the cells, have been observed in the presence of anti- $\alpha v \beta 3$  autoantibody. These findings suggest that anti- $\alpha v \beta 3$  autoantibodies might have a selective inhibitory impact on megakaryocyte adhesion and migration during ITP pathogenesis. Furthermore, using a murine model the authors reported a lower count of megakaryocytes residing in the vascular niche in ITP mice. Interestingly, bone marrow biopsies of ITP patients, which express anti- $\alpha v \beta 3$  antibody, have shown similar phenotype, corroborating the *in vitro* and *in vivo* findings. The results of this study indicate that anti- $\alpha v \beta 3$  autoantibody might reduce megakaryocyte differentiation and platelet production through inhibition of the megakaryocyte's migration ability.

Whereas conventional therapeutic approaches in ITP aimed mainly to reduce immune-mediated platelet destruction, newer treatments seek to increase thrombopoiesis and the consequent platelet production. Megakaryopoiesis suppression has been observed in ITP patients with autoantibodies targeting specifically the TPO receptor (TPO-R) c-Mpl.<sup>91</sup> As a key activator and regulator of the platelet production process, the activation of c-Mpl receptor was one of the successful therapeutic targets investigated. In fact, platelet count increases have been addressed using TPO-R agonists such as romiplostim and eltrombopag.<sup>92,93</sup> Clinical confirmation of the efficacy of these substances emerged from several randomized trials, performed during long-term treatment, which reported a 70 to 80% response in refractory ITP patients.<sup>94,95</sup> Recently, Bal et al reported an additional therapeutic effect of TPO-R agonists showing increased proliferation, mobilization and differentiation of HSCs and early cell progenitors of all three haematopoietic lineages.<sup>96</sup> Consequently, HSCs seem to be a promising tool for novel cell-based therapies in ITP.

Genetic factors with a potential association to ITP have been investigated extensively; however, it is still difficult to define a clear conclusion from the data currently available. Nevertheless, it has been reported that microRNAs (miRNAs), small non-coding RNA molecules that regulate gene expression by targeting messenger RNA (mRNA),<sup>97</sup> change under pathological conditions. Interestingly, although the

role of mRNA in ITP has not been completely elucidated,<sup>98,99</sup> several mRNAs seem to have an impact on megakaryocyte differentiation. For instance,  $\beta$ -1 tubulin R307H single-nucleotide polymorphism (SNP) was suggested to be a potential biomarker in ITP. It has been shown that 30% of ITP patients present SNP allele, and any difference observed in relation to SNP might influence a large number of patients. The relationship between changes in platelet physiology and changes in isotype of tubulin (as the main component of marginal band platelet) highlights the possible role of  $\beta$ -1 tubulin in platelet activity.<sup>100,101</sup>

### Megakaryocyte Apoptosis

An interesting open question is the role of megakaryocyte apoptosis in the ITP pathophysiology. Controversial results were presented in several studies during the last few years. In fact, it has been reported that ITP plasma can reduce megakaryocyte apoptosis.<sup>102</sup> In particular, after cultivation of HSCs from healthy umbilical cord blood with ITP plasma, a decreased percentage of apoptotic cells, reduced expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and increased expression of the anti-apoptotic protein Bcl-xL have been observed in the differentiated megakaryocytes.<sup>103</sup> In contrast, an *in vivo* study by Houwerzijl et al showed that megakaryocytes undergo apoptosis in the presence of autoantibodies displaying nuclear fragmentation, chromatin condensation and activation of caspase 3 in biopsies of ITP patients, leading to phagocytosis of the polyploid cells by macrophages residing in the bone marrow.<sup>104</sup>

Although the mechanisms of impaired megakaryopoiesis are not yet completely clear, it is well known that in bone marrow of ITP patients the number of megakaryocytes is normal or increased despite platelet production and count being reduced.<sup>105</sup> In this context, the apoptotic pathways in megakaryocytes might represent an explanation. In fact, reduced apoptosis in megakaryocytes and their consequent long survival in the bone marrow without an effective megakaryopoiesis is a reasonable hypothesis. This theory is corroborated by a more recent study where megakaryocyte apoptosis was investigated in bone marrow samples of ITP patients.<sup>106</sup> Vrbensky and coworkers have observed a decrease of megakaryocyte apoptosis in bone marrow biopsies of ITP subjects in comparison to healthy controls. In the same line, data from animal study showed that within 24 hours upon administration of different autoantibodies (anti- $\alpha$ IIb, anti- $\beta$ 3 or anti-GPIb) all of them were able to develop thrombocytopenia in recipient mice. However, only two antibodies (anti- $\alpha$ IIb and anti- $\beta$ 3) have induced an alteration of the number of megakaryocytes in the bone marrow. This suggests that not all autoantibodies affect the megakaryocyte count and consequently a different mechanism may be the cause of megakaryocyte alterations during ITP.<sup>107</sup>

