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Recent advances in the pathology of idiopathic thrombocytopenic purpura

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder in which, for reasons that remain unclear, platelet surface proteins become antigenic and stimulate the immune system to produce autoantibodies and cytotoxic T lymphocytes. This results in immune-induced platelet destruction and suppression of platelet production.[1-6] What causes the loss of tolerance to one's own platelets remains unclear and is likely to be a result of a number of different co-operating factors including genetics (polymorphism in selected genes) and environment events (virus- and bacteria-associated ITP).[3] Both acute and chronic forms of disease can be distinguished. In children acute ITP is often associated with a viral or bacterial infection and generally resolves spontaneously within 6 weeks. Approximately 20% of children with acute ITP progress to the chronic form. In contrast ITP in adults is generally chronic and often requires treatment.[1]

Increased platelet destruction

Two main mechanisms have been reported to be involved in accelerated platelet clearance in ITP: 1) antibody-mediated platelet destruction and 2) platelet lysis due to cytotoxic T lymphocytes (Figure 1a).

Antibody-mediated platelet destruction

The initial stimulation for the production of platelet autoantibodies is unknown but undoubtedly is regulated by complex cellular and soluble mechanisms, primarily involving T helper (Th) lymphocytes and antigen presenting cells (APCs). Platelets are the primary source of autoantigens which stimulate Th cells. Dendritic cells (DCs) or macrophages, which are responsible for the normal destruction of senescent platelets *in vivo*, are the initial APCs which stimulate platelet-reactive Th cells.[1] Thus, the platelet first interacts with a major histocompatibility complex (MHC) class II-positive APCs, which subsequently processes platelet glycoprotein antigens into smaller antigenic peptides. These peptides are translocated to endosomal compartments and ultimately re-expressed on the APC surface in association with MHC class II molecules. If the Th cell receptor has a sufficient affinity for the antigen-MHC complex and appropriate costimulatory events are met (CD40 (APCs)/CD154 (T cell) or B7 (APC)/CD28 (T cell)), the Th cell would be activated and would subsequently drive antigen-primed B lymphocytes to produce autoantibodies. After antibody binding, platelets can be

