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Immune Thrombocytopenia: Antiplatelet Autoantibodies Inhibit Proplatelet Formation by Megakaryocytes and Impair Platelet Production *in vitro*

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This commentary review considers the above publication, as invited by the Journal of Immunological Science. These data are already published and no additional data are provided. The paper deals with samples from 19 immune thrombocytopenia patients and was published in the journal *Haematologica*[1].

Immune thrombocytopenia (ITP) is an autoimmune bleeding condition characterised by low platelet counts (< 100 x 10^9/L; reference count 150 – 400 x 10^9/L) in the absence of other triggers that may cause thrombocytopenia[2]. Bleeding (petechiae, mucosal bleeding) is a common sign of ITP. There is a risk of internal bleeding and fatal haemorrhage has been reported[3]. ITP patients have reduced quality of life and decreased life expectancy[4]. Autoantibodies against abundant platelets antigens such as glycoproteins (GP) Ib/IX and IIb/IIIa are found in ITP patients and contribute to platelet destruction. Platelet production by their precursor cells, megakaryocytes (MKs), is also decreased in ITP[5]. This is thought to occur via autoantibody-mediated detrimental effects on MK differentiation and proliferation, which subsequently affect platelet production. These conclusions were drawn from observations of MKs cultured in the presence of ITP plasma[6, 7], antiplatelet monoclonal antibodies[8] or serum from drug-induced ITP[9]. However, a recent investigation in a murine passive model of ITP showed that most anti-platelet antibodies did not produce changes in MK numbers or morphology[10]. It should be noted that this work[10] used monoclonal or polyclonal antibodies against mouse platelets and not ITP patient's-derived IgG. Nevertheless, as will be discussed below, the observations of this mouse model study are in agreement with our conclusions using ITP antibodies on human MKs *in vitro*[1].

Treatment of ITP comprises several phases depending on severity and response. The goal of treatment is to decrease the risk of bleeding. The first line of therapy involves the use of corticosteroids or intravenous immunoglobulins. The objective of corticosteroid administration is to reduce antibody production[11]. In addition, steroid treatment also decreases platelet activation in responsive ITP patients[12]. Several mechanisms of action have been proposed for IVIg, including clearance of the autoantibodies mediated by FcRn[13, 14] and induction of the Fcγ inhibitory receptor[15]. The evidence for these mechanisms has been challenged (reviewed by[16]), and involvement of the FcγRIIIa has been proposed[16]. These therapies can produce a rapid increase in platelet counts but do not produce a lasting response in most patients. Non-responsive patients can be

treated with second line therapies such as splenectomy, which can be effective and long-lasting due to the removal of an organ that is involved in both platelet destruction and autoantibody production. Immunosuppressants such as anti CD20 (rituximab)[17], cyclophosphamide[18], cyclosporine or mycophenolate mofetil[19], which inhibit B or T cells are also used as second line therapies. More recently thrombopoietin (TPO) receptor agonists (TPO-RAs) such as romiplostim and eltrombopag have entered clinical practice[20]. These agonists act by activating c-Mpl on precursor cells and promoting MK proliferation.

A well-recognised aspect of ITP is its heterogeneity, especially patients' response to treatment. TPO-RAs, for instance have a durable response mainly in non-splenectomised patients[21]. In addition, long-lasting complete responses have been documented after discontinuation of TPO-RAs therapy[22]. Durable response is not a universal feature of these drugs and the nature of this immunomodulatory effect is yet to be described. The literature is still unclear about how these drugs increase platelet production. Leon *et al* showed an increase in MK proliferation (which is expected for TPO-RAs) leading to enhanced platelet production[23], while other authors documented increased platelet numbers despite no changes in MK mass[24].