### Defective Mesenchymal Stem Cells in ITP

Emerging studies are suggesting a contribution of the defective MSCs in the mechanism leading to ITP pathogenesis. One of the most important properties of these cells is their immunosuppressive function, involving both adaptive and

innate immune responses.<sup>108</sup> In fact, it was reported that MSCs could induce an immunosuppressive or tolerant phenotype in physiological conditions by altering the cytokine's secretion functions of dendritic cells, effector T cells (Th1 and Th2) and natural killer cells.<sup>109,110</sup> In contrast, functional impairment of MSCs was observed in different autoimmune disorders including rheumatoid arthritis,<sup>111</sup> systemic lupus erythematosus<sup>112</sup> and aplastic anaemia.<sup>113</sup> Accordingly with these observations, several investigations have demonstrated that MSCs from ITP patients are characterized by increased number of apoptotic cells and reduced capacity to inhibit the proliferation of activated T cells.<sup>114,115</sup> Given these findings, defective MSCs are currently being explored as a potential target in the treatment of ITP. A few years ago it was reported that platelet-derived growth factor (PDGF-BB) has a protective effect on MSCs. PDGF-BB was found to act against apoptosis, senescence and immunomodulatory dysfunctions. These observations were corroborated by Tao et al who demonstrated that transplantation of healthy MSCs can rescue the functional immune phenotype in a murine model of ITP. The administration of MSCs induced a significantly decreased level of T regs and an increase in platelet count in ITP mice.<sup>116</sup> These observations were further supported by a recent clinical study exhibiting a similar experimental setting that involved four patients with chronic refractory ITP. A complete remission was achieved in three patients within 12 months and for one patient after 24 months, without additional immunosuppressive drugs. Interestingly, during follow-up analysis no severe side effects were observed, suggesting that MSC transplantation seems to be a safe and efficient cell-based therapeutic approach to treat refractory ITP.<sup>117</sup>

## Conclusion

ITP is a complex and multifactorial disease. In fact, the clinical manifestations as well as patients' response to different treatments are very heterogeneous suggesting that ITP is a group of disorders sharing common characteristics, namely loss of immune tolerance toward platelet (and megakaryocyte) antigens and dysfunctional primary haemostasis. Recent studies provided significant insight into the pathophysiology of ITP including identification of the role of antigen-presenting cells, T and B cells, and their interactions. Of most importance was the discovery that the abnormal functions of B regs and T regs, which are supposed to maintain self-tolerance, are involved in modulating ITP pathogenesis. The immunological consequence of the activated B and T cell-mediated immune response is the proliferation of platelet-specific plasma and cytotoxic cells, respectively. While the latter are responsible for a direct destruction of platelet as well as megakaryocyte in ITP, IgG autoantibodies generally directed at the most abundant platelet surface GPs (GPIIb/IIIa and GPIb/IX/V) can induce thrombocytopenia in ITP by several Fc-domain dependent mechanisms including platelet phagocytosis, complement activation, apoptosis, cell lysis and inhibition of proplatelet production. Additionally, recent data suggest that surface

expression of sialic acid can be modified by autoantibodies leading to platelet clearance via Ashwell–Morell receptor (AMR) system in the liver in an Fc-independent manner. Of note, some patients show a decrease in autoantibody concentration after treatment with steroids but no significant improvement in platelet count (personal observation). Since only platelets are used in antibody tests, it could be possible that autoantibodies with higher avidity to megakaryocytes are still circulating but not detectable using standard assays. These autoantibodies with a preferential binding to megakaryocytes could induce thrombocytopenia via inhibition of thrombopoiesis. Although this hypothesis is speculative, future investigations could help understand the heterogeneity that is frequently observed in the responsiveness of ITP patients to different treatments. Future studies should also aim to investigate the increased platelet mass due to various therapies, and its role in presenting platelet autoantigens to T cells and potential effects on activating and suppressing T regs, which may elucidate novel pathomechanisms. Taking the heterogeneity of ITP into consideration, improving our knowledge of the pathogenesis of ITP is an essential cornerstone for identifying effective diagnostic tests and efficient therapeutic approaches for this clinically relevant form of acquired thrombocytopenia.

## Author Contributions

Irene Marini did the literature search and wrote the sections on alterations of megakaryopoiesis and thrombopoiesis. Tamam Bakchoul contributed to the subsections on the antibody-mediated platelet clearance. Both authors approved the final version of the manuscript.

## Conflict of Interest

I.M. has no conflict of interests.

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