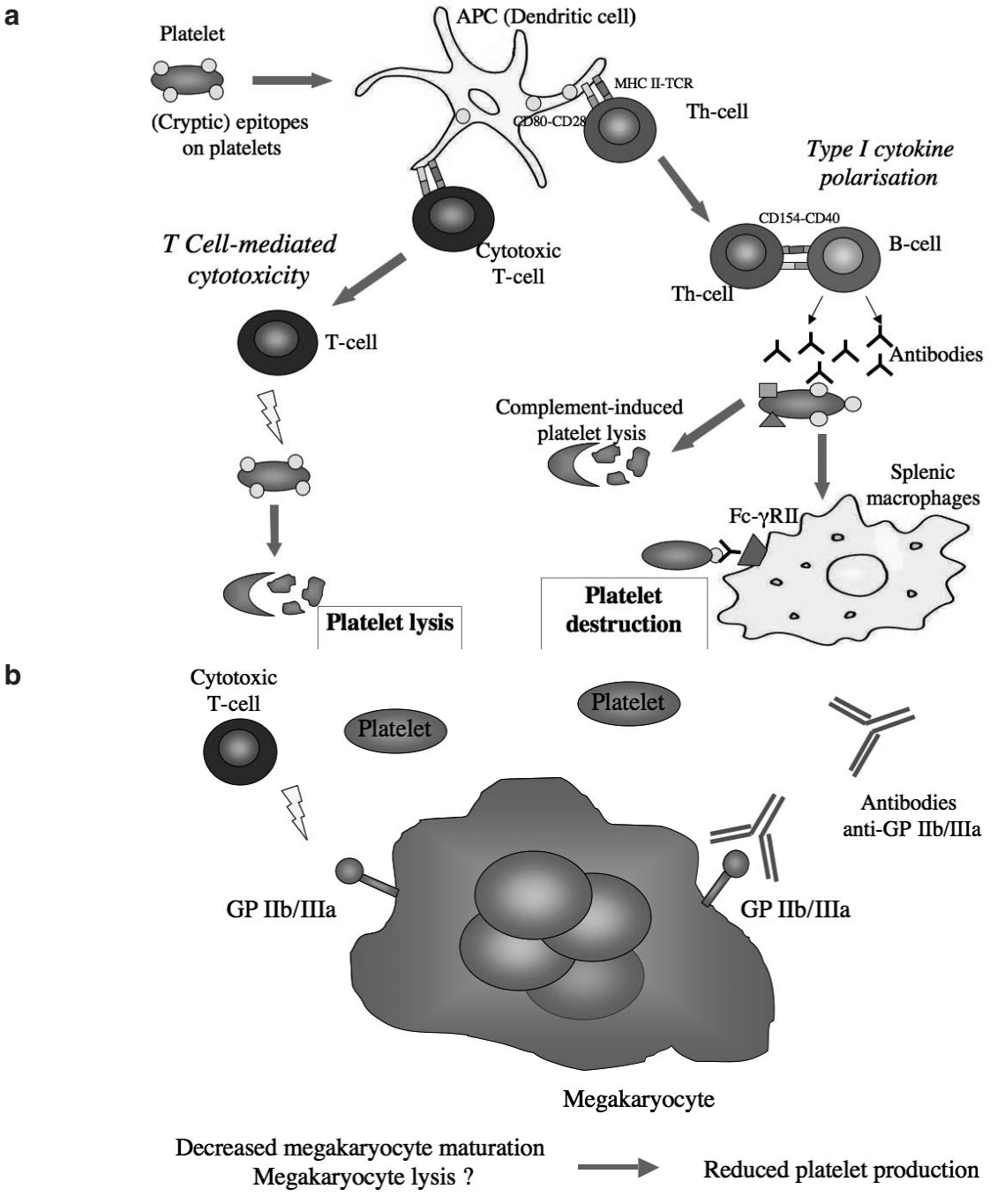

Figure 1. Pathogenesis of idiopathic thrombocytopenic purpura. a) Two mechanisms have been reported to be involved in accelerated platelet clearance: antibody-mediated platelet destruction and platelet lysis due to cytotoxic T lymphocytes. Platelets are the primary source of autoantigens. DCs or macrophages are the initial APCs which stimulate platelet-reactive Th cells. The platelet first interacts with a major histocompatibility complex (MHC) class II-positive APCs, which subsequently processes platelet glycoprotein antigens into smaller antigenic peptides. These peptides are expressed on the APC surface in association with MHC class II molecules. If the T cell receptor (TCR) has a sufficient affinity for the antigen-MHC complex and appropriate costimulatory events (CD80/CD86-CD28) are met, the Th cell would be activated and would subsequently drive antigen-primed B lymphocytes to produce autoantibodies. After antibody binding, platelets can be removed from the circulation by either phagocytosis or complement-mediated platelet lysis. Cytotoxic T lymphocytes activation results in platelet lysis. b) Ineffective platelet production. Antibodies directed against platelet glycoproteins (GPIIb-IIIa) bind to megakaryocytes resulting in decreased maturation and reduced platelet production. T cell-mediated inhibition of platelet production is also possible.

removed from the circulation by either phagocytosis or complement-mediated platelet lysis; there is experimental evidence supporting both mechanisms. Engagement of the Fcγ receptor on the surface of human macrophages by anti-GPIIbIIIa-coated platelets leads to engulfment of the opsonized platelets.[1,4]

Therefore, ITP is a complex disease that involves platelets, antigen presenting cells, T cells and B cells/antibodies.

Role of platelets

Platelets are the primary source of autoantigens in ITP. However, mechanisms leading to autoantigens generation are still to be investigated in depth. One of the suggested mechanisms in self-antigens recognition is molecular mimicry and cross-reaction due to viral/bacterial infection.[3] Another mechanism involves the generation of "criptic" epitopes.[6] Semple *et al*. found that the production of platelet-specific autoreactive T helper cells was fundamental to induce autoreactive antiplatelets antibodies.[7] However, T cells were shown to be responsive only to platelet "cryptic" self-determinants, but not to the native molecule.[8] Therefore, the question is how normally "cryptic" epitopes of platelet antigens may become visible to the immune system and may elicit a sustained pathogenic response in ITP patients.

Apoptosis occurs without maturative stimuli addressed to APCs like DCs or macrophages. However, recent studies show that apoptotic cells contain antigens able to activate a specific cytotoxic T-cell response.[9] An incorrect disposal of apoptotic cells may cause exposure of hidden antigens to the immune system.[10] The dying cells may also set an easier recognition of intracellular autoantigens by their redistribution into cell surface blebs or generate neoantigens.[11] Therefore, apoptosis might be a mechanism by which cryptic antigens are exposed and not recognized as "self". Recent experimental evidences suggest that platelets may undergo an apoptotic program. The platelet apoptotic phenotype includes cell shrinkage, membrane blebbing, cytoplasm condensation, and phospatidylserine exposure.[12,13] On these basis, we previously demonstrated that platelets from ITP patients, either fresh or *in vitro* aged, show increased apoptosis (with low levels of activation) in comparison to their normal counterparts.[14] However, whether platelet apoptosis plays a causative role in the induction of antiplatelet autoantibody production or it is the consequence of antibody activity remains a matter of speculation.