Our study[1] used MKs derived from cord blood $CD34^+$ cells to address two aspects of ITP, i) the effect on ITP patients' serum and purified IgG on MK proliferation, maturation, apoptosis, proplatelet formation and platelet release; and ii) the activity of TPO-RAs on proplatelet formation in the presence of ITP autoantibodies. The findings of the study[1] are summarised in Table 1. The principal observation was that most ITP autoantibodies inhibited proplatelet formation and this was unrelated to effects on MK proliferation, differentiation, apoptosis or ploidy status. This implies that it is the final stage of megakaryopoiesis that is targeted by ITP antibodies, most likely by the effects of these antibodies on proplatelet structure[25]. Three serum samples affected MKs in culture but this was unrelated to the presence of anti-platelet antibodies and was most likely due to other components present in these sera such as TGF-β1 , PF4 or s-FAS[1]. *In vivo*, other mechanisms such as T cell imbalance are involved in ITP and may lead to reduction of MK production (reviewed by[26]), however in this case the experiments were performed *in vitro* in the absence of other cells.

Historically, it was thought that ITP was characterized by platelet destruction, but it is now apparent that insufficient platelet production is a substantial feature of ITP pathogenesis. Our work shows that IgG from most patients decreases proplatelet formation and consequently platelet production without evident effects on other MK features such ploidy, proliferation, cell size and apoptosis[1]. Damage to proplatelet structures by ITP antibodies in the absence of obvious apoptotic changes was also documented in a study by Lev *at al*[25], which was conducted mostly concurrently with our work and is also supported by observations of normal MK numbers and morphology in a mouse model of ITP[10]. More recently, it was shown that thrombocytopenic patients (ITP and myelodysplastic syndrome) had reduced rather than increased MK apoptosis, which corroborates the *in vitro* observation of no significant apoptosis induction upon ITP antibody treatment.

TPO-RAs represent a relatively effective therapy for patients refractory to first line treatments. This in turn increases platelet production. Unexpectedly, MK proliferation has been observed in both responders and non-responders to TPO-RA administration[27], suggesting that in some patients there is no direct correlation between MK proliferation and increase in platelet counts. Our work[1] offers an explanation by the surprizing finding that TPO-RAs not only promote MK proliferation but can also neutralize the inhibitory activity of some ITP antibodies, thus increasing the number of proplatelet-bearing MKs. In addition, the response to the TPO-RAs used in our study[1] (romiplostim and eltrombopag) varied among patient samples, suggesting that some non-responders might benefit by switching TPO-RAs. This might become more pertinent once additional TPO-RAs such as avatrombopag[28] become widely available. This study does not question the view that platelet destruction is also a key feature of ITP, as documented many decades ago[29]. In fact, several mechanisms, including phagocytosis of opsonised platelets by macrophages[30], complement-mediated destruction[31],

Table 1. Effect of ITP serum or IgG on cultured MKs

	19 ITP Patients[a]			
Effect[b]	Serum[c]	*P* value[e]	IgG[d]	*P* value[e]
Decrease in Proplatelet formation	8 (42%)	<0.0001	13 (68%)	<0.0001
Reduction of MK proliferation	3 (15.7%)	0.0005	0	
Reduction of MK maturation	3 (15.7%)	<0.0001	0	
Decrease in cell size	3 (15.7%)	<0.0001	0	

[a] Anti-platelet antibodies (IgG, IgA, IgM) were present in 16 patients (84%). [b] MK were treated after 8-9 days of differentiation with serum or total IgG at 1:10 dilution and evaluated 3-5 days later. Percentages shown are relative to control serum or control IgG (n=9). [c] Serum was obtained from coagulated blood and heat-inactivated at 56°C for 30 min. [d] Total IgG was purified from ITP and normal sera with protein-G agarose beads, dialyzed and concentrated to 10mg/ml. [e] *P* represents unpaired Student t-test.

apoptosis and desialylation[32] may account for platelet depletion in ITP. Liu *et al* reported that TPO-RAs may also inhibit platelet destruction by upregulation of inhibitory FcγRII and suppression of activating Fcγ receptors[33]. This expands the potential range of TPO-RA activities to MK proliferation, increase of pro-platelet formation per MK and inhibition of platelet destruction. In conclusion, it is evident that in both platelet destruction and production are contributors to the development of ITP. Our work shows that megakaryopoiesis is largely unaffected, but antibody-mediated inhibition of proplatelet formation and platelet release is a regular feature of the pathogenesis of ITP.

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