Furthermore, recent experimental evidences described abnormal platelet phenotype in ITP and mechanisms leading to T cell response. They found that platelet HLA-DR[15] and platelet-associated CD40-ligand (CD154) expression increased in patients with ITP, suggesting a possible active role of platelets in the autoimmune process.[16] Expression of CD154 by platelets could be critical in ITP, as it would mean a coexistence on the platelet surface of an essential co-stimulus of B-cell activation. In addition, an enhanced expression of CD80 on platelets from ITP patients was also observed, implicating a role of B7/CD28 costimulation in the pathogenesis of this disorders.[17]

Role of antigen presenting cells

What triggers and regulates T cell responses in patients with ITP still remains unknown. However, in order to activate T cells, APCs are absolutely required to present processed antigens in association with MHC class II molecules. Therefore, APCs play a key role in the pathogenesis of the disease. A model has been recently advanced in which APCs are crucial in generating a number of new or cryptic epitopes from platelet glycoproteins in ITP.[1]

Dendritic cells

DCs are specialized to initiate primary immune response. Immature antigen capturing DCs undergo maturation after cytokine stimulation. As a result, antigen acquired in peripheral sites is retained, processed and well presented to T cells in lymphonodes. Several findings suggest that DCs are likely to have a role in the pathogenesis of autoimmunity.[18] In fact, it has been suggested that under conditions of cytokine imbalance, DCs are differentiated into deviant phenotypes that could meddle with the state of self-tolerance. This phenotype could include altered migration, altered release of cytokines, or altered antigen processing so that criptyc self-determinants and/or self-determinants derived from apoptotic bodies are presented.[18] Therefore, it could be generated DCs that directly prime autoreactive T cells, or fail to prime regulatory T-cell subsets, or shift Th1/Th2 balance to an unfavourable outcome. Despite their fundamental role in initiating immune response, DCs have been poorly investigated in ITP. In a previous study, we investigated whether DCs may play a role in the stimulation of the immuno-mediated anti-platelet response in ITP.[14] We found that immature DCs readily ingest apoptotic platelets. Furthermore, in ITP patients DCs, pre-pulsed with autologous/allogeneic fresh and aged platelets, are highly efficient in stimulating autologous T-cell proliferation as compared to DCs derived from healthy donors. This finding may be related to the up-regulated expression of CD86 in DCs from ITP patients and not to higher phagocytic activity. Taken together, these results suggested that DC dysfunction, together with increased propensity of platelets to undergo apoptosis, may play a role in the stimulation of the immune system in ITP.

Macrophages

Macrophages play a key role in antibody-coated platelet phagocytosis. This hypothesis is supported by many evidences. Activated macrophages are present in patients with ITP. Zeigler *et al.*[19] demonstrated significantly elevated serum levels of macrophage-colony stimulating factor (M-CSF) in patients with chronic ITP. It was suggested that the high M-CSF levels in patients with ITP may contribute to or initiate enhanced platelet destruction by affecting macrophage function.[19] This cytokine specifically supports the differentiation of monocytic lineage cells and is a potent activator of mature monocytes and macrophages; it enhances phagocytosis.[20] ITP macrophages show also enhanced interactions with platelets in ITP[21] and activated cells induce platelet abnormalities such as HLA-DR expression.[21]

Role of T cells

Tolerance to self-antigens is generated through two fundamental mechanisms: a) elimination of self-reactive cells in the thymus during selection and b) generation of a variety of peripheral regulatory cells to control self-reactive cells that escape the thymus.

It has been published that autoreactive T cells to the immunodominant epitopes within the aminoterminal portions of platelet glycoprotein (GP)IIb and GPIIIa are present in the peripheral blood of patients with ITP for a long period and are involved in the production of anti-GPIIb-IIIa antibodies.[22] Self-reactive T cells are present also in healthy individuals.[23] The finding that auto-reactive T cells found in patients with ITP are more easily activated than those from normal subjects, leads to the hypothesis that regulatory T cells (Tregs) may normally suppress the activation of self-reactive T cells and either deficient generation or reduced effector function of these cells plays a role in the development of autoimmunity. Tregs are CD4 positive cells, with high levels of cell surface expression of CD25. They can

also be identified by their expression of the forkhead family transcription factor p3 (Foxp3) and the expression of Foxp3 has been proposed to be the crucial switch factor in the induction of Tregs.[24] It has recently been proposed that, in addition to the direct effect of Treg cells on T-cell function, Treg cells might also modulate the maturation and/or function of DCs, which are required for the activation of effector T cells.[24,25]

Quantitative and qualitative alterations of Treg have been described in systemic as well as in organ-specific autoimmune disorders.[26] Recent studies, focusing on Tregs and ITP, have investigated the frequency and the suppressive activity of natural Tregs, with some contrasting results. Some authors demonstrated reduced levels of Foxp3 mRNA[27] and protein[28] in circulating mononuclear cells and abnormal Treg function in spleen biopsies.[29] Yu *et al.*[30] found comparable frequency of circulating $CD4^+CD25^{high}Foxp3^+$ Tregs in patients and controls, even though the suppressive activity was impaired. Stasi *et al.*[31] showed reduced number and defective suppressive capacity of Tregs in ITP patients compared with control individuals. We recently investigated whether abnormal Tregs interactions with effector T cells and/or DCs might play a pathogenetic role in ITP. We found that in ITP patients Tregs show lower ability to inhibit DCs maturation and that the low number of circulating Tregs may be partly due to the reduced ability of DCs to convert non-Treg cells into Tregs (*personal comunication*). Taken together, these results indicate that quantitative and qualitative abnormalities of Tregs might be involved in the pathogenesis of ITP.

Multiple T cells defects are known to be present in ITP. One consistent abnormality described by several laboratories is an increased number of activated $CD3^+$HLA-DR^+ T lymphocytes in patients with chronic ITP. This observation may have importance in autoimmune pathology since activated HLA-DR^+ T cells can significantly influence resting $CD4^+$ T cells and may modulate autoreactive recognition *in vivo*. A significantly high level of oligoclonal expansion of T cells in peripheral blood from ITP patients has been recently reported.[5,31] In addition, very recently, Olsson *et al.*[32] analyzed genes and proteins involved in T-cell trafficking. They found that ITP is associated with accumulation and activation of T cells in the bone marrow. The recruitment of T cells in the bone marrow may be facilitated through increased VLA-4 and CX3CR1 expression. Therefore, these molecules, involved in T cell homing, may be of importance in recruiting T cells into the organs where the platelet destruction takes place.

Role of B cells and autoantibodies

By showing that injection of plasma from chronic ITP patients into healthy recipients can transfer thrombocytopenia, Harrington has proven that humoral factors play an important role in ITP.[33] Consistently, ITP patients often have elevated titers of antiplatelet antibodies.[4,34] The most commonly occurring autoantibodies in patients with ITP are directed against the platelet surface glycoprotein complexes GPIIb-IIIa and GPIb-IX. However, in many patients, antibodies against more than one platelet glycoprotein can also be detected.[1] Although antibodies are primarily of the IgG subtype, IgM and IgA may be found. The autoantibodies are light-chain restricted and are generated through the expansion of B cell clones that use genetically restricted and highly specific combinations of heavy and light-chain gene products.[35] Platelets coated with IgG autoantibodies undergo accelerated clearance through Fcγ receptors that are expressed by phagocytic cells, predominantly in the spleen, liver and bone marrow.[4,6]

There is now substantial evidence suggesting that the antiplatelet autoantibodies are generated by B cells under the control of Th cells and the cytokine they produce.[36] Two major helper T-cell cytokine profiles have been described: Th1 and Th2. Th1 cells produce interleukin-2 (IL-2), interferon (IFN)-γ, granulocyte macrophage-colony stimulating factor and tumor necrosis factor-α. Th2 cells produce IL-4,IL-5,IL-10. In general, Th1 cells promote organ-specific autoimmune disorders while Th2 cells tend to protect against them. The cytokine profile secreted by $CD4^+$ cells in patients with ITP is consistent with Th-cell activation. Studies have found evidence supporting Th0/Th1 polarization of the immune response in ITP patients with active chronic disease, whereas the cytokines during remission are skewed to a Th2 pattern.[37,38]

Platelet lysis due to cytotoxic T lymphocytes

Several observations indicate the existence of antibody-independent mechanisms of thrombocytopenia in ITP. First, anti-platelet antibodies are detected in only 50-70% of ITP patients,[4,6] and not serum from all ITP patients transferred thrombocytopenia in Harrington's experiment.[33] Second, there is evidence to suggests a direct cytotoxic effects of T cells on platelets. Olsson *et al.*[39] incubated radiolabeled platelets with anti-CD3-stimulated autologous $CD14^-CD19^-$ blood mononuclear cells. Of eight patients with active ITP, significant platelet lysis was noted in six of the samples when compared with the cells from either normal subjects or ITP patients in remission. The active cells were $CD3^+/CD8^+$ lymphocytes and lysis was HLA-specific. The Authors also noted increased expression of T-cell cytotoxic genes (*Apo1/Fas, granzyme 1, granzyme 2, and perforin*), as well as genes involved in the Th1 response (*IFN-γ and IL-2 receptor B*), whereas genes from the KIR receptor family, which are involved in the downregulation of cytotoxic T lymphocytes, are increased in ITP patients in remission when compared with patients with active ITP. Thus, T cells certainly contribute to the destruction of platelets in ITP.

It is therefore apparent that immune dysfunction in multi-steps play a central role in the final outcome of platelet destruction in ITP.

Suppression of platelet production

The failure of about a third of individuals to respond to aggressive immunosuppressive therapy or splenectomy has raised the suspicion that other mechanisms may contribute to the development of the disease. Ineffective platelet production has been recently suggested in ITP (Figure 1b). This hypothesis is based on the following experimental evidences:

Reduced platelet turnover

A shorter platelet life span is consistently seen in ITP patients compared with the 8- to 10-day platelet survival duration in healthy controls. The bone marrow contains normal or increased numbers of megakaryocytes. Surprisingly, studies of platelet turnover have shown that platelet production is decreased or normal in most patients.[40,41] In addition, a recent study looking at platelet reticulocytes demonstrated that the absolute platelet reticulocyte count is substantially reduced even if the percent platelet reticulocytes are increased.[42] It has been suggested that some surface antigens (GP IIb-IIIa and GPIb-IX), which are coexpressed on platelets, megakaryocytes, and megakaryocyte precursors, are recognized by autoantibodies. Therefore the reduced platelet production is presumed to be due to a direct effect of antibodies on megakaryocyte maturation or platelet release.[4,6,43] Moreover, other mecha-

nisms, i.e. T cell-mediated inhibition of platelet production, are possible and largely unexplored and could have an effect by altering the cytokine milieu of the bone marrow.

Bone marrow megakaryocyte abnormalities

Although bone marrow megakaryocytes are typically plentiful in ITP, more detailed analysis revealed these megakaryocytes to be structurally abnormal. These abnormalities included defects in the platelet-forming membranes of maturing megakaryocytes, morphological features of apoptosis and evidence of phagocytic engulfment by macrophages. An ultrastructural analysis of megakaryocytes of patients with ITP showed that 80% of mature megakaryocytes had features of apoptosis and para-apoptosis, suggesting that the low platelet production rate or ineffective thrombopoiesis in ITP may be a result of greater apoptosis of platelet-producing megakaryocytes.[44,45]

In vitro suppression of megakaryocytopoiesis by platelet autoantibodies

Chang *et al.*[46] showed that plasma from pediatric patients with ITP (44 with acute ITP and 9 with chronic ITP) containing antibodies against GPIb and GPIIbIIIa significantly suppressed megakaryocytopoiesis *in vitro*. They proposed that platelet autoantibodies may affect megakaryocyte survival, but not maturation, leading to decreased platelet production. McMillan *et al.*[47] studied the effects of plasma from 18 adult patients with chronic ITP on megakaryocyte production of $CD34^+$ cell cultures from healthy subjects. Cells were cultured in a medium containing thrombopoietin and 10% plasma from either the ITP patients or controls. *In vitro* megakaryocyte production was significantly lower when plasma from 12 of the 18 ITP patients containing autoantibodies against GPIb and GPIIbIIIa was added to the medium. The ITP plasmas that suppressed *in vitro* megakaryocyte production reduced not only the total number of megakaryocytes but also impaired megakaryocyte maturation.

Normal circulating thrombopoietin level

Thrombopoietin (TPO) levels have been repeatedly found to be within the normal range or only slightly elevated in patients with ITP.[48] Levels of TPO could be lower as expected in ITP because of binding to c-MPL receptor on the megakaryocytic mass. Aledort *et al.*[49] found no relationship between TPO levels and platelet counts in patients with ITP.

Taken together, these findings are consistent with the hypothesis that decreased platelet production is more central to ITP than had been previously appreciated.

In conclusion, in ITP patients thrombocytopenia seems to be mediated by various mechanisms. Even though the triggering event is unclear, the above reviewed experimental evidences suggest that the low platelet counts in ITP patients is the result of both increased platelet destruction and the decreased platelet production. It is therefore apparent that ITP is a heterogeneous disease with individual patients having different causes of thrombocytopenia and probably needing different therapeuthic approaches.

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